Studies on Photohemolysis with Special Reference to Demethylchlortetracycline

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Hemolysis induced by ultraviolet radiation as well as demethylchlortetracycline (DMCT) phototoxicity have been investigated in a model using human red blood cells. Total hemolysis for UV-B and UV-C was obtained with 8.3 and 1.9 J/cm^2 respectively. DMCT was shown to have pronounced hemolytic properties causing 88% hemolysis at 50 μg/ml and 72 J/cm^2 of UV-A. No increased hemolysis rate was seen in combination with UV-B. Several factors influencing the results were studied such as incubation time, UV-A dose, drug concentration and different methods for hemoglobin detection. Photohemolysis is an accurate tool for demonstrating DMCT phototoxicity. *Key words: Phototoxicity; Tetracycline.* (Received November 10, 1983.)

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Previous studies on the phototoxic effect of drugs have mainly focused on the psoralens and the phenothiazines. The tetracyclines, on the other hand, have been less extensively studied in spite of several clinical reports of light-induced reactions in the skin and nails (1). In experimental studies on phototoxicity different models have been used including the candida albicans inhibition test, photohemolysis, growth inhibition of mammalian cells in culture and registration of phototoxic erythema and edema in animals and human volunteers (2). Tetracyclines have been tested with negative result using the candida albicans inhibition method (3). Investigations using living mammalian cells in culture showed phototoxic effects from demethylchlortetracycline (DMCT) but no such effects from tetracycline (4). Experiments using human lymphoid cells showed decreased incorporation of thymidine into nuclear DNA after addition of DMCT, which was the only tetracycline tested (5). Studies on mice demonstrated a phototoxic edema from DMCT, doxycycline and lymecycline whereas other tetracyclines were negative (6). Using the photohemolysis test, which is considered to reflect membrane damage, Kahn & Fleischaker were unable to show phototoxicity from DMCT or tetracyline (7). Swanbeck & Wennersten, on the other hand, found DMCT to have a photohemolytic effect (8). In a recent study Hasan et al. (10), testing DMCT and tetracycline, could show no photohemolysis.

In view of these conflicting results we considered it of interest to reinvestigate DMCT using a photohemolysis method. Different parameters influencing the results have been evaluated.

MATERIAL AND METHODS

A modification of the photohemolysis technique described by Kahn & Fleischaker was used (7).

Drugs

Demethylchlortetracycline HCl (Declomycine®) was provided by Lederle Laboratories, Pearl River, N.Y., USA. Chlorpromazine HCl (Hibernal®) was provided by AB Leo, Helsingborg, Sweden.

Basic experimental design

Red blood corpuscles (RBC) were obtained by venipuncture from healthy human volunteers not taking any drugs. RBC were then immediately washed in physiological saline and centrifuged for 10 min at 2000 rpm. This procedure was repeated three times, whereafter the RBC were stored no longer than 40 h before use to avoid spontaneous hemolysis.

The drug was dissolved in a buffer solution consisting of 0.075 M Na-Veronal (barbital) at pH 7.4 with saline to 290 mosmol/kg. Ten ml of the buffered drug solution was poured into plastic cups forming a liquid layer of approximately 7 mm. RBC 20 μl were added to each cup giving a dilution of 1 : 500. The cups were then irradiated with long-wave (UV-A), medium-wave (UV-B) or short-wave (UV-C) ultraviolet radiation. A metal halogen lamp (Osram-Ultramed 400 W) with an emission peak at 365 nm was used as the UV-A source. A UV-B filter consisting of 3 mm window glass was inserted between the lamp and the samples giving an output in UV-A of 10 mW/cm^2 sec at 30 cm distance as measured with a PUVA-meter (Waldmann AG, Schwenningen, GFR). For UV-B 2 fluorescent tubes (Westinghouse Sun Lamp, 40 W) emitting continuously from about 280 nm to 380 nm with a peak at 313 nm were used. These lamps have an output of 2.3 mW/cm^2 sec in the UV-B region as measured with a UV-meter (Waldmann AG, Schwenningen, GFR). For UV-C one fluorescent tube (Philips TUV 30 W) emitting at 254 nm was used, having an output of 2.1 mW/cm^2 sec as measured with a UVX digital radiometer (Ultra-violet products Inc, San Gabriel, Calif., USA).

Duplicate samples of drug and RBC, RBC only, as well as one sample containing drug only (the latter allowing compensation for possible UV induced colour changes of the drug), were irradiated and controls kept in the dark. After irradiation the samples were incubated in the dark. The volume of the samples was then measured to compensate for minor losses due to evaporation and the suspensions were transferred to test tubes and centrifuged for 10 min at 2000 rpm. Four ml of the supernatant was mixed with 1 ml of Drabkins' solution (K$_3$Fe (CN)$_6$ 200 mg, KNC 50 mg, KH$_2$PO$_4$ 140 mg, surfactant Triton×100 0.5 ml, diluted with distilled water to 1000 ml with a pH between 7.0 and 7.4) in order to convert all types of hemoglobin to methemoglobin which forms the stable pigment cyanmethemoglobin with a maximum absorbance at 540 nm. The samples were read at this wave length in a Beckman DB-GT spectrophotometer. A sample with 100% hemolysis consisted of 20 μl of RBC in 12.5 ml of Drabkins' solution, also giving a dilution of 1 : 500.

Studies on the hemolytic effect of UV-irradiation without drug

The following UV-A doses were tested (corresponding doses in J/cm^2 in brackets): 1, 2, 3 and 4 h (36, 72, 108 and 144). Incubation time 2 h.

The following UV-B doses were tested (corresponding doses in J/cm^2 in brackets): 0.25, 0.5, 0.75, 1, 1.5, 2 and 4 h (2.1, 4.1, 6.2, 8.3, 12.4, 16.6 and 33.1). Incubation time 2 h.

When UV-C was tested physiological saline was used instead of Na-Veronal buffer as the latter was shown to absorb radiation at 254 nm. The following doses were tested (corresponding doses in J/cm^2 in brackets): 1, 3, 7, 15 and 30 minutes (0.1, 0.4, 0.9, 1.9 and 3.8).

Studies on the photohemolytic effect of DMCT

The influence of variations in incubation time, UV-A dose, DMCT concentration as well as the effect of UV-B were studied.

1. *Incubation time.* RBC in the presence of 100 μg/ml of DMCT were irradiated for 2 h with UV-A. Samples were subsequently incubated for 0, 1, 2 and 3 h.

2. *UV-A dose.* RBC in the presence of 25 μg/ml of DMCT were irradiated for 0.5, 1, 2 and 3 h. Incubation time 2 h.

3. *Concentration of DMCT.* RBC in the presence of DMCT 5, 10, 25, 50 and 100 μg/ml were irradiated for 2 h with UV-A and incubated for 2 hours. This experiment was repeated at 10 different occasions to test the reproducibility of the method.

4. *Irradiation with UV-B.* RBC in the presence of DMCT 50 μg/ml were irradiated with UV-B for 7, 15 and 30 min. RBC without DMCT, irradiated with the same doses, served as controls.

Studies on the photohemolytic effect of chlorpromazine (CPZ)

RBC in the presence of CPZ 0.1, 0.5, 1, 5 μg/ml were irradiated for 2 h with UV-A and incubated for 2 h.

Studies on the effect of Drabkins' solution

RBC were irradiated with UV-B for 0.25, 0.5, 0.75, 1, 2 and 3 h without drug. For each UV-B dose four cups were used, two of which were treated with Drabkins' solution (as was the 100% control) according to the basic experimental design, the other two with 1 ml of buffer instead. For the latter two samples the 100% control was hemolysed with distilled water and not treated with Drabkin's
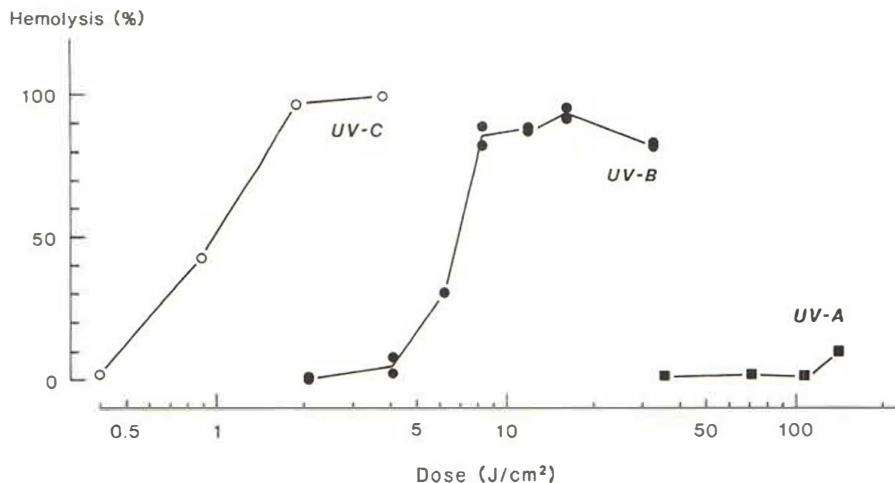
Hemolysis (%)

Dose (J/cm²)

Fig. 1. Dose-response curves for photohemolysis. RBC without drug irradiated with increasing doses of UV-C, UV-B and UV-A respectively. Incubation time 2 h. The UV-B curve represents the mean of two experiments.

solution. This experiment was repeated irradiating RBC in 10, 25 and 100 µg/ml of DMCT for 2 h with UV-A. Incubation time 2 h.

Calculations

All results are expressed as per cent hemolysis relative to the 100% hemolysed solution using the following formula:

$$\frac{(EDR-ED) \times V-D}{T} \times 100$$

EDR = optical density of exposed drug solution with RBC
ED = optical density of exposed drug solution without RBC
D   = optical density of dark control drug solution
T   = optical density of total hemolysis control solution
V   = $\dfrac{\text{volume after irradiation}}{\text{volume before irradiation}}$.

RESULTS

Studies on the effect of UV-irradiation without drug

UV-A exposure. Only the longest exposure time of 4 h (144 J/cm²) induced a slight hemolysis of 10% (Fig. 1).

UV-B exposure. An exposure time of 0.5 h or less induced almost no hemolysis. Above this level an increasing rate of hemolysis was found reaching 91% after 2 h (16.6 J/cm²) (Fig. 1).

UV-C exposure. Hemolysis was induced with 7 min of irradiation and was total (99%) with 30 min of UV-C (3.8 J/cm²) (Fig. 1).

Studies on the photohemolytic effect of DMCT

1. *Incubation time.* For RBC UV-A irradiated for 2 h together with 100 µg/ml of DMCT a photohemolytic effect was shown. A 90% hemolysis rate was reached with 2 h incubation time which was therefore chosen as the standard in the following experiments (Fig. 2).

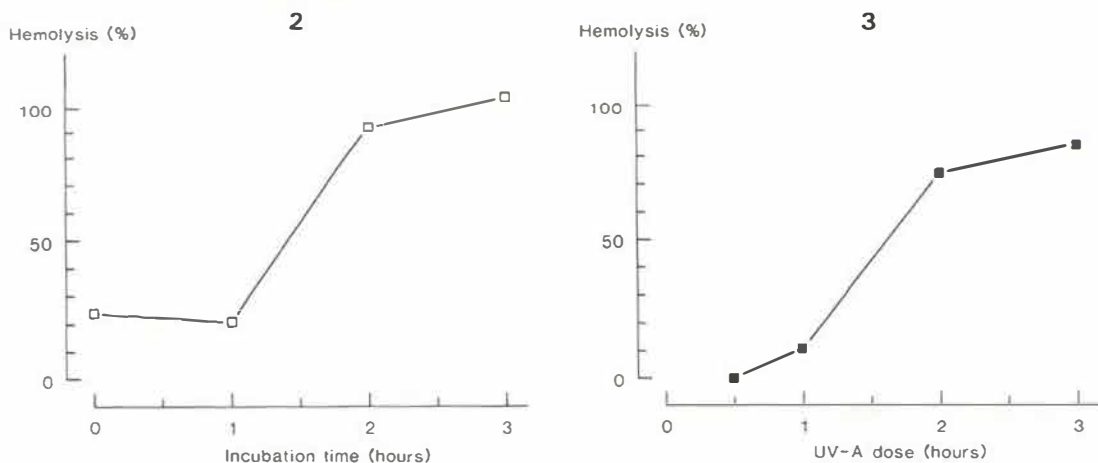   2. *UV-A dose.* When RBC were exposed to increasing UV-A doses in the presence of

Fig. 2. Photohemolysis with different incubation times for RBC irradiated with 2 h of UV-A together with DMCT 200 µg/ml.

Fig. 3. Photohemolysis with DMCT. The effect of increasing UV-A doses for RBC in DMCT 25 µg/ml. Incubation time 2 h.

25 µg/ml DMCT a dose-response curve was obtained, levelling off above 2 h exposure. A maximum of 84% hemolysis was induced with 3 h of UV-A (Fig. 3). A standard exposure of 2 h (72 J/cm^2) was used in the following experiments.

3. *Concentration of DMCT*. With 2 h of UV-A, 2 h incubation time and increasing concentrations of DMCT a dose-response curve was obtained with a maximal hemolysis of 88% at 50 µg/ml. The reproducibility was satisfying as indicated in Fig. 4.

4. *Irradiation with UV-B*. RBC irradiated with UV-B for 30 min (4.1 J/cm^2) in the presence of DMCT 50 µg/ml showed no hemolysis.
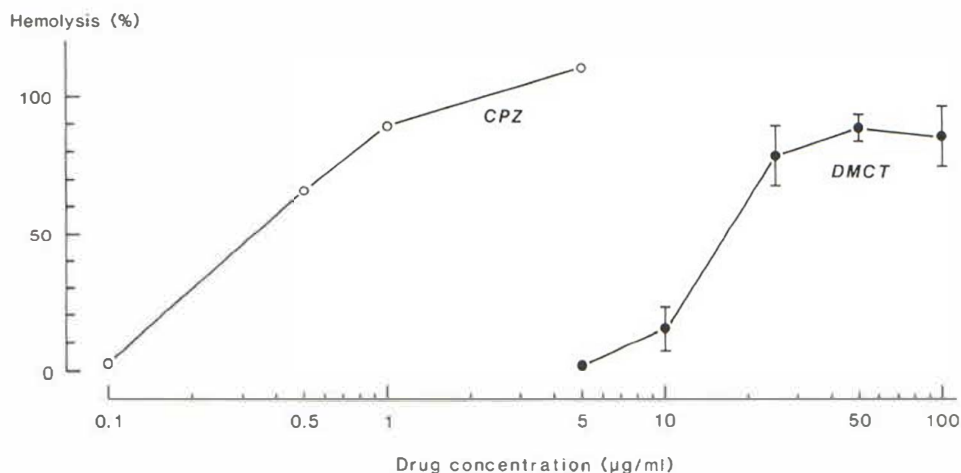

Fig. 4. Dose-response curves for CPZ and DMCT photohemolysis. RBC in increasing concentrations of CPZ and DMCT irradiated with 2 h of UV-A. Incubation time 2 h. The values for DMCT are the means of 10 experiments. Vertical bars indicate the standard deviation.
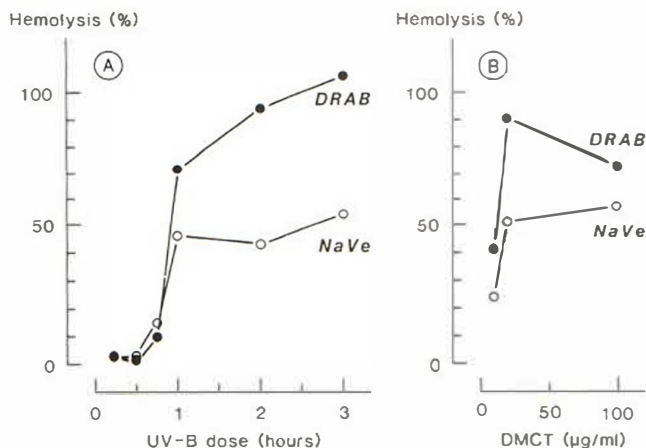
Fig. 5. Dose-response curves for photohemolysis showing the difference in hemoglobin determination with (DRAB) and without (NA Ve) the use of Drabkins' method. A: RBC irradiated with UV-B without drug. Incubation time 2 h. B: RBC together with 10, 25 and 100 µg/ml of DMCT irradiated with 2 h of UV-A. Incubation time 2 h.

Studies on the photohemolytic effect of CPZ

CPZ had a very strong photohemolytic effect causing 89 % hemolysis at 1 µg/ml and UV-A 2 h (Fig. 4).

Studies on the effect of Drabkins' solution

When RBC without drug were irradiated with UV-B for 1 h or more the addition of Drabkins' solution gave a higher value of hemolysis than the falsely low figure obtained with buffer alone. The difference increased with exposure time (Fig. 5 A). The difference after 1, 2 and 3 h of exposure was 26, 51 and 53 % respectively. With exposures up to 0.45 h no difference was noted.

When RBC with DMCT 10, 25 and 100 µg/ml were irradiated with UV-A for 2 h the addition of Drabkins' solution gave higher values for all concentrations of DMCT. A maximal difference of 39 % was estimated for DMCT 25 µg/ml (Fig. 5 B).

DISCUSSION

Exposure to UV-A alone had no hemolytic effect on RBC in doses below 108 J/cm^2. UV-B on the other hand caused marked hemolysis amounting to 91 % at a dose of 16.6 J/cm^2. UV-C also showed a pronounced effect at even lower doses raching 99 % hemolysis at a dose of 3.8 J/cm^2. UV-C thus is at least 1000 times as potent as hemolyser than UV-A. The hemolytic ability of UV-B and UV-C has been reported earlier (11).

DMCT was shown to be a strong photosensitizer in combination with UV-A, hemolysis beginning at 10 µg/ml and reaching 88 % at 50 µg/ml with 72 J/cm^2. A similar dose-response curve was obtained for increasing UV-A doses. The negative results in the UV-B experiments are in accordance with the action spectrum of DMCT, being in UV-A, as earlier reported (9). The reproducibility in repeated experiments was good. These findings support the data of Swanbeck & Wennersten (8) who also found DMCT to be photohemolytic in concentrations of 10 µg/ml or more combined with UV-A 132–264 J/cm^2. The negative results of Hasan et al. (10) may be due to the application of UV-A doses not greater than 20 J/cm^2. To further test the system CPZ was studied, giving hemolysis already at a concentration of 0.5 µg/ml, a rate of about fifty times that of DMCT.

It is important to compensate for possible colour changes of the irradiated drug solutions. UV-A exposure of DMCT causes a pronounced colour change with the photopro-

ducts absorbing at 540 nm which results in falsely high estimated hemolysis if not compensated for. This phenomenon was also observed by Kahn and Fleischaker (7).

Kahn & Fleischaker reported a phenomenon which they named the fading of hemoglobin (7). When irradiating RBC with UV-B and measuring the release of hemoglobin by reading the optical density at 540 mm they found a decreasing density with increasing exposure time. This was believed to reflect the conversion of some of the hemoglobin to methemoglobin with a new absorption peak at 630 mm. This problem can be overcome by the use of Drabkins' solution. By this method all forms of hemoglobin are converted to methemoglobin which forms a stable pigment with cyanide having an absorption maximum at 540 mm. We have shown the importance of this method in connection with UV-B exposure by finding a difference in hemolysis of 53% after 3 h of irradiation, the higher value obtained when Drabkins' solution was added. When testing Drabkins' solution in photohemolysis caused by DMCT and UV-A the difference was 39% at most.

In conclusion photohemolysis has proved to be a sensitive and accurate method for studying DMCT photoxicity as well as the effects of ultraviolet radiation. Further studies including other tetracyline derivates are in progress.

ACKNOWLEDGEMENTS

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