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Photoinactivation of Skin Fibroblasts by Fractionated Treatment with 8-Methoxypsoralen and UVA

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Guinea pig skin fibroblasts treated with low doses of 8-methoxypsoralen (8-MOP) and long-wave ultraviolet light (UVA) showed a dose-dependent inhibition of ^{3}H-Thymidine incorporation as determined by liquid scintillation counting. The minimum incubation time necessary to obtain constant inhibition rates was 60 min. By washing the drug was removed from the reactive sites within 30 min. Repeated light exposure at a constant concentration of 8-MOP caused a cumulative inhibition of DNA synthesis. Irradiation of 8-MOP-plus-UVA treated cells, from which the drug was removed, produced a small increase in photoinhibition. Split dose treatment at various time intervals (ranging from 1–48 hr) revealed inhibitory rates, which correspond to the total amount of UVA applied. No recovery effects were seen in cultures treated by single or multiple applications of 8-MOP-plus-UVA.

Photochemotherapy (PUVA) employing orally administered 8-methoxypsoralen (8-MOP) in combination with long-wave ultraviolet light (UVA) has gained attention in the treatment of various skin diseases. Dermatoses such as psoriasis, lichen planus, mycosis fungoides or urticaria pigmentosa, all of which differ greatly have been shown to be PUVA-responsive [1–8]. In skin the mechanism of action of this treatment is not fully understood. This is partly due to the complexity of skin consisting of a variety of cell populations, structures and reaction sites. *In vitro* and *in vivo*, 8-MOP as well as other psoralen derivatives are known to covalently bind to DNA forming pyrimidine dimers under the influence of UVA [9–20]. We have recently studied the effects of low dosages of 8-MOP followed by UVA in cultured skin fibroblasts [21]. Using ^{3}H-TdR incorporation and cellular plating it was found, that under dosages similar to those employed in patients, DNA synthesis and cell replication were inhibited without affecting cellular viability [21]. Such sublethal photoinactivation (SPI) raises several questions concerned with the cellular uptake of 8-MOP *in vitro*, treatment schedules with 8-MOP and UVA and the accumulation of damage. In the present paper such data will be reported.

MATERIALS AND METHODS

1. Cell Cultures

Fibroblast cultures from guinea pig skin were prepared as previously described [21]. The growth medium was McCoy's 5A medium supplemented with 10% fetal calf serum and 100 IU/100 μg/ml penicillin-streptomycin (all materials obtained from Gibco Bio-Cult). The fibroblasts were subcultivated every week into plastic tissue culture dishes (Linbro FB 16–24 TC) at a density of 3–4 × 10^4 cells/cm^2. These were kept in the dark at 37°C in a humidified atmosphere containing 5% CO_2. Weekly tests for mycoplasm contamination were negative.

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Abbreviations:
8 MOP: 8-methoxypsoralen

2. Treatment with 8-MOP and UVA

8-MOP (Xanthotoxin) was dissolved in the growth medium as described before [21] and added at concentrations ranging from 0.05–2 μg/ml for 1 hr or as indicated. The cultures were irradiated with commercial Sylvania GTE black light lamps at a fluence rate of 33.3 J m^{-2} sec^{-1} including absorption of light quanta by the cover of the culture dishes and the medium. After irradiation the medium was removed, the cells were washed and fresh medium was added. ^{3}H-TdR incorporation was not affected when growth medium plus 8-MOP were UVA-irradiated and added to the cells. Therefore, in this system the generation of photoproducts can be excluded.

3. ^{3}H-TdR Incorporation

24 hrs after the last treatment or as indicated 2 μCi of (methyl-^{3}H)-thymidine (0.4 nmol in 2 μl, specific activity 5 Ci/mmol) was added to each culture (1 ml volume) for 2 hr. After washing the cells 2 times with buffered saline, the fibroblasts were trypsinized and harvested by means of a cell harvester (Titertec, Flow Laboratories, Germany). The filter plates were dried, placed into 5 ml scintillation fluid and the radioactivity (cpm) was measured (Packard Tricarb spectrometer). The standard deviations (SD) were calculated. All experiments were done at least 2 times using quadruplicate cultures.

4. Split Dose Treatment

The cultures were incubated with 8-MOP at the indicated concentration and irradiated with 1 J/cm^2. Subsequently the medium was replaced by fresh growth medium without 8-MOP. This treatment was repeated from 1 to 4 times at time intervals shown in the tables (Table I, IV). One hour before each UVA-treatment, fresh medium containing 8-MOP at indicated concentrations was added. Control cultures (quadruplicates) were treated in the same way except for the application of UVA. Further, a dose equivalent to the cumulative dosages of UVA and 8-MOP was applied at the time of the last treatment. ^{3}H-TdR was added after 24 hr.

RESULTS

Incubation with 8-MOP for different lengths of time before irradiation showed that 8-MOP enters the cell quite rapidly (Table I). With 2 dosages (0.1 μg and 1.0 μg 8-MOP per ml) the decrease in ^{3}H-TdR incorporation remained constant after approximately 1 hr incubation time. Therefore this time period was chosen in all subsequent experiments. The time needed to remove 8-MOP from the reactions sites after 1 hr incubation time was determined in subsequent experiments. When 8-MOP containing medium was replaced by fresh medium without 8-MOP the subsequent irradiation at various time intervals (Table II) caused a biphasic response: UVA irradiation carried out within 30 min produced an inhibition of DNA synthesis (Table II), while with an interval of more than 30 min between medium change and irradiation no effects upon ^{3}H-TdR were seen. This indicates complete removal of 8-MOP from the reactive sites within the cells.

When 5 J/cm^2 were applied as fractionated doses of 1 J/cm^2 5 times in the presence of 0.5 μg 8-MOP per ml an exponential decrease of ^{3}H-TdR incorporation is seen ($r^2 = 0.94$, Fig 1, *curve A*). The rate of inhibition obtained in these experiments compares to data previously reported [21]. In addition, the effect of light application after medium change is recorded (Fig 1, *graph B*). In these experiments the cultures were exposed to 1 J/cm^2 in the presence of 8-MOP and, after the medium had been replaced by fresh medium without 8-MOP, they were further

TABLE I. *Effects of length of incubation with 8-MOP (0.1 and 1.0 μg/ml) on ^{3}H-TdR uptake following UVA exposure (2J/cm^2).*

Concentration of 8-MOP (μg/ml)	0	0.1	0.1	0.1	0.1
Length of incubation (min)	0	10	35	90	150
cpm ± SD	5133 ± 354	4035 ± 643	3356 ± 316	1865 ± 170	1808 ± 270
Concentration of 8-MOP (μg/ml)	0	1.0	1.0	1.0	1.0
Length of incubation (min)	0	10	30	60	120
cpm ± SD	9116 ± 121	3043 ± 326	2433 ± 243	1857 ± 150	1858 ± 102

TABLE II. *Effects of length of time between medium change and UVA irradiation (2J/cm^2) on ^{3}H-TdR incorporation*

Concentration of 8-MOP (μg/ml)	0	1.0	1.0	1.0	1.0	1.0
Length of time after medium change (min)	0	10	20	30	60	120
cpm ± SD	13862 ± 3757	3632 ± 568	8299 ± 2637	12712 ± 1624	12698 ± 712	11068 ± 1515

TABLE III. *Comparison between fractionated treatment at daily intervals and unfractionated 8-MOP-plus-UVA-treatment with total dosages*

Experiment No.	Concentration of 8-MOP (μg/ml)	J/cm^2	No. of treatments	cpm (% of control)	Significance
1	0.1	1	4	22.5 ± 4.4	$p < 0.001$
	0.4	4	1[a]	7.9 ± 2.1	
2	0.5	1	4	16.9 ± 0.9	$p < 0.001$
	2.0	4	1[a]	0.7 ± 0.08	

[a] applied on day 4.

TABLE IV. *Effects of fractionated treatment at constant concentrations of 8-MOP and various treatment intervals*

Exp. No.	Concentration of 8-MOP	J/cm^2	No. of treatments	Intervals between treatments (hrs)	cpm (% of control)	Significance
1	0.1	1	4	24	26.4 ± 4.2	$p < 0.1$
	0.1	4	1		30.8 ± 3.8	(n.s.)
2	0.1	1	4	1	69.1 ± 6.5	$p < 0.1$
	0.1	4	1		74.9 ± 4.1	(n.s.)
	0.4	4	1		42.2 ± 5.6	$p < 0.0001$
3	0.1[a]	1	2	48	57.8 ± 6.1	$p < 0.2$
	0.1[b]	2	1		51.7 ± 6.0	(n.s.)
4	1.0[a]	1	2	48	11.3 ± 0.9	$p < 0.2$
	1.0[b]	2	1		9.5 ± 2.7	(n.s.)

In all experiments UVA was administered 1 hr after incubating the cells with 8-MOP-containing medium. ^{3}H-TdR was added to the cultures 24 hr after the last UVA exposure. n.s. = no significance between first and second measurement.

[a] ^{3}H-TdR incorporation was determined 2 days after last treatment.

[b] ^{3}H-TdR incorporation was determined 4 days after treatment.

irradiated with UVA (1–4 J/cm^2). The time interval between the first and the second exposure to UVA lasted 15 min. The resulting curve reveals, that nearly all of the psoralen was removed by changing the medium thus confirming the results reported in Table II. There is a small but significant increase in inhibition caused by the second irradiation, as can be seen in this graph.

Since in these as well as in previous experiments [21] the 8-MOP-UVA induced photoinactivation was determined for up to one day after treatment, it seemed necessary to follow the treatment effects for a longer time period. The pooled data obtained from 5 separate experiments carried out over 6 days are shown in Fig 2. The amount of radioactivity being incorporated on day 1 after treatment (36.0 ± 6.4%) becomes further reduced on the following days (eg, 8.0 ± 3.9 on day 4) indicating the lasting effect of a single treatment. In these cultures, all of which were treated while in log phase, no evidence for cellular recovery as judged by ^{3}H-TdR incorporation was detected.

Photochemical inhibition of DNA synthesis as shown in Table III was smaller when 8-MOP and UVA were split 4 times as compared to the one time application of the total dose. For example with daily treatment with 0.1 μg/ml plus 1 J/cm^2 for 4 days the incorporated ^{3}H-TdR decreased to 22.5% whereas the single application of the cumulative dose of 8-MOP and UVA (0.4 μg/ml plus 4 J/cm^2) on day 4 reduced the precursor uptake to 7.9% (exp 1). While these data indicate that treatment fractionation causes less inhibition as compared to the total dose of 8-MOP and UVA given at one time, the effects of cumulative UVA applications at constant (noncumulative) 8-MOP concentrations were compared. Table IV shows the results of repeated treatments at constant concentrations of 8-MOP, given at various time intervals. As can be seen from this table no significant difference is found between fractionated and nonfractionated treatments in all 4 experiments. Furthermore repeated treatments given at hourly intervals (exp 2, Table IV) or longer (exp 1, 3, 4) revealed that with a constant concentration of the psoralen no difference is found whether the total amount of light is applied as a single dose or fractionated by multiple treatments. Also, the time interval between the photoinactivating treatment was of no significance.

The results constantly failed to show evidence for immediate or late recovery from damage. Further, they demonstrate that provided the concentration of psoralen is constant, the total amount of UVA light determines the degree of photoinhibition, no matter to what extent the dosage of light is split.

In order to further ascertain the latter point fibroblast cultures were subjected to multiple treatments at daily intervals (Fig 3). The plotted incorporation rates decrease exponentially ($r^2 = 0.95$) depending upon the number of treatments. The data recorded for 1 and 3 treatments compare to those found previously for 1 and 3 J/cm^2 and 0.05 μg 8-MOP/ml [21]. Thus, the figure clearly indicates the cumulative effects of multiple PUVA treatments on DNA synthesis for which the total amount of light, not however the cumulative dose of 8-MOP is responsible.

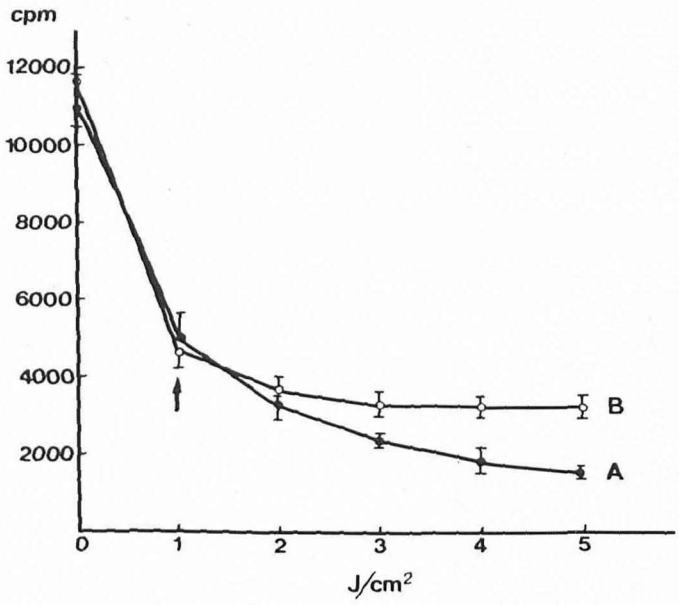

FIG 1. Inhibition of ^{3}H-TdR incorporation by multiple applications of UVA (1–5 J/cm^2) in the presence of 0.5 μg 8-MOP/ml (A, ●——●). In *curve B* (○——○) the drug was removed and replaced by fresh medium after irradiation with 1 J/cm^2 (↑). Subsequently UVA was applied as in *A*. Controls were left unirradiated. The radioactivity was determined 24 hr after the last treatment. Correlation coefficient r^2 = 0.94 (*A*).

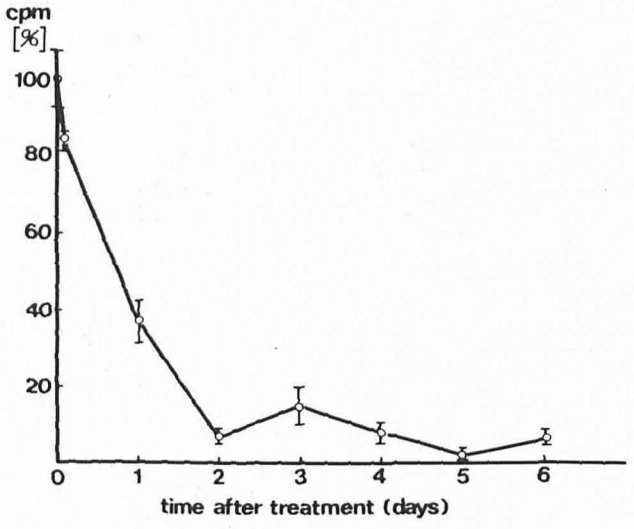

FIG 2. Inhibition of ^{3}H-TdR incorporation at daily intervals after treatment with 0.1 μg 8-MOP per ml and 2 J/cm^2 at day 0. Cells were grown while in log-phase before treatment. Data represent average values ± S.D. from 5 experiments grown in quadruplicates.

DISCUSSION

Previous experiments have shown, that in skin fibroblasts treated with low doses of 8-MOP and UVA DNA synthesis is reduced depending upon the dosage [21]. In the present report it is attempted to further define the cellular reactivity *in vitro*. The results show, that 8-MOP passes into the cell in a matter of 60 min. At this time maximum photoinhibition rates are found. Also, the drug leaves the cellular reaction sites in an even shorter time period (approx. 30 min) indicating that transmembraneous diffusion may be the principal mechanism.

Treatment with increasing amounts of UVA at a constant concentration of 8-MOP revealed an exponential regression curve (the correlation coefficient being close to unity, Fig 1). Furthermore, when the cells were briefly washed after 1 treatment, the subsequent application of light caused a moderate increase in photoinhibition (Fig 1). This again indicates rapid loss of the drug from the relevant cell compartments (eg, nucleus). In addition, it is possible, that by the subsequent application of light monoadducts are transformed into interstrand crosslinks. Ben Hur and Elkind reported 7.8 monoadducts to be present per crosslink in Chinese hamster cells after a single application of psoralen-plus-UVA [19]. While these authors irradiated their cells with small amounts of UVA (the highest dose being 0.1 J/cm^2 and 0.21 μg trioxsalen) the increased amount of UVA used here may have caused more bifunctional adducts to be formed in relation to monofunctional adducts. Therefore the moderate increase in photoinhibition produced by subsequent UVA application (Fig 1) can be explained.

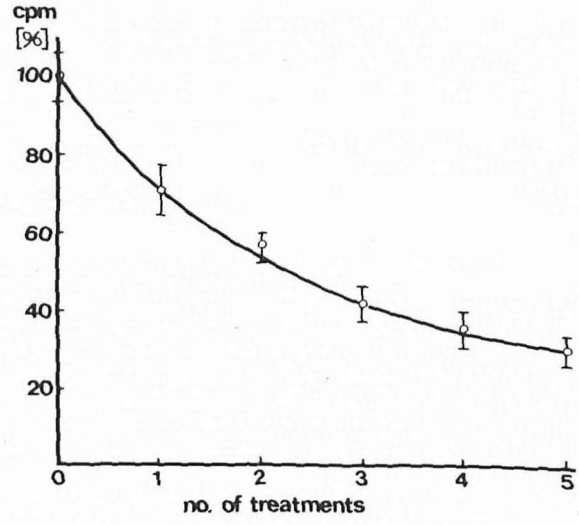

FIG 3. Inhibition of ^{3}H-TdR incorporation after repeated treatment with 8-MOP (0.05 μg/ml) and UVA (1 J/cm^2) at daily intervals. The psoralen-containing medium was replaced by fresh medium each time after irradiation. The radioactivity in treated cells as well as in the unirradiated controls was determined 24 hr after each treatment. Correlation coefficient r^2 = 0.95.

It is of interest to note, that the single streatment with 8-MOP-plus-UVA causes lasting effects which increase with time (Fig 2). Since these cultures were treated while in logarithmic growth phase, density inhibition cannot be responsible for the substantial lack of recovery. Instead, a fraction of cells seems to retain the DNA synthesizing capacity as has been shown before [21]. As further substantiated in the present paper, therefore, the majority of cells photoinactivated by PUVA is blocked from synthesizing DNA for relatively long time intervals (at least 6 days). This however is not accompanied by a loss of viability [21].

In these experiments the absence of recovery from photoinactivation is noteworthy and is further demonstrated by the split dose treatment. The results clearly show, that the time interval placed between the treatments did not influence the degree of inhibition. In fact no difference was found when cells were treated every hour, every 24 hr or 48 hr (Tables III and IV). This seems to exclude the possibility of cellular recovery taking place during the time in which the cells are left without treatment. The question of the ability of the cells to repair sublethal damage from PUVA has not been satisfactorily answered. By the use of fractionated survival curves produced by treatment at 5 hourly intervals Ben Hur and Elkind observed, that the cells "recovered some of their capacity to accumulate sublethal damage" [22]. In their experiments the difference in survival between fractionated and unfractionated exposure remained smaller than that observed after X-irradiation. In a subsequent report it was found, that 90% of bound psoralen can

be removed during 10 hr incubation time after exposure [19]. It is suggested, that at least some of the removed psoralen molecules were cross-links. Small amounts of repair DNA synthesis have also been observed by Baden et al using autoradiography [10]. In view of our present findings more specific methods need to be used to further clarify this aspects.

Finally, the fractionated treatment results indicate, that the total dose of 8-MOP and UVA applied once causes considerably more damage as compared to the fractionated application of 8-MOP and UVA. On the other hand, when cultures are treated repeatedly, the degree of photoinhibition is nearly identical to one time treatment results employing the total dose of UVA at noncumulative 8-MOP concentrations (Table IV). The experiments show, that the total amount of light determines the rate of photoinhibition at a given 8-MOP concentration. This can also be seen from the single treatment experiments using varying UVA dosages (Fig 4). Thus fractionated photoinactivation demonstrating the cumulative effectiveness of light closely resembles the conditions of PUVA therapy in vivo. It appears justified therefore to use the total dose of UVA as a measure of phototoxicity provided the degree of cutaneous UVA absorbance is known.

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