0022-202X/80/7504-0306$02.00/0
THE JOURNAL OF INVESTIGATIVE DERMATOLOGY, 75:306–310, 1980

Vol. 75, No. 4
Printed in U.S.A.

Photoinactivation and Recovery in Skin Fibroblasts after Formation of Mono- and Bifunctional Adducts by Furocoumarins-Plus-UVA

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Cultured skin fibroblasts from young male guinea pigs were irradiated with UVA light in the presence of 8-methoxypsoralen (8-MOP) or angelicin. As compared to 8-MOP 30 times higher concentrations of angelicin were needed to obtain comparative inhibition rates of DNA-synthesis. Complete cellular recovery could be observed when the cell cultures were treated with angelicin-plus-UVA (320–400 nm) or 8-MOP-plus-395 nm. Both treatment schedules are known to cause monofunctional photoreactions. In contrast to this, bifunctional photoreactions caused by 8-MOP-plus-365 nm produced an inhibition of DNA synthesis which lasted more than four days. Also, UVA (320–400 nm) applied to cells treated with ^{3}H-labeled 8-MOP resulted in a dose-dependent binding of 8-MOP molecules again lasting several days.

Application of 8-MOP-plus-UVA (320–400 nm) to cells growing in log-phase showed a characteristic change in morphology. An increasing number of polynuclear and hyperchromatic cells appeared with time after treatment. In this subpopulation of cells DNA synthesis continued without division as revealed by DNA measurements and autoradiography.

It is concluded that monofunctional adducts caused by angelicin-plus-UVA as well as 8-MOP-plus-395 nm permit cellular recovery whereas bifunctional photoadducts remained without recovery. In the latter case semiconservative DNA synthesis continued leading to hyperchromatic cells which could serve as a parameter for the presence of cross-linked nuclear DNA strands.

In skin systemic as well as local treatment with 8-methoxypsoralen (8-MOP)-plus-UVA (PUVA) is shown to be therapeutically effective in a heterogeneous group of skin diseases [1–6]. As furocoumarins (psoralens) are able to form photoadducts and interstrand cross-links with pyrimidin base pairs of DNA under the influence of UVA [7–15] the effect of PUVA in hyperproliferative skin disease (e.g., psoriasis) can be explained. Indeed, skin fibroblasts, as well as epidermal cells treated with doses used *in vivo* show a marked inhibition of DNA synthesis and growth [16–18]. This lasts for several days without signs of cellular recovery. Also, no evidence for recovery is found, when split doses of 8-MOP-plus-UVA are applied [17].

On the other hand, there is indication that in mammalian cells both types of photoreactions, namely monofunctional and bifunctional adducts, can be repaired [15,19–22]. In this report we have compared monofunctional and bifunctional photoreactions in affecting DNA synthesis, growth and recovery in skin fibroblasts.

Manuscript received December 26, 1979; accepted for publication April 23, 1980.

This work was supported by Stiftung Volkswagenwerk.

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Abbreviations:
EB: ethidium bromide
EBSS: Earle's balanced salt solution
^{3}H-TdR: methyl-^{3}H-thymidine
8 MOP: 8-methoxypsoralen

MATERIALS AND METHODS

1. *Preparation of Cultures*

Cell cultures of skin fibroblasts from young male albino guinea pigs (Pirbright) were prepared as previously described [16,17]. The growth medium was McCoy's 5A medium supplemented with 10% fetal calf serum, 100 IU/ml penicillin, 100 μg/ml streptomycin and 10 μg/ml amphotericin B. All materials were obtained from Gibco Bio-Cult. The growth medium was changed twice a week or as indicated. The cells were kept in the dark at 37°C in a humidified atmosphere (5% CO_2). Regular checks of the cultures for mycoplasm contamination were negative.

2. *Treatment of Cells with 8-MOP or Angelicin and UVA*

As previously described [16,17] 8-MOP (Basotherm, Biberach, W.-Germany) was dissolved in equal volumes of DMSO and diluted with growth medium to reach final 8-MOP concentrations of 10 to 0.01 μg/ml. The controls contained the solvent which in all samples did not exceed a final concentration of 0.1%. This was previously shown to be without effect upon the cells [16]. The cultures were incubated for 1 hr with 8-MOP or angelicin (kindly supplied by Prof. P. Chandra, Dept. of Biochemistry, University of Frankfurt, W.-Germany) and irradiated with window glass shielded Sylvania GTE black light lamps (PUVA 180, Waldmann, W.-Germany) which emitted a continous spectrum of UVA-light (320–400 nm). The fluence rate of the UVA-light was 4.44 mw/cm^2 considering the absorption rates of the growth medium and the cover of the culture plates. For narrow UV-light bands (395 nm, 365 nm) a Xenon 150 w high pressure lamp served as light source. The emitted light was focussed by 2 quartz lenses and passed a filter system (peak 395 nm (half width = 20 nm, Schott, Mainz, W.-Germany) or 365 nm (half width = 15 nm)). For reflection an UV-reflecting mirror (Spindler and Hoyer, Göttingen, W.-Germany) was used. Irradiation of the cultures with narrow band UV-light was carried out in the presence of 8-MOP dissolved in 0.9% NaCl. No UV-light absorption was detectable by spectrophotometry (Zeiss M4 Q III, W.-Germany). The fluence rates were 0.244 mw/cm^2 (λ = 395 nm) and 0.088 mw/cm^2 (λ = 365 nm). After irradiation the 8-MOP containing NaCl solution was removed, the cells were washed twice with Earle's balanced salt solution (EBSS, Gibco Bio-Cult) and fresh growth medium was added. All experiments were run at least 2 times and done in quadruplicates.

3. *Measurement of ^{3}H-TdR Incorporation and Determination of DNA Contents*

At different times after treatment 50 μl of methyl-^{3}H-thymidine (^{3}H-TdR) (1 mCi/ml, spec. act. 5 Ci/mmol, Amersham, dilution 1:25 corresponding to 2 μ Ci/ml) was added to each culture for 1 hr. After 2 washes with EBSS the fibroblasts were gently trypsinized (0.05% in EBSS) and harvested by means of a multiple sample harvester (Titertec, Flow Laboratories, Bonn, W.-Germany). The filter plates were dried for 30 min at 60°C and placed into 5 ml scintillation fluid [16]. The incorporated radioactivities were measured by means of a Packard Tricarb spectrometer and the standard deviations of quadruplicate cultures were calculated.

Quantitative determinations of the DNA content per cell after treatment with psoralen plus UVA were carried out using the method of Halprin et al [23]. In brief, the cells were incubated for 1 hr at 95°C in 0.5 N NaOH. 50 μl of the solution was added to 1.95 ml ethidium bromide (EB) solution (20 μg/ml in Tris-buffer (100 mM NaCl with 100 mM "Tris" HCl adjusted with 50% NaOH to pH = 7.4)). The fluorescence of the intercalation product of EB into DNA was measured at 620 nm (Zeiss M4 Q III spectrophotometer) after exciting the complex at 360 nm (Zeiss ZF M4). Separate experiments proved that the number of intercalated 8-MOP crosslinks into DNA did not affect the intensity of the emitted fluorescence light.

4. Autoradiography

Cells were cultured at low densities (2×10^4 cells/cm^2) on Thermanox plastic tissue culture plates (Lux Scientific Corp., California, U.S.A.). They were incubated with 8-MOP as described before and irradiated with UVA. At different times after irradiation the cells were incubated for 1 hr with 10 μ Ci/ml ^{3}H-TdR and fixed with methanol-glacial acetic acid (9:1).

After drying the culture plates were covered with Kodak AR 10 films and kept in the dark (+4°C) for 14 days. The films were developed using Kodak D 19 and the cells were stained with Giemsa. Microscopical evaluation of the specimes was carried out under oil immersion (20 microscopic fields per sample were counted).

5. Determination of Photoconjugated 8-MOP

Tritium labeled 8-MOP (kindly donated by Basotherm, Biberach, W.-Germany, spec. act. 2.09 Ci/mmol, New England Nuclear) was dissolved in DMSO after evaporation of the solvent (chloroform-ethanol 1:1). This was mixed with McCoy's 5A medium as described above. The final concentration of DMSO in the growth medium did not exceed 0.1%. Treatment of the cells was carried out as described and the cultures were harvested for determination of the cell number (Coulter Counter, Model Z.F. Coulter Electronics, England) and radioactivities at the indicated times (Fig 4). The data were calculated as cpm per cell. Control cultures were incubated with ^{3}H-8-MOP in the same manner and processed without irradiation. Medium changes were performed at daily intervals.

RESULTS

The incorporation rates of ^{3}H-TdR 24 hr after treatment are shown in Fig 1. As shown elsewhere [16] no effects on ^{3}H-TdR incorporation were seen in fibroblasts after treatment with UVA (1–3 J/cm^2) or 8-MOP (1 μg/ml) alone [16]. As can be seen from this figure the lowest concentration of 8-MOP affecting the uptake of the precursor under these conditions is close to 0.01 μg/ml and 1 J/cm^2. Linear inhibition rates are present when plotted semilogarithmically. For 8-MOP the dose-range extends over 3 orders of magnitude from the beginning of the inhibition until the complete block of ^{3}H-TdR incorporation. As shown before [16] the increase of UVA light from 1 to 3 J/cm^2 causes a similar inhibition with the 8-MOP concentration reduced by the factor 10.

At a given concentration angelicin caused inhibition rates which were much lower as compared to 8-MOP (Fig 1). Angelicin alone, up to 30 μg/ml, did not affect the incorporation rates. With 1 J/cm^2 at least a 30 times higher concentration of the sensitizer as compared to 8-MOP was needed to obtain the same inhibition rates. Similar rate differences were present when 3 J/cm^2 were applied.

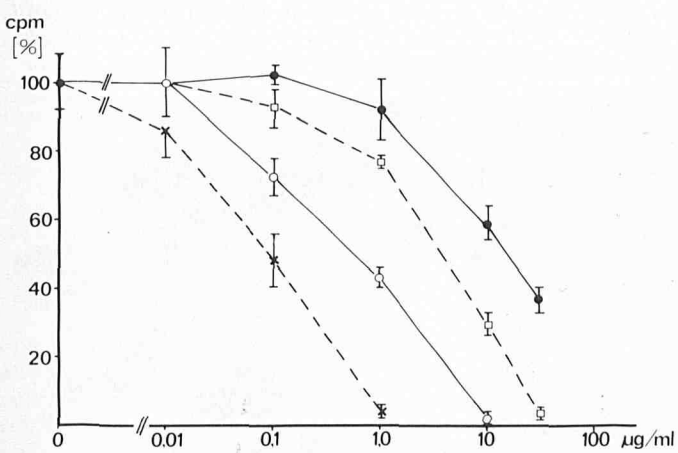

FIG 1. ^{3}H-TdR incorporation rates 24 hr after treatment with different concentrations of angelicin or 8-MOP and irradiation with UVA (angelicin: ●——● 1 J/cm^2, □——□ 3 J/cm^2, 8-MOP: ○——○ 1 J/cm^2, ×——× 3 J/cm^2). Absolute cpm value of the untreated cultures was 3021 ± 242. Data represent values (± SD) from 4 separate cultures.

The ability of the cell cultures to recover from various treatment modalities over the following days was examined in subsequent experiments (Fig 2,3). Recovery from the phototreatment with angelicin, a monofunctional photoreagent, is presented in Fig 2. As shown within 48 hr after treatment (10 μg angelicin/ml plus 1 J/cm^2) the incorporation of ^{3}H-TdR was at the level of the control cultures which were run in parallel for each value presented (Fig 2).

While cultures treated with 8-MOP-plus-395 nm show an increase of ^{3}H-TdR uptake on the following days (similar to angelicin-treated cultures (Fig 2)) cells photoinactivated by 8-MOP-plus-365 nm show a persistent inhibition of DNA synthesis. This was present for the time of observation (4 days). Similarly, when in the 8-MOP treated cultures the 395 nm irradiation (0.35 J/cm^2) was followed by 365 nm (0.35 J/cm^2) in the presence of 8-MOP the inhibition of ^{3}H-TdR uptake became persistent (Fig 3). Inhibition rates were nearly identical to those seen in cultures treated with 365 nm (0.7 J/cm^2) alone (Fig 3). In a further experiment, irradiation with 395 nm followed by a wash-out of 8-MOP and subsequent irradiation with 365 nm (0.35 J/cm^2) demonstrated persistent photoinactivation (Table I). Again ^{3}H-TdR incorporation rates decreased until day 2 and remained constant thereafter.

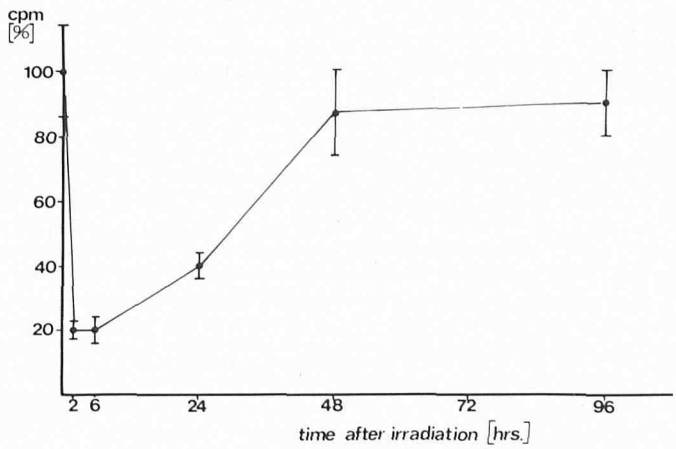

FIG 2. ^{3}H-TdR incorporation rates (± SD) of cultures at different times after treatment with 10 μg angelicin/ml plus 1 J/cm^2 (UVA). 48 hr after treatment the thymidine incorporation rates of irradiated cultures reached the 100% level of the control cultures.

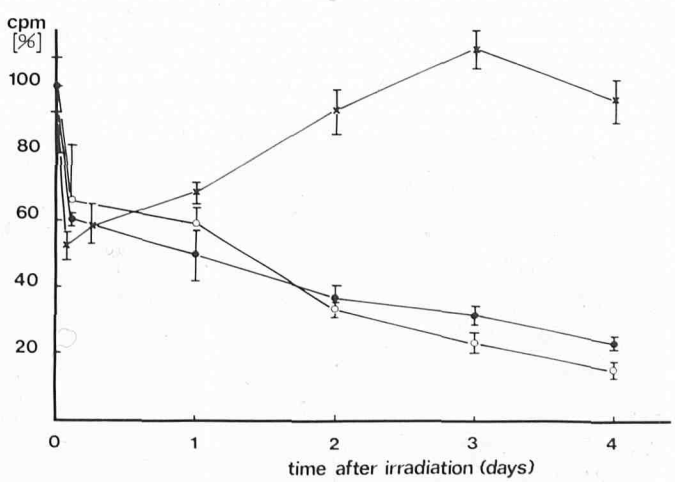

FIG 3. Inhibition of ^{3}H-TdR incorporation of cell cultures at different times after treatment with 1 μg 8-MOP/ml and irradiation (0.7 J/cm^2) at 395 nm (×——×) or 365 nm (○——○). Persistent inhibition of ^{3}H-TdR incorporation is present when the cultures were first irradiated at 395 nm (0.35 J/cm^2) followed by irradiation at 365 nm (0.35 J/cm^2) in the presence of 1 μg 8-MOP/ml (●——●).

The activities of incorporated nonremovable ^{3}H-8-MOP after application of UVA are shown in Fig 4. Using various treatment schedules the cell bound radioactivities decrease proportionate to the dosages of the drug and UV-light. One day after treatment the number of bound 8-MOP molecules per nucleus remains constant.

Microscopical examination of PUVA-treated cultures while in log phase of growth exhibited greatly enlarged cells and nuclei (Fig 5). The majority of these giant cells showed round or oval nuclei with a reticular chromatin pattern. In a number of cells up to 12 tightly packed and rounded nuclei were seen. Close to these giant cells normal appearing fibroblasts were present. Their number increased with time of culturing. Cell counts at various time intervals after PUVA treatment revealed dose-dependent rates of growth inhibition (Fig 6). While the control cultures as well as those treated with .01 μg 8-MOP per ml plus 2 J/cm^2 arrived at saturation density within 6 days, growth was significantly retarded when higher concentrations of 8-MOP were used (Fig 6).

In addition to this quantitative determination of the DNA content after 8-MOP-plus-UVA treatment in growing cultures demonstrated increasing values during the time after treatment (Table II) while treatment with UVA showed no effect on cellular DNA-content. On the average the relative DNA content per cell was more than doubled depending upon the treatment dose. During the second week after treatment the DNA content decreased. Examination of Giemsa stained cultures revealed an increasing number of normally appearing cells which grew up to saturation density (Fig 5).

While giant cells could readily be seen in low density cultures, they were absent in cultures treated at the point of saturation density. Indeed, Giemsa staining as well as quantitation of the DNA content per cell showed no deviations of DNA content from normal.

TABLE I. *^{3}H-TdR incorporation rates in cell cultures*

λ (nm)		Time after irradiation (days)				
	J/cm^2	0	1	2	3	4
(A) 365	0.35	100 ± 10.6	65.7 ± 5.9	48.1 ± 15.9		34.8 ± 1.7
(B) 395	0.35	100 ±10.3	53.4 ±6.4	83.2 ±4.2		89.2 ±7.7
(C) 395+ 365	0.35+ 0.35	100 ±8.8	71.7 ±1.6	61.5 ±9.2	65.5 ±3.5	67.1 ±11.7

Cell cultures were irradiated with 0.35 J/cm^2 at 365 nm (A) or 395 (B) in the presence of 1 μg 8-MOP/ml. In (C) irradiation with 395 nm (0.35 J/cm^2) was followed by 0.35 J/cm^2 at 365 nm 30 min after repeated washes.

Cultures photoinactivated at low density by 8-MOP-plus-UVA showed increasing labeling indices of the giant cells (diameter more than 3 times of normal cells). After 2 days the labeling indices decreased reflecting reduced DNA-synthesis of giant cells (Table III).

DISCUSSION

Photosensitizing furocoumarins (psoralens) are known to form covalent mono- or/and bifunctional adducts with DNA

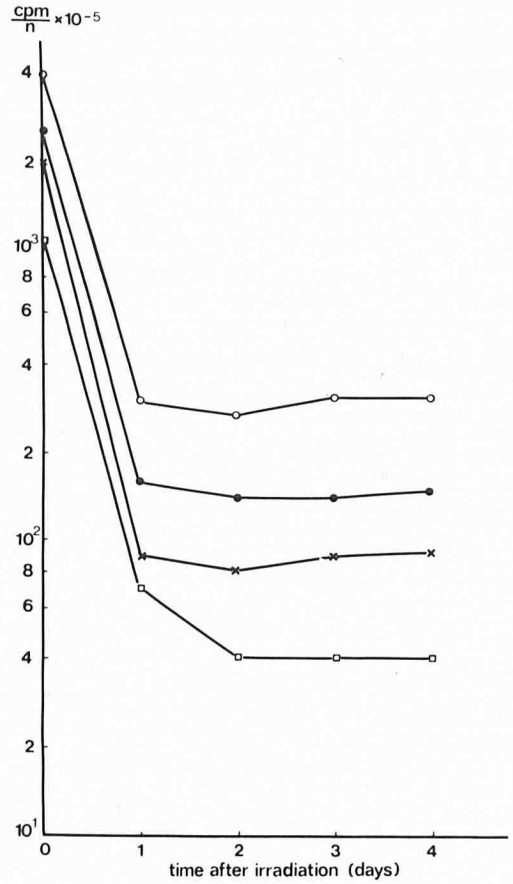

FIG 4. Incorporation of nonremovable ^{3}H-8-MOP in fibroblast cultures calculated as cpm per cell (○——○, 0.5 μg 8-MOP/ml plus 3 J/cm^2; ●——●, 1 μg 8-MOP/ml plus 1 J/cm^2; ×——×, 0.5 μg 8-MOP/ml plus 1 J/cm^2; □——□, 0.25 μg/ml plus 1 J/cm^2). The values (*ordinate*) at day 0 represent the number of all 8-MOP molecules per cell.

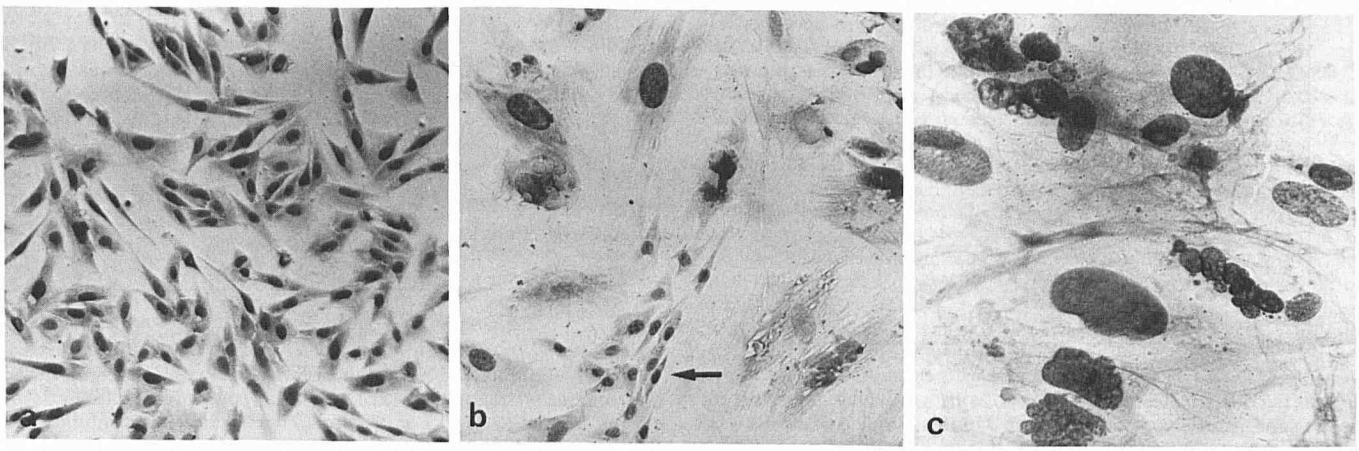

FIG 5. Morphology of 8-MOP-plus-UVA treated fibroblasts. (*a*) control cultures 2 days after seeding, reduced from ×125, (*b*), (*c*) fibroblasts 10 days after treatment with 1 μg 8-MOP/ml and 2 J/cm^2, reduced from ×125 (*b*), ×300 (*c*). Photograph (*b*) shows hyperchromatic, polynuclear cells as well as euchromatic not affected cells (*arrow*).

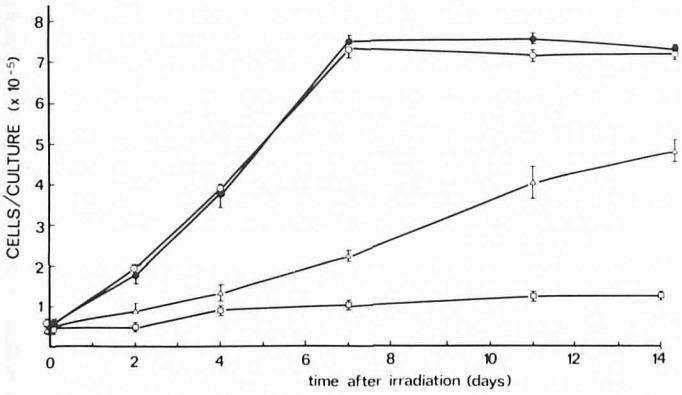

FIG 6. Number of cells per culture (± SD) after treatment with PUVA (UVA dose: 2 J/cm^2) at various concentrations of 8-MOP (○——○ control, ●——●, 0.01 μg/ml, △——△ 0.1 μg/ml, □——□ 1.0 μg/ml). Saturation density in untreated control cultures as well as in those treated with 0.01 μg/ml 8-MOP-plus-UVA is reached 7 days after treatment (age of culture at treatment: 2 days).

TABLE II. *Average DNA content (pg ± SD) of 8-MOP-plus-UVA treated cells at different times (days) after treatment*[a]

Time after irradiation	Concentration of 8-MOP (μg/ml)			
	0	0.01	0.1	1.0
0	6.20 ± 0.35	6.37 ± 0.71	5.96 ± 0.53	6.25 ± 0.06
2	6.20 ± 0.53	5.72 ± 0.30	7.32 ± 0.35	9.50 ± 1.36
4	5.55 ± 0.53	6.49 ± 0.83	9.50 ± 0.71	10.21 ± 0.94
7	5.90 ± 0.24	6.14 ± 0.53	11.62 ± 0.65	13.22 ± 0.12
11	6.08 ± 0.24	5.96 ± 0.01	8.56 ± 0.65	12.10 ± 0.06
14	5.96 ± 0.41	6.43 ± 0.18	7.67 ± 0.47	11.51 ± 1.71

[a] (UVA dosage: 2 J/cm^2). Initial cell number was 2.5×10^4/cm^2.

TABLE III. *Labeling indices of PUVA-treated fibroblasts while in log phase of growth (1 μg 8-MOP/ml plus 2 J/cm^2 UVA)*[a]

Time after treatment (days)	0	2	4	10
No. of cells per HPF[b]	10.7 ± 2.5	12.0 ± 3.0	13.7 ± 3.1	18.6 ± 2.6
% hyperchromatic[c]	0	15.0 ± 8.3	29.9 ± 9.5	36.0 ± 11.8
Labeling index	31.8 ± 10.3	13.1 ± 7.9	21.2 ± 7.8	29.2 ± 9.9
% labeled hyperchromatic cells	0	5.8 ± 2.5	5.1 ± 3.0	2.9 ± 1.6
Labeling index of UVA-treated cells	32.2 ± 8.1	24.5 ± 11.0	35.1 ± 9.3	32.6 ± 12.3

[a] Cells were pulse labeled (1 hr, 10 μCi ^{3}H-TdR/ml) at the given times after treatment.

[b] HPF means high power field.

[c] Hyperchromatic nuclei are called those nuclei in which the nuclear diameter is 3 times normal or more. Cells with more than one nucleus are also included.

20 eye-pieces (oil immersion) were counted per indicated data (average value ± SD).

under the influence of long-wave ultraviolet light [8]. In the absence of a psoralen UVA showed no effect on ^{3}H-TdR incorporation at doses ranging from 1 to 5 J/cm^2 [16]. Also, 8-MOP could readily be eluted when the cells were left in the dark [17] indicating high dissociability of psoralen-DNA complexes [25].

Photoinactivation in various cell systems including yeasts, phages, bacteria and mammalian cells is thought to be primarily based upon the presence of cross-links within doublestranded DNA. Also, skin sensitisation by some psoralens including 8-MOP in combination with UVA supposedly is due to cross-linking properties [7–9].

Previously it has been shown that sublethal photoinactivation of skin fibroblasts by 8-MOP-plus-UVA causes a lasting inhibition of DNA synthesis and growth. Also, split dose treatment of the cells performed at various time intervals revealed cumulative photoinactivation rates [17]. In these experiments indication for the repair of photoadducts was lacking. Similarly, Coppey, Averbeck, and Moreno [22] found no evidence for the repair of bifunctional adducts in African green monkey kidney cells using 8-MOP-plus-UVA. However, monofunctional adducts caused by angelicin-plus-UVA were repaired quite efficiently.

On the other hand, bifunctional adducts have been found to be repaired in bacteria [21] and to a limited degree in mammalian cells [11,13,19].

In the present study angelicin, a monofunctional photoreagent [26] was compared to 8-MOP, which forms mono- as well as bifunctional adducts depending upon the wavelength of the applied UV-light [27]. Treatment of fibroblasts with angelicin followed by broad band UVA (320–400 nm) reveals that compared to 8-MOP angelicin is approximately 30 times less efficient in inhibiting DNA synthesis. This corresponds to photosensitization rates observed in monkey kidney cells [22] and may be related to the inability of angelicin to form cross links.

Our results show that 8-MOP-treated cells irradiated at 395 nm fully resumed DNA synthesis within 48 to 72 hr. This is similar to angelicin-plus-UVA-treated cultures, which recovered from the initial inhibition within 2 days. As angelicin as well as 8-MOP (plus 395 nm light) are forming monofunctional adducts [26,27] our data indicate a significant repair of monofunctional photodamage.

There is a surprisingly long recovery period in the angelicin as well as in the 8-MOP-plus-395 nm treated cultures, lasting approximately 48 hr. This is in contrast to the repair of UVB-induced pyrimidine dimers which are rather quickly repaired by removal and subsequent "patch-ups" [15,28]. As shown in bacteria [21] the excision repair of monofunctional adducts causes DNA strand gaps which may persist for a rather long time period [22]. Therefore the slow recovery observed in our cells after monofunctional damage could be due to the persistence of open gaps after excision. Also repair processes like postreplication repair need consideration.

In accordance with previous observations [17] photoinactivation produced by 8-MOP-plus-365 nm UV light remained persistent. Also when irradiation at 395 nm was succeeded by 365 nm UV light a persistent inhibition was present. Assuming that cross-linking of psoralen molecules between DNA double strands is causing the inhibition of DNA synthesis, the 395-plus-365 nm split dose treatment indicates conversion of monofunctional adducts into cross-links. On the basis of our results their number is close to the number of cross-links produced by 8-MOP-plus-365 nm. In agreement with this is the experiment in which nonbound psoralen is washed out between the 2 applications of light (Table I). Still a persistent inhibition of DNA synthesis is present in these cultures. This is in agreement with previous observations on the strictly cumulative effects of fractionated application of 8-MOP-plus-UVA in skin fibroblasts [17].

In addition to these quantitative data morphologic examination of cells sublethally photoinactivated demonstrated a subpopulation of giant cells surrounded by normally appearing fibroblasts. These cells may resemble "phototoxic" cells occasionally seen in PUVA-treated epidermis [29] or in culture [30]. As confirmed by autoradiography the giant cells continue to synthesize DNA for several days in the absence of mitosis. While they are found in growing cultures only, being absent in density inhibited cultures, their formation appears to be related to the proliferative activity. As shown in Table III the number of these hyperchromatic cells increases up to 36% with time after treatment and also, they retain the capacity to synthesize DNA. As a result of this the average DNA content of the PUVA treated cells is more than doubled after treatment with the

higher 8-MOP dosage (Table II). It may be concluded that euchromatic cells give rise to hyperchromatic nuclei which continue to synthesize DNA. It may be suggested that these hyperchromatic cells are unable to devide because of cross-links. In this case they could serve as a parameter for the presence of cross-linked nuclear DNA.

The skillful assistance of Frl. Anne Schröder is gratefully acknowledged.

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