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In vitro and *In vivo* Effects of Photodynamic Therapy in Cutaneous T Cell Lymphoma

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Photodynamic therapy consists of the combination of photosensitizers absorbing light in the visible spectral region and irradiation with light of corresponding wavelengths. We analysed its effects in comparison to PUVA treatment on cell lines MyLA and HuT78, established from patients with cutaneous T cell lymphomas. Proliferation was reduced to 50% by exposure to 7.5 J/cm² UV-A (= ED_{50}). This effect was increased more than 10-fold in the presence of 10 μg/ml 8-methoxypsoralen. The ED_{50} for photodynamic therapy using 630 nm light emitted by a dye laser and 10 μg/ml Photosan-3 was found to be about 1 J/cm². In vivo fluorescence recordings during topical photodynamic therapy of mycosis fungoides lesions showed photobleaching and thus documented the triggering of photochemical reactions. Our observations document the capability of photodynamic therapy to inhibit proliferation of transformed T cells similar to PUVA, pointing out its potential in the treatment of cutaneous T cell lymphomas.

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PUVA, combining the administration of psoralens as photosensitizers and irradiation with UV-A light (1), is a well established treatment for a variety of immunological diseases affecting the skin (2). In contrast, photodynamic therapy (PDT) consists of systemic intravenous application of photosensitizers absorbing light of the visible spectral region, e.g. hematoporphyrin derivates, and subsequent irradiation with light of the corresponding wavelength around 630 nm (3). The photosensitizing porphyrin components accumulate preferentially in tumours rather than in normal tissues (4–6). Irradiation results in the formation of singlet oxygen, which in turn causes tumour regression due to necrosis (7, 8). This regimen has been successfully applied in the treatment of a variety of solid tumours including skin cancer (9–11).

Cutaneous T cell lymphomas are lymphoproliferative malignancies mainly of CD4+ T cells (12). PUVA is one of several well established and evaluated therapeutic approaches (13). However, its mechanism of action in this disease is still not fully understood, since responses were reported also in lesions too thick to be completely penetrated by UV-A light. Hönigsmann et al. (14) speculate that cells from deeper layers move upward once superficial tumour cells are destroyed, reaching a level where UV-A light can effectively penetrate.

Besides PUVA, PDT has also been reported to be effective in the treatment of mycosis fungoides (15, 16). We therefore investigated the effects of PDT on proliferation, morphology, and distribution of photosensitizers on a T cell line established from a patient with mycosis fungoides. These observations were compared to results obtained with PUVA therapy.

MATERIAL AND METHODS

Cell lines

T cell line MyLa was established from a patient with mycosis fungoides as described elsewhere (17, 18). Cell line HuT78 (19), derived from the peripheral blood of a patient with Sézary's syndrome, was purchased from the American Type Culture Collection.

Photosensitizers

8-methoxypsoralen (8-MOP) and hematoporphyrin derivate Photosan-3, comparable to Photofrin II, were purchased from Sigma (Germany) and Seelab (Germany), respectively. Photosan-3 is a mixture of different porphyrins like hematoporphyrin IX, protoporphyrin IX, deuteroporphyrin IX. In aqueous solution, these porphyrins are present as monomers, dimers, or higher aggregates. Eight-MOP was dissolved in 96% ethanol. Photosan-3 and dissolved 8-MOP were diluted in medium prior to adding to cell cultures.

Light sources

630 nm red light was emitted by an argon-ion laser pumped dye laser (100 mW/cm²). The UV-A sources were 14 F15T8 Sylvania lamps (Waldmann, Germany) emitting light of 315–400 nm with a peak at 365 nm (7 mW/cm²). UV-A doses were controlled with a UV meter (Waldmann, Germany). The 407 nm line of a krypton-ion laser served as excitation source for fluorescence measurements.

Cell proliferation

To determine the effects of PUVA therapy and PDT on proliferation, 2×10^5 cells per well were placed in U-bottom microtitre plates. Eight-MOP and Photosan-3 were added to a final concentration of 10 μg/ml, and the plates were then wrapped in aluminum foil and kept in the dark to prevent uncontrolled light exposure. After 6 h of incubation, cells were washed 3 times prior to and after a single exposure to either UV-A light or 630 nm light. In parallel, cell cultures not incubated with any photosensitizer were similarly treated. During the final 6 h of a 24-h culture period, ^{3}H-thymidine was added and the uptake was determined. Experiments were performed in triplicates; results are shown in percent proliferation with spontaneous proliferation set to be 100%.

Fluorescence pattern and morphological changes

To visualize the distribution of the photosensitizers within the cells we used video-intensified fluorescence microscopy with a highly sensitive silicon intensified target camera SIT (Hamamatsu, Japan) as described elsewhere (20). The same camera was used to determine morphological changes of the cells detected by phase contrast microscopy. In the case of Photosan-3 the fluorescence intensity in the spectral range 590–800 nm was detected after excitation with the 405/436 nm bands of a 50 W mercury high pressure lamp. The fluorescence intensity of 8-MOP was detected in the spectral range 520–560 nm after excitation with the UV band (356 nm). Power densities of less than 100 mW/cm² were applied to limit photochemical modifications during the detection time.

In vivo fluorescence recording

Plaque lesions in two patients with mycosis fungoides were selected for in vivo fluorescence recording. An ointment containing 3.3 mg/ml

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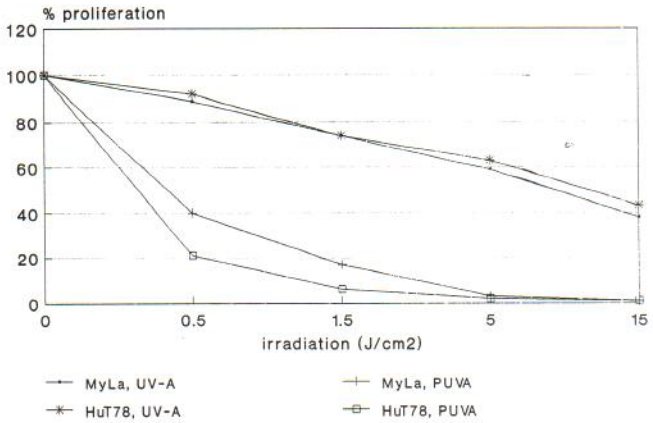

Fig. 1. Proliferation inhibition by UV-A and PUVA. The effects of UV-A light on the proliferation of T cell lines MyLa and HuT78 are indicated in the absence or presence of 10 μg/ml 8-MOP as photosensitizer.

Photosan-3 was applied topically to the selected lesion 18 and 6 h prior to irradiation. The area to be treated was then covered by a bandage until irradiation. Fluorescence spectra were recorded at the pre-treated site as well as 1 cm aside. Fluorescence was detected using a sensor for in vivo measurements and a polychromator combined with an optical multichannel analyzer as previously described (21). These recordings were repeated every 10 s for 40 s of irradiation (= 0.11, 0.22, 0.33 and 0.44 J/cm^2) of 630 nm light. For control purposes, fluorescence recordings were performed in vitro using the ointment containing Photosan-3 alone.

RESULTS

UV-A and PUVA treatment cause proliferation inhibition of malignant transformed T cell lines

UV-A irradiation and PUVA treatment resulted in similar changes in proliferation of cell lines MyLa and HuT78. Irradiation with UV-A light was found to inhibit ^{3}H-thymidine uptake in a dose-dependent way. Proliferation was decreased to 50% at a dose of 7.5 J/cm^2 (Fig. 1). Eight-MOP did not significantly alter the proliferation of the cell lines used in the applied concentration (data not shown). Pre-incubation with 10 μg/ml 8-MOP over 6 h in the dark resulted in a profound increase of the inhibitory effect of UV-A irradiation ($ED_{50} \approx 0.3$ J/cm^2).

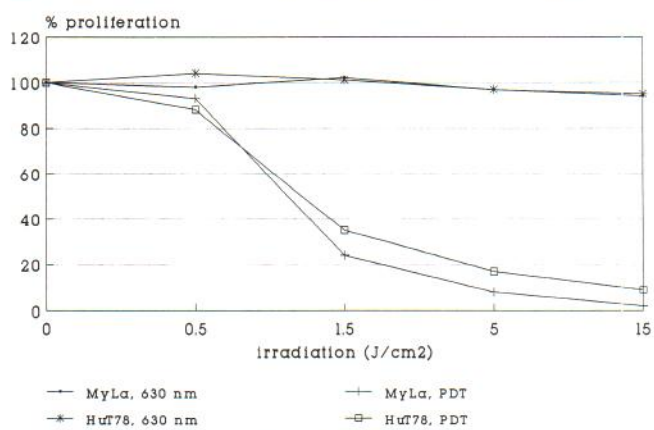

Fig. 2. Proliferation inhibition by red light and photodynamic therapy. The effects of light of 630 nm wavelength emitted by an argon-ion laser pumped dye laser are indicated in the presence or absence of 10 μg/ml Photosan-3 as photosensitizer.

PDT exhibits an inhibitory effect comparable to PUVA

Neither light of 630 nm wavelength (Fig. 2) nor incubation with 10 μg/ml Photosan-3 (data not shown) affected proliferation of MyLa or HuT78. However, irradiation of cells pre-incubated with Photosan-3 over 6 h in the dark resulted in proliferation inhibition. At about 1 J/cm^2 inhibition was half maximal (Fig. 2). Again, data obtained from cell line MyLa were similar to the results in the case of HuT78.

Eight-MOP shows increasing fluorescence intensity under irradiation

To analyse the cellular distribution pattern prior to and after irradiation, video-intensified fluorescence microscopy was performed on cell line MyLa. By means of this technique, a diffuse distribution of 8-MOP was detected within the cytoplasm before irradiation (Fig. 3a). Exposure to UV-A light resulted in a profound increase of fluorescence throughout the cell, no longer sparing the nucleus (Fig. 3b). This effect was paralleled by morphological changes: a marked swelling of the cell occurred, the regular shape was lost, and vacuoles appeared within the cytoplasm (Fig. 3c, 3d).

Irradiation of cells incubated with Photosan-3 results in photobleaching

MyLa cells incubated with Photosan-3 exhibited a fluorescence pattern similar to 8-MOP before irradiation (Fig. 4a). Here, light exposure caused a gradual decrease of fluorescence throughout the cytoplasm (data not shown). This phenomenon is known as "photobleaching". The morphological changes observed were similar to those described for 8-MOP (Fig. 4b, 4c).

Photobleaching during PDT of mycosis fungoides lesions in vivo

To determine whether photoreactions occur at all in cutaneous T cell lymphoma lesions treated topically with Photosan-3, we performed on-line fluorescence recordings during PDT of plaque lesions in two patients with mycosis fungoides. Fluorescence as a means to determine the accumulation of the photosensitizing agent was found to be strictly restricted to the area to which this agent was applied; no fluorescence was detectable 1 cm aside of its application (data not shown). Subsequently, light of 630 nm emitted by a dye laser was administered and fluorescence was determined every 10 s for 40 s of exposure (= 0.11, 0.22, 0.33 and 0.44 J/cm^2). These recordings showed a decrease in fluorescence intensity with increasing dosage (Fig. 5). This process of "photobleaching" documents the photo- and oxygen-induced reaction of the porphyrin photosensitizer (21).

Photochemical reactions take place in the tissue rather than at the skin surface

To exclude the possibility that the photochemical reactions observed occurred only at the surface of the skin but not in the lesional tissue, fluorescence was also determined for Photosan-3 in vitro. The fluorescence detected was markedly lower compared to the in vivo values. More importantly, the fluorescence spectrum also showed qualitative differences (Fig. 5): two

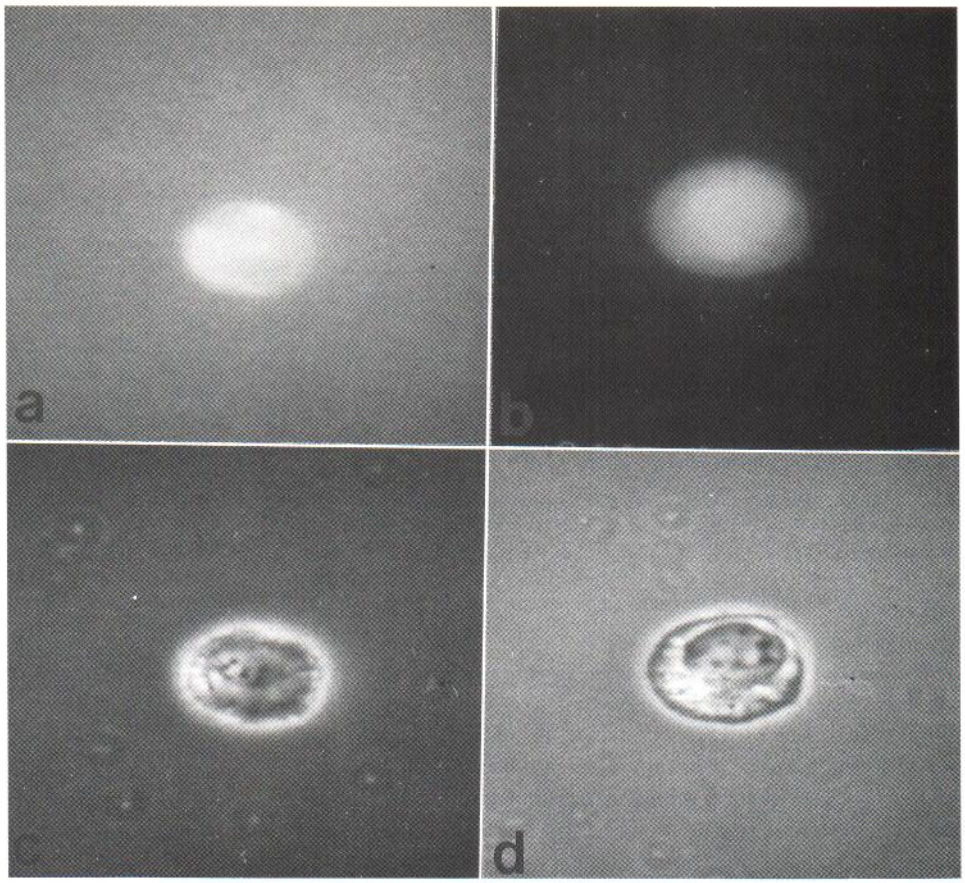

Fig. 3. Fluorescence increase of 8-MOP. Distribution of 8-MOP in MyLa before (*a*) and after (*b*) excitation with light of 356 nm and morphological changes (*c* and *d*) are documented.

maxima were observed at 613 nm and 675 nm, respectively. In contrast, the maxima in vivo were at 630 nm and 691 nm. Thus, the photoreactions documented do take place within the lesions.

DISCUSSION

The mechanisms of action are not fully understood in either PUVA or PDT. When psoralens are exposed to UV-A light two independent photoreactions take place, resulting in covalent binding to one or both strands of DNA (22). This interaction, together with the use of UV-A light for excitation, keeps the discussion about the potential risk to induce cancer by PUVA therapy alive. In contrast, the site of damage caused by PDT with Photosan-3 is not the nucleus. Damage occurs at membranes in case of short exposure, and the mitochondria are altered in case of long treatment (23). Thus, the use of PDT seems to be favourable from a theoretical point of view, since the risk of inducing malignancies can be avoided.

Our observations document the ability of PDT to inhibit proliferation of malignant transformed T cells, pointing out the potential of this regimen in the treatment of cutaneous T cell lymphomas. It needs to be stressed that at similar concentrations of the photosensitizers higher doses of irradiation are needed in the case of PDT to obtain comparable inhibition of proliferation.

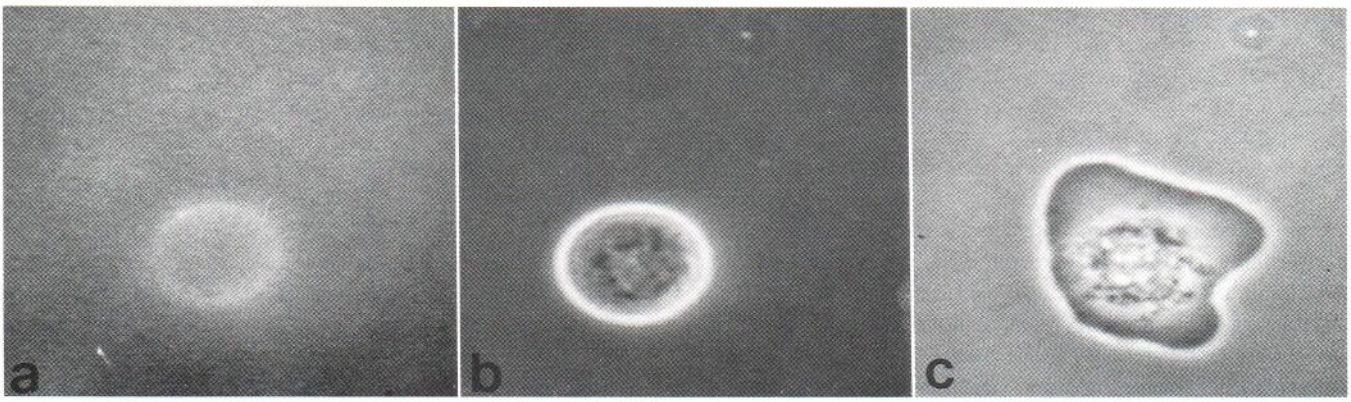

Fig. 4. "Photobleaching" of Photosan-3. Distribution of Photosan-3 in MyLa prior to irradiation (*a*) and morphological changes caused by excitation with light of 405/436 nm (*b* and *c*) are recorded.

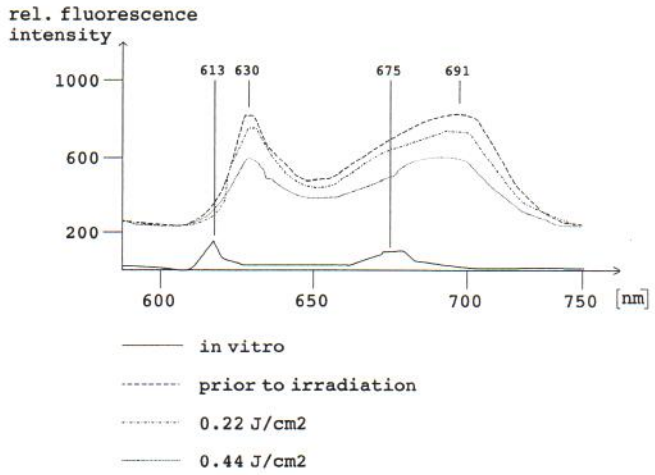

Fig. 5. On-line fluorescence recordings. Fluorescence recording in vitro as well as in a plaque lesion of a patient with mycosis fungoides prior to and under irradiation with light of 630 nm wavelength. Note the decreasing fluorescence intensity under irradiation ("photobleaching") and the qualitative differences between the in vitro and in vivo spectra.

This disadvantage in vitro, however, could be compensated by two effects in vivo: hematoporphyrin derivatives accumulate in target tissues. Kostron et al. (4) showed that direct injection into subcutaneous tumours resulted in tumour:adjacent skin ratios as high as 44:1. Moreover, light at 630 nm emitted by argon pumped dye lasers, usually used as a source for PDT, penetrates into tissues much deeper compared to UV-A light. Thus, in vivo application of PDT might even prove to be superior to PUVA therapy, since higher concentrations of photosensitizers might be reached in the target tissues and photoreactions might occur much deeper within the target lesions. Indeed, PDT has already occasionally been used in single cases of mycosis fungoides (15, 16).

Two common side effects of PDT are cutaneous photosensitizing and systemic immunosuppression (24). These severe threats can be avoided if the photosensitizer is applied topically, which is possible in the case of cutaneous lesions. Our in vivo recordings document that the accumulation of the photosensitizing agent is indeed strictly limited to the target. Thus, local PDT might be a very safe treatment regimen not carrying the burden of the severe side-effects observed in the case of systemic PDT.

The photobleaching observed, i.e. the gradual decrease of fluorescence caused by irradiation, in cells incubated with Photosan-3 represents the most common behaviour of photosensitizers (25). A fluorescence increase during irradiation, as detected with 8-MOP, was first observed with hydrophilic mesotetraphenylporphyrins (26) in addition to a fluorescence relocalization into the nucleus and nucleoli of cells. Interestingly, with 8-MOP we made the same observation, i.e. fluorescence relocalization into the nucleus and intensity increase during irradiation.

To summarize, we have shown the potency of PDT to inhibit proliferation of malignant transformed T cells in vitro similar to PUVA. Photosensitizer fluorescence measured in vivo and online during treatment in plaque lesions of patients with mycosis fungoides documents both the occurrence of photochemical reactions under irradiation in vivo as well as a strict limitation of these reactions to the target area. The lower effectiveness of PDT in comparison to PUVA in vitro might be compensated by the accumulation of hematoporphyrin derivatives in lesions to be treated and by the better tissue penetration of light with longer wavelengths.

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