# Systemic Photodynamic Therapy with Aminolevulinic Acid Induces Apoptosis in Lesional T Lymphocytes of Psoriatic Plaques

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**Photodynamic therapy (PDT) is a recently approved treatment modality that involves the sequential administration of a photosensitizer or its precursor and light to generate singlet oxygen for treating diseased tissue. The use of topical aminolevulinic acid (ALA) and blue light for nonhypertrophic actinic keratoses currently represents the only approved dermatologic application for PDT in the U.S.A. ALA is a photosensitizer precursor that is metabolized by cells into protoporphyrin IX (PpIX), which can be subsequently activated by visible light. PDT with topical ALA has been shown to improve psoriasis, but post-treatment hyperpigmentation as well as inconsistent clinical responses despite repeated PDT sessions have limited the development of this treatment approach for psoriasis. Furthermore the use of topical PDT photosensitizers becomes somewhat impractical for treating larger body surface areas in patients with extensive psoriasis. We have recently shown that oral administration of ALA induces preferential accumulation of PpIX in psoriatic plaques. The objectives of this study were to evaluate the effects of PDT with blue light on psoriatic plaques after systemic ALA administration as well as to determine whether systemic ALA–PDT induces apoptosis in lesional T lymphocytes. It has been suggested that induction of apoptosis in lesional T lymphocytes may be indicative of longer remission time following treatment of psoriasis. *J Invest Dermatol 119:77–83, 2002***

## MATERIALS AND METHODS

**Patients** This protocol involving human subjects was approved by the University of Montreal Hospital Center Ethics Committee, and informed consent was obtained from each patient before any procedures were performed. Three groups of four patients received a single oral ALA dose of either 5, 10, or 15 mg per kg. ALA in powder form was provided by DUSA Pharmaceuticals (Valhalla, NY) and mixed with orange juice immediately before administration. Patients were all asked to eat breakfast before coming to the clinic on the day of ALA administration. Baseline laboratory evaluation included complete blood count, aspartate amino transaminase (AST), alanine amino transaminase (ALT), alkaline phosphatase (AP), lactate dehydrogenase (LDH), γ-glutamyl transferase (GGT), bilirubin, creatinine, urea, and urinalysis; AST, ALT, AP, GGT, LDH, and bilirubin were repeated 24 h and 72 h after ALA administration. On the day of ALA administration patients were kept under subdued lighting at the dermatology clinic for the entire day.

**PDT session and psoriasis evaluation** For each patient a total of 15 1.5 × 1.5 cm square areas of psoriatic skin within three different plaques on the limbs were selected for PDT and exposed to blue light at 1, 3, or 6 h after ALA administration. A blue fluorescent lamp (Blue-U, DUSA Pharmaceuticals) at irradiances of 9–11 mW per $cm^2$ was used for light exposure. The maximal output of this source is at 417 nm with a full-width half-maximum of 30 nm. The irradiance was measured each morning with an International Light IL 1700 radiometer (Newburyport, MA) equipped with a SED 240 detector, a UVB-1 filter, and a "Wide Eye" Diffuser (W). The variation in irradiance according to differences in the size of limbs from patient to patient was less than 10%. The measured irradiance was multiplied by a factor of 1.65 following comparison of the International Light radiometer against a UDT S370 radiometer (Graseby Optronics, Orlando, FL). Five squares within each plaque were exposed to the following light fluences: 1, 3, 6, 12, or 20 J per $cm^2$ for patients who received 5 or 10 mg per kg and 1, 2, 4, 8, or 10 J per $cm^2$ for patients who received 15 mg per kg. Psoriasis severity was assessed separately at each treatment site at baseline and at days 1, 3, 7, and 28 following PDT according to the visible degree of erythema, scaling, and thickness. Each of these features was evaluated on a scale of 0 (absence) to 4 (severe) and then added together to give the overall psoriasis severity score, which had a maximum possible score of 12.

***In vivo* fluorescence spectroscopy** PpIX fluorescence in psoriatic and normal skin was monitored *in vivo* with a spectrofluorometer equipped with a xenon lamp and a double monochromator (Skin Skan, SPEX Instruments, Edison, NJ). Fluorescence excitation was set at 407 nm and emission intensity at 635 nm was serially measured at baseline and after ALA administration for up to 24 h. These parameters were kept constant for every patient. In addition emission spectra were also specifically recorded before ALA administration as well as at 1, 3, and 6 h before and immediately after light exposure on the squares that were exposed to the lowest and highest fluence. For all emission spectra, PpIX fluorescence before and after light exposure was evaluated by measuring the area under the emission spectra between 620 and 650 nm after subtraction of the autofluorescence background.

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Abbreviations: ALA, aminolevulinic acid; ALT, alanine amino transaminase; AP, alkaline phosphatase; AST, amino transaminase; .GGT, γ-glutamyl transferase; LDH, lactate dehydrogenase; PDT, photodynamic therapy; PpIX, protoporphyrin IX.

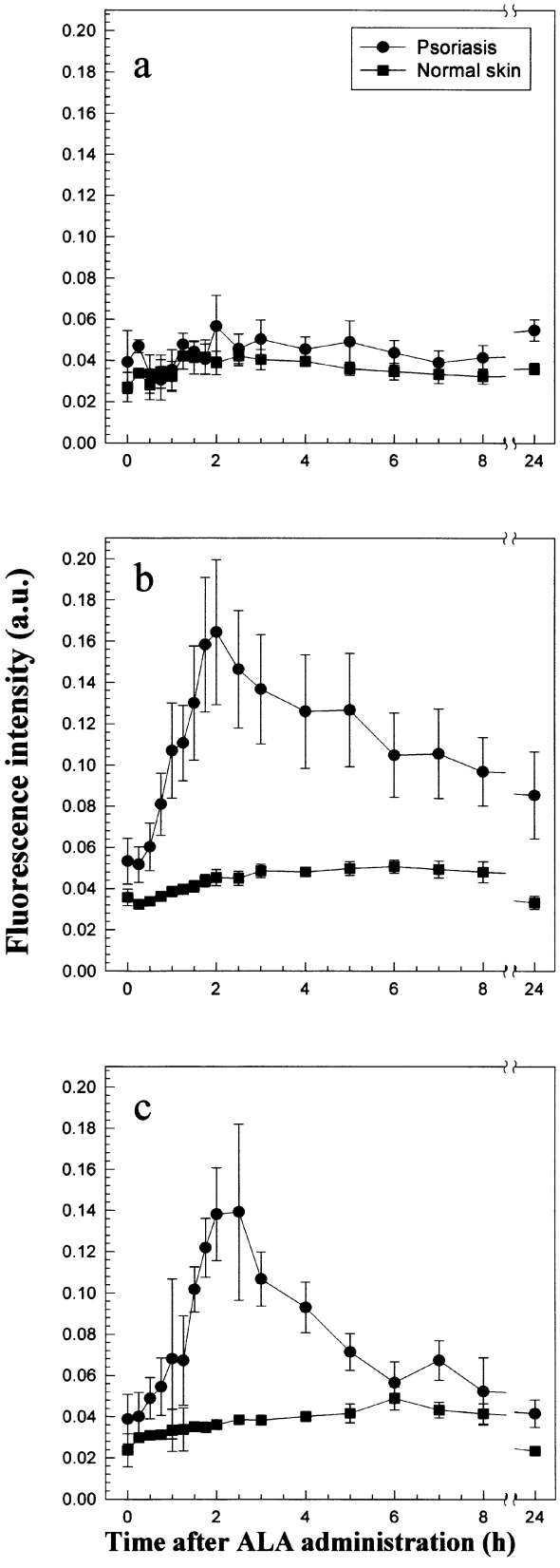


**Figure 1. Oral administration of ALA at 10 and 15 mg per kg induces preferential accumulation of PpIX in psoriatic plaques compared to normal skin.** PpIX levels were measured in normal and psoriatic skin with *in vivo* fluorescence spectroscopy at different times after administration of 5 mg per kg (*a*), 10 mg per kg (*b*), or 15 mg per kg (*c*) of ALA. *Bars*: SD; $n = 4$.

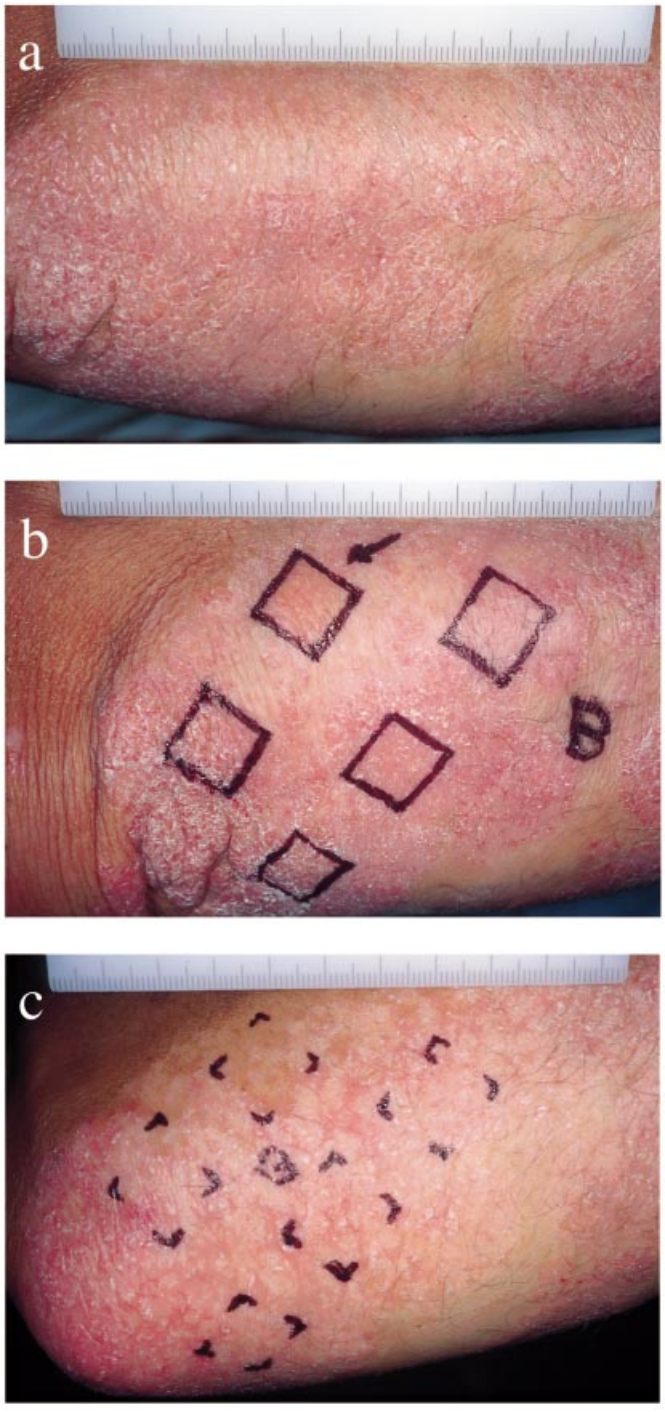


**Figure 2. PDT with 20 J per cm$^2$ 3 h after administration of 10 mg per kg of ALA induces erythema on exposed psoriatic skin.** (*a*) Before light exposure. (*b*) 24 h after light exposure erythema (*arrow*) is present on the area exposed to 20 J per cm$^2$. (*c*) 4 wk after PDT improvement is present on the area exposed to 20 J per cm$^2$ (*arrow*).

**Quantitative fluorescence microscopy** Skin biopsies of psoriatic skin unexposed to blue light were taken before and at 1, 3, and 6 h after ALA administration. These biopsies were embedded in Frozen Section Medium (Stephens Scientific, Riverdale, NJ) and snap frozen in liquid nitrogen. Sections of 10 μm thickness from each frozen sample were cut with a cryostat, taking great care to minimize exposure of the sections to light in order to minimize PpIX photobleaching. Fluorescence images were generated with a Nikon Optiphot-2 fluorescence microscope equipped with a thermoelectrically cooled CCD camera (Model

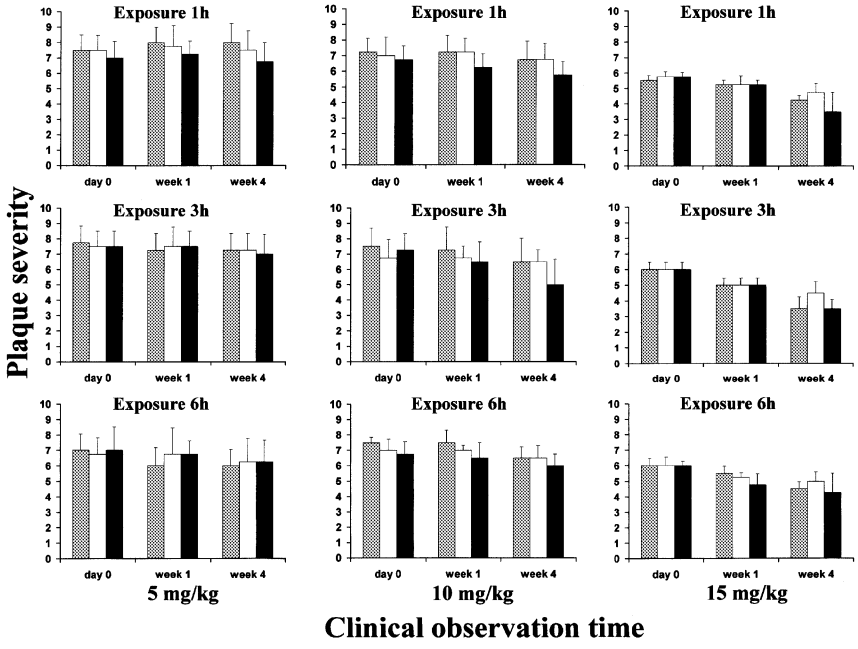


**Figure 3. PDT after oral administration of ALA decreases psoriatic plaque severity.** Plaque severity decreased following PDT on 1.5 × 1.5 cm areas of psoriatic skin after administration of 15 mg per kg of ALA. Improvement was not significant for patients who received 5 mg per kg or 10 mg per kg. Plaque severity scores are given for 1 (*gray*), 6 (*white*), and 20 J per $cm^2$ (*black*) for patients who received 5 mg and 10 mg per kg and 1 (*gray*), 4 (*white*), and 10 J per $cm^2$ (*black*) for patients who received 15 mg per kg. Plaques were exposed to blue light at either 1 h, 3 h, or 6 h after ALA administration. *Bars*: SD; $n = 4$.

DC330E, Dage-MTI, Michigan City, IN) connected to a PC computer. The sensitivity settings on the camera were identical for each slide. The integration time was 1 s. For fluorescence recording, a 480 nm short pass (480DF60) excitation filter and a 635 ± 10 nm bandpass (635DF55) emission filter were used (Harvard Apparatus, Montreal, Canada). After fluorescence imaging, the slides were stained with toluidine blue and a standard light microscope image of the same area was captured without moving the microscope stage. All images were analyzed with Clemex vision software (Version 3.0.023, Longueuil, Quebec, Canada). Using this software, the stratum corneum and the rest of the epidermis were delineated on the toluidine blue images, which were then superimposed onto the fluorescence images for quantifying fluorescence intensity. The average fluorescence intensity per pixel was calculated for the stratum corneum and the rest of the epidermis.

**Detection of apoptosis in lesional lymphocytes by double sequential staining** Twenty-four hours after ALA administration 4 mm skin biopsies were taken from one psoriatic square of each plaque exposed to light as well as from an unexposed plaque. For each PDT-treated plaque the biopsy was performed on the psoriatic square exposed to the highest fluence that did not exhibit erythema. For patients treated with 5 mg per kg all biopsies were from the squares exposed to 20 J per $cm^2$. For patients treated with 10 mg per kg all biopsies were performed on squares exposed to 20 J per $cm^2$ except three biopsies performed on plaques exposed at 3 h after ALA administration that were from squares exposed to 12 J per $cm^2$. For patients treated with 15 mg per kg all biopsies were from squares exposed to 10 J per $cm^2$ except two from the same patient that were performed on squares exposed to 8 J per $cm^2$ for plaques exposed at 3 and 6 h after ALA administration. After formalin fixation and paraffin embedding, sections were cut from each specimen. Tissue sections where deparaffinized, rehydrated in phosphate-buffered saline (PBS), and then processed to detect apoptotic cells with the TUNEL technique using the ApopTag Fluorescein *in situ* Apoptosis Detection Kit (Intergen Compagny, Purchase, NY). Fluorescence images demonstrating apoptotic cells were acquired with the Nikon Optiphot-2 fluorescence microscope and recorded with the Clemex Vision Software as previously described. The same tissue sections were then left overnight at 4°C in their mounting medium, following which they were washed in PBS and processed for anti-CD3 immunohistochemistry the next day. Briefly, after blocking with the avidin/biotin blocking kit (Vector Laboratories, Burlingame, CA) the sections where incubated for 1 h at room temperature with a polyclonal rabbit antihuman CD3 antibody at a dilution of 1:150 (Dako Diagnostic Canada, Mississauga, Ontario, Canada). After three washes in PBS, tissue sections were incubated for 30 min at room temperature with a biotin-conjugated goat antirabbit antibody diluted at 1:200 in 5% normal goat serum in PBS. Sections were washed in PBS and signal amplification and visualization were carried out using an avidin/biotin/peroxidase complex and diaminobenzidine (Vector Laboratories). Slides were dehydrated in ethanol and xylene and mounted in VectaMount. Using the recorded image of the TUNEL technique a corresponding image of the same area was recorded after anti-CD3 immunohistochemistry. The number of TUNEL + positive CD3 lymphocytes per field was counted.

**Statistical analysis** A one-way ANOVA for repeated measures was used for statistical analysis. A Bonferroni correction was applied when multiple comparisons were performed.

## RESULTS

**Oral ALA induces preferential accumulation of PpIX in psoriatic skin** Following oral administration of ALA an emission spectrum typical of PpIX was observed on psoriatic plaques of patients who received 10 and 15 mg per kg. PpIX fluorescence increased rapidly and significantly over baseline in psoriatic skin after administration of 10 mg per kg ($p = 0.002$) and 15 mg per kg ($p < 0.001$) of ALA to reach a maximum at about 2–2.5 h (**Fig 1**).

PpIX fluorescence intensity did not increase significantly after administration of 5 mg per kg of ALA. PpIX fluorescence intensity also increased slightly in normal skin after administration of ALA at 10 mg per kg ($p = 0.01$) and 15 mg per kg ($p = 0.005$) (**Fig 1**).

**PDT with oral ALA and blue light induces mild erythema followed by improvement within psoriatic plaques** Two patients who received 10 mg per kg developed mild erythema on the face 24 h after PDT. Despite clear instructions to avoid sunlight for the first 2 d after ALA administration these two patients sustained direct sun exposure for about 5 min after leaving the clinic on the day of ALA administration. Blue light exposure was well tolerated by all patients. Only one patient who received 15 mg per kg complained of a mild but tolerable burning sensation during light exposure. Increased erythema compared to nonexposed psoriatic skin was observed in one patient treated with 15 mg per kg followed by 10 J per $cm^2$ of blue light at 3 and 6 h. Increased erythema was also noted on psoriatic skin for three patients treated with 10 mg per kg followed by exposure to 20 J per $cm^2$ of blue light 3 h later (**Fig 2**).

Erythema was not observed on psoriatic skin of patients who received 5 mg per kg. Changes in plaque severity score were not statistically significant for patients who received 5 mg per kg and 10 mg per kg (**Fig 3**).

The best clinical improvement, however (**Fig 2**), was seen in a patient who received 10 mg per kg. Individual clinical improve-

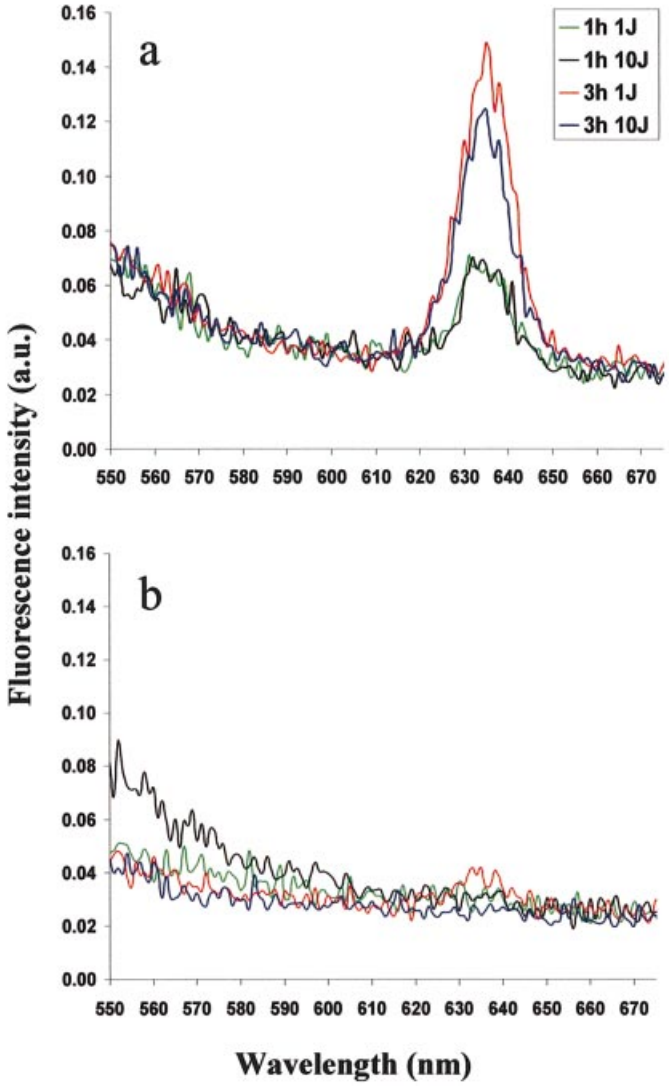


**Figure 4. PDT with blue light after oral administration of ALA induces photobleaching of PpIX.** The emission spectrum from 550 to 675 nm was collected before (*a*) and after (*b*) irradiation of psoriatic skin with 407 nm light. There is a decrease in the 635 nm PpIX peak after blue light exposure.

ment was not as marked for patients treated with 15 mg per kg. For patients treated with 15 mg per kg the maximum improvement was noted for psoriatic skin exposed to 10 J per $cm^2$ of blue light 3 h after ALA administration with a 42% improvement in plaque severity compared to baseline ($p < 0.001$) (**Fig 3**).

**PDT at the highest fluence induces photobleaching of PpIX** Immediately after blue light exposure the red emission peak of PpIX intensity decreased in intensity to a level at which it was not detectable for most patients (**Fig 4**).

Immediately after PDT performed at 3 h at the highest fluence, PpIX fluorescence intensity decreased by 100%, 97%, and 88% for patients who received 5, 10, and 15 mg per kg, respectively (**Fig 5**). This was statistically significant ($p < 0.02$) for all three ALA doses.

**Epidermal PpIX accumulation in psoriatic skin is maximal 3 h after administration of ALA** Following administration of ALA at 10 and 15 mg per kg an increase in red fluorescence was mostly present in the epidermis (**Fig 6**).

A granular fluorescence was also observed in the dermis at 6 h but the resolution of the unfixed sections was not sufficient to verify if it originated from immunocytes (**Fig 6*d***). PpIX microscope fluorescence intensity did not increase significantly in the stratum corneum or the epidermis following oral administration of ALA at 5 mg per kg (**Fig 7**).

Following 10 mg per kg of ALA PpIX intensity increased significantly in the epidermis ($p = 0.03$) to reach a maximum at 3 h,

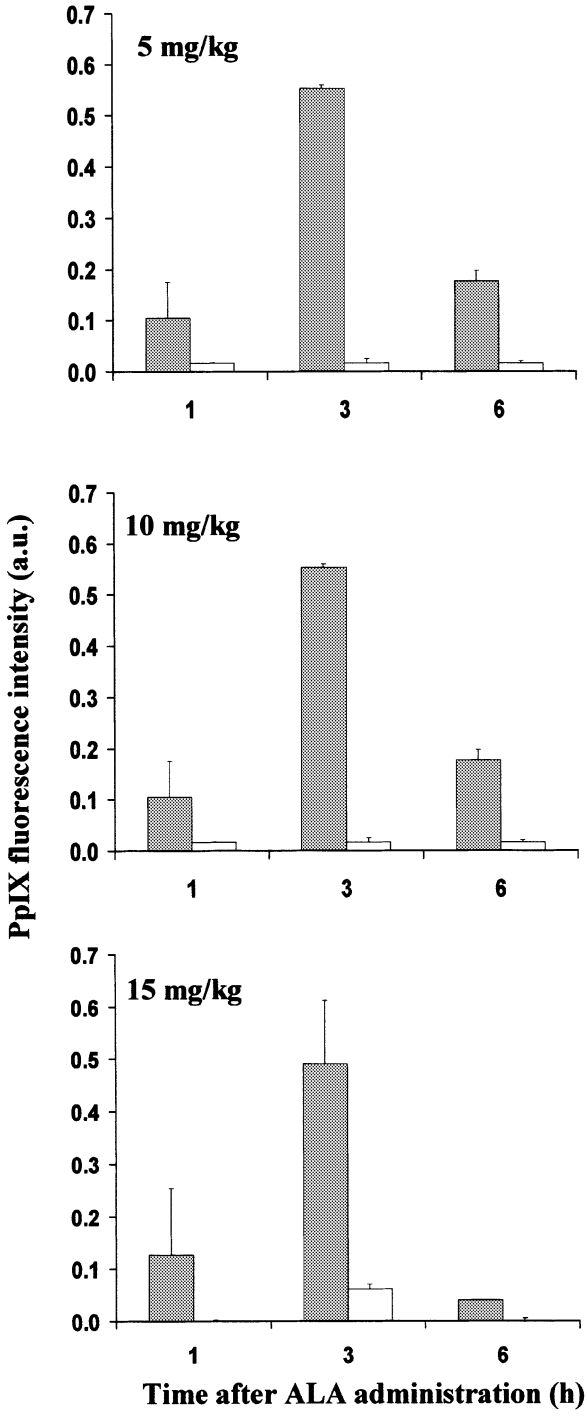


**Figure 5. PDT at the highest fluence induces almost complete photobleaching of PpIX**. PpIX levels were measured with *in vivo* fluorescence spectroscopy before and after PDT with 20 J per $cm^2$ for patients who received 5 mg per kg and 10 mg per kg, and after 10 J per $cm^2$ for patients who received 15 mg per kg. More than 85% photobleaching of PpIX was observed at all three time points for all ALA doses. A single emission spectrum was recorded from four patients for each time point. *Bars*: SD.

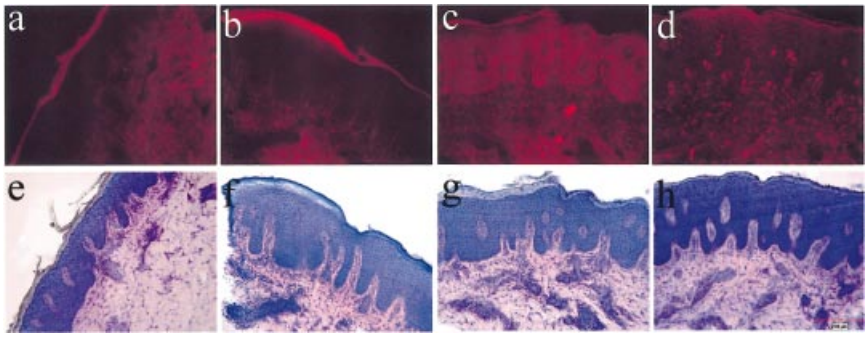


**Figure 6. Following oral administration of ALA red fluorescence accumulates mostly in the epidermis.** Fluorescence micrographs showing epidermal red fluorescence were generated before (*a*), as well as 1 h (*b*), 3 h (*c*), and 6 h (*d*) after ALA administration at 15 mg per kg. Granular fluorescence was also present in the dermis at 6 h. Corresponding sections were stained with toluidine blue after fluorescence microscopy (*e*, *f*, *g*, *h*).

whereas the levels in the stratum corneum did not demonstrate significant changes (**Fig 7**). PpIX fluorescence intensity increased by up to 2-fold in the epidermis after administration of 15 mg per kg of ALA to reach a maximum at 3 h ($p = 0.005$) (**Fig 7**). PpIX intensity also increased in the stratum corneum at 1 h ($p = 0.03$) after ALA at 15 mg per kg (**Fig 7**).

**PDT with oral ALA induces apoptosis in lesional CD3+ lymphocytes** TUNEL+ and CD3+ cells were seen in the dermis of psoriatic plaques of patients treated with ALA–PDT (**Fig 8**).

The number of TUNEL+ and CD3+ cells per field in the dermis did not differ significantly from baseline for patients treated with 5 mg per kg or 15 mg per kg of ALA (**Fig 9*a***).

For patients treated with 10 mg per kg of ALA there was a significant ($p < 0.001$) increase in the number of TUNEL+ and CD3+ cells per field in plaques exposed to light 3 h after ALA administration compared to an unexposed plaque (**Fig 9*a***). The total number of TUNEL+ cells per field in the epidermis was increased after ALA–PDT with 10 mg per kg ($p = 0.003$) and 15 mg per kg ($p = 0.005$) (**Fig 9*b***). This was significant at 1 and 6 h after PDT for 15 mg per kg as well as 3 h for 10 mg per kg. The total number of TUNEL+ cells did not increase for patients treated with 5 mg per kg.

## DISCUSSION

In this study we showed that blue light exposure of psoriatic plaques after oral administration of ALA induces apoptosis in lesional T lymphocytes under suberythemogenic conditions. T lymphocytes play a central role in the pathogenesis of psoriasis as suggested by clinical improvements reported following treatment with cyclosporine, interleukin-2–diphtheria toxin fusion protein (Gottlieb *et al*, 1995), anti-CD4 (Rizova *et al*, 1994), and anti-tumor necrosis factor $\alpha$ (Oh *et al*, 2000) monoclonal antibodies. Induction of apoptosis in lesional T lymphocytes has also been reported with other therapeutic modalities for psoriasis such as ultraviolet B (UVB) phototherapy and psoralen plus UVA (PUVA) therapy (Krueger *et al*, 1995; Coven *et al*, 1999). PUVA therapy for example induces apoptosis in lymphocytes and has been associated with one of the longest periods of remission of the existing therapeutic modalities for psoriasis (Koo and Lebwohl, 1999). In a previous study we failed to detect significant PpIX accumulation in resting circulating CD4 and CD8 T lymphocytes (Bissonnette and Haishan, 2001). Activated T lymphocytes are more sensitive to ALA–PDT-induced apoptosis *in vitro*, however, than their resting counterpart (Rittenhouse-Diakun *et al*, 1995). Activated T lymphocytes expressing CD25 are present in psoriatic skin and might therefore be sensitive to ALA–PDT (Gottlieb, 1997). In this study a significant increase in apoptotic CD3+ lymphocytes was only observed after ALA–PDT for patients treated with 10 mg per kg. This could be related to a higher photosensitizer–light combination and therefore a more important PDT effect in patients with 10 mg per kg. Patients treated with 10 mg per kg received as much as 20 J per $cm^2$ of blue light compared to 10 J per $cm^2$ for patients who received 15 mg per kg. Because of the relatively low penetration of blue light combined with light scattering from psoriatic scales it is possible that at 10 J per $cm^2$ not enough blue light reached the dermis to induce apoptosis in T lymphocytes. Clinically this resulted in an increase in erythema on the exposed plaques for three out of four patients treated with 10 mg per kg as opposed to none treated with 5 mg per kg and only one out of four treated with 15 mg per kg (light fluences were lower at that higher ALA dose). The number of CD3+ TUNEL+ cells in the epidermis was too low to allow comparison of apoptosis in T lymphocytes from unexposed and exposed plaques. In addition the use of the TUNEL assay in psoriatic epidermis is more difficult as it is associated with a higher level of false positive because of the high number of actively dividing keratinocytes (Wrone-Smith *et al*, 1997). Nonetheless we observed an increase in the total number of TUNEL+ cells in the epidermis after PDT with 10 and 15 mg per kg. These cells could be apoptotic keratinocytes and/or T lymphocytes.

The overall tolerability of systemic ALA–PDT was excellent. In contrast to topical ALA–PDT for the treatment of psoriasis, which can be quite painful (Robinson *et al*, 1999), patients did not complain of pain during light exposure. Oral ALA at 30–60 mg per kg has been associated with a high incidence of nausea, vomiting, as well as decreased blood pressure (Regula *et al*, 1995; Rick *et al*, 1997; Webber *et al*, 1997). In this study only one patient who received 15 mg per kg experienced nausea, suggesting that lower ALA doses may be better tolerated for the treatment of psoriasis. Erythema and mild edema were the only signs observed on some of the exposed psoriatic skin areas after PDT; in all cases this was asymptomatic, and the appearance of psoriatic skin returned to baseline within 3 d after light exposure.

Clinical improvement as well as induction of apoptosis in T lymphocytes were thus observed without crusting and/or necrosis, which is often present after PDT for the treatment of skin tumors. Nonspecific photosensitivity has been reported in patients treated with ALA doses of 30–60 mg per kg (Regula *et al*, 1995; Sibille *et al*, 1995). Facial erythema 24 h after administration of 30 mg per kg of ALA has also been observed even with exposure limited to ambient lighting (Bissonnette and Haishan, 2001). This was not observed in this study with the lower ALA doses administered. Slight facial erythema was observed in two patients, however, who were not compliant with sun avoidance measures, suggesting that even at 10 mg per kg patients need to avoid sunlight, especially in the first 24 h. Further studies on facial accumulation of PpIX will need to be performed in order to fully evaluate the duration of photosensitivity.

PpIX accumulation in psoriatic skin was observed using *in vivo* fluorescence spectroscopy for patients who received 10 and 15 mg per kg. This represents mostly epidermal PpIX as seen on fluorescence micrographs. Although PpIX accumulation was not detected for patients who received 5 mg per kg this may have been due to the detection limit of our *in vivo* fluorescence spectrometer. In addition the detection of PpIX levels after administration of low dose ALA can be confounded by the presence of endogenous PpIX in psoriatic scales (Bissonnette *et al*, 1998). In a previous study using a different *in vivo* fluorescence spectrometer we were able to detect

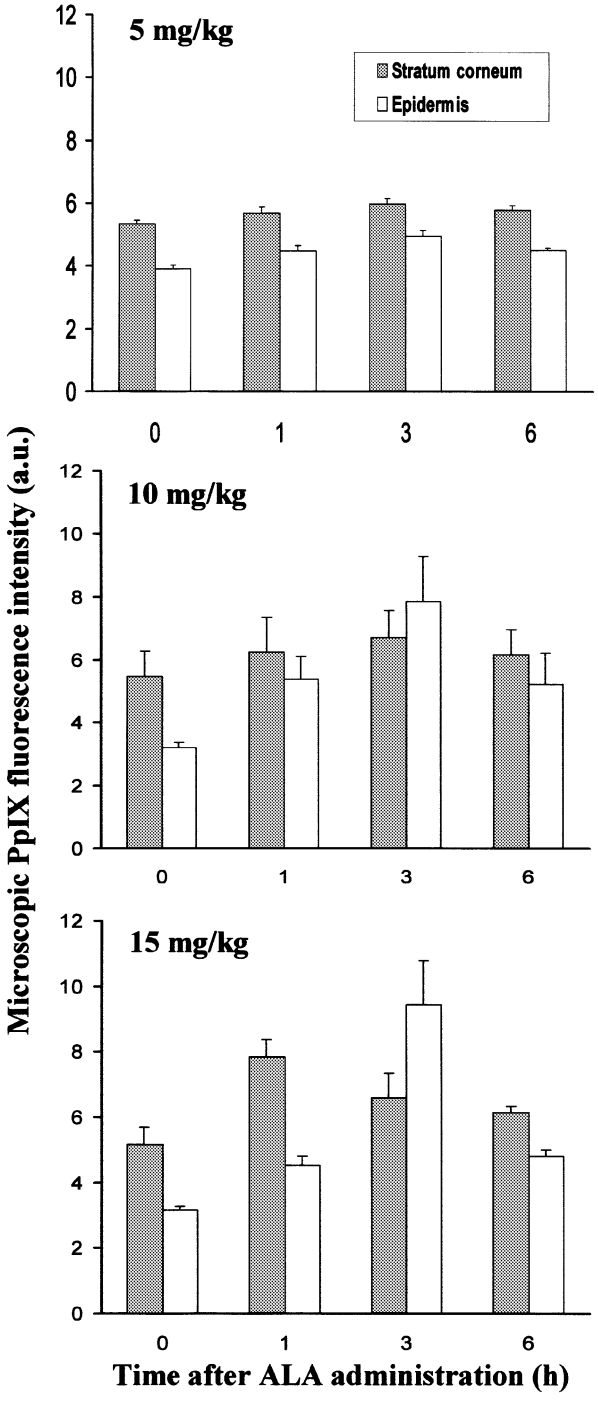


**Figure 7. Epidermal levels of PpIX are increased after administration of ALA at 10 mg per kg and 15 mg per kg**. PpIX levels were determined in the epidermis and stratum corneum with quantitative fluorescence microscopy for patients who received ALA at 5 mg per kg, 10 mg per kg, and 15 mg per kg. Values represent average pixel intensity in the stratum corneum or the rest of the epidermis. *Bars*: SD; $n = 16$ sections from four patients for each dose and each time point.

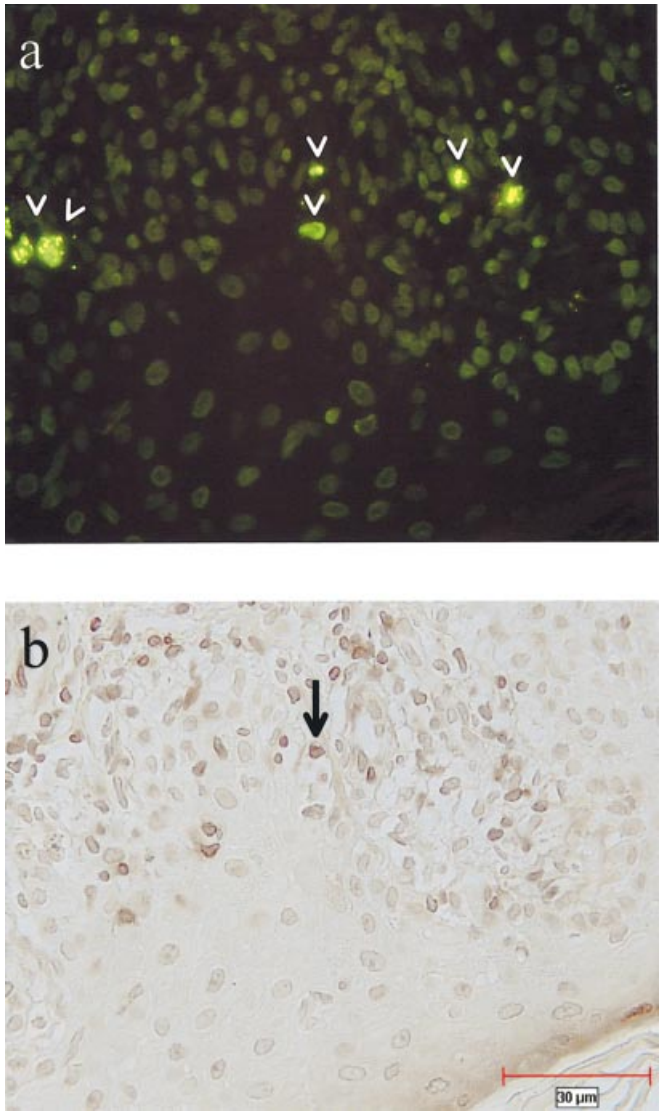


**Figure 8. Presence of TUNEL+ CD3+ cells in the dermis after ALA–PDT at 10 mg per kg.** TUNEL-positive cells are indicated by *arrowheads* (*a*) whereas CD3-positive lymphocytes on the same section are indicated by *arrows* (*b*). *Scale bar*: 30 µm.

PpIX fluorescence in psoriatic skin of patients who received 20 and 30 mg per kg but not 10 mg per kg (Bissonnette and Haishan, 2001), which demonstrates the importance of instrumental sensitivity. The absence of PpIX for patients who received 5 mg per kg also does not necessarily indicate that blue light exposure after this ALA dose would be ineffective, as *in vivo* fluorescence spectroscopy predominantly measures PpIX accumulation within the epidermis. If sufficient PpIX is present in lesional T lymphocytes after administration of ALA at 5 mg per kg to induce apoptosis upon light exposure, it would be clinically desirable to use lower ALA doses to avoid facial photosensitivity. The relatively low fluences used with 5 mg per kg in this study may also explain the absence of increased apoptosis levels for T lymphocytes.

Improvement in plaque severity was observed at the higher fluences for patients treated with 15 mg per kg. Although encouraging, the presence of improvement cannot be interpreted as definite proof of efficacy due to the limited number of patients treated and the absence of a placebo-controlled group in this study. In order to assess efficacy, further studies will need to be performed comparing ALA–PDT to placebo. The presence of apoptosis in lesional T lymphocytes after systemic ALA–PDT suggests that this therapeutic modality could be useful for other diseases where T lymphocyte activation plays a central role, such as atopic dermatitis or alopecia areata. We have recently shown that ALA–PDT induces apoptosis in malignant T cells *in vitro* (Gad *et al*, 2001). This observation coupled with reports of improvement following topical

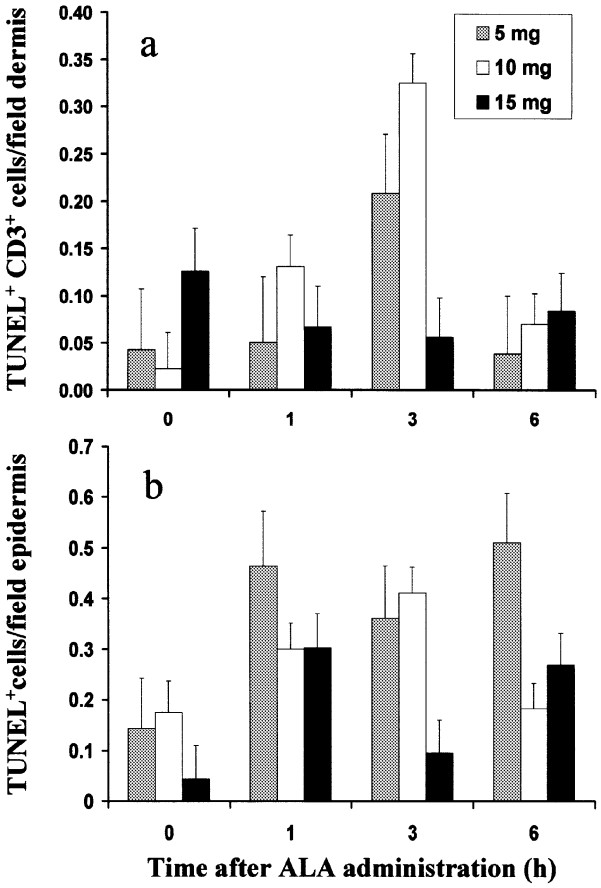


**Figure 9. The number of TUNEL+ CD3+ cells per field is increased in plaques exposed to blue light at 3 h after administration of ALA at 10 mg per kg.** The mean number of TUNEL+ CD3+ cells per field in the dermis (*a*) and the mean number of total TUNEL+ cells in the epidermis (*b*) is presented for unexposed plaques (0) as well as plaques exposed to blue light at 1, 3, or 6 h after ALA administration at either 5, 10, or 15 mg per kg. *Bars*: SD; $n = 27$ sections from a total of four biopsies for each dose and each time point.

ALA–PDT in patients with mycosis fungoides (Wolf *et al*, 1994) also suggests that systemic ALA–PDT should be further explored for this indication.

In conclusion this study showed that PDT with blue light exposure after oral administration of ALA induces apoptosis in lesional T lymphocytes. The clinical improvement observed at the highest ALA doses and fluences suggests that this therapeutic modality should be further investigated for the treatment of psoriasis.

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