

---

# Orally administered *Polypodium leucotomos* extract decreases psoralen-UVA–induced phototoxicity, pigmentation, and damage of human skin

Maritza A. Middelkamp-Hup, MD,<sup>a</sup> Madhu A. Pathak, PhD,<sup>a</sup>  
Concepcion Parrado, MD, PhD,<sup>a,b</sup> Tomas Garcia-Caballero, MD, PhD,<sup>c</sup>  
Francisca Rius-Díaz, PhD,<sup>a,b</sup> Thomas B. Fitzpatrick, MD, PhD,<sup>a,†</sup> and  
Salvador González, MD, PhD<sup>a</sup>

*Boston, Massachusetts, and Malaga and Santiago de Compostela, Spain*

**Background:** The use of psoralen-UVA (PUVA) in patients of skin phototype I to II is limited by side effects of acute phototoxicity and possible long-term carcinogenesis.

**Objective:** We sought to assess oral *Polypodium leucotomos* (PL) extract in decreasing PUVA-induced phototoxicity of human skin on a clinical and histologic level.

**Methods:** A total of 10 healthy patients with skin phototypes II to III were exposed to PUVA alone (using 0.6 mg/kg oral 8-methoxypsoralen) and to PUVA with 7.5 mg/kg of oral PL.

**Results:** Clinically, phototoxicity was always lower in PL-treated skin after 48 to 72 hours ( $P < .005$ ), and pigmentation was also reduced 4 months later. Histologically, PL-treated skin showed a significant numeric reduction of sunburn cells ( $P = .05$ ), preservation of Langerhans cells ( $P \leq .01$ ), decrease of tryptase-positive mast cell infiltration ( $P < .05$ ), and decrease of vasodilation ( $P \leq .01$ ). No differences were found in Ki-67<sup>+</sup> proliferating cells.

**Conclusions:** PL is an effective chemophotoprotector against PUVA-induced skin phototoxicity and leads to substantial benefits of skin protection against damaging effects of PUVA as evidenced by histology. (J Am Acad Dermatol 2004;50:41-9.)

---

From the Wellman Laboratories of Photomedicine, Department of Dermatology, Massachusetts General Hospital, Harvard Medical School<sup>a</sup>; Department of Morphological Sciences, Faculty of Medicine, Malaga University<sup>b</sup>; and Department of Morphological Sciences, Faculty of Medicine, Santiago de Compostela University.<sup>c</sup>

<sup>†</sup>Deceased.

Conflicts of interest: None.

Supported in part by a research grant from Industrial Farmacéutica Cantabria, S.A., Madrid, Spain.

Presented in part at the World Congress of Dermatology, Paris, France, July 1-5, 2002.

Accepted for publication July 18, 2003.

Reprint requests: Salvador González, MD, PhD, Wellman Laboratories of Photomedicine, BHX 630, Massachusetts General Hospital, Boston, MA 02114. E-mail: sgonzalez3@partners.org.

0190-9622/\$30.00

Copyright © 2004 by the American Academy of Dermatology, Inc.

doi:10.1016/S0190-9622(03)02732-4

**P** soralen-UVA (PUVA) therapy (UVA, 320-400 nm) was first reported for the treatment of psoriasis,<sup>1</sup> and its efficacy was soon confirmed by controlled clinical trials in a large patient series, both in the United States and Europe.<sup>2,3</sup> These original findings contributed to the development of photomedicine and established the clinical benefit of PUVA for more than 30 skin disorders,<sup>4</sup> especially for severe psoriasis<sup>5</sup> and early-stage mycosis fungoides.<sup>6</sup> However, in addition to cross-linking with DNA, leading to decreased epidermal proliferation and immunomodulation,<sup>7-9</sup> UVA-activated psoralens also produce reactive oxygen species (ROS) and free radicals by reacting with oxygen.<sup>10</sup> This leads to acute skin phototoxicity,<sup>11</sup> manifested as erythema, edema, pain, and patient discomfort. The hyperpigmentation that develops after PUVA therapy contributes to therapeutic tolerance of UVA and, thus, induces the need to increase the UVA dose repeatedly to maintain the therapeutic effect, leading to higher

**Abbreviations used:**

MOP:	methoxypsoralen
MPD:	minimal phototoxic dose
PL:	<i>Polypodium leucotomos</i>
PUVA:	psoralen + UVA
ROS:	reactive oxygen species
UVR:	ultraviolet radiation

total UVA irradiation doses.<sup>12</sup> PUVA also leads to Langerhans cell depletion, inducing local immunodeficiency of skin.<sup>13</sup> This may be one of the reasons why high-dose PUVA exposure has been correlated with an increased risk of epithelial skin cancer.<sup>14-18</sup> These factors are limitations in the use of PUVA therapy for skin disorders.

The tropical fern, *Polypodium leucotomos* (PL), has long been believed by Native Americans to have antitumoral and anti-inflammatory effects.<sup>19</sup> Previous studies have shown that PL acts as an antioxidant by quenching free radicals, membrane-lipid peroxidation, and ROS such as hydroxyl radical, singlet oxygen, and superoxide anion.<sup>20,21</sup> Preliminary data showing that orally administered PL decreased skin phototoxicity and resulted in Langerhans cell preservation of human skin when exposed to 8-methoxypsoralen (MOP) and sunlight were very promising as they suggested that the addition of PL to PUVA therapy might improve this therapeutic modality by decreasing phototoxicity and epidermal damage.<sup>22</sup>

The objective of this study was to investigate, under carefully controlled laboratory conditions, whether PUVA therapy could be improved by the addition of PL. We clinically assessed the effectiveness of oral PL in decreasing the acute PUVA-induced phototoxic reaction. We also studied the protective effect of PL on various histologic parameters, such as sunburn cells, Langerhans cells, mast cells, proliferating cells, and vasodilatation.

## PATIENTS AND METHODS

### Patient selection

A total of 10 healthy volunteers with skin phototype II or III were included in this open-label study after they read and signed a written informed consent form and after protocol was approved by an institutional review board of Massachusetts General Hospital in Boston, Mass. The study was conducted over a period of 1 year in our laboratories. The group consisted of 6 men and 4 women with ages ranging from 24 to 47 years. We excluded patients with a personal or family history of skin cancer, a history of photosensitivity, or taking any drug that might alter the response of skin to UV radiation

(UVR). We used the skin of the back for these studies, which had to be free of any blemishes and not exposed to sunlight or artificial UVR (tanning booth) for at least 8 weeks before the study.

### Radiation source and exposure conditions

A 1000-W high-pressure Xenon arc lamp (Oriel Corp, Stratford, Conn) emitting a collimated beam and equipped with a 2-mm filter (WG335, Schott Glas, Mainz, Germany) and a first surface mirror (Edmund Scientific, Barrington, NJ) was used as a UVA radiation source (320-400 nm). A high-velocity fan was used to eliminate any impact of heat from the infrared radiation of the lamp on the skin. The intensity of the UVA radiation was measured with a calibrated radiometer (International Light, Newburyport, Mass) before each experiment when the lamp had warmed up for 30 minutes, and after each experimental protocol to ensure stability of the UVA output, which was usually around 3 mW/cm<sup>2</sup>. Phototests were performed with adhesive UVR-reflecting aluminum stickers containing 6 exposure windows, each 3.3 cm<sup>2</sup> in size (DV Die Cutting Inc, Danvers, Mass). One phototest consisted of exposure of each skin site to UVA doses increasing with 12% to 50% to obtain an exposure range with regular incremental UVA doses. A dose equivalent to 2 times the minimal phototoxic dose (MPD) was always included in each phototest of the volunteer. From the 6 skin sites, either the first, second, or third site was exposed to the MPD dose, and either the fourth, fifth, or sixth site was exposed to 2 times the MPD. During exposures, the rest of the skin was covered by an opaque UV-protective cloth. The distance between the exposed skin and the lamp was kept constant by stabilizing the back skin against an aluminum template located at a fixed distance from the exit port of the lamp.

### Study design

Before the start of the study each patient's MPD was assessed. All patients were instructed to have no more than a low-fat light meal before the exposure. Patients received a dose of 0.6 mg/kg 8-MOP, and were exposed to UVA radiation 1 hour and 30 minutes later. The MPD was defined as the minimal dose of UVA inducing confluent erythema with 4 sharp borders of the exposed skin site. This MPD was further used throughout the following study protocol.

In the first part of the study protocol, patients were exposed to PUVA without receiving oral PL. After intake of oral 8-MOP (0.6 mg/kg), the skin of the back was exposed 4 times to the same phototest at: (1) 1 hour; (2) 1 hour and 30 minutes; (3) 2 hours; and (4) 2 hours and 30 minutes to assure optimal

phototoxicity. The phototoxic reaction of PUVA-exposed skin was evaluated 48 hours after UVA exposure. Biopsy specimens were taken from 4 patients after 48 hours and from 3 patients 72 hours after exposure.

After completion of this first part of the protocol, patients received the first dose of oral PL (7.5 mg/kg body weight) the evening before the second part of the protocol. The next day each patient received the dose of oral 8-MOP and the second dose of oral PL (7.5 mg/kg body weight), after which the same 4 phototests were repeated as performed in the first part of the protocol, ie, after 1 hour, 1 hour and 30 minutes, 2 hours, and 2 hours and 30 minutes of ingestion of 8-MOP and oral PL. Skin phototoxicity was again evaluated after 48 hours, and skin biopsy specimens were taken after 48 hours from 4 patients, and after 72 hours from 3 patients. Specifics about the biopsy specimens are given in the "Histology" section. In addition, follow-up pictures up to 4 months after the experimental protocol were obtained from 6 patients for clinical evaluation of the photoprotective effect of PL on PUVA-induced pigmentation. Oral 8-MOP (Methoxsalen or Oxsoralen Ultra, ICN, Costa Mesa, Calif) was administered to each patient in a dose of 0.6 mg/kg, as used in conventional PUVA therapy. The capsules containing PL (180 mg each) were supplied by I. F. Cantabria, S.A. (Madrid, Spain) and were administered orally in a dose of 7.5 mg/kg body weight.

### Clinical evaluation

Clinical evaluation of the phototoxic response of every exposed skin site was performed by at least 2 experienced investigators of PUVA therapy using a scoring system for the intensity of erythema and edema ranging from grades 0 to 4 (0 = no erythema; 1 = trace erythema; 2 = visible, not confluent erythema, no sharp borders; 3 = confluent erythema with 4 sharp borders and no edema, MPD; 4 = intense erythema with edema). The mean of the grades of each skin site was calculated for all patients at each time point of exposure. Differences in pigmentation of exposed sites were assessed qualitatively during the next 4 months.

### Histology

A 3-mm punch biopsy specimen was obtained during the first part of the protocol from the skin site exposed to 2 times the MPD, from the phototest at the time point of maximal phototoxicity. The biopsy specimen in the second part of the protocol was taken from the mirrored skin site, ie, exposed to the same fluence, from the same phototest time point. All skin specimens were fixed in 10% buffered formalin and embedded in paraffin for sectioning and

microscopic evaluation. Sections of 3  $\mu$ m were routinely stained with hematoxylin and eosin for histologic gross evaluation and quantitative assessment of sunburn cells.

### Immunohistochemistry

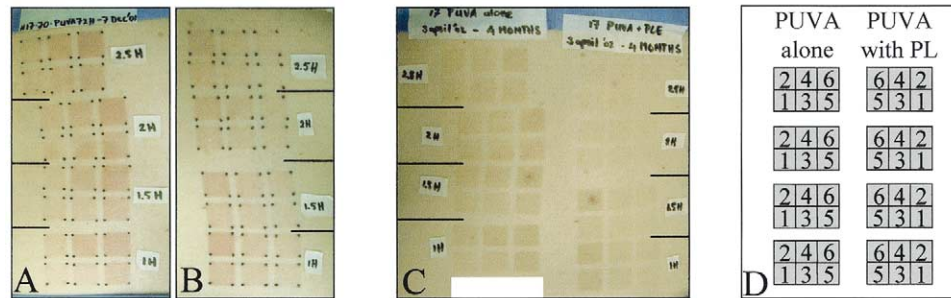
For immunohistochemical analysis, 5- $\mu$ m sections were used. They were deparaffinized with xylene and rehydrated with graded ethanols. Endogenous peroxidase was blocked with hydrogen peroxide, and the samples were then rinsed in phosphate-buffered saline. To yield adequate intensity signals with the respective antibodies, heat-induced antigen retrieval was carried out by microwave pretreatment in citric acid buffer (10 mmol/L; pH 6.0) for 20 minutes. For reduction of background labeling, the sections were blocked for 30 minutes in normal horse serum. The sections were later incubated overnight at 4°C with the commercially available antibodies: (1) prediluted anti-CD1a for Langerhans cells (clone O10, Catalog No. 1590, Immunotech, Marseille, France)<sup>23</sup>; (2) 1:150 diluted anti-Ki-67 for proliferating keratinocytes (clone MIB-1, catalog No. M72470, Dako, Glostrup, Denmark)<sup>24</sup>; (3) 1:100 diluted antitryptase for mast cells (clone AA1, catalog No. M7057, Dako)<sup>25</sup>; and (4) 1:10 diluted anti-CD31 for endothelial cells (clone JC/70A, catalog No. M0823, Dako).<sup>26</sup> After washing with phosphate-buffered saline, sections were incubated with biotinylated secondary antibody, avidin-biotin-peroxidase complex (ABC Elite, Vector Laboratories, Burlingame, Calif), and then 3,3'-diaminobenzidine (DAB). Each section was counterstained with hematoxylin, dehydrated, and covered with a coverslip. Positive and negative controls were also included in each staining run.

### Quantitative histologic analysis

All parameters listed below were quantified in a blinded fashion.

**Sunburn cells.** Sunburn cells were defined as cells with a hyper eosinophilic cytoplasm and a dense, small, dark, and irregularly formed nucleus in comparison with neighboring cells, and located in the epidermis away from areas of blistering or crush artifacts.<sup>27</sup> Sunburn cells were quantified in 4 entire sections per specimen (10-11 fields per section) with a light microscope at a magnification of 40 $\times$ . Using an eyepiece micrometer, the average number of sunburn cells/mm epidermal length was calculated.

**Proliferating cells.** Ki-67 immunoreactivity (proliferating cells) was quantified with a image analysis system (Visilog, Noesis, France). The equipment used included a microscope (Elipse E400, Nikon, Tokyo, Japan) with a 20 $\times$  objective lens, a digital color camera (Polaroid Corp, Waltham,



**Fig 1.** Phototoxic reaction 48 hours after exposure to psoralen-UVA (PUVA) alone (A) and with *Polypodium leucotomos* (PL) (B). C, Pigmentation reaction 4 months after exposure, showing skin sites exposed to PUVA alone (left) and to PUVA with PL (right). Note decreased erythema and pigmentation in PL-treated skin, indicating that PL decreases phototoxic and pigmentary response. D, Schematic representation of skin sites. Numbers in squares indicate relative UVA intensities of each site (1 = lowest UVA intensity, 6 = highest UVA intensity). Sites exposed to PUVA without and with PL are mirrored.

Mass), and image-processing and analysis software (version 5.2, Visilog). This program is able to differentiate and count hematoxylin-stained objects and Lag-red/brown (DAB-stained) objects. The percentage of proliferating epidermal cells was calculated by dividing the number of immunoreactive nuclei by the total number of epidermal nuclei.

**Langerhans cells and mast cells.** CD1a<sup>+</sup> cells (Langerhans cells) and tryptase-positive cells (mast cells) were quantified by light microscopy using a 40× objective lens and an eyepiece micrometer. A cell with a nucleus and clear immunoreactivity was considered a positive cell. In each biopsy specimen the number of positive cells was counted in 8 to 10 consecutive fields, and then calculated per square millimeter of epidermal or papillary dermal surface, respectively. To determine the number of mast cells, the micrometer and grid were aligned on one edge along the dermoepidermal junction.

**Microvessels.** Using a 40× objective lens, 8 to 10 consecutive fields were counted. CD31<sup>+</sup> cell (endothelial cell) clusters consisting of more than 2 cells and large microvessels were included in the microvessel count.<sup>28</sup> The number of microvessels per square millimeter of dermis was counted, and vasodilatation assessed as a percentage of the total surface area occupied by vessels divided by the total dermal surface area.

### Statistical analysis

Clinically scored phototoxicity grades were analyzed using the analysis of variance test. Mann-Whitney tests were performed to compare the number of sunburn cells, Langerhans cells, mast cells, proliferating cells, and microvessels and vasodilatation in PL-treated and untreated skin specimens. Analyses were performed on combined data obtained from

biopsy specimens taken at 48 and 72 hours. A *P* value of .05 or less was considered statistically significant.

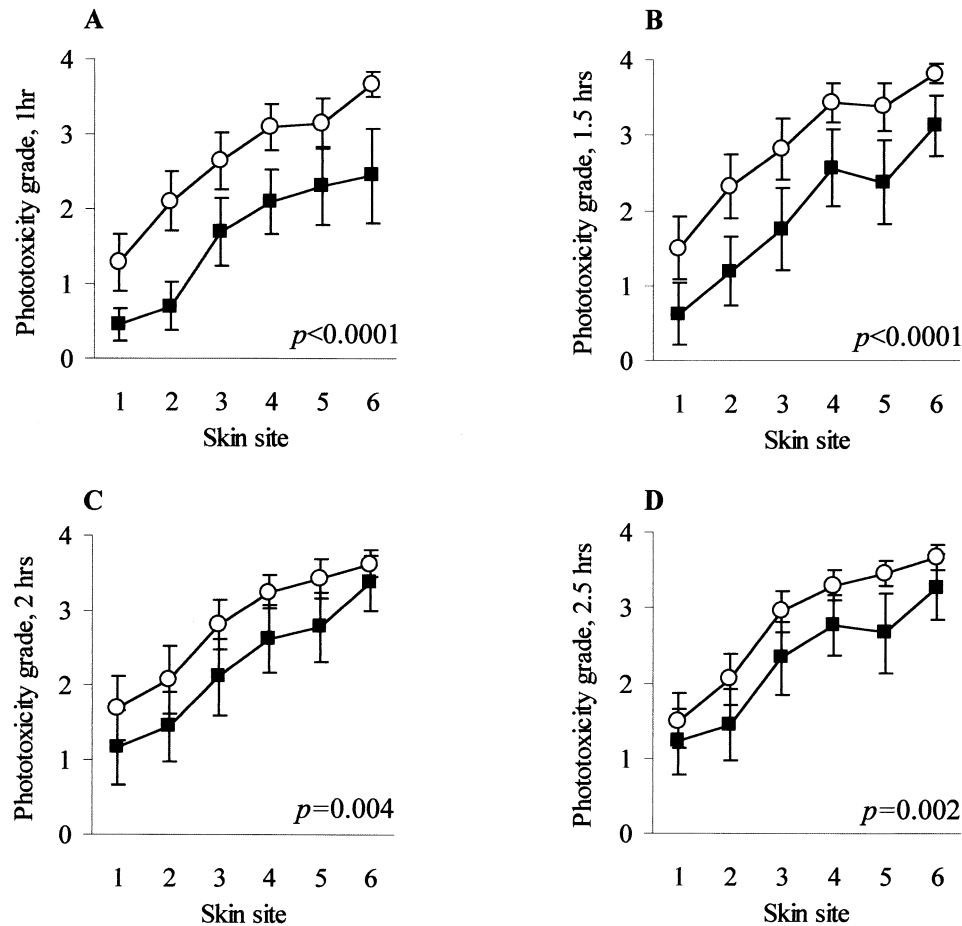
## RESULTS

### Clinical results

PL significantly decreased the acute PUVA-induced phototoxic reaction and also diminished the subsequent cutaneous pigmentary response (Fig 1). PL-treated skin sites always showed a statistically significant lower grade of erythema and edema than skin sites exposed to PUVA alone at all time points of exposure (*P* < .005) (Fig 2), indicating a decreased phototoxic reaction of PL-treated skin. The PUVA-induced hyperpigmentation was clearly decreased in 4 of the 6 patients who received a follow-up examination after 4 months (Fig 1). This decrease in pigmentation coincided with a decrease in the acute phototoxic reaction.

### Histologic results

When comparing hematoxylin and eosin-stained sections from skin exposed with PUVA alone and PL-treated skin, some gross morphologic changes became apparent. PUVA-exposed skin without PL showed noticeably more maturation disarray, microvesiculation, and vacuolization of keratinocytes, whereas these findings of epidermal damage were not as prominently found in PL-treated skin (Fig 3, A). The number of sunburn cells/mm epidermis was significantly lower in PL-treated skin when compared with PL-untreated skin (*P* = .05) (Fig 3, A). PL-treated skin showed significantly less depletion of Langerhans cells/mm<sup>2</sup> epidermis in response to PUVA when compared with PL-untreated skin (*P* ≤ .01) (Fig 3, B). In addition, Langerhans cells in PL-untreated skin were increased in size and showed a

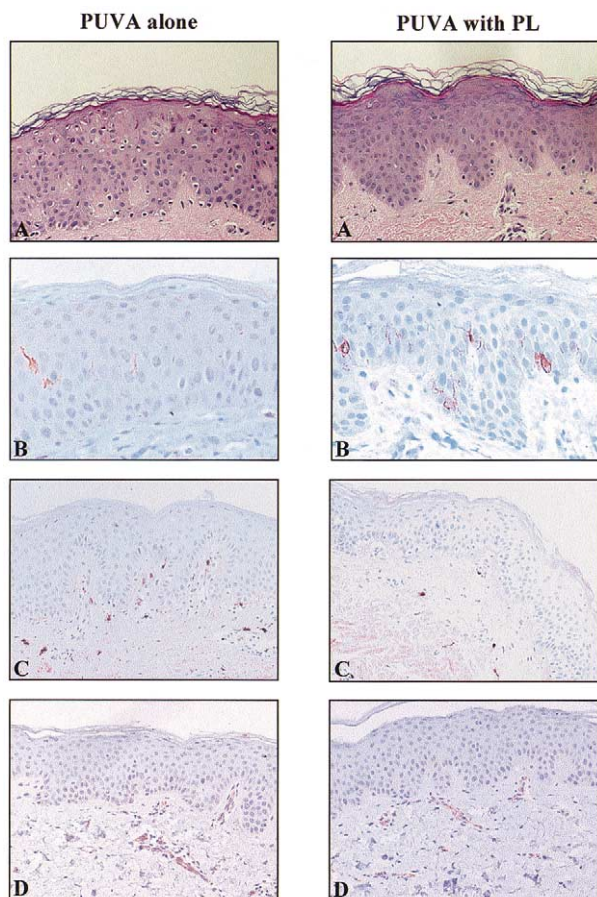


**Fig 2.** Mean of phototoxicity grade at 48 hours (*y-axis*) of each exposed skin site (*x-axis*) from 10 patients. Mean of grades of skin sites exposed to UV radiation without (*open circles*) and with (*closed squares*) *Polypodium leucotomos* (PL) after 1 (**A**); 1.5 (**B**); 2 (**C**); and 2.5 (**D**) hours of 8-MOP ingestion. PL-treated skin always shows statistically significant lower phototoxicity grades at all 4 time points of exposure. *Error bars*, SEM.

loss of dendritic morphology, whereas in PL-treated skin these cells preserved their size and dendritic appearance. PL pretreatment also resulted in a statistically significant reduction of tryptase-positive mast cells in the papillary dermis compared with PL-untreated skin ( $P \leq .05$ ) (Fig 3, C). The total number of CD31<sup>+</sup> microvessels/mm<sup>2</sup> dermis did not show a statistically significant difference in either group. However, there was a statistically significant decrease in the surface area occupied by microvessels in PL-treated versus untreated skin ( $P \leq .01$ ) (Fig 3, D), indicating that PL was able to decrease vasodilatation induced by PUVA. There was no statistically significant difference in epidermal proliferation seen by Ki-67 immunoreactivity in PL-treated versus untreated skin. Table I gives an overview of all histologic parameter counts and statistical results.

## DISCUSSION

The high popularity of PUVA therapy has somewhat declined since its discovery in the 1970s.<sup>29</sup> New developments in the area of phototherapy have made the phototoxicity and patient discomfort associated with PUVA less acceptable and attractive. When considering safety aspects it should be recognized that the long-term risks of PUVA are known because of careful prospective follow-up studies, whereas data on long-term effects of newer types of phototherapy are mainly on the basis of animal studies and mathematic models, and actual long-term effects need yet to be assessed.<sup>30,31</sup> In spite of these issues, PUVA remains a significant therapeutic option, as its effectiveness continues to be recognized,<sup>32-34</sup> and it still is the most effective UV-based therapy for patients with extensive psoriasis<sup>5</sup> and



**Fig 3.** Histology from paired biopsy specimens of skin treated with psoralen-UVA (PUVA) alone (left) and with *Polypodium leucotomos* (PL) (right). PL-treated skin shows: less sunburn cells, maturation disarray, microvesiculation, and vacuolization (A); Langerhans cell numeric and morphologic preservation (B); less dermal mast cell infiltration (C); and less blood vessel dilation (D).

severe plaque form psoriasis.<sup>35</sup> Therefore, improvement of this therapy is clinically relevant.<sup>36</sup>

In this study we combined PUVA with the oral administration of an extract of the fern, PL. PL has been used in the management of inflammatory skin diseases<sup>37-39</sup> for more than 30 years without significant side effects.<sup>40</sup> In animal models, toxicology tests have shown that PL is noncarcinogenic, nontoxic, and nonteratogenic (unpublished data).

This study shows that oral administration of PL lead to a significant reduction of phototoxicity, ie, erythema and edema, in PUVA-treated individuals ( $P < .005$ ) (Fig 1). This observation was made at all time points of PUVA exposure (Fig 2). This decrease in cutaneous phototoxicity should make PUVA considerably more tolerable and comfortable for patients. It is believed to result mainly from the antioxidative properties of PL,<sup>20,21</sup> which can also be concluded from the histologic data presented in this

**Table I.** Quantitative overview of histologic parameters and  $P$  values between skin treatments

Histologic parameter	PUVA alone mean (SEM)	PUVA with PL mean (SEM)
Sunburn cells/mm epidermis	16.6 (1.5)	11 (2.5)*
Langerhans cells/mm <sup>2</sup> epidermis	20.64 (3.68)	34.24 (3.52)†
Mast cells/mm <sup>2</sup> papillary dermis	132.16 (8.96)	108.8 (9.6)*
Number of microvessels/mm <sup>2</sup> dermis	109.41 (9.55)	100.32 (6.93)
Vasodilatation (percentage vessel surface area)	1.76 (1.24)	1.24 (0.08)†
Percentage Ki-67 <sup>+</sup> epidermal cells	16.8 (1.21)	15.9 (1.47)

PL, *Polypodium leucotomos*; PUVA, psoralen-UVA.

\* $P \leq .05$ .

† $P \leq .01$ . For each slide the number of Langerhans cells and mast cells were counted in at least 10 view fields (ocular grid, 0.0625 mm<sup>2</sup>;  $\times 400$ ).

communication (Table I, Fig 3). Oral PL caused a marked reduction in sunburn cells in PUVA-exposed skin ( $P = .05$ ), and significant numeric preservation of Langerhans cells ( $P \leq .01$ ) with conservation of morphology. PUVA exposure induces sunburn cell formation by either oxidative damage or direct DNA damage.<sup>41</sup> PUVA is also known to lead to Langerhans cell depletion in psoriatic<sup>13</sup> and normal skin<sup>42</sup> because of generated oxidative stress.<sup>43</sup> We believe that the decrease in sunburn cells and the preservation of Langerhans cells result from the potent quenching of oxidative stress by oral PL,<sup>20,21</sup> similar to the presumed working mechanism of other topically administered antioxidants.<sup>20,22,44,45</sup> ROS and free radicals can also lead to lipid peroxidation of endothelial cell membranes, resulting in vasodilatation, increased vasopermeability, and increased synthesis of eicosanoids.<sup>8,46</sup> The antioxidative ability of PL to inhibit lipid peroxidation<sup>20</sup> lead to a significant decrease in vasodilatation ( $P \leq .01$ ), clinically expressed in reduced phototoxicity, and less pain and burning sensations in PL-treated skin as spontaneously reported by our study patients. PL also reduced the dermal mast cell infiltration significantly ( $P \leq .05$ ). As previous studies show that mast cells do not play a major role in the development of the PUVA phototoxic reaction,<sup>47,48</sup> we believe that the mast cell reduction most likely did not contribute to the observed reduction in phototoxicity. The number of proliferating cells indicated by Ki-67 did not differ in the 2 treatment regimens. This may either be because the biopsy specimens were taken too early (at 48 to 72 hours) to detect any changes in Ki-67 expression, or because PUVA does not have a clear

effect on Ki-67 expression, as previously shown by repeated PUVA exposures of normal skin of patients with psoriasis.<sup>49</sup>

Our observations made in the acute phase after PUVA give rise to interesting hypotheses about the long-term benefits PUVA plus PL may have. There is an increased risk of squamous cell carcinoma developing in PUVA-exposed skin that seems to be directly related to the total cumulative UVA dose received.<sup>14-17</sup> In patients treated with high-dose PUVA, even an increased risk of malignant melanoma has been reported,<sup>50</sup> although this has not as yet been confirmed by other studies,<sup>16,18,51</sup> and the American follow-up study<sup>50</sup> did not have a control population and did not make a distinction between patients with a foregoing increased risk of melanomas.

Any long-term effect of the combination of PL and PUVA will have to be carefully studied. Nevertheless, on the basis of these study results we would like to hypothesize that the combination of PL and PUVA may contribute to improve the safety of PUVA. First, PUVA-induced DNA damage, resulting in sunburn cells, occurs either directly through cross-linking of psoralens in DNA, or through damage induced by generated ROS and free radicals.<sup>9,41</sup> The significant reduction of sunburn cells by PL may very likely be partly because of a reduction of the latter type of DNA damage. As ROS and free radicals damaging DNA have been implicated in the promotion of carcinogenesis,<sup>52,53</sup> a decrease of this damage may increase the safety of PUVA. A second argument is the significant preservation of Langerhans cells. Langerhans cell depletion is thought to play a role in the development of skin cancer, as they have been shown to be vital for the induction of tumor-specific immunity against UVR-induced tumors,<sup>54</sup> and their presence has been shown to be indispensable for tumor rejection.<sup>55</sup> Commonly accepted chemical skin cancer promoters also work by depleting Langerhans cells, giving transformed cells the chance to grow uninhibited by any immunologic surveillance.<sup>56</sup> Carcinogenesis resulting from PUVA follows the pattern induced by carcinogenesis promoters,<sup>57</sup> and it can be assumed that this is, at least in part, the result of Langerhans cell depletion. As PL leads to a significant preservation of Langerhans cells and, thus, an improved immunologic status of PUVA-exposed skin, it is possible that this will lead to a decrease of the chance of skin cancer developing when compared with PUVA alone. Preliminary studies have shown that PL reduces UVR-induced carcinogenesis in animal models.<sup>58</sup> Noteworthy is that PL decreased the hyperpigmentation typically induced by PUVA. Although clinical evaluation of the pigmentation response was not systematically

performed in all study patients, we found a decrease of hyperpigmentation in 4 of the 6 cases evaluated, always corresponding with a decrease in acute phototoxicity. Cellular membranes and DNA are 2 major UVR targets involved in melanogenesis in response to injury. DNA damage by itself increases melanogenesis,<sup>59</sup> and the observed decrease in pigmentation may reflect reduced DNA damage by quenching of ROS by PL. Exposure to PUVA also increases diacylglycerol,<sup>46</sup> which stimulates melanogenesis through activation of protein kinase c.<sup>59</sup> As oxidative stress generates diacylglycerol,<sup>60</sup> the quenching of ROS by PL can be another mechanism leading to reduced pigmentation. Reduction of this PUVA-induced hyperpigmentation is important, because when pigmentation is not the primary therapeutic goal, it can become a limiting factor by increasing the tolerance of skin to UVR.<sup>12</sup> This makes it necessary to increase UVA doses during the therapy to maintain the therapeutic response. By reducing this hyperpigmentation, the need for increasing UVA doses will also be reduced, thus, leading to a lower total UVA dose for clearance of psoriasis. As the height of the total UVA dose is considered the main risk factor for squamous cell carcinoma developing,<sup>14-18</sup> it is highly likely that the addition of PL will decrease the risk of skin cancer developing, thus, markedly improving the safety profile of PUVA. Supporting this hypothesis are the results of a preliminary study in patients with psoriasis, in which the addition of PL resulted in a significant reduction of PUVA sessions and a significant reduction in the total cumulative UVA dose required for clearance of psoriasis when compared with PUVA alone.<sup>61</sup> From this study we can also conclude that PL does not interfere with the therapeutic effectiveness of PUVA for psoriasis.<sup>61</sup>

In conclusion, PL is the first oral agent to decrease the acute phototoxicity and the subsequent development of hyperpigmentation induced by PUVA, and leads to a significant Langerhans cell preservation, decrease of sunburn cell formation, vasodilation, and mast cell infiltration. These results show that PL is an effective chemophotoprotector against PUVA-induced skin phototoxicity and leads to substantial benefits of skin protection against damaging effects of PUVA as seen by histology. The observations made in the acute phase warrant long-term follow-up studies as they suggest that the combination of PUVA and PL may result in a therapeutic modality with less long-term adverse side effects.

This article is dedicated in memory of Dr Thomas B. Fitzpatrick.

We thank John Demirs (Massachusetts General Hospital, Boston, Mass) and Carmen Rios-Barranquero (Faculty of Medicine, Malaga, Spain) for their preparation of histology slides. We also thank William Farinelli (Massachusetts General Hospital, Boston, Mass) for his technical support.

#### REFERENCES

- Parrish JA, Fitzpatrick TB, Tanenbaum L, Pathak MA. Photochemotherapy of psoriasis with oral methoxsalen and longwave ultraviolet light. *N Engl J Med* 1974;291:1207-11.
- Wolff K, Hönigsmann H, Gschnait F, Konrad K. Photochemotherapie bei psoriasis: klinische erfahrungen bei 152 patienten. *Dtsch Med Wochenschr* 1975;100:2471-7.
- Melski JW, Tanenbaum L, Parrish JA, Fitzpatrick TB, Bleich HL. Oral methoxsalen photochemotherapy for the treatment of psoriasis: a cooperative clinical trial. *J Invest Dermatol* 1977;68:328-35.
- Honig B, Morison WL, Karp D. Photochemotherapy beyond psoriasis. *J Am Acad Dermatol* 1994;31:775-90.
- Tanew A, Radakovic-Fijan S, Schemper M, Hönigsmann H. Narrowband UV-B phototherapy vs photochemotherapy in the treatment of chronic plaque-type psoriasis: a paired comparison study. *Arch Dermatol* 1999;135:519-24.
- Gilchrist BA, Parrish JA, Tanenbaum L, Haynes HA, Fitzpatrick TB. Oral methoxsalen photochemotherapy of mycosis fungoides. *Cancer* 1976;38:683-9.
- Johnson R, Staiano-Coico L, Austin L, Cardinale I, Nabeya-Tsukifuji R, Krueger JG. PUVA treatment selectively induces a cell cycle block and subsequent apoptosis in human T-lymphocytes. *Photochem Photobiol* 1996;63:566-71.
- Hönigsmann H, Szeimies RM, Knobler R, Fitzpatrick TB, Pathak MA, Wolff K. Photochemotherapy and photodynamic therapy. In: Freedberg IM, Eisen AZ, Wolff K, Austen KF, Goldsmith LA, Katz SI, et al, editors. *Fitzpatrick's dermatology in general medicine*. 5th ed. New York: McGraw-Hill, 1999. p. 2880-900.
- Cech T, Pathak MA, Biswas RK. An electron microscopic study of the photochemical cross-linking of DNA in guinea pig epidermis by psoralen derivatives. *Biochim Biophys Acta* 1979;562:342-60.
- Pathak MA, Joshi PC. Production of active oxygen species ( $^1O_2$  and  $O_2^-$ ) by psoralens and ultraviolet radiation (320-400 nm). *Biochim Biophys Acta* 1984;798:115-26.
- Carraro C, Pathak MA. Studies on the nature of in vitro and in vivo photosensitization reactions by psoralens and porphyrins. *J Invest Dermatol* 1988;90:267-75.
- Bech-Thomsen N, Wulf HC. Photoprotection due to pigmentation and epidermal thickness after repeated exposure to ultraviolet light and psoralen plus ultraviolet A therapy. *Photodermatol Photoimmunol Photomed* 1996;11:213-8.
- Friedmann PS. Disappearance of epidermal Langerhans cells during PUVA therapy. *Br J Dermatol* 1981;105:219-21.
- Stern RS, Lunder EJ. Risk of squamous cell carcinoma and methoxsalen (psoralen) and UV-A radiation (PUVA): a meta-analysis. *Arch Dermatol* 1998;134:1582-5.
- Hannuksela-Svahn A, Pukkala E, Laara E, Poikolainen K, Karvonen J. Psoriasis, its treatment, and cancer in a cohort of Finnish patients. *J Invest Dermatol* 2000;114:587-90.
- McKenna KE, Patterson CC, Handley J, McGinn S, Allen G. Cutaneous neoplasia following PUVA therapy for psoriasis. *Br J Dermatol* 1996;134:639-42.
- Maier H, Schemper M, Ortel B, Binder M, Tanew A, Hönigsmann H. Skin tumors in photochemotherapy for psoriasis: a single-center follow-up of 496 patients. *Dermatology* 1996;193:185-91.
- Lindelof B, Sigurgeirsson B, Tegner E, Larko O, Johannesson A, Berne B, et al. PUVA and cancer risk: the Swedish follow-up study. *Br J Dermatol* 1999;141:108-12.
- Horvath A, Alvarado F, Szocs J, de Alvarado ZN, Padilla G. Metabolic effects of calagualine, an antitumoral saponine of *Polypodium leucotomos*. *Nature* 1967;214:1256-8.
- Gonzalez S, Pathak MA. Inhibition of ultraviolet-induced formation of reactive oxygen species, lipid peroxidation, erythema and skin photosensitization by *Polypodium leucotomos*. *Photodermatol Photoimmunol Photomed* 1996;12:45-56.
- Gomes AJ, Lunardi CN, Gonzalez S, Tedesco AC. The antioxidant action of *Polypodium leucotomos* extract and kojic acid: reactions with reactive oxygen species. *Braz J Med Biol Res* 2001;34:1487-94.
- Gonzalez S, Pathak MA, Cuevas J, Villarrubia VG, Fitzpatrick TB. Topical or oral administration with an extract of *Polypodium leucotomos* prevents acute sunburn and psoralen-induced phototoxic reactions as well as depletion of Langerhans cells in human skin. *Photodermatol Photoimmunol Photomed* 1997;13:50-60.
- Krenacs L, Tiszalvics L, Krenacs T, Boumsell L. Immunohistochemical detection of CD1A antigen in formalin-fixed and paraffin-embedded tissue sections with monoclonal antibody 010. *J Pathol* 1993;171:99-104.
- Key G, Becker MH, Baron B, Duchrow M, Schluter C, Flad HD, et al. New Ki-67-equivalent murine monoclonal antibodies (MIB 1-3) generated against bacterially expressed parts of the Ki-67 cDNA containing three 62 base pair repetitive elements encoding for the Ki-67 epitope. *Lab Invest* 1993;68:629-36.
- Irani AM, Bradford TR, Kepley CL, Schechter NM, Schwartz LB. Detection of MCT and MCTC types of human mast cells by immunohistochemistry using new monoclonal anti-tryptase and anti-chymase antibodies. *J Histochem Cytochem* 1989;37:1509-15.
- Miettinen M, Lindenmayer AE, Chaubal A. Endothelial cell markers CD31, CD34, and BNH9 antibody to H- and Y-antigens—evaluation of their specificity and sensitivity in the diagnosis of vascular tumors and comparison with von Willebrand factor. *Mod Pathol* 1994;7:82-90.
- Kane KS, Maytin EV. Ultraviolet B-induced apoptosis of keratinocytes in murine skin is reduced by mild local hyperthermia. *J Invest Dermatol* 1995;104:62-7.
- Massi D, Franchi A, Borgognoni L, Paglierani M, Reali M, Santucci M. Tumor angiogenesis as a prognostic factor in thick cutaneous malignant melanoma: a quantitative morphologic analysis. *Virchows Arch* 2002;440:22-8.
- Housman TS, Rohrback JM, Fleischer AB Jr, Feldman SR. Phototherapy utilization for psoriasis is declining in the United States. *J Am Acad Dermatol* 2002;46:557-9.
- Slaper H, Schothorst AA, van der Leun JC. Risk evaluation of UVB therapy for psoriasis: comparison of calculated risk for UVB therapy and observed risk in PUVA-treated patients. *Photodermatol* 1986;3:271-83.
- Young AR. Carcinogenicity of UVB phototherapy assessed. *Lancet* 1995;345:1431-2.
- Pabsch H, Rutten A, Von Stemm A, Meigel W, Sander CA, Schaller J. Treatment of childhood mycosis fungoides with topical PUVA. *J Am Acad Dermatol* 2002;47:557-61.
- Gordon PM, Diffey BL, Matthews JN, Farr PM. A randomized comparison of narrow-band TL-01 phototherapy and PUVA photochemotherapy for psoriasis. *J Am Acad Dermatol* 1999;41:728-32.
- Boer J, Hermans J, Schothorst AA, Suurmond D. Comparison of phototherapy (UV-B) and photochemotherapy (PUVA) for clearing and maintenance therapy of psoriasis. *Arch Dermatol* 1984;120:52-7.
- Spuls PI, Bossuyt PM, van Everdingen JJ, Witkamp L, Bos JD. The development of practice guidelines for the treatment of severe plaque form psoriasis. *Arch Dermatol* 1998;134:1591-6.
- Hobbs J, Lebowitz M, Lim HW. Improving the safety profile of long-term PUVA therapy. *J Am Acad Dermatol* 1999;41:408-13.



37. Padilla HC, Lainez H, Pacheco JA. A new agent (hydrophilic fraction of *Polypodium leucotomos*) for management of psoriasis. *Int J Dermatol* 1974;13:276-82.
38. Jimenez D, Naranjo R, Doblare E, Munoz C, Vargas JF. Anapsos, an anti-psoriatic drug, in atopic dermatitis. *Allergol Immunopathol (Madr)* 1987;15:185-9.
39. Mohammad A. Vitiligo repigmentation with Anapsos (*Polypodium leucotomos*). *Int J Dermatol* 1989;28:479.
40. Spanish Ministry of Health. *Polypodium leucotomos* extract (Difur) in the treatment of psoriasis and atopic dermatitis. Assigned registration No. 55,757. January 5, 1982.
41. Godar DE. Light and death: photons and apoptosis. *J Invest Dermatol Symp Proc* 1999;4:17-23.
42. Koulu L, Jansen CT. Effect of oral methoxsalen photochemotherapy on human Langerhans cell number: dose-response and time-sequence studies. *Arch Dermatol Res* 1982;274:79-83.
43. Horio T, Okamoto H. Oxygen intermediates are involved in ultraviolet radiation-induced damage of Langerhans cells. *J Invest Dermatol* 1987;88:699-702.
44. Yuen KS, Halliday GM. Alpha-tocopherol, an inhibitor of epidermal lipid peroxidation, prevents ultraviolet radiation from suppressing the skin immune system. *Photochem Photobiol* 1997;65:587-92.
45. Baba T, Nakano H, Tamai K, Sawamura D, Hanada K, Hashimoto I, et al. Inhibitory effect of beta-thujaplicin on ultraviolet B-induced apoptosis in mouse keratinocytes. *J Invest Dermatol* 1998;110:24-8.
46. Punnonen K, Jansen CT, Puntala A, Ahotupa M. Effects of in vitro UVA irradiation and PUVA treatment on membrane fatty acids and activities of antioxidant enzymes in human keratinocytes. *J Invest Dermatol* 1991;96:255-9.
47. Ikai K, Danno K, Horio T, Narumiya S. Effect of ultraviolet irradiation on mast cell-deficient W/W<sup>v</sup> mice. *J Invest Dermatol* 1985;85:82-4.
48. Kumar JR, Haberman HF, Ranadive NS. Comparative studies on the tolerance to photoinduced cutaneous inflammatory reactions by psoralen and rose bengal. *J Photochem Photobiol B* 1997;37:245-53.
49. Hannuksela-Svahn A, Paakko P, Autio P, Reunala T, Karvonen J, Vahakangas K. Expression of p53 protein before and after PUVA treatment in psoriasis. *Acta Derm Venereol* 1999;79:195-9.
50. Stern RS. The risk of melanoma in association with long-term exposure to PUVA. *J Am Acad Dermatol* 2001;44:755-61.
51. Hannuksela-Svahn A, Sigurgeirsson B, Pukkala E, Lindelof B, Berne B, Hannuksela M, et al. Trioxsalen bath PUVA did not increase the risk of squamous cell skin carcinoma and cutaneous malignant melanoma in a joint analysis of 944 Swedish and Finnish patients with psoriasis. *Br J Dermatol* 1999;141:497-501.
52. Imlay JA, Linn S. DNA damage and oxygen radical toxicity. *Science* 1988;240:1302-9.
53. Cerutti PA. Prooxidant states and tumor promotion. *Science* 1985;227:375-81.
54. Cavanagh LL, Sluyter R, Henderson KG, Barnetson RS, Halliday GM. Epidermal Langerhans' cell induction of immunity against an ultraviolet-induced skin tumour. *Immunology* 1996;87:475-80.
55. Grabbe S, Bruvers S, Gallo RL, Knisely TL, Nazareno R, Granstein RD. Tumor antigen presentation by murine epidermal cells. *J Immunol* 1991;146:3656-61.
56. Halliday GM, MacCarrick GR, Muller HK. Tumour promoters but not initiators deplete Langerhans cells from murine epidermis. *Br J Cancer* 1987;56:328-30.
57. Strauss GH, Bridges BA, Greaves M, Hall-Smith P, Price M, Vella-Briffa D. Inhibition of delayed hypersensitivity reaction in skin (DNCB test) by 8-methoxypsoralen photochemotherapy. Possible basis for pseudo-promoting action in skin carcinogenesis? *Lancet* 1980;2:556-9.
58. Alcaraz MV, Pathak MA, Rius F, Kollias N, Gonzalez S. An extract of *Polypodium leucotomos* appears to minimize certain photoaging changes in a hairless albino mouse animal model: a pilot study. *Photodermatol Photoimmunol Photomed* 1999;15:120-6.
59. Gilchrist BA, Eller MS. DNA photodamage stimulates melanogenesis and other photoprotective responses. *J Invest Dermatol Symp Proc* 1999;4:35-40.
60. Wang XT, McCullough KD, Wang XJ, Carpenter G, Holbrook NJ. Oxidative stress-induced phospholipase C-gamma 1 activation enhances cell survival. *J Biol Chem* 2001;276:28364-71.
61. De las Heras ME, Ledo E, Gonzalez S, Rocamora A, Ledo A. An extract of *P. leucotomos* (Difur) as an adjuvant in PUVA therapy. *Med Cutan Iber* 1997;25:103-7.