Normalized UV induction of Langerhans cell depletion and neutrophil infiltrates after artificial UVB hardening of patients with polymorphic light eruption

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Abstract

UVB hardening has been widely used as a prophylactic treatment of polymorphic light eruption (PLE). Recent investigations have shown that in PLE patients Langerhans cells (LCs) and neutrophils display less migration from and to the epidermis after an intense UVB irradiation compared to controls.

Aim of this study was to investigate the effect of UVB hardening of PLE patients on their cell migratory responses after intense UVB exposure. Also, the relation between UVB provocation and the impact of UV hardening was investigated.

Ten PLE patients were recruited and UVB provocation testing was performed before entering the study. Among these patients, five developed PLE rash upon UVB provocation (‘UVB-P’) and the other five did not respond (‘UVB-NP’). Eleven age/sex-matched controls were included. Buttock skin of all included individuals was exposed to 6 minimal erythemal doses (MED) (TL12 lamps). Biopsies were taken after 24 hrs and 48 hrs, together with one control biopsy of unirradiated skin. Patients received total body UVB-hardening therapy consisting of 12 irradiations, on average rising from 10% to 140% of the initial MED in 6 weeks. Subsequently, MEDs were re-assessed and biopsies were taken from newly irradiated (6 MED UVB) and unirradiated buttock skin. Skin sections were stained for the presence of LCs, macrophages and neutrophils. The cross sectional area (in %) of positively stained cells within the epidermis was assessed from patients before and after hardening and compared with controls. Before therapy, epidermal LC depletion and neutrophil influx at 48 hrs after 6 MED were most significantly reduced in ‘UVB-P’ patients (p= 0.025 and p= 0.006 resp.) when compared to controls. ‘UVB-NP’ patients did not differ significantly from controls. After therapy, there were no longer any significant differences in the cell numbers among these three groups.

UVB hardening significantly improves UV-induced cell migratory responses in PLE patients. UVB provocability of PLE appears to be most strongly linked to reduced UVB-induced trafficking of LCs and neutrophils, and ‘UVB-P’ patients show normalisation of these responses after UVB hardening.
Introduction

Polymorphic light eruption (PLE) is the most common ultraviolet (UV)-induced dermatosis. It is characterised by subacute, itchy, erythematous papules, vesicles or plaques on sun-exposed skin sites. However, the rash in an individual patient is usually monomorphic. The skin rash can last from one day to several weeks. Most of the PLE patients are sensitive to broadband UVA sources, although a substantial number of patients also react to UV-B dominated sources.

In healthy individuals, ultraviolet radiation (UVR) can induce immunosuppression of reactivity to a contact sensitizer through different mechanisms that primarily affect antigenic presentation. The pathogenetic mechanism of PLE is still unknown, but the available data have led to the assumption that PLE is the result of a reduced UVB-induced immunosuppression allowing a delayed type contact hypersensitivity reaction to UV-induced neo-antigens. The putative antigen is yet to be discovered. Investigations focussed on the pathogenetic mechanisms of PLE have revealed that UV-induced cellular responses in PLE are aberrant. One of the cells that displays abnormal responses in PLE is the Langerhans cell (LCs), the primary antigen-presenting cell of the epidermis. In healthy individuals, LCs disappear from the epidermis after UVB irradiation. In PLE patients, it was found that LCs persisted in the epidermis after UVB irradiation as a result of lower migration. A disturbance in LCs behaviour (migration and antigen presentation) could therefore be a fundamental component of reduced UV-mediated immunosuppression.

Another cell of interest is the neutrophil, which migrates into the epidermis after UV-irradiation. Infiltrating epidermal neutrophils stain with antibodies against IL-4, CD11b and elastase. Kölgen et al. found that the IL-4+/anti-elastase+ neutrophils infiltrated the epidermis after UVB irradiation in lower numbers in PLE patients than in healthy controls. This finding has also recently been confirmed by Schornagel et al., who found a lesser dermal influx already at 6 hrs after UV exposure to 3 MED.

The third cell of interest is the macrophage. In healthy subjects, CD11b+ macrophage-like cells (which include both macrophages and neutrophils) infiltrate the dermis and epidermis after UVB exposure. CD11b+ and class II MHC+ monocyctic-macrophage-like cells play an important role in the induction of suppression and tolerance of delayed type hypersensitivity (DTH) after UVB irradiation. Kölgen et al. described CD11b+ neutrophils to display abnormal UV-induced cell responses in PLE. Since the macrophages within the CD11b population have the ability to express MHC class II on their surface, a possible role of these cells in disturbed UV-induced immunosuppression in PLE should also be taken into consideration.

One of the ways to prevent PLE is to treat patients with repeated low-dose UVB irradiations (UV hardening) in early springtime. UV hardening with artificial UV radiation was first described by van der Leun et al. in 1975, but has become
widely used since the early 80’s. Some patients become completely free of symptoms in the subsequent sunny season, while others benefit from this therapy by a slightly increased tolerance to sun exposure. Unfortunately, the benefits of UV hardening are only limited to one season, as the effect will be lost during wintertime. Apart from the normal skin reactions that occur after sun exposure, such as thickening of the epidermis and stimulation of pigmentation, the precise mechanisms that are responsible for the improvement of PLE after hardening therapy have never been investigated.

In this study we measured immunologically relevant UV-induced cellular responses in PLE patients before and after one course of UVB hardening, and compared them with those in healthy controls. Furthermore, we investigated whether the appearance of PLE rash, induced by UVB provocation, was connected with aberrant UV-induced cellular migration. We demonstrate that both the UV-induced Langerhans cell depletion and epidermal neutrophil infiltration in PLE patients are corrected after one course of UVB hardening. Most specifically, the ratio of epidermal neutrophils over macrophages in PLE differed significantly between controls and PLE patients and normalised after hardening. Moreover, we demonstrate that there is a difference in UV-induced epidermal leucocyte trafficking between PLE patients who develop a PLE rash upon UVB provocation (UVB-provocable; ‘UVB-P’) and patients who do not develop such a rash (UVB-non-provocable; ‘UVB-NP’). In the ‘UVB-P’ patients, UV-induced cellular migratory responses are more aberrant before hardening and improve more after the therapy.

Methods

Overall experiment
In brief, 13 PLE patients and 11 age/sex-matched controls were compared. To characterise the photosensitivity of the PLE patients, UVA and UVB provocation tests were performed on the forearms. Six MED UVB irradiation was applied to the buttocks and biopsies were taken after 24 and 48 hrs together with one control biopsy from unirradiated buttock skin. Subsequently, PLE patients underwent hardening therapy. Within two weeks after therapy, MEDs were re-assessed on the previously UV-treated buttocks and biopsies were taken after 6 MED UVB irradiation. Biopsies were kept frozen until further use. The numbers of epidermal immune cells in irradiated skins of PLE patients before and after hardening were compared with those from control biopsies. A distinction was made between UV-induced cellular reaction patterns of ‘UVB-P’ patients and ‘UVB-NP’ patients. The appearance of PLE rash during hardening treatment and the disappearance of rash at the end of hardening was recorded. For a detailed description of the methods, see below.

Patients
In early springtime of 2003, 13 patients with PLE entered the study for UV-hardening therapy (see table 1). Their diagnosis of PLE was based on a detailed history with typical symptoms in relation to UV exposure, seasonal dependency and healing without scars. In some cases the diagnosis was confirmed by photoprovocation. Serologic ANA and A-ENA testing were performed before the patients’ inclusion. Patients with positive anti-Ro/SSA and/or anti-La/SSB antibodies were excluded. Other exclusion criteria were pregnancy and lactation, the use of systemic immunosuppressive drugs, atopic history and chronic dermatoses such as psoriasis. Informed consent was obtained from patients and healthy volunteers. The medical ethical committee of the Leiden University Medical Center in Leiden approved the study.

Table 1. Patient characteristics

<table>
<thead>
<tr>
<th></th>
<th>PLE patients</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>age (y)</td>
<td>48 (24-64)</td>
<td>48 (25-64)</td>
</tr>
<tr>
<td>male ; female</td>
<td>1 ; 12</td>
<td>1 ; 10</td>
</tr>
<tr>
<td>UVB provocation</td>
<td>7 ‘UVB-P’</td>
<td>n.a.</td>
</tr>
<tr>
<td></td>
<td>6 ‘UVB-NP’</td>
<td>n.a.</td>
</tr>
<tr>
<td>UVA provocation</td>
<td>6 ‘UVA-P’</td>
<td>n.a.</td>
</tr>
<tr>
<td></td>
<td>7 ‘UVA-NP’</td>
<td>n.a.</td>
</tr>
<tr>
<td>therapy</td>
<td>11 UVB, 1 PUVA</td>
<td>n.a.</td>
</tr>
<tr>
<td>MED before therapy (mJ/cm²)</td>
<td>131 ± 59 (57-265)</td>
<td>122 ± 55 (76-270)</td>
</tr>
<tr>
<td>MED after therapy (mJ/cm²)</td>
<td>201 ± 65 (105-331)</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

Data as mean ± standard deviation and/or with range between brackets. Abbreviations: PLE: polymorphic light eruption; ‘UVB-P’ : development of PLE rash upon UVB provocation; ‘UVB-NP’ : no development of PLE rash upon UVB provocation; ‘UVA-P’ : development of PLE rash upon UVA provocation; ‘UVA-NP’ : no development of PLE rash upon UVA provocation; MED: minimal erythemal dose; n.a.: not analysed. NB: 3 PLE patients did not complete the whole study. Therefore, MEDs were tested and biopsies were taken from 13 patients before therapy and from 10 patients after therapy.

Phototesting procedure

For the determination of the minimal erythemal dose (MED), a phototesting device designed by Diffey et al. was purchased from PJ Saunders, Regional Medical Physics Dept, University Hospital of North Durham, Durham, United Kingdom. This device is made of 2 flexible metal foils, containing 10 mesh attenuators giving ten irradiance levels incremented in 26% steps and resulting in a 8:1 ratio from the highest to the lowest exposure. The device was slightly modified to maintain a constant distance of 3 mm from the skin. The MED tests were performed with broadband UVB TL 12 tubes (Philips Lighting, Eindhoven, the Netherlands). MEDs were first determined before the provocation test on the ventral side of the upper arm. Before 6 MED irradiation of the buttocks, MED was tested again on the contralateral and previously unexposed buttock skin. The device was placed on
the skin and irradiated for 4 minutes, giving an irradiance ranging from 30 to 240 mJ/cm². MED reading was done exactly 24 hrs later. MEDs of the PLE patients were within the normal range and were the same as the MEDs found in our control group. Re-tested MEDs after hardening were on average 53% higher than the initial MEDs. For the results of MED testing see table 1.
Chapter 4

**UVA and UVB provocation test**
All PLE patients were provoked with broadband UVA (Philips Cleo Performance lamps) and UVB (Philips TL12 lamps) on the flexor side of the forearms with a maximum of 3 daily exposures. The dose of UVB given per daily irradiation was one MED. The dose from the UVA lamp was standardised to 20 J/cm². A positive provocation was defined as the development of clearly visible and palpable erythematous papules or vesicles on the irradiated field. Seven patients had a positive UVB provocation and six had negative outcome. These will be further referred to as ‘UVB-P’ and ‘UVB-NP’ patients, respectively. Six patients exhibited a positive UVA provocation (‘UVA-P’).

**UVB challenge of the buttocks**
After reading the MED on the buttocks, a field of 2 x 2 cm on unexposed buttock skin was irradiated with 6 MED. Biopsies were taken from the irradiated buttock skin 24 hrs and 48 hrs later, together with one biopsy from unirradiated skin. Eleven age and sex-matched healthy individuals were also exposed to 6 MED and biopsies were taken at the same intervals. Within two weeks after finishing the hardening therapy, MED testing and 6 MED challenge in PLE patients were repeated. Biopsies were again taken after 24 and 48 hrs, together with one control biopsy from unirradiated skin. All the biopsies were embedded in O.C.T. compound (Tissue-Tek, Sakura, Zoeterwoude, the Netherlands), snap frozen in liquid nitrogen and stored at -80 °C until further processing.

**Hardening therapy**
From March until May 2003, eleven PLE patients received whole body UVB hardening therapy. One patient refused UVB therapy and received psoralen plus UVA (PUVA) since she was used to be treated successfully with PUVA. However, the patient was still willing to participate and remained included. This particular patient presented with the same PLE symptoms as the whole group and was both ‘UVB-P’ and ‘UVA-P’. One patient did not show up for hardening. UVB-treated patients were irradiated 12 times in 6 weeks, using a standard protocol. Patients with skin types I and II received a starting dose of 0.01 J/cm² and patients with skin types III and IV 0.02 J/cm². If the patients did not develop any skin rash, dose increments of 20% were given. Most patients ended their hardening course with a dose of 1.4 times their initial MED. If patients developed a rash (erythema and itch and/or papules) during therapy, one irradiation session was either skipped or the previous dose was repeated, depending on the severity of the rash.

**Antibodies and immunohistochemical detection**
The primary antibodies we used were monoclonal CD1a antibody, clone NA 1:34 (DAKO diagnostics BV, Amsterdam, The Netherlands) diluted 1:60 in PBS, antineutrophil elastase antibody (clone NP57) diluted 1:100 in PBS and mouse anti-human monoclonal CD68 antibody (clone EBM11) diluted 1:100, purchased from
DAKO. Biotinylated goat anti-mouse immunoglobulin, diluted 1:200 (Vector, Burlingame, CA, USA) was used as the second antibody. As a detecting reagent, horseradish peroxidase (HRP)-conjugated avidin-biotin complex (DAKO, diluted 1:100) was used. Frozen skin sections (6μm) were immunostained as previously described. 25

Quantification of epidermal immune cells
The epidermis was photographed at a magnification of 10 x 20 using a light microscope (Nikon Eclipse E-800, Tokyo, Japan) with a standard eye piece. A digital image analysis program (Image-Pro Plus software, MediaCybernetics, Silver Spring, MD USA), was used for the detection of epidermal stained cells. The area covered by red stain was expressed as a percentage of the area of interest (manually contoured epidermis) and imported in a file of Excel 2000 for Windows (Microsoft, Redmond, WA, USA). The whole epidermis was photographed resulting in three to five pictures per biopsy. Epidermal areas around follicular orifices were avoided, because they serve as epidermal reservoirs of Langerhans cells. 26

Statistical analysis
For statistical analysis, SPSS 10 for Windows (SPSS Inc., Chicago, IL, USA) was used. To compare the means, a student’s T-test was performed. For comparison of the effect of UVB at different times in different patient groups, a Mann-Whitney U-test was utilised. To compare epidermal cell stains before and after hardening therapy in individual patients, a non-parametric test for 2 dependent samples was performed. Results were considered significant if p < 0.05.

Results
The unirradiated control biopsies from both patients and controls displayed similar numbers of LCs, neutrophils and macrophages.

UV-induced immune cell migration before hardening therapy
Our experiments confirmed the phenomenon of diminished LC depletion in the epidermis of PLE patients after a single high UVB exposure. As depicted in figure 1A, the number of epidermal LCs steadily decreased in the irradiated skins of healthy controls after 24 and 48 hrs. In PLE patients, more LCs persisted in the epidermis 24 hours after 6 MED irradiation than in controls (resp. 2.1% vs 0.9%, p= 0.002) but not after 48 hrs. This effect was more pronounced in ‘UVB-P’ PLE patients (see figure 1B). In this particular group of patients, the higher number of epidermal LCs was present both 24 and 48 hrs after the irradiation when compared to controls (resp. 2.3% vs 0.9%, p= 0.005 and 1.8% vs 0.6%, p= 0.025, see figure 1B). Moreover, the number of LCs in ‘UVB-P’ patients at 48 h was
significantly different from ‘UVB-NP’ patients (p= 0.045) and the ‘UVB-NP’ did not differ from controls.

As shown in figure 2A, epidermal neutrophil infiltration after 6 MED was less in PLE patients, but the difference with controls did not reach statistical significance (p= 0.16). However, when we focussed only on ‘UVB-P’ PLE patients, significantly lower neutrophil numbers were observed at 48 hrs compared to controls (resp. 1.3% vs 4.2%; p= 0.006, see figure 2B). Although also low at 24 hrs after 6 MED, neutrophil numbers in ‘UVB-P’ PLE did not reach statistical significance (p= 0.051) at that time point. The number of neutrophils in ‘UVB-P’ PLE patients at 48 hrs after 6 MED were significantly lower than that in ‘UVB-NP’ patients (p= 0.019).

Figure 3A shows similar increase in CD68+ macrophages in controls and PLE patients after 6 MED. [Although, macrophage infiltrates at 48 hrs in ‘UVB-P’ patients did not differ from controls, they were somewhat, but significantly less than in ‘UVB-NP’ patients (p= 0.015)].

UV-induced immune cell migration after hardening therapy

As shown in figure 1A, 6 MED irradiation after hardening resulted in lower LC numbers than before hardening in PLE patients and they were no longer significantly different from controls. This effect was especially pronounced in ‘UVB-P’ patients (see figure 1B). The unirradiated skin of PLE patients after hardening displayed similar LC numbers as healthy controls.

As represented by the grey bars in figure 2B, hardening caused an increase in infiltrating neutrophils after 6 MED of UVB in PLE patients. This improvement was especially observed in ‘UVB-P’ PLE patients. In this group, neutrophil infiltration was significantly increased compared to untreated ‘UVB-P’ patients (p= 0.043) and was no longer different from controls.

Before hardening we did not observe abnormalities in infiltrating macrophages in PLE. After hardening, no changes in the responses were observed except from a slightly lower number of epidermal infiltrating macrophages in hardened PLE patients compared to the untreated patients, but this was not a significant decrease (see figure 3A).

Ratios of infiltrating neutrophils over macrophages are different in PLE after UVB overexposure and normalise after hardening therapy

Ratios between epidermal neutrophils and macrophages were calculated in PLE patients and controls. Before the therapy, the ratios in PLE patients (0.9) were significantly different from those of controls (3.8, p < 0.05), but only after 48 hrs. Hardening therapy caused a clear increase in the epidermal neutrophil/macrophage ratio in PLE, which no longer differed significantly from controls (2.0 vs control 3.8, p= 0.19).
Figure 1. A: Epidermal Langerhans cells (LCs) after 6 MED UVB in PLE patients and healthy controls before and after hardening therapy. B: LCs in patients who develop a PLE rash upon UVB provocation ("UVB-P") and in controls.

A

B
Figure 2. A: Epidermal neutrophils after 6 MED UVB in PLE patients and healthy controls before and after hardening therapy. B: Neutrophils in patients who develop a PLE rash upon UVB provocation (‘UVB-P’) and in controls.

A

B
Figure 3. A: Epidermal macrophages after 6 MED in PLE patients and healthy controls before and after hardening therapy. B: Macrophages in patients who develop a PLE rash upon UVB provocation (‘UVB-P’) and in controls.
Figure 4. Development of rashes caused by provocation test or by UVB hardening.

Abbreviations: ‘UVB-P’: development of PLE rash upon UVB provocation; ‘UVB-NP’: no development of PLE rash upon UVB provocation

Discussion

Our results confirm the findings of Kölgen et al. that more epidermal LCs persist in the epidermis of PLE patients after a UVB overdose. After hardening we observed a normalised depletion of epidermal LCs in PLE at 24 and 48 hrs, most likely as a result of improved UV-induced LC migration in PLE. These effects were more pronounced among ‘UVB-P’ PLE patients.

Our experiments also confirmed the findings of lower infiltrating neutrophil numbers in PLE epidermis after UVB exposure. In the present study and in published work of Kölgen et al. a challenge of 6 MED was used, while a 3 MED exposure described by Schornagel et al. was also effective to elicit decreased neutrophil skin infiltration in PLE. In our case, this phenomenon was restricted to the group of ‘UVB-P’ patients and it normalised after the hardening.

A novel finding was the lower ratio between epidermal infiltrating neutrophils and macrophages in UV irradiated skin of PLE patients compared to controls (0.9 vs 3.8). This ratio reached normal values after the hardening therapy, and was independent of the UVB-provocability of PLE patients. The change of this ratio in PLE patients after hardening, is mostly due to the increase of neutrophil infiltration. The macrophages display a more or less stable UV-induced infiltration in PLE. This ratio indicates a disturbed balance among CD11b+ immune cells after UV irradiation in PLE patients, and could be a useful early indicator of PLE, which requires further confirmatory studies dealing with interaction between neutrophils and macrophages in the skin of PLE patients.

Another intriguing finding was the difference in UV-induced cellular reactivity between ‘UVB-P’ and ‘UVB-NP’ patients. Some patients exhibited positive provocation reaction only during the provocation test while others only
during irradiations for hardening or sometimes at both or neither occasions. To illustrate the presence of rash at any stage of phototesting or phototherapy a flowchart was produced (see figure 4). From this flowchart we can learn that the development of PLE rash upon UVB provocation does not necessarily mean a development of rash during therapy and vice versa. We could not find any correlation between the size or nature of UV-induced cellular infiltrates in PLE and the appearance of rash during hardening. Only distinguishing the patients according to the development of PLE rash upon UVB provocation has proved helpful to trace larger deviations in cellular responses.

How do these findings fit into the hypothesis of reduced immunosuppression after UVR in PLE? In both human in vitro models and murine in vivo adoptive transfer studies, induction of tolerance to a contact sensitiser applied on the skin receiving a single UV dose is due to the infiltration within the UV-irradiated skin of class II MHC+ CD11b+ monocyte/macrophage-like cells. Our finding of the normalised LC depletion, more neutrophil infiltration and an increased neutrophil/macrophage ratio after 6 MED irradiation in UVB-hardened PLE patients indicates a normalisation of the response. We infer that this normalisation in epidermal leucocytes responses after UV-hardening stems from a rebalancing of inflammatory and immunosuppressive signals (cytokines patterns), thereby improving the course of the disease.

Based on our present findings, it may be important to distinguish ‘UVB-P’ patients as a subcategory of PLE with more pronounced aberrant cellular responses to UVB exposure. As evidenced by the present hardening therapy, these distinguishing features of ‘UVB-P’ patients are easily lost after a series of mild UV exposures. Our findings are only directly valid for patients with a severe form of PLE who seek care of a university clinic. Although we expect the basic pathogenetic mechanisms to be similar in the general group of PLE patients, the effects will be difficult to detect in milder forms of PLE, because one has to provoke them in their most susceptible period.

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