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Reduced IL1Ra/IL1 ratio in UVB-exposed skin of patients with polymorphic light eruption

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submitted

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Abstract

Polymorphic light eruption (PLE) is a putative delayed type allergic reaction to (solar) UV exposure. Inadequate immune suppression after UVB-induced sunburn appears to be associated with reduced trafficking of Langerhans cells (LCs) out and neutrophils into the epidermis of PLE patients. Since underlying mechanisms are unknown, we investigated whether pro-inflammatory and chemotactic cytokines are differentially expressed in UVB-irradiated skin of PLE patients and healthy controls.

IL-1α, IL-1β, IL-1Ra, IL-4, IL-8, TNF-α, MIP-1α, MIP-1β and MCP-1 were measured by flow fluorimetry of multiplexed sandwich-ELISAs on beads in suction blister fluid raised 16 hrs after exposure to 0, 3, and 6 minimal erythema doses of UVB. Cytokine levels in unirradiated versus UVB-irradiated skin of PLE patients versus healthy controls were measured.

Cytokine levels in unirradiated skin appeared similar in both groups, except for IL-1Ra, which was significantly lower in PLE patients (p < 0.05). IL-8 and TNF-α levels increased strongly upon UVB irradiation in both groups. No differential shifts in cytokine profiles were found that could explain a reduced trafficking of LCs and neutrophils in PLE patients. Although UVB irradiation caused significant increases in IL-1α in both groups, the levels of IL-1α and IL-1β were 2 fold higher in the PLE group than in the control group. Accordingly, the ratios of IL-1Ra over IL-1α and over IL-1β were overall lower in the skin of PLE patients.

The shift of cytokines in UVB-irradiated skin of PLE patients reveals an amplified early pro-inflammatory cytokine response, which may contribute to the allergic reaction to UVB.
Introduction

Polymorphic light eruption (PLE) is the most common idiopathic photosensitivity disorder, having a prevalence among the European population of 15-20%. The disease presents itself with papules, vesicles or plaques, some hours to two days after sun irradiation on exposed skin areas. The pathogenesis of PLE is still unclear, but it is thought to be a delayed type hypersensitivity response against putative ultraviolet (UV)-induced de novo antigens. The skin reaction is probably allowed to develop because of an inadequate UV-induced immune-suppression in PLE. Functional proof of this inference has recently been provided in experiments that showed diminished UV-induced suppression of contact hypersensitivity in PLE patients. In earlier studies, we found that after a single high dose of UVB radiation much less Langerhans cells (LCs) were depleted from the epidermis of PLE patients compared to healthy controls. In addition, infiltration of the dermis and epidermis with neutrophils, which normally takes place after UVB irradiation, was found to be reduced in PLE patients. Remarkably, these aberrations of immune cell migration were more pronounced in those PLE patients who specifically developed PLE lesions after UVB provocation, compared to patients who react only to UVA sources, or not at all.

Leukocyte migration in the skin is regulated by a complex system of adhesion molecules on endothelial cells and on leukocytes, and by the migratory activity of leukocytes in reaction to chemotactic factors released from the target tissue. These chemotactic factors comprise chemokines and other cytokines that guide different types of leukocytes. The combinatorial diversity in responsiveness to chemokines ensures proper tissue targeting by distinct leukocyte subsets under normal and inflammatory conditions.

Only a few investigations have been published on the cytokine levels of unirradiated and UV-irradiated skin of healthy humans in vivo. Information on cytokine levels in UV-irradiated skin of PLE patients in vivo is not only limited but also contradictory. One immunohistochemical study showed fewer cells expressing interleukin 4 (IL-4), tumour necrosis factor-α (TNF-α) and IL-10 after UVB irradiation in skin sections of PLE patients compared to healthy controls. Another group was not able to confirm these differences, although in this study mRNA levels were measured.

LC migration from the epidermis is induced and enhanced by cytokines like TNF-α and IL-1β and IL-1α which are up regulated in human epidermis after UVB irradiation. Chemokines like IL-8 (CXCL8) and MIP-1α (CCL3) are chemotactic for neutrophils (through activation of CXCR1/2 and CCR-1 respectively) and could be likely candidates for the recruitment of these cells in UVB-exposed skin.

In this study we tested the hypothesis that the development of PLE may be attributed to an imbalance between inflammation promoting versus down regulating responses. In the UV-induced allergic skin reaction seen in PLE the
cytokine profile seems to be skewed toward inflammation. The reduced post-UV trafficking of LCs and neutrophils in PLE patients is indicative of an inadequacy in the immunosuppressive branch of the response. The basic premise for this study is that all leukocyte migratory responses are driven by cytokines, and therefore, any disturbance in the responses may be reflected in cytokine profiles. We focussed on early inflammatory cytokines (IL-1α, IL-1β, and antagonist IL-1Ra), on cytokines related to the migration of LCs (TNF-α, IL-1α, IL-1β) and neutrophils (IL-8, MIP-1α), and on chemokines more generally involved in leukocyte migration (MIP-1β, MCP-1, RANTES). IL-4 was included as marker of Th2 skewing (i.e. suppression of Th1-cellular immunity), which was found to be increased after UVB irradiation. In this study we demonstrated no differences in cytokine profiles at baseline or after UVB exposure between PLE patients and healthy controls, except for significantly reduced levels of IL-1Ra and increased levels of IL-1α and IL-1β in response to UVB in the skin of PLE patients. Resulting reduced ratios of IL-1Ra over IL-1α and IL-1β in PLE-prone individuals may contribute to the pro-inflammatory condition colloquially called ‘sun allergy’.

Methods

Patients and healthy controls
This study was conducted during a period of the year when ambient UV radiation was low in order to minimize the interference of sunlight with the artificial UVB exposure used in the study. The diagnosis of PLE was based on the (a) characteristic course of the disease in relation to sun exposure, (b) morphology of skin rash, (c) exclusion of other photosensitivity disorders and the absence of SSA and SSB antibodies. All PLE patients underwent UVB provocation tests on the flexor side of the forearm as previously described. Only patients in whom PLE rash was elicited by UVB provocation were included in this study, since previous studies of our group had shown that reduced LC and neutrophil migration upon UVB irradiation was most pronounced in this particular group of PLE patients. Our study included 1 male and 5 females with PLE (mean age 47.7 yrs; range 33-58). Six age- and sex-matched controls were recruited (mean age 49.2 yrs, range 32-63). Exclusion criteria were pregnancy and lactation, atopic history, the use of photosensitising drugs, a history of auto-immunity and the use of sun beds or sun bathing within 3 months before the participation in this study. The study was approved by the medical ethics committee of the Leiden University Medical Centre and all clinical investigation was conducted according to declaration of Helsinki principles. All patients gave their written informed consent before they took part in the study.

Photo testing and irradiation
In line with previous and comparable studies the minimal erythema dose (MED) was determined on non-UV adapted, non-lesional buttock skin with broadband
UVB TL 12 tubes (Philips Lighting, Eindhoven, the Netherlands)\textsuperscript{6,7}, using a device with a graded series of perforated holes.\textsuperscript{6,24} The mean MED in the PLE group was 104 mJ/cm\textsuperscript{2} (range 60-151 mJ/cm\textsuperscript{2}) and in the matched controls 112 mJ/cm\textsuperscript{2} (range 60-265). After determination of the MED, patients and healthy individuals were irradiated with 3 and 6 MED (two spots per dose with a diameter of 2 cm each) on previously unexposed buttock skin. These doses were intended to generate firm erythema, but no PLE lesions, consistent with prior studies, to observe the early cytokine responses to UV in the skin of PLE patients.\textsuperscript{6,7} In this study, no PLE lesions were observed after the irradiation.

**Blister formation**

Sixteen hours after 3 and 6 MED irradiation suction blisters were raised. Blisters with a diameter of 10 mm were raised in each of the irradiated fields and in unirradiated buttock skin (6 blisters in total per individual). To this end, cups with 10 mm openings were attached to the skin, and under-pressurized by 200 mmHg. The blister fluid was collected with a syringe and stored at -20\textdegree C; blister volumes collected ranged between 50 and 150 µl. The average time to raise full blisters was 2 hrs, but the blisters developed faster in the skin after 6 MED irradiation than after 3 MED or without UVB exposure. There was no difference in blister appearance time between patients and healthy subjects.

**The multiplex assays**

For cytokine measurement, we utilised the Luminex Lab xMAP technology 100 system (Luminex Corporation, Austin, TX) which uses specific antibody-coupled microspheres as solid support for a sandwich immunoassay. The fluorescent microspheres are subsequently analysed in a flow cytometer.\textsuperscript{25} Multiplex bead kits were purchased from BioSource International, Inc. (Camarillo, CA, USA), including cytokine antibodies directed at IL-1\textalpha, IL-1\beta, IL-1Ra, IL-4, IL-8, TNF-\alpha, MIP-1\alpha, MIP-1\beta, MCP-1, and RANTES and reagents for human Multiplex Luminex™. Each multiplex assay was performed according to the manufacturer's specifications. Calibration curves for each cytokine were generated by titration in PBS using the reference cytokine concentrations supplied by the manufacturer (calibration in spiked blister fluid was practically impossible, because of scarcity of blister fluid and because of ethical considerations). Accuracy and reproducibility of the standard was tested by adding multiple series of titration standards, 2 preceding the blister samples and 1 after, set up in reverse order at the end of the 96-wells plate. For each standard, the accuracy and precision of an analytical run was considered to be acceptable if the results of repeated standards agreed within a margin of 25%.

The Luminex system was able to detect IL-1\textalpha, IL-1\beta, IL-1Ra, MIP1-\textalpha, MIP-1\beta, MCP-1, IL-8, and TNF-\alpha in blister fluid samples, but the inter-individual variability of cytokine concentration was large. In the majority of samples, blister fluid failed to produce any detectable fluorescence signals for RANTES.
Concentrations of IL-4 were at the lower end of the calibration curves (< 5 pg mL\(^{-1}\)) and were therefore regarded as unreliable. Raw data (mean fluorescent intensity) were analysed by STarStation (Applied Cytometry Systems, Sheffield, UK) to obtain concentration values. Ratios of the functionally competitive cytokines IL-1Ra versus IL-1\(\alpha\) and IL-1\(\beta\) were calculated in every blister fluid sample.

**Comparison between conventional ELISA and Luminex multiplex**
Results obtained with the Luminex-based method were compared with the results from the same blister fluid specimens analysed by a solid phase sandwich ELISA kit for IL-8 and an ultra sensitive ELISA kit for TNF-\(\alpha\) (both from BioSource International, Inc. Camarillo, CA). The assays were carried out according to the instructions supplied by the manufacturer, again with calibration by titration standard in PBS.

**Statistical analysis**
Unpaired and paired and non-parametric tests (Mann-Whitney-U tests and Wilkinson's signed rank test, respectively) were performed to compare patients with controls at different UVB dose levels, using SPSS 12 for Windows (SPSS Inc., Chicago, IL, USA). We carried out trend analyses on UVB dose and simultaneously tested for group differences by mixed linear model analysis on log-values of the cytokine concentrations. Results were considered significant if \(p < 0.05\).

**Results**

**Reduced IL-1Ra levels in unirradiated skin of PLE patients.**
The unirradiated skin of healthy controls showed low levels of IL-1\(\alpha\), IL-1\(\beta\), IL-8 and MIP-1\(\alpha\) with average concentrations between 10 and 60 pg mL\(^{-1}\) (see table 1). MIP-1\(\beta\), MCP-1 and TNF-\(\alpha\) levels were between 220 and 630 pg mL\(^{-1}\). IL-1Ra concentration was much higher with concentrations of 36 \(\pm\) 9.5 ng mL\(^{-1}\).

In skin of PLE patients the concentration of IL-1Ra was significantly lower than in healthy controls (22 \(\pm\) 9 vs 36 \(\pm\) 9.5 ng mL\(^{-1}\) respectively, \(p= 0.05\)), and IL-1\(\alpha\) showed a much larger spread in PLE patients up to concentrations 8 fold higher than in healthy controls, but this difference was however not significant. Other cytokine concentrations in unirradiated skin were similar in both groups.

**Increased IL-1\(\alpha\) and IL-1\(\beta\), levels in UVB-irradiated skin of PLE patients compared to healthy individuals.**
In healthy controls, concentrations of IL-1\(\alpha\), IL-1\(\beta\), IL-1Ra, IL-8, TNF-\(\alpha\), MIP-1\(\alpha\), MIP-1\(\beta\) and MCP-1, as measured with the Luminex assay, were not significantly changed at 16 hrs post-irradiation when comparing non-irradiated skin with skin exposed to 3 and 6 MED of UVB. Although IL-8 and TNF-\(\alpha\) showed a dose-dependent increasing trend, this was not significant. Like the healthy controls,
cytokine levels did not significantly change in PLE patients upon UVB exposure, with the exception of IL-1α, which was significantly increased after UVB irradiation (about 3-fold after 3 and 6 MED; both p= 0.043). Further, the average IL-1α concentration in UVB-exposed skin of PLE patients was significantly higher than in irradiated skin of healthy controls (p= 0.02).
Table 1. Cytokine concentrations in suction blister fluid in healthy individuals and PLE patients before and after UVB irradiation.

<table>
<thead>
<tr>
<th></th>
<th>PLE patients (n=6)</th>
<th>Healthy individuals (n=6)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Luminex</td>
<td>ELISA</td>
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<tr>
<td><strong>Cytokines</strong></td>
<td><strong>No UV</strong></td>
<td><strong>3 MED</strong></td>
</tr>
<tr>
<td>IL-1α</td>
<td>29 (37)</td>
<td>88 (76)</td>
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<tr>
<td>IL-1β</td>
<td>15 (12)</td>
<td>22 (11)</td>
</tr>
<tr>
<td>IL-1Ra</td>
<td>22 (9)</td>
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<td>MIP-1α</td>
<td>92 (99)</td>
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<tr>
<td>MIP-1β</td>
<td>367 (231)</td>
<td>526 (175)</td>
</tr>
<tr>
<td>MCP-1</td>
<td>434 (308)</td>
<td>598 (421)</td>
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<tr>
<td>IL-8</td>
<td>68 (97)</td>
<td>296 (118)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>114 (148)</td>
<td>769 (804)</td>
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</table>

**Values are given as means (SD), in pg mL⁻¹, except for IL-1Ra, which is expressed as ng mL⁻¹. MED: minimal erythema dose.**

When measured with ELISA, IL-8 and TNF-α levels increased significantly upon UVB-irradiation in the two groups (an average 9-fold and nearly 5-fold increase, respectively; p < 0.05, see figure 1) without significant differences between both groups. Because the results in figure 2A through F suggest trends in the IL-1 family of cytokines, albeit with a large spread in data points, we performed a trend analysis on the dose effects in both groups simultaneously, thus including all data points of one cytokine in one analysis.

IL-1α increased upon UV irradiation in both controls and PLE patients (by about 30% per MED; p= 0.002). This cytokine was significantly higher in the PLE group over the whole dose range (by a factor 2.2; p= 0.03). For IL-1β, there was no significant dose-dependent UVB effect, but this cytokine was significantly higher in the PLE group over the whole dose range (by a factor 2.2; p= 0.004). Analysis of IL-1RA indicated a UV dose dependency difference between the two groups, with a tendency to increase in PLE and to decrease in healthy controls.

**Low ratios of IL-1Ra over IL-1α and over IL-1β in PLE patients**

Because of the functionally competitive relationship between IL-1Ra versus IL-1α and IL-1β, ratios of IL-1Ra over IL-1α and over IL-1β were calculated. 12,26-28

The ratio of IL-1Ra over IL-1α decreased significantly with increasing UVB dose in PLE patients and controls (by 20% per MED; p= 0.002). Further, this ratio was significantly lower in the PLE group (by 60%; p= 0.015), compared to the
control group. The ratio of IL-1Ra over IL-1β was not significantly influenced when monitored.

Figure 1. IL-8 (A and C) and TNF-α (B and D) in blister fluid of UVB irradiated skin of PLE patients (A and B) and controls (C and D) measured with ELISA.

When measured with ELISA, a significant increase of IL-8 after 3 and 6 MED irradiation was found in PLE patients ($p= 0.028$, 1A) and also in controls ($p= 0.046$, 1C). The increase of TNF-α is only significant after 6 MED in PLE ($p= 0.028$, 1B); and after both 3 and 6 MED in controls ($p= 0.043$, 1D). A comparison of IL-8 and TNF-α between PLE patients and controls did not meet statistical significance.

at each individual UVB dose, but was significantly lower in PLE patients when analyzed over the whole dose range (by 60%; $p < 0.001$).

**Discussion**

In the present study we have investigated in situ the UVB-induced cytokine response of a selected set of pro-inflammatory and chemotactic cytokines in PLE patients and healthy controls in order to determine a possible role for any of these factors in the pathogenesis of PLE. Despite considerable variation in cytokine levels between individuals, we found a clear pro-inflammatory shift in the
balance between IL-1α and IL-1β versus IL-1Ra in the skin of PLE patients. IL-1Ra appeared to be expressed at significantly lower levels in the unirradiated skin of these patients compared to
Figure 2. IL-1α (A and D), IL-1β (B and E) and IL-1Ra (C and F) in blister fluid of PLE patients (A, B and C) and controls (D, E and F) after UVB irradiation, measured with Luminex.

Cytokines were measured in suction blister fluid of unirradiated and UVB irradiated skin of PLE patients and healthy control subjects, 16 hrs after irradiation. IL-1α is upregulated from 0 to 3 and 6 MED in PLE (p= 0.043, 2A) and is higher when compared to controls after 3 MED (p= 0.02, 2A vs 2D). IL-1β after 3 MED is higher in PLE than in controls (p= 0.016, 2B vs 2E). IL-1Ra in PLE patients at 0 MED is lower than in controls (p= 0.05, 2C vs 2F).
healthy individuals. A strong rise in IL-1α after UVB exposure occurred in PLE patients, and ratios of IL-1Ra over IL-1α and IL-1β after UV exposure were systematically lower in PLE patients than in healthy controls. Although levels of IL-1β did not change significantly upon UVB exposure, levels were significantly elevated in PLE when compared to healthy controls. Because recent investigations have shown a reduced epidermal LC depletion and neutrophil infiltration in the skin of PLE patients after a single high dose of UVB radiation\(^6;^7;^10\), we focused on chemotactic cytokines involved in the migration of LCs and neutrophils. However, no aberrant levels of these cytokines were found in the cytokine profile of PLE patients. TNF-α and IL-8 levels increased upon UVB exposure in PLE patients and control subjects without significant differences between both groups, and no significant UVB-induced changes in MIP-1α, MIP-1β and MCP-1 levels were observed.

To date only limited data are available on cytokine levels in suction blister fluid obtained from healthy individuals, whereas our study is the first report on cytokines in suction blister fluid of PLE patients. Concentrations of IL-1α, IL-1β and IL-1Ra of unirradiated skin of healthy controls\(^12;^29\) and of IL-8 and TNF-α levels after UVB irradiation\(^9;12-14\) reported in earlier studies are consistent with our observations in the present study. However, the unchanged levels of IL-1β and IL-1Ra in healthy controls do not correspond with a previously described increase after 3 MED solar simulated radiation.\(^12\) We did not confirm the increased ratio of IL-1Ra/IL-1 after UVB irradiation in healthy individuals as reported by Terui et al.\(^28\) However, in this study a considerably different time frame (days in stead of hours after UV) and method of sample collection (isolation from tape stripped stratum corneum in stead of blister fluid) was used.

In a study on cytokine expression in cryostat sections from PLE skin, a lower number of cells expressing TNF-α was detected compared to normal skin.\(^16\) However, we were not able to confirm similar differences in blister fluid. This discrepancy might stem from differences in cell-contained versus interstitial freely available cytokines and chemokines.

Large inter subject variation in levels of cytokines measured in blister fluid appears common to all studies. The differences in absolute concentrations between different published studies must be largely attributable to differences in standards supplied by manufacturers. The use of Luminex technology enabled us to analyse ten different cytokines in a single small sample volume, and this increased the chance to detect a difference in one of the many cytokines that are involved in LC and neutrophil migration. However, when comparing Luminex results with conventional ELISA, concentrations of IL-8 and TNF-α differed substantially between the methods despite the use of antibodies from the same manufacturer. Although absolute values differed, the overall trends were similar between the two methods (i.e., relative measurements obtained with each method appear to be reliable; although the micro-beads surface fluorescence in
the Luminex may have introduced larger variations than the macro-surface to volume substrate reaction in the conventional ELISA). Unfortunately, the number of investigated patients in our study was by necessity small: a result of the high burden to participants in this study. Since our patients had been selected on the basis of previous UVB-reactivity, the number of suitable patients was further reduced.

Our results indicate a pro-inflammatory skewed response in PLE skin upon UVB irradiation, but these results offer no explanation of reduced leukocyte migration in PLE patients. Our observations of a higher IL-1β level and similar TNF-α levels in PLE patients compared to controls, are seemingly in conflict with lower LC migration in PLE.18 Aberrant epidermal neutrophil infiltration in PLE patients after UVB irradiation could not be attributed to IL-8, MIP-1α, MIP-1β or MCP-1, as we did not observe any differences in these chemokines between patients and controls. Furthermore, as investigated by another group, neither the intrinsic chemotactic activity of peripheral blood neutrophils towards IL-8 and C5a, nor the expression of ICAM-I and E-selectin on endothelial cells could explain the migratory differences between PLE patients and controls. 10

The higher IL-1α and -1β concentrations and lower ratios of IL-1Ra over IL-1α and over IL-1β indicate that the pro-inflammatory response upon UVB exposure will run stronger in PLE patients than in healthy individuals. The mechanisms underlying the abnormal LC and neutrophil migration in UVB-exposed skin remain as yet unexplained. The observed cytokine responses in irradiated PLE skin seem not to be related to the disturbed migration of these cells. We conclude that PLE patients who are susceptible to UVB provocation show higher IL-1 and lower IL-1Ra levels before and after UVB exposure when compared to healthy controls. This implies an enhanced pro-inflammatory skewing, which may well contribute to the development of PLE.

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