The puzzle of polymorphous light eruption

patients and pathogenesis

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Chapter 1

General Introduction

PREFACE

Polymorphous light eruption (PLE) is a common idiopathic photodermatosis. Research has focussed on the clinical appearance of the disease, the provocation of PLE by phototesting procedures and the therapeutic options. No general accepted definition and diagnostic phototesting protocol for PLE exists. Only recently, studies were performed on severity assessment in PLE by subjective questionnaires and objective interpretation of phototesting results (1).

Rather few studies have focussed on the pathogenesis of PLE and presented new leads. The established hypothesis describes a DTH-like response to ultraviolet (UV)-induced neoantigens, because of the clear delay between sunlight exposure and the onset of skin lesions (2-4). However, the neoantigens responsible remain elusive. Another, more recent theory claims the role of singlet oxygen in UVA mediated inflammation and PLE (5).

Overall, PLE remains an intriguing puzzle. In this thesis it is pursued to put some pieces of the puzzle in the right place.

INTRODUCTION

POLYMORPHOUS LIGHT ERUPTION

Clinical characteristics

In 1798 Robert Willian described an erythemato-vesicular eruption on exposed skin areas in the summer season due to irritation of direct sunlight and named it 'eczema solare'. This is the first description of what is nowadays named polymorphous light eruption (PLE) (6). The term PLE was introduced in literature by Rasch in 1900 (7). PLE belongs to the idiopathic photodermatoses together with actinic prurigo, hydroa vacciniforme, chronic actinic dermatitis (CAD) and solar urticaria (8,9). They are separated from other skin disorders which can be aggravated by sun exposure (e.g. lupus erythematosus (LE), dermatomyositis, atopic dermatitis (AD) and psoriasis). Some studies have claimed subsets of PLE, like benign summer light eruption (10) and polymorphic light eruption sine eruptione (11). On the other hand, some authors consider actinic prurigo and hydroa vacciniforme to be included in the term PLE, rather than a distinctive entity (12). Even to date, an unambiguous definition for PLE does not exist.

Attempts have been made to subdivide PLE into morphologic variants. The papular and vesicular variants are most common (13-15). Other variants include excessive erythema and oedema (8,16), the plaque type, the erythema exsudativum multiforme-like type and the rare hemorrhagic type and insect-bite-like type (17). All morphologic variants are accompanied by intense pruritus. If PLE morphology is typical papular or vesicular, no problems in diagnosing PLE exist. It is the severe forms and overlap cases (e.g. CAD, photosensitive LE) that cause the most difficulty (18). Futher discussion on the definition of PLE is about the time of delay between the sunlight exposure and the onset of PLE skin lesions. Authors agree that there is a typical delay in time, which separates PLE from for instance solar urticaria. Solar urticaria develop within minutes after sun exposure whereas PLE develops after "several hours to days". Lindmaier and Neumann (16) determined that 38% of PLE lesions were visible within 5 hours after sun exposure and Fesq *et al.* (19) measured 70% onset of lesions between 2 and 24h after sun exposure. Rarely, PLE can develop within 30 minutes after sun exposure and some authors permit preceding pruritus within minutes after sun exposure in the definition of PLE (18, 20).

Most authors agree on the other clinical characteristics of PLE. The duration of the PLE exacerbations is from 24 hours up to 2 weeks. The lesions heal completely, without leaving scars. The PLE eruption occurs in a cyclic fashion, beginning in spring and resolving by autumn. In severe cases of PLE, eruptions may be present throughout the

year. In very mild PLE, often called benign summer light eruption, PLE lesions only develop after extensive sunbathing (10,18). Mild patients tend to develop tolerance toward sunlight exposure during the summer season which is called hardening. PLE eruptions are predominantly located on sun-exposed skin areas, with predilection places such as the V-shaped area of the neck and the arms. The hands and face can be less affected, because of natural hardening. Females are two to four times more often affected than man. PLE can begin at all ages, but is most frequently reported (60-90%) to start in the second and third decade of life (overview characteristics adapted from ref (8,10,13-19,21-23)). The prevalence of PLE is estimated to range from 5 to 20% depending on the country and latitude. PLE is supposed to be more common in temperate climates than nearer the equador. Pao *et al.* (24) determined an incidence of 5% in Ballat, Australia (37.5°S) and 15% in London (51.5°N). Work from Ling *et al.* (25) showed that increasing latitude had a negative effect on the quality of life (QOL) in the spring season, probably due to the more abrupt seasonal change in countries of higher latitude. However, in summer season, latitudinal impact on QOL dissapeared.

Prognosis and genetic factors

PLE, although changing with the seasons, follows a chronic course in life. Hasan *et al.* (26) determined from a 32 year follow-up study that 24% of the PLE patients experienced spontaneous total remission, 51% experienced milder symptoms over the years and 24% experienced equal or increase in symptoms.

A positive family history of PLE is surprisingly common (46%), suggesting a possible genetic component (27,28). Millard *et al.* (29) determined a significantly higher PLE prevalence in first-degree relatives of patients with cutaneous LE, suggesting a shared pathogenesis. Furthermore, 50% of the photosensitive LE patients experience concomitant symptoms consistent with PLE or PLE preceded the onset of their cutaneous LE (30). However, an increased risk of developing LE after developing PLE was not found (31,32).

Phototesting characteristics

PLE can be provoked by artificial light sources. Success rates of phototesting have increased over the years, starting from 10-30% in early studies up to 60-95% in later studies (33). There are unexplained contradictory results regarding the most effective wavelength for provoking PLE. Some studies show that UVA (315-400nm) is the most successful wavelength (17,34,35). However, evidence points out that UVB (280-315nm) can be as effective as UVA (36,37). In severe cases, PLE lesions can also be provoked by visible light (22). The minimal erythema dose (MED) is usually normal in PLE patients, but can be lowered (22,38,39). By phototesting, PLE can be

Table I. Diagnostic photoprovocation tests in polymorphous light eruption

Author	Light Source	Nr irrad.*	Dose	Test result
Epstein (36)	Hanova Aero Kromayer Lamp model 2223	5	3-5 MED	86% pos test
	Carbon arc lamp (Bausch and Lomb)			
Jansen (101)	Medium pressure mercury lamp	1	1-12 MED UVA+B+C	72% pos test (lamps combined)
	Xenon arc lamp	1	1-12 MED UVA+B+C	
Diffey et al. (102)	900 W Xenon arc lamp with WG 305 filter	>1	3 MED UVA+B	87.5% pos test
Ortel et al. (34)	Sellas Sunlight 2001 with 5mm glass filter	8	0.8 MED with increments of +/- 20%	49% pos test; 56% UVA,
	Ultra-Vitalux Lamp (Osram GmbH) or			17% UVB, 26% UVA+B
Hönigsmann et al. (103)	Sellas Sunlight 2001 Ultra-Vitalux Lamp (Osram GmbH) or Sellasol (Sellas)	8	? MED with increments of +/- 20%	60% pos test; 50% UVA, 20% UVB, 30% UVA+B
Verheyen et al. (3)	Philips TL09 PUVA cabin	5	12-25 J/cm^2 UVA	100% pos test UVA
	Philips TL12	3	3.3 MED UVB	0% pos test UVB
Barnadas et al. (104)	Solar light simulator with Xenon arc lamp	4	10-25 J/cm^2 UVA+B	30% pos test
Miyamoto (37)	Dermaray M-DMR-1	3	2 -5 MED UVB	57% pos test
Lindmaier et al. (105)	SUPUVASUN 30000 (Mutzhas)	4-8	48 J/cm^2	37% pos: 45% UVA, 20% UVB,
	Ultra-Vitalux Lamp (Osram GmbH)	3	0.8 MED with increments of 20-40%	35% UVA+B
Van Praag et al. (14)	Sellas Sunlight 2000	3	60-90 J/cm^2 UVA	84% pos test; 69% UVA,
	Philips TL 20W/12		1.5 MED UVB	19% UVB, 12% UVA+B
Lambert et al. (106)	Philips TL 09 (355 nm)	5	15-25 J/cm^2 total body UVA+B	86.7% pos test (PLE+BSLE)
	Philips TL 20W/12	3	3.3 MED UVB	
Van der Pas et al. (33)	Solar light simulator with Xenon arc lamp	4	0.25-0.5-0.75-1.0-1.25-1.5 MED	68% pos test
Salomon et al. (107)	Supersun 5000 (Mutzhas)	3	100 J/cm^2 UVA1	74% pos test : 53% UVA1,
	Philips TL 20W/12	3	1.5 MED UVB	18% UVB, 63% UVA1+B
Mastalier et al. (23)	Sellas Sunlight 2001 with 5mm glass filter Sellas 1200 UVB source	5	0.7 MED with increments of +/- 20%	57% pos test; 59% UVA, 23% UVB, 18% UVA+B
Leroy et al. (108)	2500W Xenon arc XBO OSRAM lamp	3	1.3 - 3.3 MED UVA+B	52.5% pos test
	UVASUN 3000/5000	3	1.2 - 7.2 J/cm^2 total dose UVA	
Boonstra et al. (22)	UVASUN 3000 (Mutzhas)	6	2 MED UVA	-
	Philips TL 20W/12	6	2 MED UVB	
	CMH45 20 filtered (ORC, Japan)	6	2 MED visible light	
Roelandts (41)	Polychomatic solar simulator or Philips 20W/TL12 or Toshiba FL20SE30	4	1-2 MED UVA+B	-
	Philips Cleo performance or Sylvana F85 or Toshiba FL20BLB	5	60-100 J/cm^2 total body UVA+B	
	whole body Philips TL 01 narrowband	4	1-2 MED UVB	

*Maximum number of irradiations, provocation is stopped if lesions occur

Table partly conducted from Van de Pas et al. (33)

distinguished from solar urticaria (14). In overlapping cases such as photoaggravated LE and CAD, besides phototesting, auto-antibody serology and histology might be useful. An airborne contact dermatitis or allergic reaction to sunscreens or drugs can lead to photodistributed skin lesions. Patch and photopatch testing is essential if an allergic reaction is suspected (18,34).

Phototesting procedures

Phototesting is performed in many institutions, and several diagnostic procedures have been presented over the last decades. However, no universally accepted protocol has been established. Inconsistencies in the definition of MED, artificial light sources used, phototesting protocols, and in the interpretation of results make it difficult to compare epidemiological data (14,20,21,31,40,41). Most protocols were developed from experience in the clinical field throughout the years. There is general agreement that provocation on previously affected skin is most successful and irradiation sites should be rather large (minimal 2x2cm) and repeatedly irradiated (14,17,22,42,43). Table I gives an overview of the different used diagnostic photoprovocation test protocols. The protocol used in the photodermatology department in Utrecht is described by Boonstra *et al.* (22) and Schornagel *et al.* (chapter 3 of this thesis).

Severity assessment

Most patients do not have active skin lesions when presented to the dermatologist, thus patient history is an important feature. In literature, phototesting in PLE is done to reproduce skin lesions and to determine the responsible UV spectrum. Sometimes, phototesting is performed to evaluate treatment and to follow sensitivity in time (41). However, in daily practice, especially in photodermatology units, patients are advised and treated based on both history and phototest results (7,22,44). Thus, phototest results are also, directly or indirectly, used to determine severity of disease. Recently, a study by Palmer *et al.* (1,45) was performed in which the number of irradiations necessary to provoke PLE lesions was used as an objective indicator of disease severity. The fewer irradiations necessary to test positively indicated more severe PLE. Since phototesting is time consuming, it would be convenient to assess PLE severity from patient history and restrict phototesting only for severe or difficult cases.

Treatment options

The pathogenesis of PLE is still elusive. Prevention is the key treatment. This can be established in the majority of patients by using protective clothes and broad spectrum sunscreens. Furthermore, restricted sun exposure during the day and carefully building exposure time in spring is quite effective (19,21). If these measures are not sufficient, photo(chemo)therapy to induce hardening, can be helpful (46-48).

Symptomatic treatment in mild to moderate PLE with local or short term oral steroids is effective (19,20). In severe PLE, symptomatic treatment with cyclosporin A or azathioprine proved successful (49,50).

ULTRAVIOLET RADIATION AND THE SKIN

UV radiation can be divided into three categories, dependent on wavelength: UVC (200-280 nm), UVB (280-315 nm) and UVA (315-400 nm). From there, the solar radiation spectrum includes visible light (400-760nm) and infrared (760nm and more). Stratospheric ozone effectively filters out the UV wavelengths shorter than 290 nm, together with 70-90% of the UVB radiation (51). Sunlight exposure on the skin has beneficial effects such as vitamin D production, tanning and hardening. However, UV exposure may also lead to acute sunburn, photoaging, ocular damage and skin cancer (52). UVB is most responsible for these adverse properties of sunlight (53). Besides photocarcinogenesis, UVB has well-known immunomodulatory properties which will be discussed in more detail below. Little is known about the immunomodulatory effects of UVA (51,54).
The initial photobiological reaction responsible for triggering the cascade of events leading to PLE is unknown. Since PLE can be induced by both UVA and UVB, a common trigger seems plausible.

ULTRAVIOLET INDUCED MODULATION OF THE SKIN IMMUNE SYSTEM

The first observations of the immunosuppressive effect of UV on the immune system were discovered in transplantation experiments in mice more than three decades ago. It was found that UVB-induced skin tumours are highly immunogenic and therefore rejected when transplanted into normal syngeneic mice. However, when the recipient mice were therapeutically immunosuppressed or irradiated with UVB before, the UVB-induced transplanted tumour grew progressively (55). It was demonstrated that in lymphoid tissues of mice with UV-induced skin cancers CD8+ T suppressor lymphocytes were present (56).
The phenomenon of UV-induced immunosuppression can also be observed in another immunologic *in vivo* model, the induction of contact hypersensitivity (CHS). CHS is a special experimental form of a delayed type of hypersensitivity (DTH) response to an allo-antigen, in which haptens are applied epicutaneously to the skin. DTH and CHS are both T cell-mediated immune responses and both can be suppressed by UVB radiation (57). In mice, low-dose UVB induces inhibition of the local sensitization

phase of a CHS response. High-dose UVB, on the other hand, induces inhibition of the systemic sensitization phase of a CHS response and induces inhibition of the DTH response to an allo-antigen (reviewed in (58)). The antigen presenting cell (APC) in the skin, the Langerhans cell (LC) is thought to be important in the induction of the local immunosuppression (59,60). Upon UVB, LC migrate from the epidermis to the draining lymph nodes (61). LCs of UV-exposed skin have abnormal morphology, reduced expression of MHCII, ICAM-1 and co-stimulatory molecues such as B7, and associated alteration in antigen presenting activity (62). In the lymph nodes, the UV-irradiated LC can efficiently present antigen to Th2 type cells, but do not stimulate Th1 type cells (63,64). Systemic immunosuppression can be induced by soluble factors (e.g. cytokines, chemokines, urocanic acid or reactive oxygen species) and not by LC, since LC are not affected at the unirradiated site (57). The field players known in UV-induced modulation of the skin immune system are reviewed in Table II.

Table II. Mediators of UV-induced modulation of the skin immune system

Final result after UV	Mediated by	Specific result	Produced by	Ref
Inflammatory sunburn response	IL-6↑	IL-1↓, TNF-α↓, IL-1RA↑	KC, LC, Th2	(109)
Langerhans cell migration and maturation	IL-1(α / β)↑	PG↑, TNF-α↑, IL-6↑, selectins↑	KC, MØ, LC, N	(110-111)
	TNF-α↑	apoptosis KC, selectins↑, C3↑	KC, MØ, LC, N, Th1, Th2, F, MC	(111-113)
Immunosuppression in skin (CHS↓/ DTH↓)	IL-4↑	LC migr↓, Th2↑, E-selectin↓	N, Th2, B, MC	(114)
	IL-10↑	IL-1↓, TNF-α↓, IL-12↓, IFN-γ↓, IL-1RA↑, LC migr↓, Th2↑	KC, MØ, N, Th2, M	(110,115,116)
	IL-12↓	IL-10↑, TNF-α↓, Th1↓, Th2↑, IFN-γ↓	KC, MØ, DC, N, Th1	(62,110)
	IFN-γ↓	IL-12↓, Th2↑	MØ, Th1	(69)
	IL-1RA↑	IL-1↓	KC, N	(54)
	PGE2↑	IL-4↑, IL-10↑	KC, MØ	(117)
	UCA↑	IL-1↓, LC migr↑, DTH↓	KC	(79,121)
	complement C3↑	CHS↓, CD11b+ influx	KC, EC	(118)
	reactive oxygen species (ROS)	LC migr↑, LC-Ag pres↓	KC, LC	(57)
Inflammatory cell influx	IL-8, Gro-(α / β), MIP-2β↑	chemotaxis N, MØ, B	KC, EC, MØ, N	(123-125)
	MIP-1(α / β)↑	chemotaxis MØ, NK, Th1	N	(120)
	ICAM-1, VCAM-1, E-selectin↑	chemotaxis leukocytes, incl T cells	KC, EC, MØ, LC, N, Th1, Th2	(2,122)
	desmosomal/cytoskeletal proteins↓	making cell movement possible	KC	(119)
Protection, hardening	conified envelope proteins↑	thickening stratum corneum↑	KC	(119)
	elastase↑	KC proliferation↑	N	(119)

CHS=contact hypersensitivity; DTH=delayed type hypersensitivity

KC=keratinocyte; MØ= macrophage; LC=langerhans cell; N= neutrophil; Th1=type1Tcell; Th2=type2Tcell; F=fibroblast; M=melanocyte; EC=endothelial cell; NK=natural killer cell;

B= basophil; MC=mast cell

UROCANIC ACID AND OTHER PHOTORECEPTORS

When UV light hits the skin, part is remitted by reflection and scattering and part is absorbed. Light absorbing molecules are called chromophores and molecules which have undergone structural changes induced by light absorption are called photoproducts. UVB is absorbed by chromophores in the upper layers of the skin, the epidermis (57). To date, three chromophores have been identified that mediate immunosuppressive effects: epidermal DNA, membrane lipids and *trans*-urocanic acid (UCA).

DNA

Kripke *et al.* (65) provided the first evidence that DNA is a photoreceptor for UV-induced immunosuppression. They showed in murine skin that adding liposomes containing T4 endonuclease V increased the rate of repair of UV-induced pyrimidine dimers in DNA, which reversed the UV-induced CHS suppression to DNFB. Ichihashi and Ramsay (66) studied the excision repair of DNA in UV-irradiated fibroblasts of PLE patients by measuring ^{3}H thymidine incorporation. They did not detect any abnormality in excision repair of DNA in PLE patients compared with healthy controls.

Lipid membranes

UVB may affect lipid membranes when striking the skin surface. It generates hydrogen peroxides and other reactive oxygen species (ROS). These photoproducts can directly trigger different surface receptors on keratinocytes to produce immune regulatory cytokines (58,67,68). Studies with anti-oxidant treatment in mice showed abrogation of the UV-induced CHS reaction, blocked the UV-induced impairment of antigen presentation and interfered with the induction of tolerance (reviewed by Ullrich *et al.* (69)). In PLE patients indirect evidence was presented that ROS might play a role in the pathogenesis. Therapeutically in PLE patients, combining a broad-spectrum sunscreen with a potent antioxidant was more effective in preventing PLE lesions than sunscreen alone or placebo (70). Furthermore, Guarrera *et al.* (71) showed a 30% decrease of catalase concentration, a natural antioxidant in the epidermis, in unirradiated non-lesional skin of PLE patients compared with healthy controls.

Urocanic Acid

UCA is synthesized as the *trans*-isomer through deamination from histidine by the histidase enzyme in the stratum corneum. The absence of the catabolizing enzyme urocanase in the epidermis results in accumulation of *trans*-UCA (72-74). On UV radiation, photoisomerization from *trans*- to *cis*-UCA takes place instantly in a dose-dependent fashion until a photostationairy state is reached, with approximately equal

quantities of the two isomers. The most effective waveband for photoisomerization is 310-320nm (UVB range), although 315-400nm (UVA range) wavelengths can also induce *cis*-UCA transformation (75,76). Broad-spectrum sunscreens are able to prevent *cis*-isomerization effectively (77). However, it is presumed that the sunscreening properties of UCA have little clinical relevance, since UV sensitivity is not correlated with the concentration UCA on the skin surface (78). *Cis*-UCA can mimic the immunosuppressive effects of UVB, suggesting that UCA is an important mediator of UV-induced immune modulation. *Cis*-UCA has been shown to have a suppressive effect on CHS, DTH and allograft rejection (79,80) Furthermore, functions of LC, monocytes, T lymphocytes, NK-cells, neutrophils and peripheral sensory nerves can be modulated by *cis*-UCA (79,81-88). UCA isomerization upon UV exposure was studied in PLE patients, without comparing patients with healthy controls. It was concluded that PLE patients can isomerise *cis*-urocanic acid on their skin during ultraviolet hardening therapy (89).

ULTRAVIOLET INDUCED MODULATION IN PATIENTS WITH PLE

Histology in PLE

In UV-induced PLE lesions, a dense perivascular upper dermal infiltrate of predominantly lymphocytes is visible already within 5 h after UV exposure and reaches a peak at 72 h post irradiation. In the first 72 h a predominance of CD4+ cells is present. Thereafter, CD8+ cells dominate the infiltrate (3,90). The histological appearance, together with the delay in onset of skin lesions in PLE after UVB, gave rise to the idea of PLE being a DTH-like response to UV-induced neoantigens (2,4,31) The T-cell infitrates may be accompanied by endothelial swelling, edema of the papillary dermis and, although less evident, influx of neutrophils, mast cells, macrophages and even eosinophils (90).

Cell migration in PLE

Intercellular adhesion molecule-1 (ICAM-1), vascular adhesion molecule-1 (VCAM-1) and E-selectin are essential for leukocyte migration into the skin. In PLE patients ICAM-1 was expressed focally on basal KC particularly above the areas of intense dermal leukocytic infiltrates (2,91). This focal increase in KC induced ICAM-1 expression in PLE is probably the result of IFN-γ release by the activated lymfocytic infiltrates and is not restricted to PLE, but has also been demonstrated in other inflammatory skin diseases such as AD and psoriasis (92).

E-selectin on endothelial cells is upregulated after UV in both PLE patients and healthy controls. The time course for E-selectin expression parallels the neutrophil

accumulation in skin in PLE patients as well as healthy controls (2,93). VCAM-1 is only slightly upregulated after UV in both PLE patients and healthy controls (91,94). From these studies it can be concluded that both endothelial ICAM-1 and E-selectin are involved in the recruitment of leukocytes to inflamed skin in PLE patients.
LC migration in PLE was investigated by Kölgen *et al.* (95,96). It was demonstrated that LC in the skin of PLE patients persisted after 6 MED overexposure to UVB compared with healthy controls. In addition, in suction blisters of PLE patients less migrating UV-damaged LC were detected compared with healthy controls after UV irradiation. Furthermore, Kölgen *et al.* (95) demonstrated that in 6 MED UVB-exposed skin of PLE patients less CD11b+, elastase+ cells infiltrated the dermis compared with healthy controls. These CD11b+, elastase+ cells were the main source of TNF-α and IL-4 production in the dermal compartment and IL-10 in the epidermal compartment (97).
Norris *et al.* studied the role of cytokines and chemokines in lymphocyte migration after solar simulated radiation (SSR) in PLE patients (98). It was concluded that in PLE, like other inflammatory skin diseases, IL-6 and IL-8 play a role in lymphocyte influx into the skin.

Complement deposition in PLE

Complement deposition in PLE patients was studied by Muhlbauer *et al.* (99). They demonstrated extensive perivascular fibrin deposits and slight vascular deposition of C3 and IgM in some, but not all, PLE patients. This complement activation and immunoglobulin deposition is not exclusive for PLE, but very common in many inflammatory cutaneous diseases, such as psoriasis, AD and lamellar ichthyosis (100).

OUTLINE OF THIS THESIS

In the puzzle of PLE, the aim of the thesis was twofold. First, to obtain a practical grip on severity assessment and phototesting a patient suffering from PLE. Second, to determine the pathogenetic field players in the early induction phase of PLE.
Patient history is important since lesions are transient and often not present at time of consultation. Phototesting is done to reproduce the PLE skin lesions. Furthermore, information is obtained about the responsible action spectrum and the UV dose necessary to elicit skin lesions. With this information the severity of the disease is assessed and adequate advice and treatment is given. However, phototesting is a time consuming diagnostic procedure. Therefore, we wondered whether it would be possible to assess severity of disease based on the patient history alone. The results of this study are presented in **chapter 2**. Many different phototesting protocols exist,

resulting in very different success rates. As presented in **chapter 3**, we determined an optimal, yet practical, phototesting procedure.
PLE is suggested to be characterized by a failure of UV-induced immunosuppression. PLE is, due to the clear delay between sunlight exposure and the onset of skin lesions, often regarded as a DTH-like response to an UV-induced neoantigen. However, PLE lesions can be visible even within a few hours after UV exposure. Therefore, we hypothesized that another, earlier, induced mechanism is responsible for the initiation of PLE. For this, we determined the migration of cells and investigated mRNA expression of genes regulating a broad spectrum of proteins involved in UV-induced inflammation of skin in PLE and healthy controls during the first 18 hours after UVB-exposure. The results are presented in **chapters 4** and **5**. Since urocanic acid has an immunomodulatory effect, we tested whether UCA concentration and isomerization upon UV was different in PLE patients compared with healthy controls (**chapter 6**). Finally, the results of the studies described in this thesis are combined and discussed in **chapter 7**, presenting an up-to-date view on the early pathogenesis in PLE.

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Chapter 2

Is severity assessment in polymorphous light eruption possible?

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ABSTRACT

Polymorphous light eruption (PLE) is a very common photodermatosis and patient history is highly specific. Phototesting is used to confirm the diagnosis and to determine the action spectrum and the severity of disease. In daily practice and in research studies it would be convenient to assess disease severity by patient history only.
Assessment of PLE disease severity by patient history questionnaire and confirmation by phototesting.
Sixty-one patients with PLE were asked 10 standard questions and all were phototested. The answers to the standard questions were coded with linear scores ranging from 0 to 10. The score of each question was plotted as independent variable in a multiple linear regression model against the score of the phototest (minimal number of irradiations necessary to elicit a positive skin lesion, with a maximum of 6 irradiations) as dependent variable using a stepwise approach. Furthermore, the scores of the separate questions were added to form a total score, the PLE severity assessment score (PLE-SAS). The medians of these PLE-SAS's were compared with the result scores obtained by phototesting. Phototesting was done with UVA and UVB.
Fifty-seven of 61 patients had a positive test result (93%). Using the multiple linear regression model, the severity assessment by patient history compared with the result of phototesting showed 2 significant, contributing questions ($p < 0.05$) but with a regression coefficient of 0.2. A significantly difference in median scores with the severity assessment (PLE-SAS) between patients testing positive after 1-3 irradiations compared to those testing positive after 4-6 irradiations was present ($p<0.05$). However, the overlap in quartile range between both groups was such that the PLE-SAS has little predictive value in individual patients.
We showed that severity assessment in PLE based on patient history was not possible. Two significantly contributing questions were not discriminating enough to be used as predicting questions to assess severity. Accurate patient history proved to be a reliable method to diagnose PLE. Phototesting is useful to determine the responsible UV action spectrum and to exclude differential diagnoses like photosensitive eczema, lupus erythematosus or chronic actinic dermatitis. In PLE, disease severity as determined using the PLE-SAS did not reliably predict severity as assessed by phototesting.

INTRODUCTION

Polymorphous light eruption (PLE) is the most common idiopathic photodermatosis. It is characterized by pruriginous polymorphous skin lesions, although monomorphous in the individual patient. Lesions include papules, vesicles, plaques and also excessive erythema (1). Lesions occur predominantly several hours after sun exposure mainly on light exposed skin areas and resolve after sun avoidance within several days to weeks without leaving scars. Most PLE patients exacerbate during springtime and summer, whereas no complaints occur during winter (2). Phototesting of PLE patients, by means of artificial light sources, is done to establish the diagnosis and determine the action spectrum responsible. Phototesting can also provide an assessment of the severity of the PLE (3,4). Recently, a study by *Palmer et al.* (5) was performed in which the number of irradiations necessary to provoke PLE lesions was used as an objective indicator of disease severity. The fewer irradiations necessary to test positive indicated more severe PLE. The choice of therapy is depended on both patient history and results from phototesting. Patients with mild reactions will be advised to use suncreen, which can be chosen dependent on the action spectrum of the responsible UV wavelength. More sensitive patients can benefit from profylactic phototherapy to induce tolerance (6). PLE patient history is highly specific. Confirmation of the diagnosis by phototesting is reported up to 85% (7,8). Since phototesting is a time consuming method, it would be convenient to assess PLE severity from patient history and preserve phototesting only for severe or difficult cases. Furthermore, the results of research studies may depend on the severity of disease among the patients studied. Therefore, based on specific patient history questions we assessed the severity of PLE and used the phototest results in all patients as confirmation test to determine severity.

PATIENTS AND METHODS

All patients who visited the photodermatology outpatient clinic of the University Medical Center Utrecht, The Netherlands between August 2002 and January 2005 for the first time and met the clinical description of PLE (as mentioned in the introduction part) were phototested. Besides phototesting, these patients answered a series of 10 standard questions (Table I). PLE exacerbations had to be present for at least one year so that patients could answer questions about summertime in comparing to wintertime properly. Patients with a relevant contact or photocontact dermatitis, as well as patients with lupus erythematosus or chronic actinic dermatitis, were excluded from the study.

Table I. Interview questions (score translation and number of patients each group)

Question	Answer	Score	Total patients
1. Which part of the body is affected when exposed to sunlight?	uncovered skin only	0	43
	sometimes slight expansion to thin coverd skin aereas	5	18
	always expansion to (also thick) covered skin areas	10	0
2. What is the first month of the year in which	July, August, September	0	9
PLE lesions usually start?	June	2.5	6
	April, May	5	39
	Februari, March	7.5	5
	Whole year around	10	2
3. How much sun exposure can you tolerate?	> 5 hours	0	10
(without getting PLE rash later on)	3-5 hours	2.5	3
	1-3 hours	5	10
	30 minutes to 1 hour	7.5	7
	<30 minutes	10	31
4. After sun exposure, after what time do the PLE lesions develop?	> 1 day	0	5
(not just itching, but typical PLE lesions like papules or vesicles)	next day	2.5	1
	that evening or night	5	40
	30 minutes - 3 hours	7.5	10
	< 30 minutes	10	5
5. After development of PLE lesions, how lang does it usually	< 4 days	0	24
take before they disappear?	4-7 days	2.5	13
(without further exposure to sunlight or topical/oral medication)	7-14 days	5	9
	2-4 weeks	7.5	6
	>4 weeks	10	9
6. Do you experience PLE complaints during wintertime?	no	0	50
(not lesions during vaction in sunny areas including skiing)	yes, but far less than spring or summertime	5	10
	yes, comparable like summer time	10	1
7. Do you experience natural hardening over the summer period?	yes, clearly	0	12
	yes, but slightly	5	5
	no	10	44
8. Do you experience PLE complaints on cloudy summer days?	no	0	46
	yes, but less than on a sunny day	5	7
	yes, no difference between sun or clouds	10	8
9. Do you experience PLE complaints under a parasol or in the shade?	no	0	13
	yes, but less than during full sun exposure	5	18
	yes, equally as in full sun exposure	10	30
10. Do you develop PLE lesions sitting behind a glass window	no	0	22
or using a tanning bed?	only tanningbed	5	13
(not just itching, but typical PLE lesions like papules or vesicles)	glas (and tanningbed)	10	26

The open answers to the ten questions in the study questionnaire were subsequently put into a category and coded with linear scores ranging from 0 to 10, with higher scores indicating more severe PLE (Table I). The linear scoring system mimicked the scoring system used by Palmer *et al.* (5).

Phototesting was performed as described in detail by Schornagel *et al.* (7). Briefly, the patients were irradiated with UVA (Mutszhas UVASUN 3000S) and UVB (Philips TL20W/12 tubes) light separately. Phototesting was performed on previously affected skin, such as the arms and the V-shaped area of the neck. Area test size was 5x10 cm. The first irradiation was started with approximately 1.7 minimal erythema dose. Each following daily irradiation, up to a maximum of 6, was done with an incremented dose of about 20%. The minimal number of irradiations necessary to elicit a positive PLE skin reaction was determined (from 1 until and including 6, or negative). A lower phototesting score indicated more severe PLE. Since no score could be given to patients with a negative phototest, these patients were excluded from the analysis.

Data were analyzed for UVA irradiation alone (regardless the result of UVB phototesting), for UVB irradiation alone (regardless the result of UVA phototesting) and for UVA and/or UVB irradiation. In the latter this meant that the first positive skin reaction to UVA or UVB determined the score on the scale from 1 to 6.

The resulting data were analyzed using a multiple linear regression model (SPSS). The score of the answers to each standard question was plotted as independent variable against the number of irradiations necessary to elicit a positive response to UV as dependent variable using the stepwise approach. Variables with coefficients differing significantly ($p < 0.05$) from zero were considered to contribute significantly to the predictive linear model. Furthermore, scores to each individual question were added to make a PLE-severity assessment score (PLE-SAS) for the tested patients. The medians of the PLE-SAS were compared between groups of patients (group 1; 1-3 irradiations and group 2; 4-6 irradiations needed before a positive reaction occurred) by the Mann-Whitney U test.

In 10 PLE patients the questionnaire was repeated several weeks after the first to measure the within-subject variability (using Spearman rank correlation test).

The study was approved by the local medical ethical committee.

RESULTS

Patient characteristics

Sixty-one patients were included in the study. There were 42 females (mean age of 42 y, range 19 to 76 y) and 19 males (mean age of 47 y, range 30 to 75 y). Mean duration of PLE exacerbations was 11 y. By patient history, it was determined that after sun-

exposure 49% of all PLE patients developed papules, 39% developed papules and vesicles, 5% developed papules and plaques and 7% developed excessive erythema and oedema.

The success of phototesting

The diagnosis of PLE was confirmed by phototesting in 57/61 patients (93%). Of these 57 patients 23% reacted to UVA irradiation alone, 21% to UVB alone and 56% to both UVA and UVB irradiation. Thus, reaction to UVA was induced in 45 patients (79%) and reaction to UVB in 43 patients (75%). The cumulative increase of positive testing patients to UVA and/or UVB was 7% after 1 irradiation, 31% after 2 irradiations, 67% after 3 irradiations, 89% after 4 irradiations, 92% after 5 irradiations and 93% after 6 irradiations. The morphological PLE type by patient history was correctly reproduced by phototesting in 75% of all patients. In 18% of all cases, patient history described papules and vesicles, whereas phototesting only reproduced papules. In the patients which tested negative (7%), patient history described papules. UVA and UVB reproduced the different morphological PLE types equally accurate and the distribution of the different PLE types in patients testing positive for UVA or UVB was comparable.

The severity assessment model for UVA and/or UVB

The results of the linear regression analysis of the questionnaire from all patients with a positive phototest result for UVA and/or UVB (n=57) are shown in Table II and Fig 1. Only question 5 ("time until clearance of developed PLE lesions") and question 10 ("complaints through glass / from a tanningbed") from the questionnaire contributed significantly to the prediction of the number of irradiations needed for a positive

Table II. Multipel linear regression model for severity assessment in UVA and/or UVB positive tested patients

		Coefficients	
Question		**p-value**	**95% CI**
1.	Affected body part	0.73	-0.11 0.15
2.	Seasonal start lesions	0.51	-0.17 0.09
3.	Sun tolerance	0.39	-0.12 0.05
4.	Time to develop lesions	0.72	-0.15 0.10
5.	**Clearance of developed lesions**	**0.01**	**-0.19 -0.03**
6.	Complaints during winter	0.36	-0.20 0.07
7.	Hardening during summer	0.42	-0.05 0.11
8.	Complaints on cloudy days	0.45	-0.05 0.11
9.	Complaints in shadow	0.09	-0.01 0.13
10.	**Complaints through glass / from tanningbed**	**0.04**	**-0.13 -0.02**

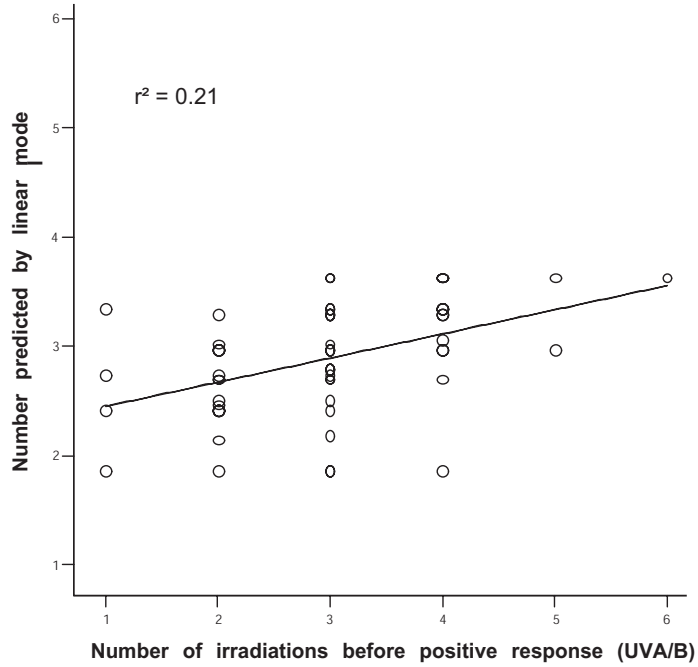

Figure 1. Relation between the objective severity score determined by phototesting and the predicted score of the significant questions in the model obtained by linear regression. (1-6: number of irradiations necessary to elicit a positive skin reaction). r^2 = regression coefficient.

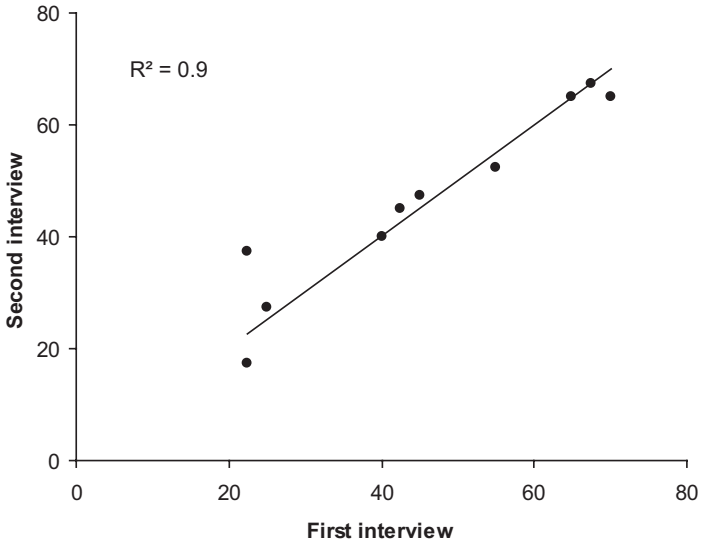

Figure 2. The inter-subject variability in the answers to the questionnaire between the first and the second interview

reaction to occur. However, the regression coefficient of the equation containing the responses to questions 5 and 10 was low (r^2 = 0.21). Data analysis using phototest results for UVA alone (n=45) showed that the answers to question 5 ("time until clearance of developed PLE lesions") and question 9 ("complaints in the shadow") contributed significantly to the prediction of the number of irradiations needed for a positive response, but also with a low regression coefficient (r^2= 0.26, data not shown). Analysis using phototest results for UVB alone (n=43) showed no answers that contributed significantly (r^2= -0.07, data not shown).
Analysis of the model parameters revealed no anomalies. Notably, the distribution of the residues was approximately normal and there were no apparent problems with co-linearity (Variance Inflation Factors were below 2). In other words, the study model was valid and was not disturbed by dependent variables. The questionnaire was repeated in 10 patients. The inter-subject variability between the first and the second interview was low with a regression coefficient r^2 = 0.9 (Fig 2).

The PLE - severity assessment score (PLE-SAS) for UVA and/or UVB

The PLE-SAS is obtained by summation of the scores on all 10 questions in the questionnaire. Therefore, severity assessment based on the PLE-SAS showed comparable

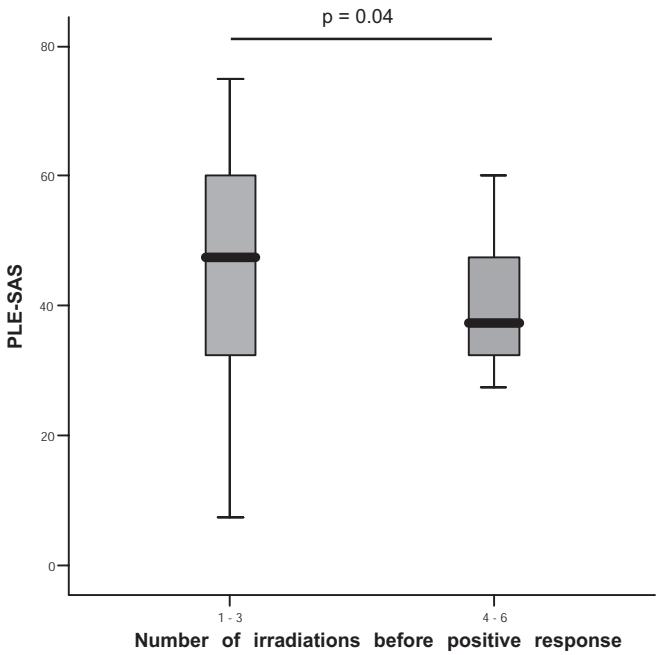

Figure 3*A*. Median of the PLE-SAS in the patient group with a positive skin reaction after 1-3 irradiations versus the group with a positive skin reaction after 4-6 irradiations . Box plots show the 25%, 50% and 75% quartiles, together with the range in minimal and maximal score. No outliers were present in this study.

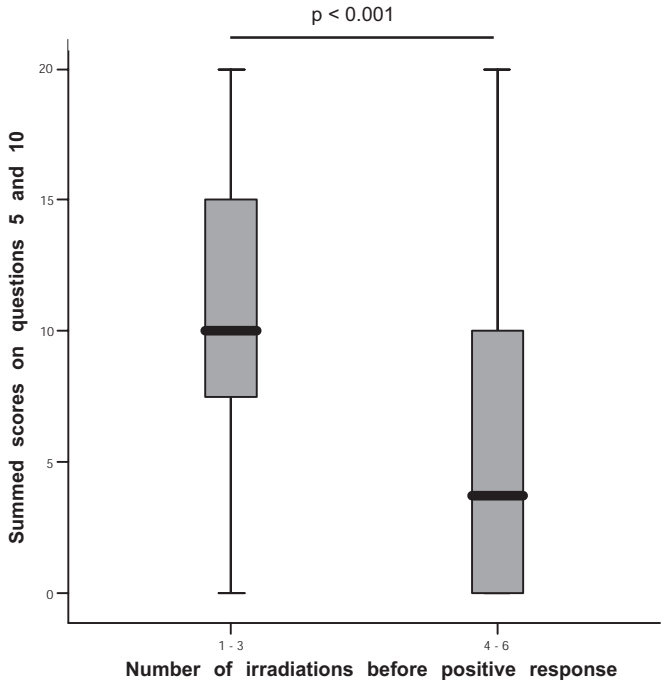

Figure 3*B*. Median of the total score of significantly contributing questions 5 ("time until clearance of provoked lesions") and questions 10 ("complaints through glass / from a tanningbed") distracted from the PLE-SAS in the two groups ("1-3" versus "4-6"). Box plots show the 25%, 50% and 75% quartiles, together with the range in minimal and maximal score. No outliers were present in this study.

results as results from the 10 questions in the multiple linear regression model. In both cases, regression coefficient was low (data not shown).The PLE-SAS had an approximately normal distribution (confirmed with the Kolmogorov-Smirnov test, p= 0.05) with a mean ± SD of 43.7 ± 14.9 (range 7.5 – 75). Palmer *et al.* (5,9) recently, described the influence of a higher PLE severity index score on the probability of a successful provocation. We reproduced this study using our own PLE-SAS data set. A significant difference in median scores between patients testing positive with UVA and/or UVB after 1-3 irradiations compared to those testing positive after 4-6 irradiations was apparent (p= 0.04; Fig 3*A*). We also analysed the 2 significant questions (number 5 and 10) in the PLE-SAS for UVA and/or UVB using the Mann-Whitney Test. The difference in median scores between patients testing positive after 1-3 irradiations and patients testing positive after 4-6 irradiations was significant (p< 0.001; Fig 3*B*). If the data were analysed without grouping the patients (1-3 or 4-6 irradiations), no significant difference between median PLE-SAS scores or scores of the 2 significant questions was found (Kruksal-Wallis test).

DISCUSSION

In this study we reproduced PLE lesions with artificial light sources in 57/61 patients (93%). Therefore, phototesting is a very accurate method to reproduce skin lesions. The cumulative increase in percentage of patients testing positive with each successive irradiation was optimal after 4 irradiations and numbers were comparable with our previous study by Schornagel *et al.* (7).

Analysis of the questionnaire of 57 positive tested PLE patients showed that, although questions 5 ("time until clearance of developed lesions") and 10 ("complaints through glass / from a tanningbed") significantly contributed to the severity assessment, the predictive value of these questions 5 and 10 was low (regression coefficient r^2 = 0.21). This means that only 20% of the variation in the phototest results (number of irradiations before a positive reaction) was explained by the scored answer to the questions in the questionnaire, which makes it unreliable for practice use (Fig 1). Also, similar results were found using the total PLE-SAS score. This was expected, since the PLE-SAS is the sum of the scores on all 10 separate questions. According to the validation study of *Palmer et al.* (9), patients with a positive phototest after 1, 2 or 3 irradiations were clustered. They compared the "1-3" group with a group with a negative phototest result. We compared the "1-3" group with the patient group testing positive after 4-6 irradiations. By using only patients with a positive phototest, which is 93% of the total group, no discussion about "true" PLE diagnosis existed in our study. A statistically significant difference was found between the "1-3" and "4-6" group in both the PLE-SAS and also the sum of the 2 significant questions (5 and 10) (Figs 3*A* and *B*). However, the large overlap in quatrile range between the "1-3" and "4-6" group indicates that neither the PLE-SAS nor the sum of the 2 significant questions will have any predictive value for the individual patient.

We believe that, despite statistically significant results, severity assessment by patient history in our population patients was not relevantly reflected by the results from phototesting. Like most photodermatology departments with facilities to test PLE patients, a patient referral bias may be present in the study population. Most patients with mild PLE will never seek help or will be treated by general physicians. It would be interesting to assess the predictive value of the questionnaire or the PLE-SAS in mild PLE patients. Regarding the questionnaire, possibly, the questions asked to assess severity of PLE are not or partially valid. To date no validated questionnaire exists to assess PLE severity. In our opinion, the only objective method to measure severity remains phototesting. Therefore, all included patients had complete questionnaires and all were phototested. Furthermore, in this study we used the minimal number of irradiations necessary to elicit a positive skin reaction as objective indicator to score severity. The type of skin lesion reproduced and the course of expanding and

clearance of the elicited skin lesions were not taken into account in this and other studies undertaken so far. So no data are available about the predictive value of these variables.

In conclusion, we showed that severity assessment of PLE based on patient history did not reliably predict severity severity as assessed by phototesting. Two significantly contributing questions were not discriminating enough to be used as predicting questions to assess severity. Accurate patient history proved to be a reliable method to diagnose PLE. Phototesting is useful to determine the responsible UV action spectrum and to exclude differential diagnoses like photosensitive eczema, lupus erythematosus or chronic actinic dermatitis. Furthermore, phototesting remains the most objective method to assess severity of PLE and cannot be replaced by the PLE-SAS.

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Chapter 3

Diagnostic phototesting in polymorphous light eruption: the optimal number of irradiations

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ABSTRACT

Diagnostic phototesting in patients with polymorphous light eruption (PLE) is performed to confirm the diagnosis and to determine the action spectrum of the disease. No universally accepted protocol for diagnostic phototesting has been established.
The aim of this study was to determine the optimal number of irradiations necessary to elicit skin reactions in diagnostic phototesting of PLE patients.
In total, 134 PLE patients which were phototested were studied. The cumulative number of irradiations necessary to elicit skin lesions was determined retrospectively in 94 patients. Furthermore, in a successive prospective study, another 40 patients were studied more detailed with an adjusted protocol.
In the retrospective as well as the prospective study it was shown that the number of patients testing positive to UVA and/or UVB irradiation increased steadily with each exposure during the first four irradiations. Continuing exposures, the number of patients testing positive after 5 or 6 irradiations decreased steadily. Furthermore, all new visible skin reactions after 5 or 6 irradiations were already initiated during the preceding 4 irradiations.
In diagnostic photoprovocation for patients with PLE, a maximum of 4 daily irradiations is optimal to elicit typical skin lesions. More extensive test protocols have no clinical implications. However, fewer exposures may lead to substantial loss of positive test results.

INTRODUCTION

Polymorphous light eruption (PLE) is an idiopathic photodermatosis with a prevalence of 10-20% in temperate climate zones (1,2). It is characterized by pruriginous polymorphous skin lesions, although monomorphous in the individual patient (3) Lesions occur mainly on light exposed skin areas and resolve after sun avoidance without leaving scars (4). Females are more affected than males and the age of onset of disease is most prevalent in the second decade of life (5). The diagnosis of polymorphous light eruption is based on patient history, morphology of the lesions, and the reproduction of lesions by phototesting (6,7). Elicitation of skin lesions can occur by irradiation with UVA, UVB and even visible light. Sunburn sensitivity (minimal erythema dose or MED) is usually normal, but can also be lowered (8). Histopathology and laboratory results can be useful to exclude differential diagnoses like lupus erythematosus, lymphocytic infiltration of the skin and porphyria.
Although phototesting is performed in many institutions, and several diagnostic procedures have been presented over the last decades, no universally accepted protocol has been established. Inconsistencies in the definition of PLE, as well as MED, used artificial light sources, phototesting protocols, and in the interpretation of results make it difficult to compare epidemiological data (9-13). Recently, Van de Pas *et al.* (14) presented an overview of the different used provocation test protocols since the 1980s. Most protocols were designed from experience in the clinical field throughout the years. In the current study we set out to determine the number of irradiations necessary to elicit typical PLE skin lesions in an optimal and suitable provocation protocol. Therefore a retrospective study was performed to evaluate the optimal number of irradiations necessary to elicit a pathological skin response. Based on these results, a subsequent prospective study was designed, in which the clinical outcome after a maximum of 6 daily irradiations was compared with the clinical outcome of 4 daily irradiations.

PATIENTS AND METHODS

Patients

Patients with a clinical PLE appearance as already described by Millard and Hawk (1) were included in the study. Briefly, typical PLE lesions are papular, vesicular and / or plaques-type. Furthermore, excessive erythema and oedema without visible papules may occur. In both the retrospective and prospective study a representative non-selected patient cohort was evaluated.

Standard phototesting procedure

Minimal Erythema Dose

Prior to phototesting, the sunburn sensitivity (minimal erythema dose or MED) in the individual patient was determined for UVA and UVB. A specifically designed automatic device with 9 small windows (3 x 10 mm each) was placed on the skin of the patient. The windows were exposed sequentially to doses increasing in a geometrical progression with dose increments of 41% between successive fields. Result of the MED test was assessed 24 hours later by visual observation. MED was defined as the dose that produced a just perceptible erythema observed 24 hours after exposure (15,16).

Phototesting procedure

Standard phototesting (as illustrated in Table I and Fig 1*A*), by means of a maximum of 6 daily irradiations with artificial light sources, was performed preferentially on a part of the skin where previous lesions had occurred. This included also the area of the face and the neck, with the only exception that visible light tests were not performed standard in facial areas because of lack of space. Large separate skin areas (5 x 10 cm) were irradiated with approximately 1.7 MED UVA and UVB. For visible light a starting dose of 70 Jcm^{-2} was used. The non irradiated skin sections were covered with a cotton cloth. The borders of the irradiated areas were marked, so that the cloths could be placed on exactly the same place with each daily irradiation. The irradiated sites were observed visually 24 hours after exposure. With exception of the area irradiated with visible light, the test sites were exposed daily to increased

Table I. Diagnostic photoprovocation test protocol for patients with polymorphous light eruption as used in the University Medical Center Utrecht, The Netherlands

UV source	Wavelength	Template Size	Location	Exposures and Dose
Mutzhas UVASUN 3000S	UVA	MED:	MED:	MED:
		9 windows of 3x10mm each	previously affected area	41% increments
			(except facial areas)	between successive fields
		phototesting:	phototesting:	phototesting:
		5x10 cm (facial areas 2x3 cm)	previously affected area	1.7 MED with ca. 20% increment
				for max 6 days (or until pos result)
Philips TL20W/12 tubes	UVB	MED:	MED:	MED:
		9 windows of 3x10mm each	previously affected area	41% increments
			(except facial areas)	between successive fields
		phototesting:	phototesting:	phototesting:
		5x10 cm (facial areas 2x3 cm)	previously affected area	1.7 MED with ca. 20% increment
				for max 6 days (or until pos result)

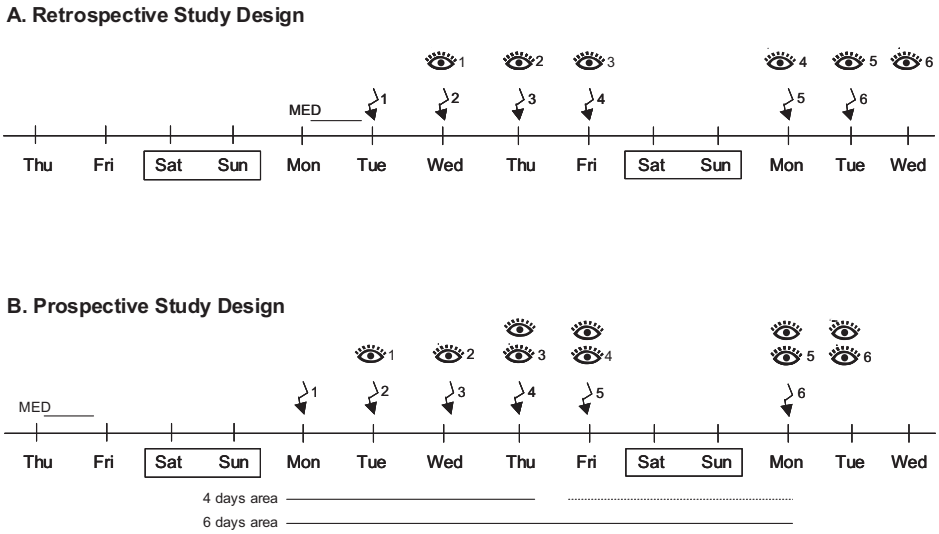

Figure 1. Time scale for MED testing and photoprovocation testing using the standard protocol in the retropective study (A) and the adjusted protocol in the prospective study (B). MED is tested and evaluated 24 h later (MED____). Irradiations ( ) are numbered 1-6. Skin reaction after irradiation was scored visually 24 h later ( ) and numbered 1-6. In the prospective study two independent observers scored the skin reaction.

doses. Usually a 20% increment was used, but this could be more or less (0-40%) depending on the erythema reaction in the individual patient. This procedure was repeated up to a maximum of 6 daily irradiations, or until a positive skin reaction occurred. Patients were not irradiated during the weekend. A positive provocation test was defined as reproduction of specific PLE skin lesions after irradiation with artificial UV-sources (papules and/or vesicles or plaques or excessive erythema and oedema on the irradiated site).

Radiation sources

The radiation sources used have been described previously by Boonstra *et al.* (8). Briefly, the UVA source (Mutzhas UVASUN 3000 S) consisted of a high-pressure mercury arc with special filtering to filter out all UVB (resulting in 340-450nm emission spectrum). The UVB source consisted of 4 Philips TL20W/12 tubes with a continuous emission spectrum (280-360nm) with a maximum around 305nm. These light sources for UVA and UVB were used for both MED and phototesting. For testing visible light the super high-pressure mercury arc with suitable filters (CMH 45-20 of ORC, Japan) was used. The output of the light sources was measured with a calibrated thermopile from Optronic Laboratories Inc., model 742, UV-Visible Spectro-radiometer.

Retrospective study

Data of 94 representative non-selected adult patients suspected for PLE that underwent diagnostic photoprovocation testing during 1999 and 2000 were re-evaluated. Patients were tested using the phototesting procedure with a maximum of six daily irradiations, as described above and illustrated in Fig 1*A*. After each irradiation the number of positive reacting patients was determined. This was done separately for the results on irradiation with UVA and UVB. This means that data were analysed for one wavelength, irrespective of the test result of the other wavelength. Furthermore, data on UVA and UVB irradiation were combined. The minimal number of irradiations necessary to elicit typical skin lesions for either one of the wavelengths (UVA and/or UVB) was noted as positive test result. Data on visible light provocation were listed separately.

Prospective study

Based on the outcome of the retrospective data a prospective study was designed. Forty representative non-selected adult patients suspected for PLE that underwent diagnostic photoprovocation testing from July 2002 until December 2003 were included. In contrast with the standard procedure (retrospective study), patients which were tested in facial areas or on the hands needed to be excluded from the prospective study because of lack of space (larger irradiated areas required). MED was determined in advance and phototesting always started on Mondays, so that the fourth irradiation could be evaluated on Friday (Fig 1*B*). During phototesting, the first four daily irradiations were done in conformity with the 6 irradiations provocation protocol. After the fourth irradiation half of the UVA irradiated area and half of the UVB irradiated area was covered and the other half was irradiated with 2 more daily irradiations (to a maximum of 6 irradiations). Which part of the area was covered was chosen randomly. Based on the results from the retrospective study, provocation with visible light sources was done using only the 6 days protocol. Two independent observers scored the visible skin reaction on the UVA and UVB irradiated areas, as shown in Fig 1*B*. Visible scoring was done 24 hours after irradiation with exception of the fifth irradiation, which was scored after 72 hours (weekend). The clinical appearance of the irradiated sites (erythema, papules, vesicles, plaque or no pathology) was scored. Furthermore, differences in clinical appearance and severity between the 4 times and 6 times irradiated site in the individual patient was scored. Inter-observer variability in scoring was determined (with a chosen maximum of 5% difference allowed). Also in this study irradiation was stopped when a positive skin reaction was elicited on a particular wavelength. The visual scoring of the skin continued, irrespectively of discontinuation of the provocation test.

The minimal number of irradiations necessary to elicit PLE skin lesions was determined, as was done in the retrospective study.

RESULTS

Retrospective study

A group of 94 patients, comprised of 64 females and 30 males (ages between 18 and 84 y) was studied. The diagnosis PLE could be confirmed in 80 of the 94 patients after 6 daily exposures with UVA and UVB. In 14 patients no positive reaction could be elicited by standard phototesting. Of all PLE patients tested positive, 14 of the 80 patients reacted to UVA alone, 19 patients reacted to UVB alone and 47 patients reacted to both UVA and UVB. Within the latter group 2 patients also reacted to visible light. MED, calculated as geometric mean, was for UVA 28.2 Jcm^{-2} (95% CI; 9.3-84.5 Jcm^{-2}) and for UVB 51.2 $mJcm^{-2}$ (95% CI; 9.1-289.0 $mJcm^{-2}$). Provocation testing was started with 1.6± 0.1 MED UVA and 1.9±0.2 MED UVB. Fig 2 shows the results of the cumulative percentage of patients reacting to UVA irradiation, to UVB irradiation or to UVAand/orUVB irradiation with each subsequent exposure. It is shown that the number of patients with a positive test increased equally during the first 4 irradiations. Thereafter, the increase in positively tested patients decreased. Since data on patients in the group reacting to UVA and/or UVB are situated on a straight line, statistical analysis could be performed. The increase of patients testing positive in this group is

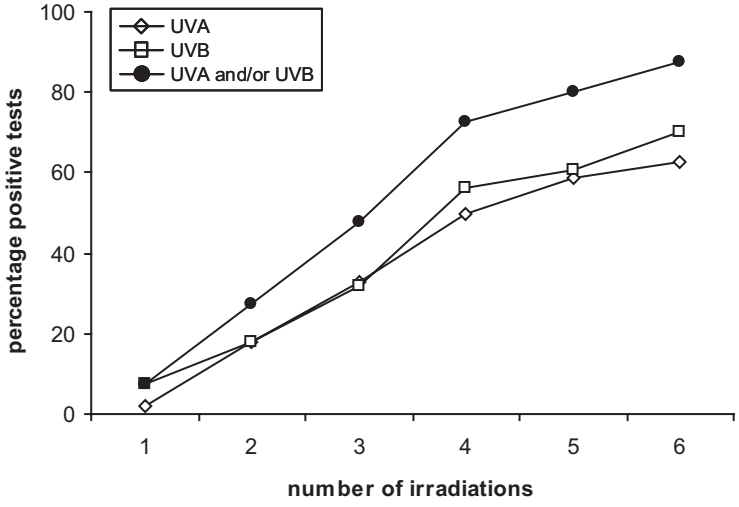

Figure 2. Cumulative increase in percentage of PLE patients in tme testing positive during diagnostic pgototesting (retrospective study).

21% per irradiation during the first 4 irradiations. However, during the fifth and sixth irradiation this increase was only 7% per irradiation.
Most patients clinically displayed the papular or papulo(vesicular) form of PLE. Positive reproduction by phototesting resulted in 85% papular PLE followed by 9% (papulo)vesicular PLE and 6% excessive erythema. Although a relation was found between patient's history and PLE lesions reproduced by phototesting for papular PLE, this was not true for the other PLE forms. No different pattern in PLE type was observed after exposure to UVA irradiation or UVB irradiation.

Prospective study

A group of 40 patients, comprised of 27 females and 13 males (ages between 22 and 79 y), was studied. The diagnosis PLE could be confirmed using standard phototesting procedure in 38 of the 40 patients. In 2 patients no positive reaction could be elicited. Of all patients tested positive 8 of the 38 patients reacted to UVA alone, 8 patients reacted to UVB alone and 22 patients reacted to both UVA and UVB. No patients within each of the groups reacted to irradiation with visible light. MED was calculated by geometric mean, for UVA 32.6 Jcm^{-2} (95% CI; 12.1-87.4 Jcm^{-2}) and for UVB 56.2 $mJcm^{-2}$ (95% CI; 13.0-242.2 $mJcm^{-2}$). Provocation testing was started with 1.5± 0.1 MED UVA and 2.0±0.1 MED UVB. Fig 3 shows the results of the cumulative percentage of patients reacting to UVA irradiation, to UVB irradiation or to UVAand/orUVB irradiation with each subsequent exposure. In all groups represented, the

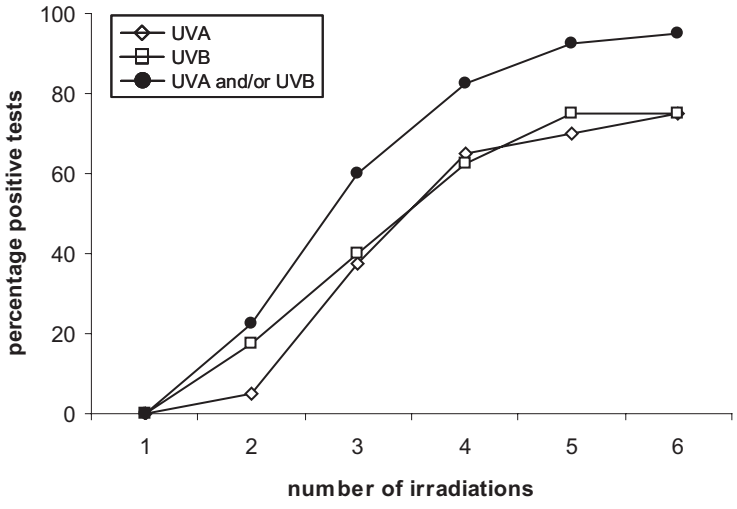

Figure 3. Cumulative increase in percentage of PLE patients in time testing positive during diagnostic phototesting (prospective study)

number of patients testing positive during each irradiation increased equally during the first 4 irradiations and levelled off with continuing the irradiations up to 6 times. Also in this prospective study, most patients suffered from the papular or papulo(vesicular) form of PLE. Positive reproduction by phototesting resulted in 71% papular PLE followed by 14% (papulo)vesicular PLE, 3% plaque-type PLE and 3% excessive erythema. Although again a relation was found between patient's history and PLE lesion reproduced by phototesting for papular PLE, this was not true for the other PLE forms. No different pattern in PLE type was observed after exposure to UVA irradiation or UVB irradiation.

Prospective study – 4 irradiations versus 6 irradiations

Table II shows the difference in skin reaction for UVA and UVB as scored by the independent observers between the area that was irradiated 4 times and the area that was irradiated 6 times. Most important, no patients developed a positive skin reaction solely on the area irradiated for 6 times.

For UVA irradiation, in 3 patients the 6 times irradiated area showed a stronger reaction than the 4 times irradiated area, but both areas developed a positive skin reaction. The same was seen in 2 patients with a positive skin reaction on UVB irradiation. For 1 patient with a late positive skin reaction after UVA and 3 patients with a late positive skin reaction after irradiation with UVB, the visual appearance of both areas was equal in reaction, although one part was irradiated 2 times more than the other area. For the late UVA and UVB reactions, in 2 patients the 2 independent observers scored the skin reaction after 4 irradiations different but equal after 5 and 6 irradiations (observer 1: 6 days area slightly more positive skin reaction than 4 days area; observer 2: both areas equal positive skin reaction). Each observer scored 40 patients four times (160 observations per observer) in only 8 cases there was a slight inter-observer variability (5%). This inter-observer variability was only seen in severity score of the elicited skin lesions and not in the presence or absence of skin lesions.

Table II. Observed skin reaction in patients with a positive phototest result after 5 or 6 irradiations.

Skin score	UVA	UVB
6+/4-	0	0
6>4	3	2
4=6	1	3
4>6	0	0

6+/4-, positive skin reaction only elicited on the area irradiated 6 times; 6>4, both areas reacted with a positive skin reaction, only reaction on area irradiated 6 times was stronger than on area irradiated 4 times; 6=4, both areas reacted with equally strong positive skin reaction; 4>6, both areas reacted with a positive skin reaction, only reaction on area irradiated 4 times was stronger than on area irradiated 6 times

DISCUSSION

Phototesting is a time-consuming diagnostic procedure. To achieve a more standardized and rational protocol for diagnostic photoprovocation testing we determined the optimal number of irradiations necessary to elicit skin lesions in PLE. From this retrospective and prospective study we conclude that the optimal phototesting protocol consists of 4 daily irradiations. More extensive protocols have no clinical implications. However, fewer exposures may lead to substantial loss of positive test results.

Retrospectively, it was shown that a cumulative increase of patients tested positive per irradiation was seen during the first 4 daily irradiations with UVA and UVB. Continuation of daily irradiations up to 6 times resulted in a reduced increase of patients tested positive (Fig 2). From these data it was concluded that a phototesting protocol of 4 daily irradiations seems optimal. After 4 irradiations 71% of the patients developed a positive skin reaction on UVA and/or UVB irradiation. In our retrospective study, the fourth irradiation is given on Fridays (Fig 1*A*). The evaluation of the fourth irradiation was done after the weekend (72 hours). We hypothesized that this could partially explain the drop in the cumulative increase in positive tested patients after the fourth irradiation. To overcome this problem a prospective study was designed in which the fourth irradiation was evaluated on Fridays (Fig 1*B*). The cumulative number of patients tested positive in this prospective study increased equally per successive irradiation during the first four irradiations. Again continuation of daily irradiations up to six times resulted in a reduced increase of patients tested positive (Fig 3). We showed that regardless of the weekend break, 4 daily irradiations were optimal. The number of patients testing positive after 5 or 6 irradiations was low in both studies (retrospective 14 out of 80 patients and prospective 5 out of 38 patients). We evaluated their history. All patients with late positive skin reactions suffered minor complaints (data not shown).

In literature, most departments performing phototesting were able to reproduce lesions in 30-100% of the PLE patients within a maximum of 5 daily irradiations (7,12,14,17-27). Difference in numbers might be explained by differences in light sources used, maximal number of irradiations, provocation regime and definition of PLE. We believe that scoring positive test results per successive irradiation showed to be a good parameter to compare different provocation regimes. With regard to this, the study by van de Pas *et al.* (14) was by our knowledge the first study that recorded the cumulative number of positive skin reactions per irradiation. Using solar simulator radiation (output approximately 290-400 nm) about 60% positive skin reactions could be reproduced after 3 or 4 irradiations, where no additive effect was shown from the fourth irradiation. Using separate UVA and UVB exposures, we were able to reproduce PLE skin lesions for UVA and/or UVB after 4 irradiations in 73%

(retrospective study) and 83% (prospective study) cases. After 3 irradiations, numbers in our study were comparable with their results. Comparing the two provocation protocols, besides different UV-lamps, increments in irradiation dose and thus higher total dose may explain our increased additive effect of the fourth irradiation (start approximately 1.8 MED with 20% increment with each successive dose, compared with 4 times 1.5 MED). We believe that increasing the dose with each irradiation may prohibit effects of hardening.

Furthermore we demonstrated, as shown in Table III, that all skin reactions which developed after 5 or 6 irradiations were already initiated during the preceding 4 irradiations. This implies that observing skin reactions up till a few days after discontinuation of phototesting can be informative. We did not study this phenomenon comparing 3 daily irradiations with 4 daily irradiations to further support our data that the fourth irradiation has a surplus value.

In our retrospective and prospective study most PLE patients (about 60%) reacted during phototesting to both UVA as well as UVB irradiation. About 20% of the patients reacted to UVA alone, or UVB alone. The number of solely UVB positive patients in our retro- and prospective study was comparable with studies from literature. The number of UVA positive patients (UVA alone and (UVA+UVB) positive) in our retro- and prospective study was 75%, which is comparable to the described studies in literature. The smaller numbers solely UVA positive patients can therefore be explained by a shift of patients to the group of patients testing positive for both UVA and UVB. Provocation with visible light resulted in only few positive results. Al patients which reacted to visible light also reacted strongly to UVA and UVB. Therefore, provocation with visible light as a part of the standard procedure is debatable.

In conclusion, a maximum of 4 daily irradiations using UVA and UVB light sources is suitable and optimal for diagnostic phototesting in the majority of the PLE patients. More extensive test protocols bare no clinical implications, however less extensive protocols may lead to substantial loss of positive test results.

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Chapter 4

Decreased neutrophil skin infiltration after UVB exposure in patients with polymorphous light eruption

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ABSTRACT

UV radiation, in particular UVB, suppresses the skin immune response. In patients with polymorphous light eruption (PLE) the skin immune response seems activated after UV exposure. Typical PLE skin lesions can occur as early as several hours after UV exposure. In healthy volunteers, neutrophils infiltrate the skin shortly after UV exposure. The kinetics and mechanisms of neutrophil infiltration in the skin of PLE patients after UVB exposure was studied. Skin biopsies at 0, 3, 6 and 18 h were taken from fivePLE patients and six healthy controls after irradiation with three MED UVB. Furthermore, neutrophils were isolated from blood of five PLE patients and six healthy controls to test their chemotactic activity.

Immunohistochemical analysis showed a significant decreased neutrophil infiltration in PLE skin after UVB irradiation compared with healthy controls ($p<0.05$). In both healthy controls and PLE patients, after UVB irradiation, ICAM-1 and E-selectin expression on endothelial cells increased at 6 h after irradiation. Blood neutrophil chemotactic response towards IL-8 and C5a, as well as the expression of cell surface markers involved in adhesion and chemotaxis, was not different between PLE patients and healthy controls. In conclusion, PLE is marked by a decreased skin infiltration of neutrophils after UVB irradiation, possibly leading to a diminished neutrophil-induced suppression.

INTRODUCTION

Polymorphous light eruption (PLE) is a photosensitivity disorder of which the pathogenesis is not fully understood. PLE patients develop pruritic erythematous, papular, vesicular or plaque-like lesions on sunlight-exposed areas of the skin within several hours to days after UV exposure. Because of the clear delay between sunlight exposure and the onset of skin lesions, PLE is often suggested to be a delayed-type (type IV) of hypersensitivity (DTH) reaction. The histological appearance of dense superficial and perivascular dermal lymphocytic infiltrates in skin of PLE patients further supports this (1-3).

UV radiation, in particular the UVB range (280-315 nm), suppresses the skin immune response (4). In PLE patients, however, UV exposure seems to result in an activation of the skin immune response. PLE lesions can be elicited already within 1 hour after UV exposure, where a classical DTH response takes more time to develop (5). This suggests that in PLE early pathogenic mechanisms, independent of DTH, are active.

It is known for a long time that neutrophils migrate to the skin shortly after UV exposure (6). The aim of this study was to analyze the kinetics and mechanisms of neutrophil infiltration in the skin of PLE patients during the first hours after UVB exposure.

MATERIALS AND METHODS

Subjects

For immunohistochemical analysis, five patients (three males and two females, ages between 28 and 44 y, mean age 35 y) with moderate-to-severe PLE with normal minimal erythema dose (MED) and six healthy controls (four males and two females, ages between 19 and 39 y, mean age 24 y) were studied. PLE patients with a positive skin reaction on UVB irradiation were included. A positive skin reaction was defined as elicitation of typical PLE skin lesions after a maximum of four daily increasing exposures with UVB. Patients who received UVB hardening therapy or used oral immunosuppressive medication were excluded. The buttock skin of patients and healthy controls needed to be restricted from sunlight or tanning bed exposure for at least 6 weeks.

For chemotaxis and flow cytometric analysis of neutrophils, five UVB sensitive, moderate-to-severe, PLE patients (ages between 39 and 84 y, mean age 55 y) and six healthy controls (ages between 24 and 40 y, mean age 35 y) were included. The medical ethical committee of the University Medical Center Utrecht approved all described studies and the participants gave their written consent.

Phototesting procedures

The MED of the buttock skin was determined using a Philips (Philips, Eindhoven, The Netherlands) TL12 lamp (57.7% of UV output in UVB (280-315nm)), as described by Boonstra *et al.* (7). Subsequently, the unaffected buttock skin was exposed to three MED UVB. Four-millimeter punch biopsies (under local anesthetics using 2% xylocaine with adrenalin) were obtained 3, 6 and 18 h after irradiation, together with a control biopsy from the unirradiated buttock skin. Biopsies were snap-frozen in liquid nitrogen and stored at –80°C until use.

Reagents

Levamisol, naphthol AS-BI / AS-MX phosphate, Fast Blue BB Salt, N,N-dimethylformamide (DMF) and 3-amino-ethyl-carbazole (AEC) were all purchased from Sigma (St Louis, Missouri). New Fuchsine was purchased from Merck (Darmstadt, Germany). IL-8 was purchased from Peprotech INC. (Rocky Hill, New Jersey) and C5a from Sigma. Incubation buffer contained 20 mM Hepes, 132 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1.2 mM KH_2PO_4, supplemented with 5 mM glucose, 1.0 mM $CaCl_2$ and 0.5% (vol/vol) human serum albumin (HSA). FACS-buffer contained phosphate buffered saline (PBS), supplemented with 5% fetal calf serum and 0.5% NaN_3. Ficoll-Paque was purchased from Amersham Biosciences (Uppsala, Sweden). Weigert solution contained [1% (vol/vol) haematoxylin in ethanol/70 nM acidic $FECl_3$, 1:1 (vol/vol)].

Antibodies

For immunohistochemical stainings, monoclonal antibodies against elastase (clone NP57, DAKO A/S, Glostrup, Denmark, diluted 1:40), E-selectin (CD62E; clone BB16-E4(5D11), R&D Systems, Minneapolis, Minnesota, diluted 1:20), ICAM-1 (CD54; Genzyme, Cambridge, Massachusetts, diluted 1:25), VCAM-1 (CD106; DAKO A/S, diluted 1:25) were used. The biotinylated-ULEX (europaeus lectin-type 1) antibody was purchased from DAKO A/S (diluted 1:50). Biotinylated horse anti-mouse immunoglobulin (Vector, Burlingame, California, diluted 1:800) was used as secondary antibody. Alkaline phosphate-labeled Streptavidin (DAKO A/S, diluted 1:300) or horseradish peroxidase-conjugated avidin-biotin complex (DAKO A/S, diluted 1:50) were used as detecting reagents. FACS analyses were performed using monoclonal antibodies against the following cell surface markers: DREG56 (anti L-selectin; CD62L) was isolated from the supernatant of a hybridoma obtained from the American Type Culture Collection (Rockville, Maryland); PL-1 (anti PSGL-1, CD162) was purchased from Endogen (Boston, Massachusetts). CXCR1 (anti IL8R1, clone 5A12) and CXCR2 (anti IL8R2, clone 6C6) were purchased from Pharmagen (San Diego, California). Direct-labeled antibodies FITC-conjugated CD11b, PE-conjugated CD15s (both from Immunotech, Marseille, France) and FITC-conjugated CD18 (DAKO A/S) were used.

PE labeled goat anti-mouse immunoglobulin (Southern Biotechnology, Birmingham, Alabama) was used as secondary antibody. Control antibody W6/32 (anti-HLA-A,B,C) was isolated from the supernatant of a hybridoma obtained from the American Type Culture Collection. Non-specific isotype matched control antibodies IgG1-FITC, IgG-PE (Becton Dickinson, San Jose, California) and IgG1 (Southern Biotechnology) were used as negative controls.

Isolation of neutrophils

Citrate-anti-coagulated blood was obtained from healthy volunteers and PLE patients diluted 1:1 with PBS. Mononuclear cells were removed by centrifugation over isotonic Ficoll-Paque (1.076 g per mL). After lysis of the erythrocytes in isotonic ice-cold NH_4Cl solution, the granulocytes were washed and resuspended in incubation buffer or isotonic FACS-buffer. All preparations contained >97% neutrophils.

Migration assay with use of Boyden chamber

Neutrophil migration was measured in the modified Boyden chamber assay as described by Coffer *et al.* (8). Briefly, chemotaxins IL-8 and C5a were added to the lower compartments of the chamber and resting granulocytes were placed in the upper compartment. The chamber was incubated for 45 min at 37°C. All migratory responses were quantified using an automatic microscope (Leitz DMRXE, Leica, Wetzlar, Germany) and an image analysis system (Quantimet 570C, Leica Cambridge).

Flow cytometric analysis of neutrophil cell surface markers

Granulocytes were resuspended in isotonic FACS-buffer at a concentration of 2×10^6 cells per mL. Antibodies were added in an optimal concentration of 5 µg per mL for 30 min. Subsequently, cells were washed in FACS-buffer. PE-coupled goat anti-mouse antibody was added for 30 min, when an unconjugated detecting antibody was used. Antibody binding was determined by flow cytometry (FACSvantage, Becton Dickinson). Neutrophils were identified according to their specific side-scatter and forward-scatter signals (9).

Immunohistochemistry

Frozen skin sections (6 µm) on 3-amino-propyltriethoxysilane-coated glass slides were used for all experiments. The glass slides were fixed for 10 min in dry acetone at room temperature and incubated for 20 min in a 10% blocking reagent [PBS, containing 10% normal human serum and 10% normal horse serum] to prevent non-specific binding. The skin sections were incubated at room temperature for 1 h with the primary antibody, diluted in 1% blocking reagent [PBS containing 1% normal human serum and 1% normal horse serum]. The glass slides were washed with PBS,

containing 0.05% Tween 20. Subsequently, skin sections were incubated for 45 min with a biotinylated horse anti-mouse antibody (diluted in 1% blocking reagent) followed by an incubation with alkaline phosphate-labeled streptavidin (diluted in 1% normal mouse serum) for 45 min. Antibody binding was visualized by incubating the sections in 100 mL Tris-HCL buffer (pH 8.4) containing 10.2 g $MgCl_2$, 9.6 mg $NaNO_2$, 250 µL New Fuchsine, 35 mg levamisol and 50 mg naphthol-ASBI (in 1 mL DMF). The skin sections were counter-stained with Mayer's hematoxylin.
For double stainings, primary antibody (E-selectin, ICAM-1 or VCAM-1) together with a biotinylated ULEX antibody was used and handled as described by de Vries *et al.* (10). All antibody incubations were performed in a humidified chamber.

Quantification of staining and statistical analysis

The skin sections were evaluated using a light microscope at x 400 magnification with a standard eyepiece. Single staining of neutrophils was quantified by counting absolute number of cells per mm^2. In double stainings, the absolute number of dermal endothelial cells (EC) expressing adhesion molecule binding was counted in number of cells per mm^2. Increase in EC expressing adhesion molecules was calculated as change compared to t= 0 h. Statistical analysis was done using a Mann-Whitney *U* test.

Table I. Minimal erythema dose (Philips TL12, UVB) and erythema response in time after 3 MED UVB irradiation in healthy controls and PLE patients

		MED	Visual erythema score (after UVB)			
		(mJ/cm2)	0 h	3 h	6 h	18 h
controls	1	150	-	-	+/-	+
	2	75	-	+	+	+
	3	105	-	ND	ND	ND
	4	75	-	+/-	+/-	+
	5	75	-	+/-	+/-	+
	6	53	-	+/-	+	+
mean controls		**88.8**				
PLE	1	80	-	-	+/-	ND
	2	56	-	+	+	+
	3	112	-	+/-	+	+
	4	80	-	-	+	+
	5	112	-	+/-	+	+
mean PLE		**88.0**				

(-) negative, (+/-) slight erythema, (+) clear erythema, (ND) not determined

RESULTS

Phototesting

After irradiation with three MED of UVB on unaffected buttock skin, erythema reaction was scored at t= 3, 6 and 18 h. Table I shows the MED and observed erythema in time for most healthy controls and PLE patients. The mean MED in healthy controls was 88.8 ± 34.4 mJ per cm^2 and in PLE patients 88.0 ± 24.0 mJ per cm^2. None of the patients developed a positive buttock skin reaction after this single exposure with three MED UVB.

UVB irradiation induces significant fewer infiltrating neutrophils in the skin of PLE patients

In unirradiated buttock skin no neutrophils, visualized by their elastase content, were found in healthy controls, as well as PLE patients. After UVB irradiation, neutrophils were present in the dermis of two of six healthy controls 3 h after irradiation and were significantly increased in all healthy controls at 6 h ($p<0.05$). The number of infiltrating neutrophils after UVB irradiation was significantly less in PLE patients compared with healthy controls ($p<0.05$ at 18 h). (Figs 1 and 2).

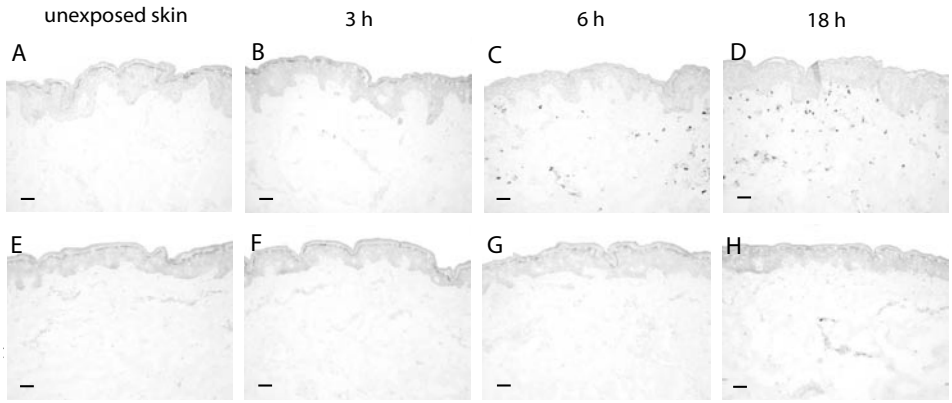

Figure 1. Neutrophil elastase staining in the dermis of a healthy control and PLE patient after three MED UVB (A+E) Unexposed (-UV), (B-D+F-H) hours after UVB exposure (+UV 3h, 6h, 18h). UVB dose given in these volunteers was equal. *Scale bar: 50 µm*

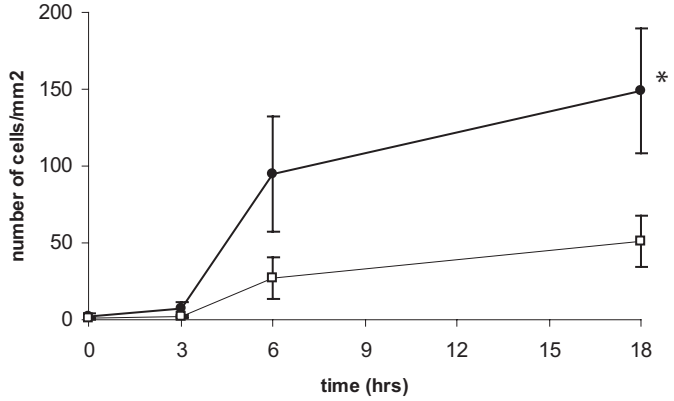

Figure 2. Number of elastase positive neutrophils in the dermis in time after three MED UVB (—●—) Controls (n=6), (—□—) PLE patients (n=5). Mean number of cells /mm^2 ± SEM, statistical significance *p<0.05, at t= 18 h, between control group and PLE group

The intrinsic properties of neutrophils to react to a chemotactic stimulus are not impaired in PLE patients

In Fig 3*A* it is shown that the reaction of peripheral blood neutrophils towards an increasing concentration of IL-8 shows a dose dependent response. The response towards IL-8 is similar in neutrophils of PLE patients and healthy controls. Besides IL-8, the chemotactic response of blood neutrophils towards C5a was tested (Fig 3*B*). Again, no difference was observed between neutrophils of PLE patients and healthy controls. Several cell surface markers on blood neutrophils, involved in adhesion and chemotaxis, were stained and analyzed by flow cytometry (Fig 4). No difference was found in cell surface marker expression on the neutrophils between PLE patients and healthy controls.

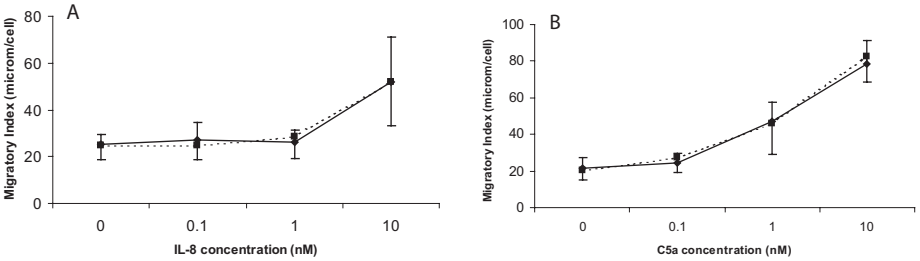

Figure 3. Chemotactic response of neutrophils towards IL-8 and C5a (*A,B*). (—●—) Controls (n=6), (·····■·····) PLE patients (n=5). Migratory Index (µm/cell), ± SEM, calculated for triplicate samples.

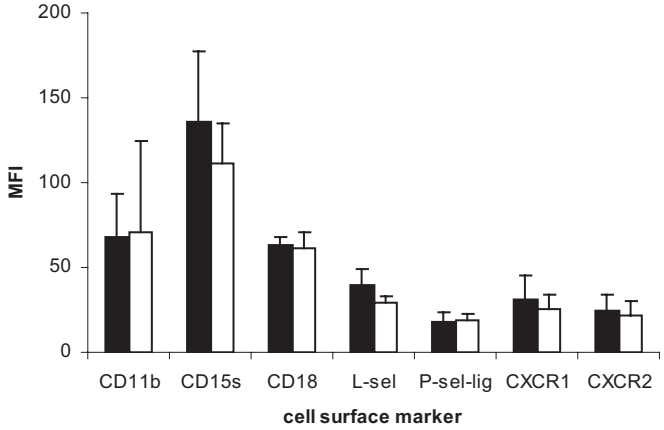

Figure 4. Cell surface markers on human neutrophils. (■) Controls (n=6), (□) PLE patients (n=5). Data are expressed in corrected mean fluorescence intensity (MFI), calculated by subtracting MFI for isotype controls. Data are based on geometric mean ± SEM.

Expression of E-selectin after UVB irradiation in PLE patients

Figs 5*A* and *B* show endothelial cells (EC) expression of, respectively, ICAM-1 and E-selectin after UVB irradiation in time. ICAM-1 was constitutively expressed on EC in unirradiated skin (mean (±SEM) for healthy controls 44.7 ± 3.0 cells per mm^2, for PLE patients 57.3 ± 3.3 cells per mm^2)(data not shown). The ICAM-1 protein expression was increased at 6 and 18 h after UVB irradiation, similar in both PLE patients and healthy controls (Fig 5*A*). The number of E-selectin expressing EC was low in unirradiated skin (mean (±SEM) of healthy controls (1.5 ± 0.5 cells per mm^2), or PLE patients 2.8 ± 1.5 cells per mm^2)(data not shown). At 6 h after UVB irradiation the number of E-selectin expressing EC increased significantly in both healthy controls and PLE patients ($p<0.05$). The trend of increase in PLE patients is lower, but not significantly lower, compared with healthy controls. (Fig 5*B*). VCAM-1 was not expressed in unirradiated skin of healthy controls and PLE patients. After UVB irradiation, no increase in VCAM-1 expressing EC was found in both healthy controls and PLE patients (data not shown).

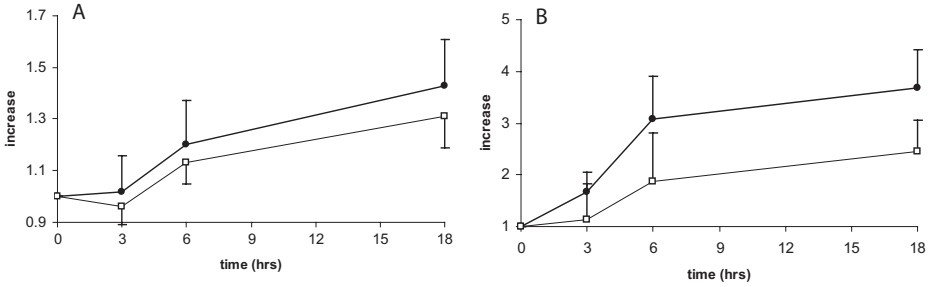

Figure 5. Increase in endothelial cells expressing ICAM-1 (A) and E-selectin (B) after three MED UVB irradiation (—●—) Controls (n=6), (·····□·····) PLE patients (n=5). Increase is calculated as change compared to number of adhesion molecule expressing endothelial cells at t= 0 h. Data are displayed as mean ± SEM.

DISCUSSION

The kinetics and mechanisms of neutrophil skin infiltration in the onset of PLE was studied. Our results clearly demonstrated a significant impaired infiltration of neutrophils into the skin of PLE patients compared to healthy controls after irradiation of buttock skin with three MED UVB (Fig 2). $CD4^+$ T cells and macrophages enter the UV exposed skin, but at a later time point than neutrophils (11,12). We also stained $CD3^+$ T-cells, which are mainly $CD4^+$ T-cells in the unirradiated skin (12). The increase in the number of $CD3^+$ T-cells 6 h after UVB irradiation was equal in healthy controls and PLE patients (data not shown). $CD68^+$ macrophages showed the same kinetics as the influx of T cells, and also no difference was found between healthy controls and PLE patients (data not shown). Neutrophils present in UV exposed skin are recruited from the peripheral blood by a coordinated repertoire of adherence to vascular endothelium, diapedesis out of the bloodstream, and subsequent migration into the skin towards the UV challenged site (13). The intrinsic chemotactic capacity of blood neutrophils towards IL-8 and C5a, as well as the expression pattern of cell surface receptors, was similar in PLE patients and healthy controls (Figs 3 and 4). We could not determine the subsequent chemotactic capacity of neutrophils once inside the skin. Since four of six healthy controls and three of five PLE patients showed the presence of neutrophils in the epidermis 18 h after irradiation (data not shown), however, we hypothesize that once in the tissue, the migration capacity of the neutrophils is similar in PLE patients and healthy controls. Leino *et al.* (14) showed reduction in the phagocytosis and adhesive function of leukocytes in healthy human volunteers after whole-body irradiation with UVB, possibly due to down-regulation of cell-surface receptors. Furthermore, de la Fuente *et al.* (15), showed both decreased phagocytosis and IL-8 induced chemotaxis in polymorphonuclear cells of patients with systemic lupus erythematosus (SLE). Compared with changes in

leukocyte function after whole-body irradiation, PLE lesions can be elicited on small UV irradiated skin areas. Therefore, it seems unlikely that reduced neutrophil infiltration in PLE skin is caused by impaired function of circulating neutrophils. However, unlike SLE, PLE has never been described as a systemic disease, and decreased numbers of infiltrating neutrophils will probably not be due to systemic effects, but more due to modulation of the skin immune response.

Neutrophils enter the skin via binding of adhesion molecules expressed on the dermal endothelium (13). We showed that 18 h after UVB irradiation an increase in ICAM-1 expressing EC was similar in PLE patients and healthy controls (Fig 5*A*). E-selectin expression on EC was increased 6 h after UVB irradiation in both healthy controls and PLE patients. Possibly due to small numbers, the lower increase in E-selectin expression on EC in PLE patients was not significantly diminished from healthy controls (Fig 5*B*). E-selectin expression after UVB irradiation may play a role in the recruitment of neutrophils and thus indirectly in the early pathogenesis of PLE. It has been shown before that E-selectin expression on EC is rapidly increased within 6 h after UVB irradiation and mimics the kinetics of neutrophil influx into the skin (1,16). The same holds true for our data (Figs 2 and 5*B*).

After UV irradiation, keratinocytes can produce tumor necrosis factor-α (TNF-α) and IL-8 (16,17). Furthermore, TNF-α can upregulate the expression of E-selectin and IL-8 by EC (1,18). Using immunohistochemical techniques we were not able to reliably quantify epidermal and dermal TNF-α expression nor IL-8 expression after UVB irradiation.

We hypothesize that the decreased number of neutrophils, or neutrophil products, in the skin of PLE patients after UVB irradiation may alter the local cytokine milieu in the skin and thus play a role in the induction of PLE. For a long time, neutrophils that infiltrated UV exposed skin were thought to be mainly involved in repair processes of UV damaged skin (6,16). It was not until recently that it was discovered that neutrophils can produce a variety of cytokines with an immune suppressive effect (like IL-4 and IL-10) and thereby may play a regulatory role in immune reactions (11,12). Less neutrophil infiltration in PLE skin may lead to impaired local production of IL-4 and IL-10, thereby altering the local skin milieu after UVB irradiation, eventually leading to activation of the skin immune response instead of suppression. In conclusion, in this study we investigated the early, possibly initiating, immunological changes after UVB exposure to the skin in PLE patients. We showed that in PLE patients significantly less neutrophils infiltrated the skin after irradiation compared with healthy controls, most probably due to local and not systemic pathogenic mechanisms.

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Chapter 5

Time dependent mRNA expression in UV-induced inflammation of skin after a single dose of UVB. A comparison between healthy controls and patients with polymorphous light eruption

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ABSTRACT

Ultraviolet (UV) radiation suppresses immune responses in human skin, leading to changes in cell-composition and cytokine levels. Patients with polymorphous light eruption (PLE) develop a pruritic rash within a few hours after UV exposure. Repeated challenges with UVB induce a pathological skin reaction. In PLE patients significantly less neutrophils infiltrate the skin at 18 h after a single UVB exposure. This suggests a dysregulation during the early phase of the UVB-induced immune response in patients with PLE.

The aim of the study was to determine *in vivo* the sequential changes in mRNA regulating a broad spectrum of proteins involved in UV-induced inflammation of the skin within the first hours after UVB exposure. Furthermore, we compared these expression patterns between healthy controls and PLE patients, in order to unravel possible mechanisms underlying the early phase of the pathological response to UVB in PLE.

Skin biopsies were collected from 16 healthy controls and 7 PLE patients at 0, 3, 6 and 18 hours after a single UV exposure of 3 MED UVB together with skin biopsies from lesional PLE skin (n=6). mRNA was extracted and gene regulation of IL-1β, TNF-α, IL-20, IL-8, Gro-all, E-selectin, ICAM-1, MMP-1, Desmoplakin-1, IL-24, IL-1RA, IL-4, IL-10, IL-12, IFN-γ, CD25 and FOXP3 was quantified by RT-PCR.

Gene specific expression time-dependency was observed after UVB exposure in healthy controls. At 3 and 6 h after UVB irradiation, no distinctive mRNA expression pattern between PLE patients and healthy controls was shown. However, at 18 h after UVB exposure, PLE patients showed significantly increased mRNA expression of ICAM-1, CD25 and FOXP3 compared with healthy controls. Furthermore, in unirradiated skin of PLE patients the expression of IL-1β was decreased.

UVB exposure of human skin leads to early sequential changes in mRNA regulating a broad spectrum of proteins involved in UV-induced inflammation of the skin. Our results suggest that there is no difference in the early inflammatory response to a single exposure of 3 MED UVB between patients and healthy controls. Eighteen hours after UVB exposure an increased mRNA expression of ICAM-1, CD25 and FOXP3 was observed in PLE patients in contrast to healthy controls. However, these results do not explain the differences found in neutrophil skin infiltration between PLE patients and healthy controls during the first hours after UVB exposure.

INTRODUCTION

Ultraviolet (UV) radiation, in particular the UVB range (280-315 nm), suppresses immune responses in animals and humans (1,2). Exposure of human skin to ultraviolet UVB leads to changes in the cell composition and cytokine levels in the skin (3,4). UV exposed keratinocytes produce ICAM-1, IL-1, IL-6, IL-8, IL-10,TNF-α and PGE_2, which can result in the onset of UV-induced erythema and attraction of leukocytes to the skin (5-7). Within a few hours after UV exposure neutrophils migrate into the skin, followed by an influx of macrophages. Upon UV-exposure, neutrophils contain IL-4 and IL-10 protein, whereas macrophages contain IL-10 (8,9). Furthermore, UV exposure results in an initial depletion of epidermal T-cells followed by a selective influx of CD4+ T-cells after 2 days (10). These CD4+ cells express CD25 and may release IL-10 upon stimulation in the skin (11).

In contrast to the influx of leukocytes, Langerhans cells (LC), which are the major antigen-presenting cells of the epidermis, migrate out of the skin to the draining lymph nodes after UV exposure (12,13). In the lymph nodes, the UV-irradiated LC can selectively present antigen to Th2 type cells, without stimulating Th1 type cells (14). The modified cellular composition and concomitant cytokine production in the skin after UV exposure promotes a Th2-skewed response, which benefits in UV-induced immunosuppression.

Polymorphous light eruption (PLE) is an idiopathic photosensitivity disorder of which the pathogenesis is not fully understood. PLE patients develop pruritic erythematous papular, vesicular or plaque-like lesions on sunlight exposed areas of the skin several hours to days after UV exposure. In UV-induced PLE lesions, a dense perivascular upper dermal infiltrate of predominantly T-lymphocytes is visible in the upper part of the dermis already within 5 h after irradiation which reaches a peak at 72 h after irradiation. In the first 72 h a predominance of $CD4^+$ cells is present. Thereafter, $CD8^+$ cells dominate the infiltrate (15,16). The histological features, together with the delayed onset of pathological PLE skin lesions after UVB, gave rise to the hypothesis of PLE being a DTH-like response to UV-induced neoantigens (15-19). However, since PLE lesions can be elicited already within a few hours after UV exposure, an earlier pathogenic mechanism might be responsible for the initiation of PLE (20,21). Previously, it was shown that the influx of neutrophils, which contain IL-4 and TNF-α, was significantly decreased in 3 and 6 MED UVB-exposed skin of PLE patients compared with healthy controls at 18 and 14 h after UVB exposure (21,22). Furthermore, after 6 MED exposure to UVB an increased number of LC persisted in the skin of PLE patients compared with healthy controls (23).

The aim of our study was to determine *in vivo* the sequential changes in mRNA regulating a broad spectrum of proteins involved in UV-induced inflammation of

the skin in the first 18 hours after UVB exposure. Furthermore, we compared the expression patterns of healthy controls with those of PLE patients.

MATERIAL AND METHODS

Subjects

In total, 16 healthy controls (12 males and 4 females, ages between 19 and 52 y, mean age 28 y) and 7 moderate to severe PLE patients (4 males and 3 females, ages between 28 and 44 y, mean age of 36 y) with a normal minimal erythema dose (MED) were studied. PLE patients with a positive skin reaction to UVB irradiation, irrespective of a reaction to UVA irradiation were included. A positive skin reaction was defined as elicitation of PLE skin lesions after a maximum of 4 daily increasing exposures with UVB as described previously (24). Patients who received UV hardening therapy or used oral immunosuppressive medication were excluded. The buttock skin of patients and healthy controls needed to be restricted from sunlight or tanningbed exposure for at least 6 weeks. The study was approved by the local medical ethical committee and the participants gave their written consent.

Phototesting procedure

The MED of buttock skin was determined using a Philips (Philips, Eindhoven, The Netherlands) TL12 lamp (57.7% of UV output in UVB (280-315nm)) as described by Boonstra *et al.* (25). Subsequently, the unaffected buttock skin was exposed to a single dose of 3 MED UVB. Four-millimeter punch biopsies were obtained under local anaesthetics, using 2% xylocaine with adrenalin. Biopsies were taken at 3 and 18 hours after UVB irradiation, together with a control biopsy of the unirradiated buttock skin. In 7 healthy controls and 5 PLE patients an additional biopsy was taken 6 hours after UVB irradiation. Due to technical difficulties in obtaining good quality mRNA, not all consecutive biopsy time-points in all subjects could be included in the study. An overview of subjects included in the different analyses is shown in Table I. Furthermore, we obtained biopsies of lesional PLE skin in 6 patients, together with a control biopsy of unirradiated non-lesional skin. Lesional PLE skin (papular type in all patients) was elicited upon repetitive irradiation with UVB. Number of irradiations and total UVB dose to elicit PLE differed between patients.

Table I. Included subjects

	controls	PLE patients
total number	n = 16	n = 7
RT-PCR	total studied#	total studied##
unexposed skin	n = 16	n = 7
3h after UVB	n = 15	n = 7
6h	n = 7	n = 5
18h	n = 13	n = 7

exept IL-4, IL-10, IL-12: 0h=7, 3h=7, 6h=7, 18h=5

exept IL-4, IL-10, IL12: all timepoints n=5

Biopsies	n=6
L- and NL-PLE skin	IL-4, IFN-γ

L= lesional; NL= non-lesional

RNA isolation and cDNA synthesis

Frozen tissue sections (80-100 of 20 μm) were homogenized in ice-cold RNeasy Lysis Buffer (RLT) containing β-mercaptoethanol (Sigma, St Louis, MO). RNA was isolated with the RNeasy micro kit (Qiagen, Hamburg, Germany) and treated with DNAse, according to the manufacturer's protocol. Reverse transcription was performed with 200 ng total RNA using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA), according to the manufacturer's protocol.

Real-time PCR

Real-time PCR was performed using a MyiQ real-time PCR detection system (Biorad, Hercules, CA, USA) using SYBR Green I (Biorad). Primers (Sigma-Genosys, The Woodlands, TX, USA) were designed using Primer 3 software of the Whitehead Institute/ MIT centre for genome Research (26) (Table II). Amplification was performed using cDNA, SYBR Green I mastermix and 300 nM of each primer, according to the following conditions: 3 minutes at 95°C to activate DNA polymerase, 45 cycles of 10 seconds at 95°C, 20 seconds at 61°C and 25 seconds at 72°C. PCR was checked for a-specific products by performing dissociation curves after each PCR run. The signal of SYBR Green I was measured against the passive signal of the reference dye, fluorescein, to normalize for non-PCR-related fluorescence fluctuations occurring from well to well. Each assay included a standard curve of four serial concentrations of a pool of all cDNA samples to determine linearity and PCR efficiency. Results were normalized for PCR efficiency and for the reference genes β-actin, GAPDH and HPRT1 using ΔΔCT method (27).

Table II. Sequences of primers used for real time PCR

Name	Forward primer sequence 5′-3′	Reverse primer sequence 5′-3′
IL-1ß	GCTTATTACAGTGGCAATGAGGAT	GGTGGTCGGAGATTCGTAGC
IL-1RA	GAAGATGTGCCTGTCCTGTGTC	CGCTTGTCCTGCTTTCTGTTC
CD25	GCGGAGACAGAGGAAGAGTAGAA	CGACCATTTAGCACCTTTGATT
IL-4	AGCAGTTCCACAGGCACAAG	ACTCTGGTTGGCTTCCTTCAC
IL-8	AGCTCTGTGTGAAGGTGCAGTT	GGGTGGAAAGGTTTGGAGTATG
IL-10	GAGAACCAAGACCCAGACATCA	GTGGAGCAGGTGAAGAATGC
IL-12	ATGCCGTTCACAAGCTCAAGT	GGTGGGTCAGGTTTGATGATG
IL-20	GCCAATTCCTTTCTTACCATCAA	GCTGTATTTCTTCATTGCTTCCTC
IL-24	CAACCCAGTCAAGAAAATGAGATG	GCATCCAGGTCAGAAGAATGTC
TNF-a	CTCCAGGCGGTGCTTGTT	GCCAGAGGGCTGATTAGAGAGA
IFN-g	TTTGGGTTCTCTTGGCTGTTACT	GTTCCATTATCCGCTACATCTGAAT
Gro-all	CACCCCAAGAACATCCAAAG	TGTGGCTATGACTTCGGTTTG
E-selectin	ACATCTCAGGGACAATGGACAG	CTTCAGGACAGGCGAACTTG
ICAM-1	GCCAACCAATGTGCTATTCAAA	GCCAGTTCCACCCGTTCT
Desmoplakin-1	CCGCTGGCAAAGGATAGA	CGGCGTTTAGCATCATAGAGC
MMP-1	CCAGATGCTGAAACCCTGAA	TGAGGACAAACTGAGCCACATC
FOXP3	AAACAGCACATTCCCAGAGTTC	GCGTGGCGTAGGTGAAAG
β—Actin	AGCCTCGCCTTTGCCGA	CTGGTGCCTGGGGCG
GAPDH	AGAAGGCTGGGGCTCATTT	GAGGCATTGCTGATGATCTTG
HPRT1	TGGTCAGGCAGTATAATCCAAAGA	TCAAGGGCATATCCTACAACAAA

Quantification of data and statistical analysis

PCR-data were expressed as fold-regulation compared with the mean expression of unirradiated healthy control skin, which was arbitrarily set at 1. A minimum of 1.5 fold-change mRNA expression was determined suitable for statistical analysis. All PCR data are expressed as the mean ± 95% Confidence interval (CI) and differences between values were compared by the Wilcoxon Rank test (paired in lesional versus not lesional skin). Differences between study groups were tested using Mann-Whitney *U* test. Statistical difference was defined as $p<0.05$.

RESULTS

Phototesting

The MED's of all participants were in normal range and did not differ between controls and patients. The mean MED in healthy controls was 93.4 ± 35.2 mJ/cm^2 and in PLE 80.6 ± 24.5 mJ/cm^2. After a single exposure with 3 MED UVB, none of the patients developed a pathological skin reaction.

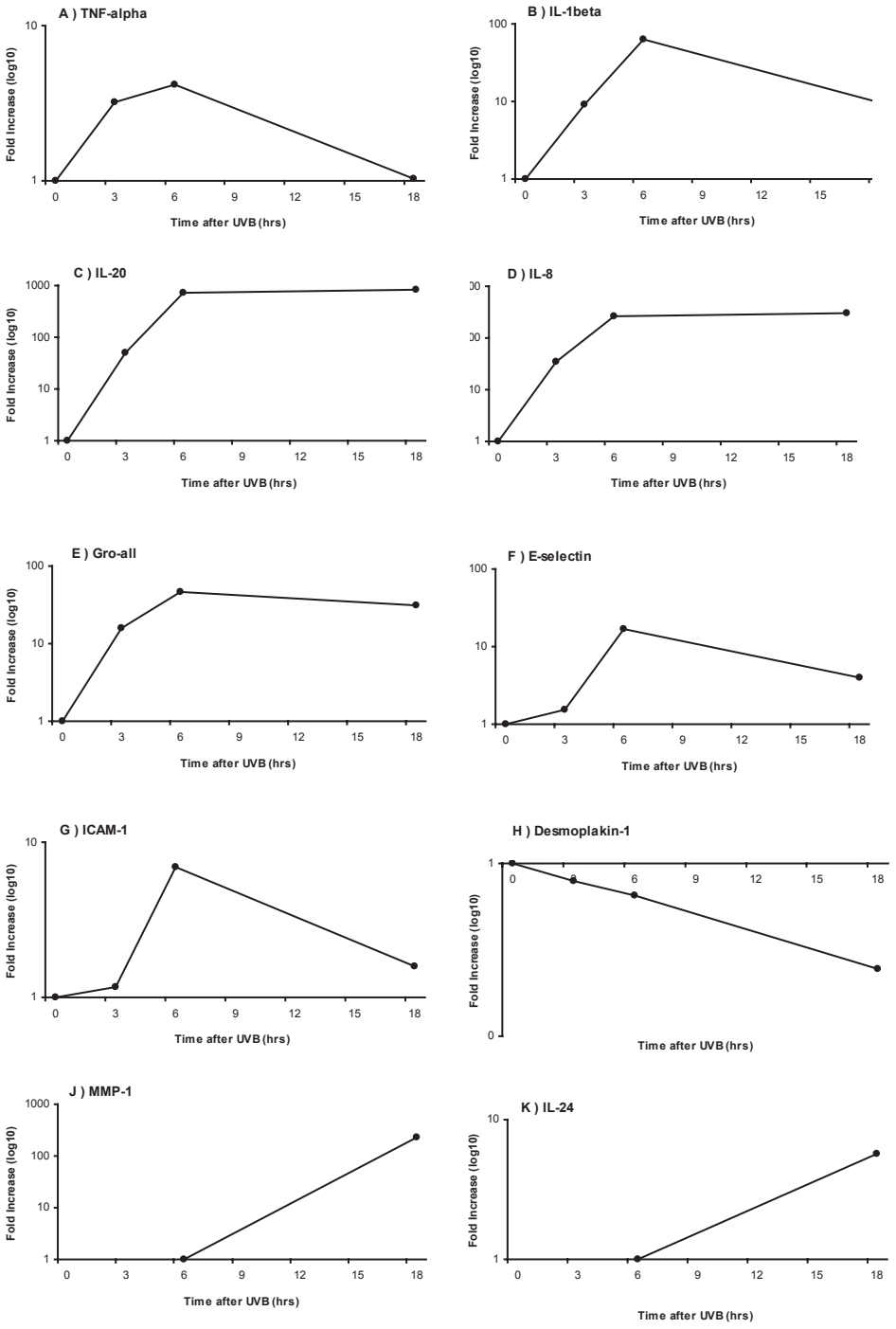

Figure 1. mRNA expression depicted in (log10)fold regulation in time (hrs) after 3 MED UVB irradiation in healthy control skin. A: TNF-α, B: IL-1β, C: IL-20, D: IL-8, E: Gro-all, F: E-selectin, G: ICAM-1, H: Desmoplakin-1, J: MMP-1, K: IL-24.

Time-related mRNA expression, the early induced genes after UVB exposure in skin of healthy controls

TNF-α, IL-1β and IL-20 were the first cytokines upregulated 3h after UVB exposure (Figs 1*A,B,C*). The maximum expression was demonstrated at 6 h after UVB exposure and expression of TNF-α and IL-1β diminished thereafter, whereas IL-20 expression was sustained. UV-exposure stimulated cells to produce a chemotactic milieu for infiltrating leukocytes. Already 3 h after UVB, upregulation of chemokines IL-8 and growth-related oncogene α, β, γ (Gro-all) was observed (figure 1*D,E*). Comparable to the gene expression of IL-20, IL-8 and Gro-all did not decline after reaching its peak at 6 h, but persisted during the test period.

The second wave of UV-induced mRNA expression in skin of healthy controls: adhesion molecules

The early upregulation of TNF-α and IL-1β was followed by that of E-selectin and ICAM-1 starting at 6 h post UVB (figure 1*F,G*). E-selectin and ICAM-1 expression declined again at 18 h after UV-exposure.

The third wave of expression in skin of healthy controls: cell-coherence and immunoregulatory genes

Kinetics of matrix-metallo proteinase (MMP)-1 and desmoplakin-1, involved in dermal (MMP-1) and epidermal (Desmoplakin-1) cell-coherence was determined. MMP-1 expression was not detectable until 6 h after UVB exposure, but at 18 h post UVB a clearly visible upregulation of MMP-1 was detected (figure 1*H*). Desmoplakin-1 expression was slowly downregulated after UVB exposure by more than 1.5 fold decrease at 18 h after UVB exposure (figure 1*J*). In addition, IL-24 expression was determined. Its UV-induced upregulation in time mimicked that of MMP-1 expression (figure 1*K*)

After UVB exposure, expression of anti-inflammatory (IL-10 and IL-1RA) and immunoregulatory (CD25 and FOXP3) genes could be shown, however the level after UVB exposure did not differ from unirradiated skin. IL-10 expression was only detected at 18 h after UVB exposure. Th1 (IL-12 and IFN-γ) and Th2 (IL-4) skewing cytokines were not detectable in the first 18 h after UVB exposure. Table III shows the fold-increase regulation of all tested genes.

Decreased IL-1β expression in unirradiated PLE skin, but early and second wave UV-induced mRNA expression does not differ in PLE patients

In general, UVB-induced effects on expression levels were comparable between healthy controls and PLE patients during the first 6 h after UVB irradiation (Table III). However, it was shown that PLE patients have highly significant down-regulation of IL-1β mRNA ($p=0.0008$) in unirradiated buttock skin compared with healthy controls (Table III).

PLE patients showed increased regulation of ICAM-1, CD25 and FOXP3 mRNA at 18h after UVB exposure (third wave)

At 18 h after UVB expsoure, ICAM-1, CD25 and FOXP3 mRNA expression was significantly more upregulated in PLE patients compared with healthy controls (ICAM-1, CD25, FOXP3: $p<0.05$). However, in the first 6 h after UVB expsoure, the concentration and kinetics of these genes was similar in PLE patients and healthy controls.

Lesional PLE skin shows Th1 skewing

In the first 18 h after UVB exposure, no Th1 or Th2 skewing cytokines were expressed above detection level. To detect UV-induced IL-4 and IFN-γ expression in skin of PLE patients, lesional PLE skin was analyzed and compared with non-lesional PLE skin (using paired Wilcoxon Rank test). IL-4 could not be detected in either non-lesional or lesional skin. IFN-γ, however, was significantly upregulated in lesional PLE skin compared with non-lesional skin ($p=0.03$), indicating a Th1 skewed milieu (data not shown).

DISCUSSION

UV-induced immunosuppression is a result of an altered microenvironment in the skin. UV-induced mRNA expression of cytokines and chemokines has been described before (4,28). However, we tested simultaneously a broad panel of genes involved in skin inflammation upon UVB exposure *in vivo* using PCR technique. mRNA expression of genes was tested at 3, 6 and 18 h after a single exposure to 3 MED UVB, resulting in 3 time-dependent gene expression waves.

UVB is able to penetrate the epidermis, but only a small fraction will reach the upper dermal compartment directly (29). Therefore, it is most likely that the earliest wave in gene expression (TNF-α, IL-1β, IL-20, IL-8 and Gro-all; within 3 h after UVB exposure) will be mainly produced by epidermal cells such as KC and LC. TNF-α and IL-1β are expressed by KC and LC; both cytokines are involved in LC emigration and upregulation of IL-6, prostaglandin, complement and selectins, thereby facilitating leukocyte infiltration upon UV exposure (28,30). Next to KC, also EC, macrophages and neutrophils can produce IL-8 and Gro-all chemokines. This may explain the sustained expression levels of IL-8 and Gro-all at 18 h after UVB exposure, when influx of inflammatory cells (neutrophils, macrophages and T-cells) in UV-irradiated skin has been initiated (31-33).

IL-20 is a pro-inflammatory cytokine, produced by KC and skin macrophages upon many different stimuli, including UV-exposure (34,35). Furthermore, in response to IL-20, KC express other pro-inflammatory genes including TNF-α, which leads to

Table III. mRNA fold-regulation after 3 MED UVB exposure in healthy controls and PLE patients

	Healthy controls (time after UVB)				PLE patients (time after UVB)			
	0 hrs	**3 hrs**	**6 hrs**	**18 hrs**	**0 hrs**	**3 hrs**	**6 hrs**	**18 hrs**
Chemokines								
IL-8	1	35.4 [16-77]	258.1 [116-575]	308.5 [148-643]	1.3 [0-4]	25.9 [7-98]	165.8 [68-406]	157.2 [65-381]
Gro-all	1	16.0 [10-27]	46.0 [26-82]	30.9 [21-47]	1.4 [1-3]	12.9 [5.8-29]	36.7 [17-80]	28.8 [15-54]
Pro-inflammatory								
IL-1ß	1	9.0 [5-15]	62.3 [32-120]	9.7 [6-17]	**0.2 [0.1-0.4]***	7.1 [7-98]	37.9 [68-406]	10.2 [65-381]
IL-12	nds	nds	nds	nds	nds	nds	nds	nds
IL-20	1	49.8 [26-95]	733.3 [0-3x108]	831 [471-1472]	0.5 [0.2-1.5]	32.9 [10-112]	504.7 [121-2103]	754.6 [248-2300]
IL-24	nds	nds	1	5.7 [3-11]	nds	nds	0.4 [0.1-3]	8.6 [5-16]
TNF-a	1	3.2 [3-4]	4.2 [3-6]	1.0 [1-1]	1.0 [1-1]	4.4 [3-6]	3.8 [3-5]	1.0 [1-1]
IFN-γ	nds	nds	nds	nds	nds	nds	nds	nds
Adhesion molecules								
E-selectin	1	1.5 [1-3]	16.7 [8-34]	4.0 [2-8]	0.9 [0.2-3.2]	2.1 [1-6]	12.9 [4-46]	7.2 [5-12]
ICAM-1	1	1.2 [1-1]	6.9 [4-11]	1.6 [1-2]	1.63 [1-2]	1.7 [1-2]	6.5 [4-10]	**2.5 [2-3]****
Cell-coherence								
Desmoplakin-1	1	0.8 [0.6-1]	0.7 [0.6-1]	0.3 [0.2-0.4]	1.2 [1-2]	1.3 [1-2]	0.9 [0.6-1]	0.3 [0.3-0.4]
MMP-1	nds	nds	1	226.8 [115-449]	nds	nds	1.1 [0.3-5]	288.0 [157-529]
Immunosuppressive								
IL-1RA	1	0.7 [0.5-0.8]	0.6 [0.5-0.8]	0.6 [0.4-0.7]	0.8 [0.6-1]	0.9 [0.7-1]	0.7 [0.4-0.7]	0.2 [0-1.2]
IL-4	nds	nds	nds	nds	nds	nds	nds	nds
IL-10	nds	nds	1	1.4 [0.5-4]	nds	nds	1.7 [0.7-4]	0.7 [0.3-2]
Immunomodulatory								
CD25	1	0.8 [0.6-1]	1.4 [1-3]	1.5 [1-2]	1.3 [1-2]	1.2 [1-2]	2.1 [1-4]	**2.8 [2-5]****
foxp3	1	0.9 [0.6-2]	1.2 [1-2]	1.0 [0.7-1]	0.6 [0.2-2]	1.3 [1-2]	1.7 [1-3]	**1.9 [1-3]****

Numbers are presented as fold-regulation ± 95% CI compared with the mean regulation of healthy control skin at 0 hrs, which is arbitrarily set at 1 Gene upregulation: numbers larger than 1 ; gene downregulation: numbers smaller than 1 nds= no detectable signal; *p<0.001; **p<0.05; significance between PLE patients and healthy controls

activation of NF-κB (36). In our study, IL-20 expression was found at 3 h after UVB exposure and levels remained high during the first 18 h (figure 1C).

The second wave of gene expression (between 3 and 6 h post-UVB) was formed by E-selectin and ICAM-1. As expected, E-selectin and ICAM-1 were upregulated at a later timepoint than TNF-α and IL-1β, starting at 6 h after UVB exposure and, as shown before, E-selectin expression mimicked the kinetics of neutrophil influx in the skin (21). The third wave of gene expression (18 h after UVB exposure) included

genes coding for proteins involved in cell-coherence and immunoregulation. Expression of matrix metallo proteinase (MMP)-1 and desmoplakin-1 was determined since these genes are involved in KC cell-coherence and degradation of extracellular matrix (ECM) proteins. Loss of cell-coherence will facilitate cell movement (37). Next to degradation of the ECM, MMPs can indirectly regulate chemokine and cytokine activity, thereby providing further evidence that MMPs play an active role in immunomodulation (reviewed by Parks *et al.* (38)). We could not detect MMP-1 expression at 3 and 6 h after UVB exposure, however there was a strong expression at 18 h after UVB exposure. MMP-1 can be expressed by KC, fibroblasts and infiltrating leukocytes upon a variety of stimuli including IL-1, which might explain the time delay in expression after UVB exposure (39,40). Desmoplakin reduction is supposed to play a role in metastases of tumour cells (41). To our knowledge, this is the first report of UV-induced down-regulation of desmoplakin-1. We hypothesize that this down-regulation might facilitate migration of cells into and out of the epidermis after UV-exposure.

Furthermore, during the third wave also immunoregulatory genes were expressed, however, at low levels (IL-1RA, IL-10, FOXP3, CD25) or not detectable at all (IL-4, IL-12, IFN-γ). Recently, Averbeck *et al.* (42) showed that after 3 MED UVB exposure in the first 8 h both Th1 and Th2 cytokines at protein level were detectable (IL-2, IL-12, and IL-4). At 24 h post-UVB, the Th1 cytokine protein amount decreased in contrast to Th2 cytokines, indicating a switch from a mixed Th1/Th2 milieu to a Th2 milieu upon UVB exposure. IFN-γ, however, was not detected at all timepoints. In their study, protein levels were measured from extracellular microdialysate samples collected over periods of 8 h. Since we determined gene expression at specific timepoints in stead of collectively sampling different timepoints, this might explain why we not able to detect any of the Th1 and Th2 cytokines. However, their findings support the idea that UV-exposure skews the skin milieu to Th2.

The kinetics of IL-10 expression in our study might be explained by skin infiltrating neutrophils, macrophages and T-cells several hours post-UVB (3,43,44). Furthermore, we observed that IL-24 gene expression was detectable at 6 h after UVB exposure and upregulated at 18 h. Macrophages and Th2 type cells, which start to infiltrate the skin at 18 h after UVB are the most likely source of IL-24 expression. The precise biological function of IL-24 is still elusive, but it appears to involve pro-inflammatory effects on KC by activating Stat-1 and Stat-3 transcription factors (45). Summarizing, we tested mRNA regulation of a broad spectrum of proteins involved in UV-induced inflammation of skin and this regulation showed gene specific time-dependency.

PLE patients were shown to have a significant downregulation of IL-1β mRNA in unirradiated buttock skin compared with healthy controls (Table III). IL-1β can be produced by KC, monocytes, macrophages, LC and dendritic cells (46,47). IL-1β

is involved in UV-induced migration of LC, which seems to be impaired in PLE patients (23,48). Furthermore, IL-1β can upregulate E-selectin expression, leading to neutrophil skin infiltration, which seems also to be impaired in PLE patients (21,49). However, IL-1β expression in PLE patients directly after UVB exposure holds an equal trend with healthy controls, indicating that there is no defect in IL-1β regulation upon UVB. It has to be further investigated whether a decreased amount of IL-1β gene expression in unirradiated skin of PLE patients might be partly responsible for an impaired LC and neutrophil migration in PLE patients. Therefore, efficiency of IL-1β mRNA translation to protein should be quantified.

After UVB exposure, also in PLE patients three waves of mRNA expression kinetics could be shown. Strikingly, the first and second waves of gene expression were similar to those observed in healthy controls, indicating no general defect in UV-induced gene regulation in the first 6 h after UVB exposure in PLE patients. The similarity in mRNA regulation of E-selectin, IL-8 and Gro-all in PLE patients compared with healthy controls after UVB exposure, does not explain the previously shown decreased number of skin infiltrating neutrophils in PLE (21). Therefore, another immunological or even non-immunological factors must be involved, such as urocanic acid, membrane lipids and DNA or members of the innate immune system (complement, Toll-like receptors) (50,51).

In contrast to the expression levels during the first 6 h after UVB exposure, after 18 h (third wave) differences could be observed in mRNA expression levels between PLE patients and healthy controls. Adhesion molecule ICAM-1 expression was increased in both healthy controls and PLE patients at 6 h after UV exposure. The expression declined at 18 h post-UVB, but to a significantly lesser extent in PLE patients compared with healthy controls ($p<0.05$). We were not able, using whole biopsy mRNA, to determine the responsible cells for the increased ICAM-1 expression in PLE patients, but most likely at this timepoint, endothelial cells and infiltrating leukocytes are the main source (52). In contrast, Norris *et al.* (17), found increased ICAM-1 protein expression in basal KC of pathological PLE lesions, particularly the epidermal sites overlying areas of dermal leukocyte infiltrates. They suggested that the ICAM-1 expression in KC was induced by IFN-γ released by immunologically activated T-cells in lesional PLE skin. However, since we did not induce pathological PLE lesions during the first 18h after UVB exposure in our test model and T-cell influx has yet to start at this timepoint, we do not expect that KC are responsible for the increased ICAM-1 expression. In addition to the results found by Norris and co-workers (17), we determined T-cell activation in skin of PLE patients and observed a significantly increased mRNA expression of CD25 and FOXP3 in comparison with healthy controls at 18 h after UV-exposure (both $p<0.05$). We also stained FOXP3 (eBioscience, San Diego, CA) and CD3 (BD Biosciences, San Jose, CA) protein in skin biopsies of PLE

patients and healthy controls. CD3 numbers increased equally in PLE patients and healthy controls at 18 h after UVB exposure, (data not shown). Furthermore, we found that the percentage of FOXP3 positive T-cells varied randomly between 1-6% of the population CD3 positive cells in both PLE patients and healthy controls and this percentage was not influenced by UVB exposure (data not shown). Therefore, we believe that T-cells expressing a regulatory phenotype (Tregs) make up a fixed proportion of the residual and skin infiltrating T-cell population and are not overexpressed in the total CD3 population upon UVB exposure. We did test the functional potency of the skin infiltrating Tregs in UV-exposed skin of PLE patients.

Th1 and Th2 cytokine gene expression could not be detected in the first 18 h after UVB exposure in PLE patients. However, in biopsies of 6 PLE patients with induced lesional PLE skin by repetitive UVB exposures, a Th1 response could be detected since IFN-γ expression was significantly upregulated in contrast to IL-4 expression being undetectable (IFN-γ $p<0.05$ using paired Wilcoxon rank test; data not shown). The fact that IL-4 mRNA expression could not be detected after UV-irradiation might be due to the fact that the number of infiltrating neutrophils in both healthy and PLE skin was too low to find a IL-4 mRNA signal. Furthermore, it might also be possible that neutrophils are not able to upregulate IL-4 mRNA once inside the skin and that the observed IL-4 protein expression reflects IL-4 protein storage in infiltrating neutrophils.

In conclusion, we showed a time-dependent expression of mRNA regulating a broad spectrum of proteins involved in UV-induced inflammation of skin after a single dose of 3 MED UVB. In general, no distinctive early mRNA expression pattern between PLE patients and healthy controls was found, except for a decreased IL-1β mRNA expression in unirradiated PLE skin. We did find an increased mRNA expression of ICAM-1, CD25 and FOXP3 in skin of PLE patients compared with healthy controls at 18 h after UVB exposure, indicating differential regulation of genes. However, these results do not explain the differences found in neutrophil skin infiltration between PLE patients and healthy controls during the first hours after UVB exposure.

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Chapter 6

Increased urocanic acid isomers in both unirradiated and UVB exposed skin of patients with polymorphous light eruption

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ABSTRACT

The aetiology of polymorphous light eruption (PLE) is still unknown. PLE patients can develop skin lesions within a few hours after UV exposure, indicating that an early UV-induced mechanism is responsible for the initiation of disease. *Cis*-urocanic acid (*cis*-UCA), a mediator of immunosuppression, is instantly formed from *trans*-UCA upon UV-exposure in the epidermis. The purpose of this study was to measure the concentrations of *trans*- and *cis*-UCA after UVB irradiation on buttock skin of PLE patients and healthy controls to determine whether UCA may play a role in the pathogenesis of PLE. Buttock skin of 18 healthy controls and 16 PLE patients was irradiated with 0.5, 1 and 2 MED and patch filters were applied for 24 hours. UCA concentrations in the filters were quantified by HPLC analysis.

The results show that the concentration of *trans*-UCA in unirradiated skin was significantly increased in PLE patients compared with healthy controls ($p<0.05$). *Trans*-UCA concentration decreased after irradiation with UVB in a dose dependent manner in both groups. However, at all MED values tested, the concentration of *trans*-UCA in PLE skin remained significantly increased compared with healthy controls ($p< 0.005$). The concentration of *cis*-UCA in unirradiated skin in PLE patients and healthy controls is similar and approximately 1 nmol/ml. C*is*-UCA concentration increased in a UVB dose-dependent manner and at 2 MED c*is*-UCA levels were significantly increased in PLE skin compared with healthy controls ($p< 0.05$).

INTRODUCTION

Ultraviolet B (UVB) is known to induce modulation of immune responses in the skin (1,2). Urocanic acid (UCA) is a major UV-absorbing component of the skin and exists in two isoforms. *Trans*-UCA is formed from filaggrin protein breakdown products in the granular layer of the epidermis by histidase. Histidase is only present in the liver and the epidermis. In the liver *trans*-UCA can be further catabolized by urocanase and metabolites are excreted with urine. Due to the absence of urocanase in the skin, *trans*-UCA accumulates in the stratum corneum (3-5). Upon UV radiation, photoisomerization from *trans*- to *cis*-UCA takes place instantly in a dose-dependent fashion until a photostationairy state is reached, with approximately equal quantities of the two isomers. The most effective waveband for photoisomerization is 310-315nm (UVB range), although 315-400nm (UVA range) can also induce UCA photoisomerization (6,7).

Patients with polymorphous light eruption (PLE) can develop pruritic erythematous papules, vesicles or plaque-like lesions on sunlight-exposed areas of the skin within several hours after UV exposure. Patients can be sensitive to UVA, UVB but rarely to visible light. The aetiology of PLE is unknown. Due to the clear delay between sunlight exposure and the onset of skin lesions, it is often regarded as a delayed-type hypersensitivity (DTH) response to a UV-induced, yet to be discovered, neo-antigen (8-10). Previously, it was shown that UVB-induced cellular responses in PLE were altered. In PLE patients, Langerhans cells (LC) persisted in the epidermis after UVB irradiation (11,12). Furthermore, PLE is marked by a decreased skin infiltration of neutrophils after UVB irradiation (12-14). Remarkably, PLE patients develop skin lesions within only few hours after UV exposure. This led us to hypothesize that another, very early mechanism after UV exposure is responsible for the onset of PLE. Like UVB, *cis*-UCA has been shown to have a suppressive effect on contact hypersensitivity (CHS), delayed type of hypersensitivity (DTH) responses, and allograft rejection (15-17). Furthermore, functions of LC, $CD11b^+$ cells, T lymphocytes, NK-cells and neutrophils can be modulated by *cis*-UCA (18-22). However, the receptor or binding mechanism for UCA to cells has not been clarified (23). PLE patients are able to form *cis*-UCA after UV exposure (24), but studies comparing UV-induced *cis*-UCA levels in PLE patients with those in healthy controls have not been conducted.

The aim of this study was to measure the total concentration of the two UCA isoforms (*trans*- and *cis*-) after UVB irradiation on buttock skin of PLE patients and healthy controls to determine whether UCA may play a role in the pathogenesis of PLE.

MATERIALS AND METHODS

Subjects

UCA concentrations were measured in skin of 18 healthy controls and 16 PLE patients. PLE patients with a positive skin reaction to UVB, regardless the reaction to UVA, were included. A positive skin reaction was defined as elicitation of typical PLE skin lesions after a maximum of four daily increasing exposures with UVB, as described earlier (25). Patients who received hardening therapy or used oral immunosuppressive medication were excluded. The study was carried out from 22 November 2005 to 8 May 2006. Overall, the buttock skin needed to have been restricted from sunlight or tanning bed exposure for at least 6 weeks prior to the study. The study was approved by the local medical ethical committee and the participants gave their written consent.

Reagents

Trans-UCA was purchased from Sigma-Aldrich Chemie bv (Zwijndrecht, The Netherlands). *Cis*-UCA was purchased from BioCis Pharma (Turku, Finland). All other laboratory reagents were of analytical grade

UVB exposure and collection of UCA samples

Minimal erythema dose (MED) of buttock skin was determined using a Philips (Eindhoven, The Netherlands) TL12 lamp (55% of UV output in UVB (280-315nm)), as described by Schornagel *et al.* (25) MED was defined as the just perceptible erythema 24 hours after irradiation with UVB. Subsequently, 3 separate areas (2x2 cm) on unaffected buttock skin were exposed to consecutively 0.5, 1 and 2 MED.

A modified non-invasive UCA sampling technique was used as described by Kammeyer *et al.* (26). Briefly, patch tests (1 cm^2 filter papers covered with a 3 cm diameter plastic sheet (Silverpatch™, van der Bend, Brielle, The Netherlands) were moistured with 25 µl 0.1*M* potassium hydroxide. Directly after UV irradiation the filters were firmly attached to the irradiated areas using Fixomull stretch tape (BSN Medical GmbH&Co, Hamburg, Germany) for 24 hours. A similar treated filter placed on an unirradiated buttock site served as control. After 24 hours the filters were collected and stored at -20°C until further processing. Prior to analysis the filter papers were soaked in 475 µl 0.1*M* potassium hydroxide and vigorously shaken for 30 seconds. Subsequently, the filters were removed and the medium was acified to pH 7 with 2 µl 98% phosphoric acid followed by stirring for 30s. After passing the epidermal extract through a 0.22 µm membrane filter, 20 µl was injected into a HPLC system.

High Performance Liquid Chromatography

The *trans*- and *cis*-UCA were separated on a 4.6x250 mm Aqua C_{18} 5µm column (Phenomenex, Torrence, CA) with a flow of 0.8 ml/min, delivered by Jasco PU 1580 HPLC pumps (Jasco Corp., Tokyo, Japan). Samples were injected by a Promis II auto sampler (Spark Holland, Emmen, The Netherlands) and chromatographic data were recorded with chromatography software of Jasco Borwin (JMBS developments, Le Fontanil, France). An UV detector (Applied Biosystems, model 759A, Foster City, CA) was set for 268nm detection. Isocratic elution was performed with 20 m*M* ammonium formate buffer, pH 5.0.

RESULTS

Demographics and phototesting

MED was normally distributed in both PLE patients and healthy controls (calculated by Kolmogrov-Smirnov test; P=0.2). Table I shows the demographics of the tested subjects, together with the distribution in MED, the concentration total-UCA in unirradiated skin and the corresponding percentage of *cis*-UCA. *Cis*-UCA levels were positively correlated with the MED, indicating that individuals with higher MED values showed higher *cis*-UCA levels, which observation was in agreement as described before (7,27). No correlation was found between UCA concentrations and age or month of testing in healthy controls or PLE patients (Spearman's Rank correlation, r^2= 0.02 and 0.3 respectively).

PLE patients have increased concentrations of trans-UCA in unirradiated skin and increased cis-UCA levels in UVB-irradiated skin

Fig 1 shows the total concentration of UCA isomers (nmol/ml) present in the epidermal extract. The total concentration of UCA was significantly higher in PLE patients compared with healthy controls at all MED doses ($p< 0.05$ at unirradiated sites (0 MED), and $p< 0.01$ at the irradiated sites; two tailed student's T-test). The total concentration of UCA extracted from the filters was equal in all tested MED sites within one group (PLE or healthy control), indicating no loss of UCA due to sampling procedure. Fig 2*A* shows the total concentration (nmol/ml) of the two isomers *trans*- and *cis*-UCA analysed separately. The concentration of *trans*-UCA in unirradiated skin was significantly increased in PLE patients compared with controls ($p<0.05$). *Trans*-UCA concentration decreased after irradiation with UVB in a dose dependent manner in both groups. However, at all MED values the concentration of *trans*-UCA in PLE skin was significantly increased compared with healthy controls ($p< 0.005$). The same holds true for the increase in *cis*-UCA after UVB. The concentration of *cis*-UCA in

Table I. Demographics subjects, MED and Urocanic acid levels

Control	No	Test month	Sexe (M/F)	Age (y)	MED (mJ/cm^2)	total-UCA (nmol/mL) 0 MED	*cis-UCA* (%of total-UCA) 0 MED
	1	Nov	F	27	82.5	158.5	4.3
	2	Nov	M	28	81	89.2	0
	3	Nov	F	24	119	54.5	2.1
	4	Nov	F	29	76	103.1	1.3
	5	Nov	M	32	77.5	79.3	1
	6	Feb	M	29	70	87.6	0
	7	Feb	M	44	140	114.3	0
	8	Feb	F	37	37	69.2	1
	9	Mar	F	47	127	112.9	0
	10	Mar	M	35	74	141.1	0.5
	11	Mar	F	31	95.9	79.6	0
	12	Mar	F	29	98.7	58.6	2.1
	13	Mar	M	32	73	74.8	0
	14	Mar	F	44	141	154.7	0.6
	15	Mar	M	29	102.2	76.7	0.9
	16	Apr	M	32	100.8	119.9	2.1
	17	Apr	M	60	101.5	108.9	1.6
	18	Apr	M	56	71	74.6	0.9
Mean ± SD			M:10/F:8	36 ± 10	92.7 ± 26.9	97.6 ± 31.2	1.0 ± 1.1
PLE	**No**	**Test month**	**Sexe (M/F)**	**Age (y)**	**MED (mJ/cm^2)**	**total-UCA (nmol/mL) 0 MED**	***cis-UCA* (%of total-UCA) 0 MED**
	1	Nov	F	44	101.5	147.9	2.8
	2	Dec	F	54	148	190.8	1.2
	3	Nov	M	65	33.75	63.5	1.8
	4	Jan	F	42	150	77.6	1.1
	5	Feb	F	49	115.5	105.1	1.4
	6	Feb	M	37	97.3	125.9	1.6
	7	Mar	F	38	52.5	105.9	1.8
	8	Feb	M	59	67.5	77.2	0
	9	Feb	F	61	53.9	106.4	0
	10	Mar	F	63	65	121.1	0
	11	Mar	F	46	126	186.6	0
	12	Mar	M	72	74	105.4	1.6
	13	Apr	M	35	101.5	99.8	1.9
	14	May	F	28	100.8	162.1	1.5
	15	May	F	56	50.4	161.2	1.1
	16	May	F	45	72	141.6	0.7
Mean ± SD			M:5/F:11	50 ± 12	88.1 ± 35.1	123.6 ± 38.3	1.2 ± 0.8

0 MED=unirradiated skin; M=male; F=female; SD=standard deviation

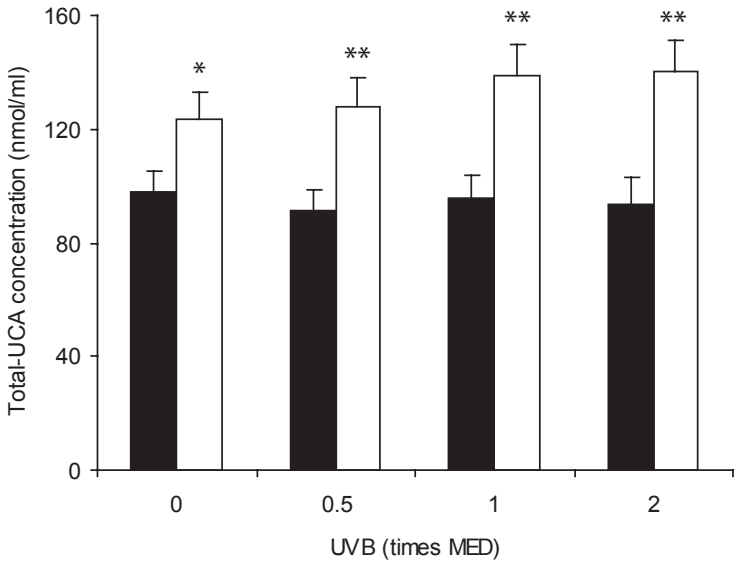

Figure 1. The total-UCA (sum of *trans*- and *cis*-UCA) concentration ± SEM in buttock skin of 18 healthy controls (■) and 16 PLE patients (□). Irradiation was done with 0.5, 1 and 2 MED UVB and compared with unirradiated skin (0). Significance, *p< 0.05, **p<0.01

unirradiated skin in PLE patients and healthy controls is similar and approximately 1 nmol/ml. C*is*-UCA concentration increased in a UVB dose-dependent manner and at 2 MED c*is*-UCA levels were significantly increased in PLE skin compared with healthy controls ($p< 0.05$).

Fig 2*B* shows *trans*- and *cis*-UCA concentrations in percentages, in which the UCA concentration at the unirradiated 0 MED site was set at 100% for *trans*-UCA and at 0% for *cis*-UCA. From this figure it can be concluded that the percentage of photo-isomerization from *trans*- to *cis*-UCA was equal between both groups. This implies that there was no isomerization defect in PLE patients compared with healthy controls.

DISCUSSION

In this study we demonstrated that PLE patients have significantly higher concentrations of total- and *trans*-UCA in their unirradiated buttock skin compared with healthy controls (Fig 2*A*). After UVB exposure the *trans*-UCA isomer is photo-isomerised to *cis*-UCA equally efficient in both PLE patients and healthy controls as shown in figure 2b. The total concentration of *trans*-UCA remained significantly increased in PLE patients, at all MED doses tested. Furthermore, the concentration of *cis*-UCA formed

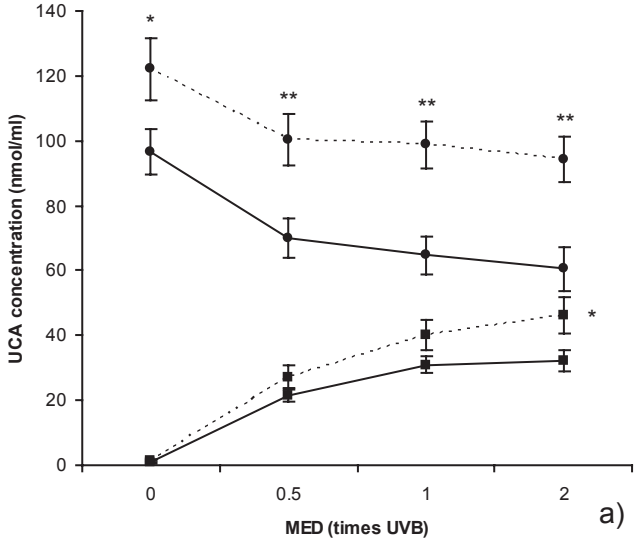

Figure 2*A*. MED dose dependent UVB-induced isomerization of *trans*-UCA to *cis*-UCA. Concentration (nmol/mL) of both isomers ± SEM is given at 0 (unirradiated skin), 0.5, 1 and 2 MED UVB. (—●—) *trans*-UCA control, (- - ● - -) *trans*-UCA PLE patient, (—■—) *cis*-UCA control, (- - ■ - -) *cis*-UCA PLE patient. Significance, *p< 0.05, **p<0.01

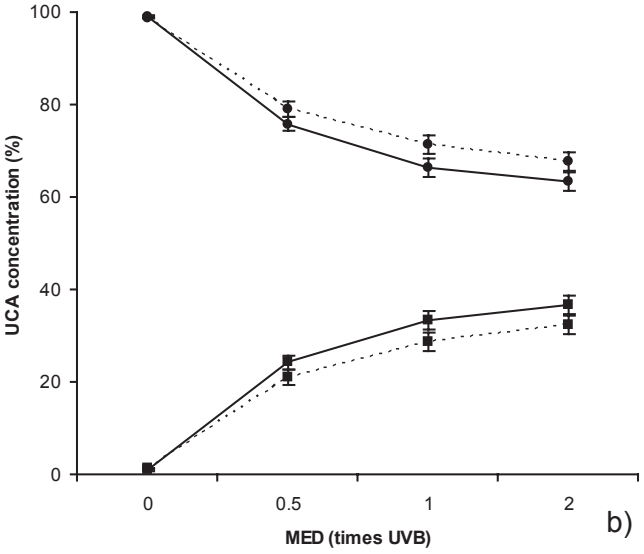

Figure 2*B*. Percentage (%) of UV-induced isomerization of *trans*-UCA to *cis*-UCA ± SEM at 0 (unirradiated skin), 0.5, 1 and 2 MED UVB. (—●—) *trans*-UCA control, (- - ● - -) *trans*-UCA PLE patient, (—■—) *cis*-UCA control, (- - ■ - -) *cis*-UCA PLE patient.

after UVB exposure is significantly increased at 2 MED UVB in PLE patients (Fig 2*A*).

UCA, and especially *cis*-UCA, has been shown to have immunomodulatory effects, *in vivo* and *in vitro* studies, which resemble the suppressive effect of UVB on the skin immune system (15). In PLE patients, however, UV exposure seems to result in inflammation suggesting an activation of the skin immune response. For that reason one would expect lower levels of UCA in the epidermis of PLE patients than that of healthy controls. It remains unclear why PLE patients have more UCA in their stratum corneum. Interestingly, we reported a decreased neutrophil skin infiltration in PLE patients compared with healthy controls (13). It has been shown, that both *cis*- and *trans*-UCA isomers were able to partially block the upregulation of CR1 and CR3 receptor in FMLP-stimulated human neutrophils (22). Furthermore, Rinaldi *et al.* (28) determined recently, that *cis*-UCA, but not *trans*-UCA, selectively inhibited the respiratory burst activity and generation of extracellular reactive oxygen species (ROS) in bovine neutrophils. These data showed that *cis*- and possibly *trans*-UCA can have an immunomodulatory effect on neutrophils. This might link the decreased UCA levels to decreased neutrophil infiltration in PLE patient. It might be interesting to determine the effect of UCA-isomers on neutrophil function and neutrophil migration in PLE patients. It has been described that patients with increased proliferation of the epidermis, such as in psoriasis, or protein malnutrition status, such as kwashiorkor, have more epidermal UCA (29). However, hyperkeratosis is no feature of PLE and we have no indication that PLE patients have deficiencies. Another explanation for the increased UCA concentration might be that PLE patients avoid sun-exposure. This might lead to overexpression of *trans*-UCA, since hardly any UV-induced isomerization to *cis*-UCA takes place. This photo-isomerization from *trans*- to *cis*-UCA is a mechanism to decrease the total UCA amount, since *cis*-UCA diffuses rapidly into the circulation (26). A seasonal variation in UCA levels has been described, with increased *cis*-UCA levels in the summer months, when sun-exposure is high (30). However, this study was conducted from November until early May. In addition, both PLE patients and healthy controls included in our study, did not have any sun or tanning bed exposure at least 6 weeks before testing and in all subjects the percentage of *cis*-UCA in unirradiated buttock skin was very low, indicating that we tested all subjects on UV-naïve buttock skin. *Cis*-UCA concentration is elevated immediately after a single total body dose UVB and returns to basic levels after 3 weeks (26). De Fine Olivarius *et al.* already described that the total UCA concentration in skin is unrelated to age, skin type, pigmentation, body site and stratum corneum thickness (30,31). Previoulsy, no differences in *cis*-UCA and total-UCA concentration was found in buttock skin of PLE patients compared with healthy controls skin (32). Unfortunately, only *cis*-UCA and not *trans*-UCA concentrations were measured and UCA samples tested were of

mixed group of controls tested in summer and winter, whereas UCA samples of PLE patients were only conducted in winter.

In conclusion, the data of this study show that PLE patients have significantly increased concentrations of total- and *trans*-UCA in unirradiated buttock skin and significantly increased concentrations of *trans*- and *cis*-UCA in UVB irradiated skin compared with healthy controls. Since UCA is immunomodulatory, these data might shed new light on the mechanism underlying the pathogenesis of PLE.

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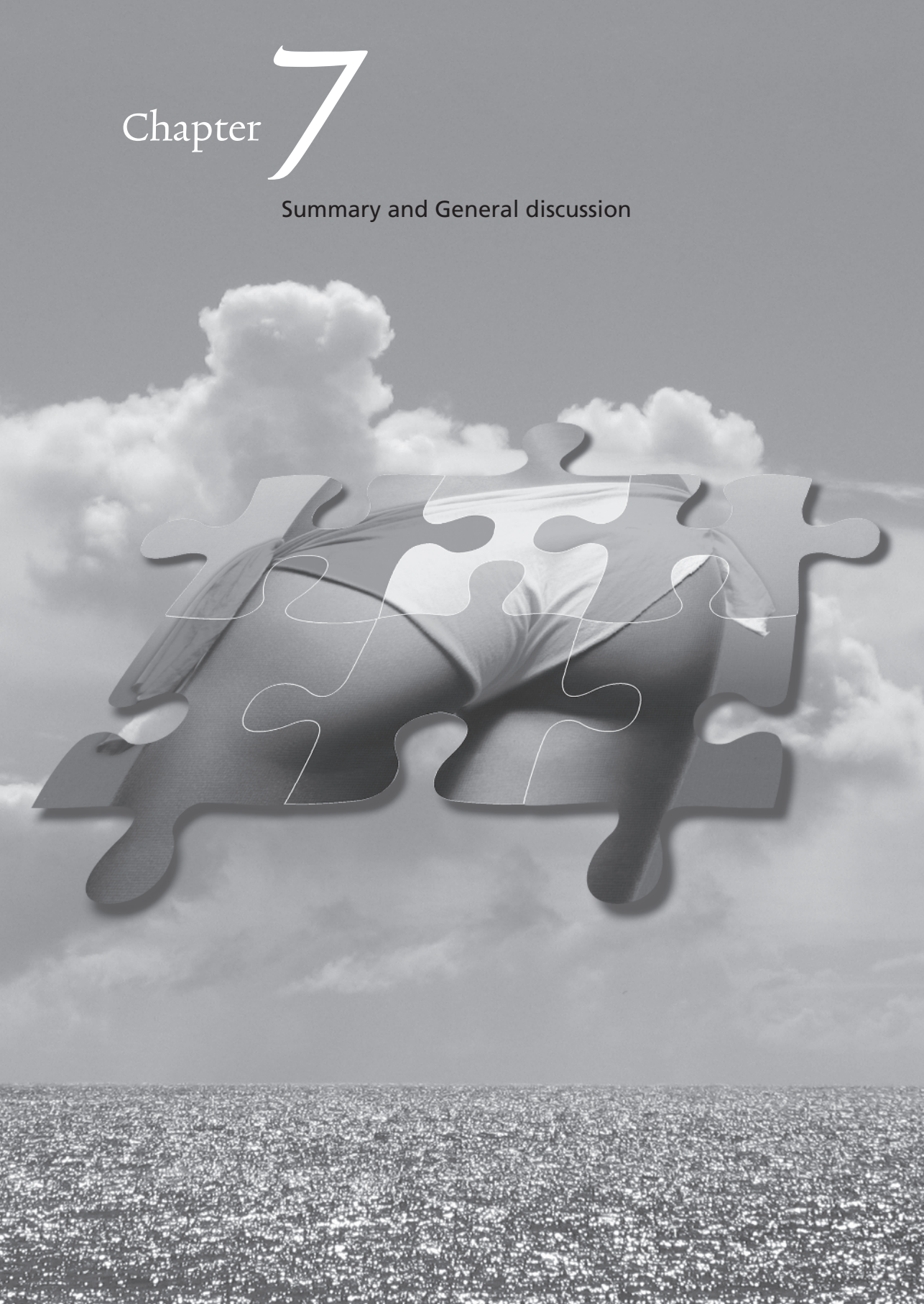
Chapter 7

Summary and General discussion



The aim of this thesis was twofold. First, to obtain a practical grip on severity assessment and phototesting a patient suffering from polymorphous light eruption (PLE). Second, to determine the pathogenetic field players in the early induction phase of PLE.

In the clinical study, we induced PLE lesions by repetitive UV irradiation. In the pathogenesis study, we used an *in vivo* UVB model to investigate the early skin response to UVB in skin of PLE patients. In this model UV-naïve buttock skin of PLE patients and healthy controls was irradiated with a single dose of 3 minimal erythema dose (MED) UVB. Subsequently, 4 mm biopsies were taken at 3, 6 and 18 h after UVB exposure. This single irradiation model with 3 MED UVB did not induce macroscopic pathological PLE lesions, but the microscopic inflammatory response differed between PLE patients and healthy controls.

In *chapter 2,* we showed that the severity assessment in PLE based on patient history did not correlate with the severity as assessed by phototesting. A PLE-severity-assessment-score (PLE-SAS) cannot replace phototesting for determining the severity of PLE. In *chapter 3,* it was demonstrated that a maximum of 4 daily irradiations using UVA and UVB light sources is suitable and optimal for diagnostic phototesting in the majority of the PLE patients. More extensive test protocols have no clinical implications, however less extensive protocols may lead to substantial loss of positive test results.
In *chapter 4,* we showed that in PLE patients significantly less neutrophils infiltrate the skin at 18 h after 3 MED UVB irradiation compared with healthy controls, most probably due to local and not systemic pathomechanisms. The intrinsic chemotactic capacity of neutrophils, as well as the expression pattern of cell surface receptors, was similar in PLE patients and healthy controls, indicating that the reduced neutrophil infiltration in PLE skin is most probably not caused by an impaired function of circulating neutrophils.
In *chapter 5,* sequential changes in mRNA regulating a broad spectrum of proteins involved in UV-induced inflammation of the skin in the first 18 hours after UVB exposure were found. After a single dose of 3 MED UVB, a gene specific expression time-dependency was observed in skin of PLE patients and healthy controls. At 3 and 6 h after UVB irradiation, no distinctive early mRNA expression pattern between PLE patients and healthy controls was shown. However, 18 h after UVB exposure, PLE patients showed a significantly increased mRNA expression of ICAM-1, CD25 and FOXP3 compared with healthy controls. Furthermore, we found that the expression of IL-1β was decreased in unirradiated skin of PLE patients. The increased mRNA expression of CD25 and FOXP3 suggests that T-cells expressing a regulatory phenotype (Tregs) play a role during the early phase of the UVB-induced response in PLE patients.

In *chapter 6,* we observed that PLE patients have significantly increased amounts of total- and *trans*-urocanic acid (UCA) in unirradiated buttock skin. After UVB irradiation significantly increased concentrations of *trans*- and *cis*-UCA were found in the skin of PLE patients. We hypothesize that the increase in *cis*-UCA might be responsible for a decreased neutrophil skin infiltration after UVB.

SEVERITY ASSESSMENT IN PATIENTS WITH PLE

PLE is the most common idiopathic photodermatosis with an estimated prevalence ranging from 5 to 20%, depending on the country and latitude (1). This high prevalence indicates that many general practitioners and dermatologists will be addressed by these patients. Since PLE lesions are transient, diagnosis is often based on patient history only. Phototesting of PLE patients, by means of artificial light sources, is done to establish the diagnosis and to determine the action spectrum responsible. Furthermore, phototesting can also be used to provide an assessment of the severity of PLE (2,3). Since phototesting is a time-consuming method, it would be convenient if severity of PLE disease could be assessed from patient history, to offer suitable advice and treatment. Subsequently, phototesting is only necessary in patients who require specialized care as a consequence of the severity of their disease. Patients with severe PLE can be characterized as insufficiently improving on sunscreen therapy combined with hardening therapy, worsening upon hardening therapy and needing chronic systemic treatment or extra protection measures, such as window glass UV-filters. In *chapter 2,* severity assessment by phototesting was used as a golden standard and was determined as the minimal number of UVA and/or UVB irradiations necessary to provoke a PLE reaction. In that study, the use of a non-validated questionnaire (PLE-severity assessment score or PLE-SAS) could not predict the severity of PLE, since PLE-SAS did not reliably predict the results of phototesting (UVA and UVB). However, recently, Palmer *et al.* (4) found a significant correlation between a questionnaire (PLE severity index also named PLESI) and the minimal number of irradiations necessary to elicit a positive skin reaction by phototesting with solar-simulated radiation (SSR). The questions listed in their questionnaire were comparable with our PLE-SAS and are also non-validated. Palmer *et al.* (4) used the PLESI score as golden standard for severity of PLE and correlated the PLESI score with the results from phototesting. The PLESI score was determined in 80 PLE patients, but phototesting was only performed in 9 patients, of whom only 5 patients showed a positive phototest result. In our study (*chapter* 2), we determined the PLE-SAS in 61 patients and all patients were also phototested. In addition, Palmer *et al.* (5) tried to validate the PLESI score by testing the relationship between PLESI and the probability of successful provocation. They showed by analyzing 36 patients, that hav-

ing a higher PLESI score correlated with a higher probability of positive tests compared with having a lower PLESI score. In contrast with their first study, phototest results were not scored as the minimal irradiations necessary to elicit a positive skin reaction, but all patients with a positive reaction were clustered and compared with patients with a negative phototest reaction. When we tested their method of analysis with our data set (*chapter 2*), a significant ($p<0.05$) positive correlation could be detected between a high PLE-SAS score and the number of positive tests after 1-3 irradiations. However, the large overlap in quartile range between the group tested positive within 1-3 irradiations and the group tested positive after 4-6 irradiations has little predictive value for the severity assessment in the individual patient. Palmer *et al.* (5), did not show the distribution range. This raises some questions on the strength of the correlation between PLESI and phototests. We investigated if other objective phototest-induced characteristics of PLE, such as intensity of the provoked lesions and the time of clearance, are involved in severity assessment, but did not find any correlation between the outcome of the phototests and the PLE-SAS. Therefore, it was concluded that PLE severity assessment based on patient history does not reliably predict severity as assessed by phototesting. Quality of life (QOL) assessment can be conducted by validated questionnaires, such as the QOL and Dermatology Life Quality Index (DLQI) (6). These questionnaires are designed to assess how patients are affected by the burden of their disease. Ling *et al.* (7) developed the PLE severity score (PLESS) based on results of both a non-validated questionnaire and the number of UVA irradiations necessary to elicit a PLE reaction. They found a correlation between DLQI-score and PLESS in springtime, but not during the summer when DLQI- score did not differ between patients with mild and severe PLE. This suggests that PLE can be a socially invalidating disease even in mildly affected patients (having only symptoms during springtime).
In conclusion, we believe that phototesting and not questionnaires provides the most objective assessment for the severity of PLE.

A CLINICAL DECISION PROTOCOL FOR PATIENTS WITH PLE

An unambiguous clinical definition for PLE does not exist, especially regarding the morphology of lesions (reviewed in *chapter 1*). It was shown in the retrospective part of the study conducted in *chapter 3* that clinically suspected PLE could be reproduced in 85% of all cases tested. In contrast, in prospectively designed studies, such as the prospective part of the study conducted in *chapter 3* and the severity study conducted in *chapter 2*, the clinical definition of PLE could be better monitored. It was shown in these studies that PLE could be reproduced in 95% and 93% of all tested patients. This implies that strictly defined “typical” PLE can nearly always be reproduced by

Table I. Definition of typical polymorphous light eruption

morphology*	papules, vesicles, plaques, excessive erythema/oedema. Itch is present
localization	uncovered skin areas; sometimes slight expansion to thin covered skin areas
appearing lesions	spring or summer; rarely before April
complaints in wintertime	none (with exception of winterhollidays in sunny areas, including skiing)
development of skin lesions after sun exposure	mostly following evening/night; rarely within 3 hours or more than 1 day
clearance of lesions	mostly within 2 weeks; sometimes the entire summer, implicating that one eruption follows the other. Clearance is scarless
complaints on cloudy summer days	none; sometimes yes, although less than during full sun exposure
Suspicion of photocontact allergic reaction in PLE patients	
consider photopatch testing**	atypical PLE reactions occur (e.g. eczematous)
	PLE reactions are inconsistently elicited in spring or summer season
	PLE reactions are only elicited when working in the garden
	sunscreens or own products might have adverse effect

* see also ref (8); ** see also refs (40-42)

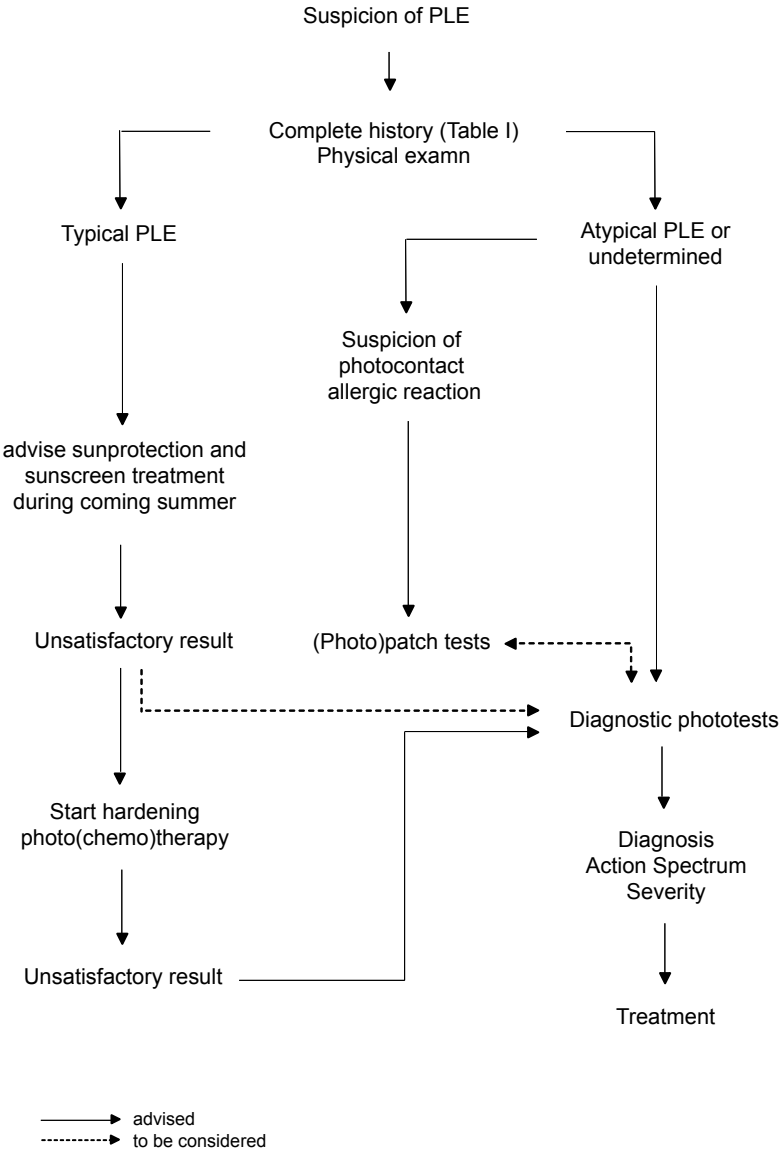
phototesting. Table I shows the characteristics of what we define as typical PLE. This definition is based on the characteristics of the studies presented in *chapters 2* and *3*. The morphology of typical PLE in Table I is in agreement with the morphological definition of PLE described by Millard and Hawk (8), in a way that typical PLE does not include UV-induced eczematous lesions, but does include excessive erythema and swelling. We believe that eczematous and plaque-like PLE are more seen in overlap cases, such as photosensitive atopic dermatitis, LE or photocontact allergic dermatitis, and should therefore not be considered typical PLE.

Subsequently, a clinical decision protocol (Fig 1) is proposed indicating when to test a PLE patient. It can be concluded from this guideline, that phototesting is not necessary for the diagnosis of typical PLE. It should be performed only in atypical PLE cases and to exclude differential diagnoses, such as solar urticaria, photosensitive atopic dermatitis, lupus erythematosus, chronic actinic dermatitis or photocontact allergic reactions.

In typical PLE patients, adequate advice about sun protection together with broad-spectrum sunscreen should be the first treatment in PLE patients. Nowadays, good quality broad-spectrum sunscreens, when applied properly, can offer protection from both UVA and UVB induced PLE, thereby reducing the necessity for determining the responsible action spectrum beforehand (9,10). If these measurements have shown inadequate improvement, UVB or PUVA hardening therapy can be started. If the result of hardening combined with sunscreen treatment remains unsatisfactory, the diagnosis of typical PLE should be reconsidered and phototests (including photopatch tests) should be performed.

Phototesting is a time-consuming procedure. Therefore, we designed an optimal diagnostic phototesting protocol for PLE patients (*chapter 3*). It was shown that PLE

Figure 1. A clinical decision protocol for patients with polymorphous light eruption



lesions could be elicited in equal numbers by UVA (78%) and UVB irradiation (80%). Approximately 21% reacted to UVA alone, 22% to UVB alone and most patients reacted to both wavelengths (58%). Positive reactions to visible light were rare and were always accompanied by strongly positive reactions to both UVA and UVB irradiation. Therefore, diagnostic phototesting should include UVA and UVB, leaving visible light testing only necessary if severe reactions to both UVA and UVB are present.

We found that the optimal diagnostic phototesting protocol for PLE patients is based on maximal 4 daily incremental irradiations with UVA and UVB (*chapter 3*). Furthermore, we observed that all skin reactions that developed after 5 or 6 irradiations had already been initiated during the preceding 4 irradiations, showing that reading phototests two days after phototesting can be informative. It would be interesting to investigate if 2 or 3 irradiations could also be sufficient to start a PLE reaction after 4-6 days, thereby assessing the minimal threshold dose for UVA and UVB.

In conclusion, by optimizing the diagnostic route for PLE patients it was perused to improve overall patient care. However, since the pathogenesis of PLE is still elusive, management of PLE remains prevention of exacerbations or symptomatic treatment when lesions do occur. Knowledge of the underlying pathomechanism in PLE might provide new treatment options. Therefore, the second aim of this thesis was to determine early mechanisms responsible for the onset of PLE.

DECREASED NEUTROPHIL SKIN INFILTRATION AFTER UVB IN PLE

It was shown that at 18 h after 3 MED UVB, a decreased number of neutrophils infiltrated the skin in PLE patients compared with healthy controls (*chapter 4*). This observation was supported by other studies using 6 MED UVB exposure of skin of PLE patients (11,12). Neutrophils present in UV-exposed skin are recruited from the peripheral blood by a coordinated repertoire of adherence to vascular endothelium, diapedesis through the endothelial layer and subsequent migration into the skin towards the UV-challenged site (13). The specific tissue recruitment of neutrophils might differ between organs. In alveolar endothelium, neutrophils pass the endothelium in capillaries at very low velocity, diminishing the need for selectin attachment. In other organs, such as the skin, neutrophils transmigrate in the postcapillary venules (14). The initial low-affinity interaction of neutrophils to the endothelial cell (EC) is mediated by selectins (L-selectin, E-selectin and P-selectin) and their ligands (CD15s, P-selectin-ligand-1), allowing neutrophils to roll along the surface of the postcapillary venules. Thereafter, a high-affinity adhesive interaction between the neutrophil and the EC is mediated by β2 integrins (CD11a/CD18, CD11b/CD18 and CD11c/CD18) and their ligands (ICAM-1 for CD11a/CD18 and CD11b/CD18 and ICAM-2 for CD11a/CD18). This high-affinity and increased avidity interaction can be established by activation of integrins via signals derived from chemokines (such as IL-8) present at the UV-induced inflammatory site. This process is called inside-out signaling (15). Integrins in high affinity and/or avidity state bind to their substrates and deliver outside-in signals (such as Src-family tyrosine kinases) back into the cell which result in changes to the cytoskeleton of cells, making

flattening and diapedesis of neutrophils possible (16). Diapedesis of neutrophils through the endothelium is poorly understood. Molecular interactions involved in diapedesis include interactions between endothelium and neutrophils and within EC junctions. Junctional adhesion molecules (JAM), CD99, VE-cadherin and platelet-endothelial cell adhesion molecule (PECAM)-1, play a role in neutrophil diapedesis (17). In addition to intercellular adhesion, neutrophils require a chemoattractant gradient (such as C5a, IL-8 and Gro-α, β, γ) to complete the process of transmigration (overview conducted from Seely *et al.* (18)).

The decrease in neutrophil skin influx after UVB in PLE did not seem to be the result of an underlying intrinsic defect of neutrophils function since: (i) cell surface expression of several markers involved in adhesion and chemotaxis (CD11b, CD18, CD15s, L-selectin, P-selectin-ligand, CXCR1 and CXCR2) were comparable in peripheral blood neutrophils of PLE patients and healthy controls, (ii) PLE is not characterized by recurrent bacterial skin infections, suggesting that neutrophil skin recruitment can be adequate in PLE patients and (iii) no *in vitro* migration defect of PLE neutrophils was detected towards the chemokines IL-8 and C5a (*chapter 4*). In addition, chemokines IL-8 and Gro-all mRNA were similar upregulated in the skin of PLE patients and healthy controls at 3 h after UVB exposure (*chapter 5*) Also, the fact that PLE neutrophils, once in the tissue, are able to migrate to the epidermis supports the finding that no chemotactic defect is present. In our study up to 18 h after UVB exposure, equal numbers of neutrophils in the epidermis were detected in PLE skin compared with healthy control skin (data not shown). However, other studies determined less epidermal infiltrating neutrophils in PLE skin 24 h after UV exposure (11,12). We believe that it is most likely that the decreased epidermal infiltration is a result of overall decreased neutrophils skin infiltration in PLE and not of selective decreased epidermal infiltration. Third, in *chapter 4*, a decreased trend (p=0.08) in E-selectin protein expression in PLE patients compared with healthy controls was observed, suggesting a possible explanation for the decreased neutrophil skin infiltration in PLE. However, in *chapter 5* it was shown that E-selectin mRNA expression was not differently regulated after UVB exposure in PLE patients compared with healthy controls. Preliminary results on lesional PLE skin biopsies showed capillaries filled with neutrophils, suggesting that neutrophils of PLE patients are able to attach to EC. From this it may be concluded that not the adherence to EC but the transmigration through endothelium is disturbed in PLE patients. It was previously shown that monoclonal antibodies against PECAM-1 can arrest leukocyte transmigration through endothelium by 70-90% without interfering with normal leukocyte adhesion to endothelial monolayers (19). Hasan *et al.* (20) determined that PECAM-1 positive endothelial cells were observed in UV-induced lesional PLE skin comparable to levels found in UV-irradiated control skin. These data most likely rule

out a complete defect in endothelial PECAM-1 regulation, although early PECAM-1 regulation upon a single UV-dose was not tested.
In summary, a decreased neutrophil infiltration was observed in skin of PLE patients at 18 h after a single 3 MED UVB exposure. It is unlikely that the characteristics of peripheral blood neutrophils or the primary selectin-induced binding to endothelium are hampered in PLE patients. Moreover, there is UV-induced production of chemokines in the skin which does not differ between PLE patients and healthy controls.
It has to be further investigated if, for instance, inside-out and outside-in signaling might be altered in PLE patients upon a specific UV-derived chemotactic signal. If the UV-induced signaling defect in PLE patients originates in the EC, *in vitro* experiments with dermal EC from PLE patients should be informative.

THE EARLY INFLAMMATORY RESPONSE IN SKIN UPON UVB EXPOSURE IS SIMILAR IN PLE PATIENTS AND HEALTHY CONTROLS

As described above, we used a single exposure to 3 MED UVB as an *in vivo* model to investigate the differences in UVB-induced inflammatory response between PLE patients and healthy controls. In *chapter 5* an extensive study was done to determine the kinetics in mRNA expression within the first 18 h after UVB exposure. A time-dependent regulation of changes in genes regulating a broad spectrum of proteins involved in UV-induced inflammation of the skin could be detected by RT-PCR technique. It was shown that there is no distinctive mRNA expression pattern between PLE patients and healthy controls during the early UV-induced inflammatory response (at 3 and 6 h after UVB irradiation). Therefore, these results do not explain the decreased neutrophil skin infiltration at 18 h after UVB exposure in PLE patients compared with healthy controls.
Neutrophils in UVB-irradiated skin are a source of IL-4, which can skew the skin towards a Th2 immunosuppressive milieu (21). Previously, it was shown that less IL-4 protein-containing neutrophils infiltrate the skin of PLE patients after a single dose of 6 MED UVB, thereby disturbing the balance between pro- and anti-inflammatory signals (12). However, it was shown in *chapter* 5 that during the first 18 h after UV exposure, mRNA levels of Th1 (IFN-γ) and Th2 (IL-4) skewing cytokines were below detection level in both PLE patients and healthy controls after a single irradiation with 3 MED UVB. IL-4 mRNA expression in human skin shortly after a single dose of UVB has, to our knowledge, never been described in literature, suggesting that neutrophil-derived IL-4 mRNA expression can not be detected. Di Nuzzo *et al.* (22) were able to measure IL-4 mRNA upregulation at 48 h and 14 days after a single dose of 4 MED UVB in skin of healthy controls. However, since neutrophil skin infiltration has its peak at 24 h

after UVB exposure, IL-4 mRNA expression at 48 h and 14 days after UVB exposure is probably not neutrophil, but T-cell derived (21). The fact that IL-4 mRNA expression could not be detected in our UV-irradiation model might be due to the fact that too few infiltrating neutrophils are present in both healthy and PLE skin to detect a proper IL-4 mRNA signal. Furthermore, lack of detectable IL-4 mRNA signals upon UVB exposure might also be explained by a failing capacity of neutrophils to transcribe IL-4 mRNA once inside the skin. In that case, the observed IL-4 protein expression might be a result of IL-4 protein storage.
In summary, the decreased number of skin infiltrating neutrophils after 3 MED UVB in PLE patients cannot be explained by differential mRNA expression of TNF-α, E-selectin, IL-8 and Gro-all. Therefore, another immunological or non-immunological factor must be responsible. To address this problem, we attempted to determine the simultaneous expression of a large number of genes using micro-array technique. However, it was not possible to obtain enough mRNA from 4 mm skin biopsies, probably due to the low number of activated and/or infiltrating cells in unirradiated and early UVB-irradiated skin. A solution to solve this problem might be to take larger size biopsies or pooling mRNA samples.

INCREASED CD25 AND FOXP3 AFTER UVB IN PLE

It was shown that UV exposure upregulates CD25 and FOXP3 mRNA expression in PLE patients; this upregulation significantly differs from healthy controls at 18 h after UVB exposure (*chapter 5*). In addition to our RT-PCR data, we stained CD3 and FOXP3 on frozen skin sections. Unfortunately, CD25 could not be stained in this population, due to insufficient material. At 18 h after UVB exposure an, although not significant, increase in CD3 positive cells in the skin was observed, suggesting that the UV-induced T-cell skin influx starts between 6 and 18 h after UVB exposure (data not shown). However, skin infiltrating CD3 positive cell numbers were equal in PLE patients and healthy controls. The percentage of FOXP3-positive T-cells varied randomly between 1-6% of the total CD3 population at all timepoints after UVB exposure in both healthy controls and PLE patients. This might imply that T-cells expressing a regulatory phenotype (Tregs) make up a fixed proportion of the residual and infiltrating skin T-cell population, and are not differentially expressed in the total CD3 population upon UV-exposure.
On the other hand, we found significantly increased FOXP3 and CD25 mRNA expression in PLE patients at 18 h after UVB exposure, suggesting that the activation status of Tregs, rather than the number of Tregs, is increased in PLE patients compared with healthy controls. In synovial fluid of patients with rheumatoid arthritis (RA) both increased numbers and increased activation of $CD4^+CD25^+$ Tregs were observed (23).

It was suggested by the authors that functionally active Tregs are trying to suppress the inflammatory reaction in RA, but that they do not succeed. In our *in vivo* 3 MED UVB-model a decreased number of infiltrating neutrophils after a single dose of UVB may result in a decreased concentration of IL-4 and IL-10, thereby modulating the immunosuppressive milieu in PLE patients. We hypothesize that activation of Tregs compensates this lack of immunosuppression. Furthermore, this may explain the absence of a macroscopic reaction in skin of PLE patients after 3 MED UVB. It would be interesting to investigate the number of Tregs at different timepoints during provocation of a pathological PLE reaction. It is not known, however, whether the concentration of CD25 and FOXP3 mRNA expression per cell is correlated with the level of functional suppressive activity. To answer this, it is necessary to measure Treg function in T-cells isolated from UV-exposed skin.
In summary, we found increased CD25 and FOXP3 mRNA expression at 18 h after a single exposure with 3 MED UVB in PLE patients compared with healthy controls. We hypothesize that Tregs substitute for a decreased immunosuppressive function of neutrophils in UV-exposed skin of PLE patients.

INCREASED UROCANIC ACID CONCENTRATION IN PLE

As described above, the similarity in the early UV-induced mRNA regulation between PLE patients and healthy controls cannot explain the decreased number of skin infiltrating neutrophils in PLE patients. Therefore, another immunological or non-immunological factor might be responsible. The UV chromophore urocanic acid (UCA), previously shown to possess immunosuppressive properties, might be a candidate (24). In *chapter 6*, it was demonstrated that PLE patients have significantly higher concentrations of total- and *trans*-UCA in unirradiated buttock skin compared with healthy controls. After UVB exposure the *trans*-UCA isomer is photo-isomerized to *cis*-UCA equally efficient in both PLE patients and healthy controls, but the total concentration of *trans*-UCA remained significantly increased in PLE patients, at all MED doses tested. Furthermore, the concentration of *cis*-UCA formed after UVB exposure is significantly increased at 2 MED UVB in PLE patients. Especially c*is*-UCA has been shown to have immunosuppressive properties (25). It has been hypothesized that *cis*-UCA and UVB share similar mechanisms resulting in immunosuppression in the skin (26,27). However, differences between UVB and *cis*-UCA have also been described. El-Ghor and Norval (28) showed that topical application of *cis*-UCA reduced the density of LC in mouse epidermis, but did not cause an increase in dendritic cell (DC) numbers in the draining lymph nodes, while UVB exposure caused both LC depletion from the epidermis and accumulation in draining lymph nodes (29). In addition to differences in the immunosuppressive

mechanism by *cis*-UCA and UVB, the immunosuppressive activity of *cis*-UCA appears to be restricted to *in vivo* models, since *in vitro* models often fail to demonstrate *cis*-UCA mediated immunosuppression (30-32). This suggests that *cis*-UCA somehow initiates an immunosuppressive or modulatory cascade *in vivo*, rather than exerting a direct immunosuppressive effect. Moreover, studies are hampered by the fact that the receptor or binding mechanism for UCA to cells has not been identified so far (33).
It is surprising that PLE patients have an increased concentration of *cis*-UCA upon a single exposure of 2 MED UVB. In line with the hypothesis of diminished immunosuppression after UV in PLE patients, one would, however, expect a decreased *cis*-UCA level upon UV-exposure in PLE patients.

DO NEUTROPHILS, UROCANIC ACID AND IL-1β CONTRIBUTE TO THE PATHOGENESIS OF PLE?

Seventy percent of PLE skin lesions occur between 2 and 24 h after sun exposure (34). This was also shown in Table I, *chapter 2*. As demonstrated in *chapter 4,* and confirmed by others, neutrophils enter the skin after UVB radiation, but fewer neutrophils infiltrate in the skin of PLE patients compared with healthy controls after a single dose of UVB (11). The neutrophil infiltration in skin of PLE patients seems to be hampered from the start of infiltration at 3-6 h after UVB, but numbers were significantly lower only at 18 h after UVB exposure. Furthermore, UV-induced skin of PLE patients is characterized by a persistent number of LC, remaining in the epidermis at 24-48 h after 6 MED UVB (11,35) and a dermal CD4 positive T-cell infiltrate starting 24-48 h after UV-exposure (20). Skin infiltrating neutrophils are a major source of IL-4, thereby stimulating a Th2 type response upon UVB exposure, resulting in UV-induced immunosuppression (21,36). Decreased neutrophil infiltration in UV-irradiated skin of PLE patients may lead to a decreased expression of IL-4, thereby inducing a Th1 shift, resulting in skin inflammation (12). As shown in *chapter 5*, it was not possible to detect either Th1 or Th2 cytokine mRNA expression in the first 18 h after UVB exposure. Recently, Averbeck *et al.* (37) quantified protein expression of cytokine release in 3 MED UVB exposed skin *in situ*, by combining dermal microdialysis and protein micro-array technique. UVB irradiation initiated a rapid pro-inflammatory response in the dermal compartment (increased TNF-α and IL-6), followed by a mixed Th1/Th2 response (IL-2, IL-12, IL-10, IL-4, TGF-β) at 8 h post-UVB in which ultimately immunosuppressive cytokines IL-4 and IL-10 pre-dominated after 24 h. IFN-γ could not be detected across all timepoints. These results suggest that in PLE patients less IL-4 and IL-10 is produced upon UV-irradiation, which might abrogate the mixed Th1/Th2 response to become a full Th2/T-suppressor skewed milieu. If the latter hypothesis is true, a decreased number of infiltrating neutro-

phils in PLE skin after UVB may be important for the onset of PLE. The only difference observed upstream of skin infiltrating neutrophils in PLE patients is an increased *trans*- and *cis*-UCA concentration in unirradiated and in UVB irradiated skin together with a decreased IL-1β concentration in unirradiated PLE skin. Kivisto *et al.* (38) observed that both *cis*- and *trans*-UCA isomers were able to partially block the upregulation of CR1 and CR3 receptor in FMLP-stimulated human neutrophils. Furthermore, Rinaldi *et al.* (39) determined recently, that *cis*-UCA, but not *trans*-UCA, selectively inhibited the respiratory burst activity and generation of extracellular reactive oxygen species (ROS) in bovine neutrophils. These data suggest that *cis*- and possibly *trans*-UCA have an immunomodulatory effect on neutrophils. This might link the increased UCA levels in PLE patients to the decreased neutrophil infiltration in PLE patients. More extensive studies, however, are required to investigate the effect of UCA on neutrophil migration. Tapestripping of the skin removes the greater part of the stratum corneum including *trans*-UCA and subsequently, hardly any *cis*-UCA can be formed upon UVB exposure. For that reason, it might be interesting to investigate *in vivo* the UV-induced neutrophil migration in skin of PLE patients and healthy controls after tapestripping.
PLE patients show a significantly decreased concentration of IL-1β mRNA expression in unirradiated skin. However, in UV-exposed skin of PLE patients and healthy controls there is no difference in IL-1β mRNA expression level. IL-1β has an effect on LC emigration and selectin upregulation, thereby regulating leukocyte skin infiltration. Since both LC emigration and neutrophil influx are impaired in PLE skin, it remains tempting to speculate whether a decreased IL-β expression in unirradiated skin is responsible for this defect. Furthermore, the decreased concentration of IL-1β and increased concentration of total-UCA in unirradiated buttock skin of PLE patients might reflect a primed status of the skin. This primed status might be responsible for a PLE reaction upon UV exposure.

CONCLUDING REMARKS

In this thesis we have focussed on severity assessment and phototesting in PLE and on possible field players in the early inflammatory skin response upon exposure to 3 MED UVB.

It was shown that the severity assessment of PLE by patient history did not correalte with the severity as assessed by phototesting. Therefore, phototesting remains the diagnostic procedure of choice to determine severity in PLE. We found that the optimal protocol for phototesting PLE patients is 4 daily irradiations with UVA and UVB. By using this protocol, we were able to elicit pathological skin reactions in more that 85% of all PLE

patients tested. Furthermore, we found that PLE lesions could develop up to 2 days after the fourth UV-irradiation. In future experiments, it is worthwhile to investigate the minimal threshold dose or number of irradiations necessary to reproduce PLE skin lesions. Furthermore, we designed a guideline for deciding which PLE patients should be phototested.

Studying the early pathogenesis study of PLE, we used an *in vivo* model with a single dose of 3 MED UVB. UV irradiation with 3 MED UVB, did not result in a macroscopic pathological PLE skin reaction in any of the patients. However, in this model we did observe microscopic differences between PLE patients and healthy. Skin of PLE patients showed significantly decreased numbers of infiltrating neutrophils. So far, there is no evidence that the characteristics of peripheral blood neutrophils or the primary selectin-induced binding to endothelium are hampered in PLE patients. Moreover, chemotactic signals in the skin do not differ between PLE patients and healthy controls and peripheral blood neutrophils of PLE patients react adequately to chemotactic stimuli *in vitro*. It might, however, be possible that endothelial cells of PLE patients react differently upon UVB-induced signals, thereby diminishing diapedesis of neutrophils into the inflamed skin.
Furthermore, we found increased mRNA expression of ICAM-1, CD25 and FOXP3 at 18 h after UVB exposure in skin of PLE patients. UVB-induced skin infiltrating neutrophils are a source of IL-4 and IL-10 and may be partly responsible for the immunosuppressive milieu in UV-irradiated skin. We hypothesize that the diminished immunosuppressive capacity (due a decreased number of skin infiltrating neutrophils) is compensated by increasing the immunosuppressive properties of Tregs. Unirradiated skin of PLE patients contains less IL-1β mRNA expression and more *trans*-UCA compared with unirradiated skin of healthy controls. This suggests that the skin of PLE patients is already primed. Furthermore, increased *cis*-UCA may be responsible for a decreased migratory capacity of neutrophils.
The results of our study show that patients with PLE do have an aberrant skin immune response very early after UV exposure. In our study model, this aberrant response is compensated since no clinical PLE reaction occured. However, repeated UV exposure probably breaks this compensating mechanism and will result in a pathological skin reaction. It might be worthwhile to search for therapeutic targets present in this early phase.

Fig 2 represents a model of the aberrant immunological response to UVB exposure in PLE patients, integrating the data of both our study and described literature.

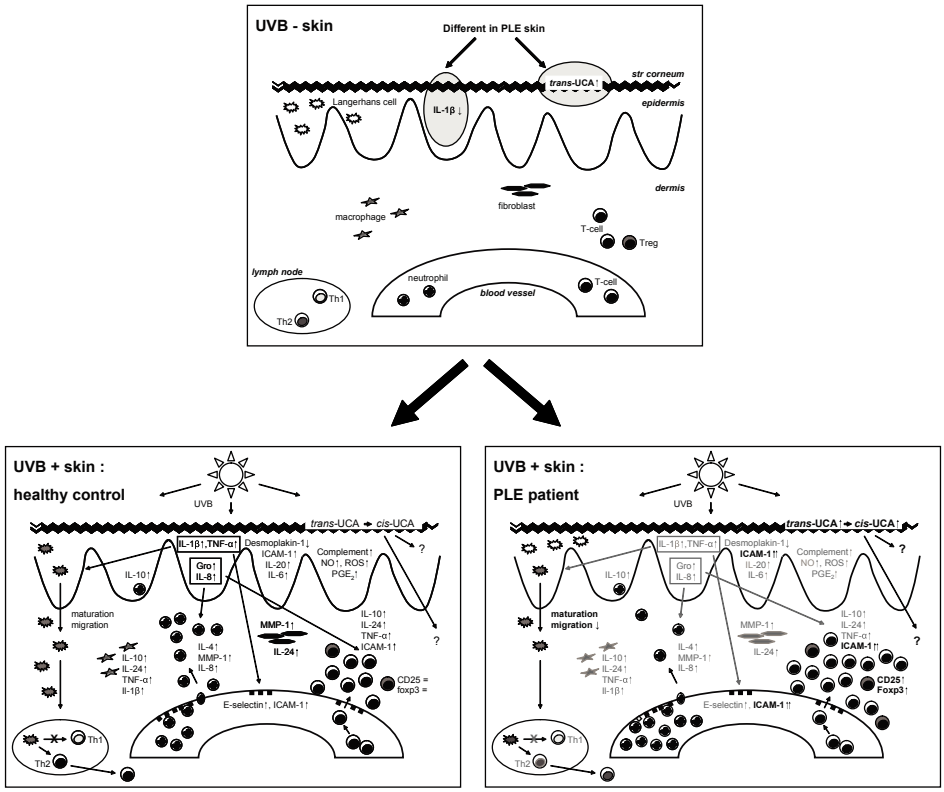


Figure 2. Before UVB exposure (UVB – skin; top panel) PLE patients have decreased IL-1β mRNA expression and increased concentration of trans-urocanic acid (UCA) in their skin. The skin cell composition and mRNA expression of other genes besides IL-1β, regulating proteins involved in UV-induced immunosuppression are similar in PLE patients and healthy controls. After UVB exposure a series of events occur. In the bottom left panel (UVB + skin: healthy control) it is shown that a broad panel of genes is up- (↑) or down- (↓) regulated, or unaltered (=) upon UVB exposure. Furthermore, cell composition changes upon UVB exposure in healthy controls, including maturation and migration of Langerhans cells (LC) from the epidermis to the draining lymph nodes. UV-irradiated LC has abnormal morphology and reduced expression of MHCII, ICAM-1 and B7. In the lymph nodes, UV irradiated LC are not able to stimulate Th1 type cells, but can only stimulate Th2 type cells. Furthermore, neutrophils enter the UVB-exposed skin from the peripheral blood via selectins, adhesion molecules and chemotaxins and are able to express IL-4 and IL-10 protein. Besides LC emigration and neutrophil influx, macrophages, $CD4^+$ and $FOXP3^+$ T-cells infiltrate the UVB-exposed skin. Various cytokines are released by these cell types. In the epidermis after UVB exposure, mRNA expression of various proteins involved in UV-induced inflammation and immunosuppression are produced. Moreover, in the stratum corneum trans-UCA is photoisomerized to cis-UCA. In the bottom right panel (UVB + skin: PLE patient) the differences found in UVB-irradiated skin of PLE patients compared with healthy controls are shown in black. All identical UVB-induced mechanisms between PLE patients and healthy controls are shown in grey. In PLE patients less LC migrate from the epidermis to the lymph nodes. It has not been investigated if stimulation by PLE-irradiated LC of T-cells is different from stimulation observed in healthy controls. Furthermore, less neutrophils infiltrate the UVB exposed skin of PLE patients resulting in less IL-4 protein expression. However, similar expression of chemokines, selectins and endothelial derived adhesion molecules is demonstrated in PLE patients. $CD4^+$ T-cells infiltrate the skin of PLE patients resulting finally in massive infiltrates in pathological PLE skin. A stable percentage of $CD4^+$ infiltrating T-cells is FOXP3 positive, similar in healthy controls and PLE patients. However, increased mRNA expression of CD25 and FOXP3 is observed in UVB-irradiated skin of PLE patients. Furthermore, increased expression of ICAM-1 mRNA is observed, although the responsible cell type is elusive. In the stratum corneum, PLE patients had already an increased concentration of trans-UCA before UVB exposure. After UVB-irradiations trans-UCA level decreased in PLE patients due to photoisomerization to cis-UCA, but remained higher compared with healthy controls. The total concentration of cis-UCA was increased after 2 MED UVB exposure compared with healthy controls. Furthermore, IL-1β, which was expressed at a decreased level in unirradiated skin of PLE patients, was upregulated after UVB exposure to a similar degree as found in healthy controls.

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NEDERLANDSE SAMENVATTING

Inleiding

Patiënten

Chronisch polymorfe licht dermatose (CPLD) wordt in de volksmond "zonneallergie" genoemd. Tussen 5 en 20% van de mensen ontwikkelt CPLD in de loop van hun leven. Het exacte percentage hangt mede af van de ligging van het land waarin de patiënt woont. Er wordt aangenomen dat landen in gematigde streken, zoals Nederland, een hoger percentage CPLD patiënten hebben dan landen met een (sub)tropisch klimaat. De mogelijke oorzaak hiervan is een hoge ultraviolet (UV) belasting in het voorjaar en de zomer, afgewisseld met een lage UV belasting in de winter in landen in gematigde streken. In de winter van landen in gematigde streken, als de sterkte van de zon nauwelijks invloed heeft (lage UV belasting), raakt de huid ontwend aan zonlicht. Als in het voorjaar de zon gaat schijnen (UV belasting omhoog), kan de huid van CPLD patiënten niet snel of goed genoeg wennen aan de zon, zodat er huidafwijkingen ontstaan. CPLD wordt gekenmerkt door het ontstaan van jeukende bultjes en blaasjes op de aan zonlicht blootgestelde huid enkele uren na zon expositie. De huidafwijkingen verdwijnen bij verdere zonvermijding binnen enkele dagen tot weken, zonder achterlating van littekens. De klachten beginnen vaak in het voorjaar en soms is er natuurlijke gewenning aan de zon gedurende de zomer. Veruit de meeste CPLD patiënten hebben geen klachten in de winter, als de zonintensiteit laag is. Vrouwen zijn vaker aangedaan dan mannen (gemiddeld 3:1) en de klachten beginnen met name tussen het 20ste en 30ste levensjaar. Hoewel de ernst van de huidafwijkingen verschilt per seizoen, is CPLD een chronische aandoening. In ongeveer 45% van de gevallen komt CPLD familiair voor.

De huidafwijkingen die ontstaan bij CPLD patiënten na blootstelling aan de zon kunnen met behulp van kunstmatige lichtbronnen worden nagebootst (lichttesten). De kans dat met behulp van deze lichttesten de diagnose CPLD kan worden gereproduceerd wisselt sterk tussen verschillende instituten en ziekenhuizen en ligt tussen de 60 en 95%. De spreiding in percentages wordt verklaard door de verschillen in gebruikte testapparatuur, testprotocollen en de exacte definitie van een CPLD patiënt.

Het ultraviolette (UV) deel van zonlicht zorgt voor de huidafwijkingen van CPLD. Het grootste deel van het UV-licht dat door de zon wordt uitgestraald wordt tegengehouden door de ozonlaag boven onze aarde. Het UV-licht dat tot het aardoppervlak doordringt kan worden ingedeeld in UVB (280-315nm, grof gezegd het UV-licht waar je van verbrandt) en UVA (315-400nm, grofweg het UV-licht waar je bruin van wordt). CPLD patiënten kunnen overgevoelig zijn voor UVA, UVB of beide golflengten. In

ernstige, zeldzame gevallen, kan ook zichtbaar licht (400-760nm) klachten veroorzaken.
De oorzaak, of pathogenese, van CPLD is nog niet opgehelderd. Het voorkómen van klachten is nog steeds de beste behandeling. De meeste CPLD patiënten hebben baat bij het vermijden van de meest intensieve zonexpositie op het midden van de dag en langzame gewenning aan de zon in het voorjaar. Verder helpen beschermende kleding en het gebruik van antizonnebrandmiddelen die gericht zijn tegen UVA en UVB. Als deze maatregelen niet afdoende zijn, kan lichtgewenning met behulp van speciale UV-lampen uitkomst bieden. Als er toch een huidreactie in de zon ontstaat, kunnen lokale corticosteroïden worden gesmeerd en in ernstigere gevallen kan prednison, ciclosporine of azathioprine verlichting bieden.

Pathogenese

De huid kan zich verdedigen tegen invloeden van buitenaf, onder andere via activering van het afweersysteem (immuunsysteem). UVB kan het natuurlijke afweersysteem van de huid onderdrukken (immuunsuppressie).
Zonlichtgeïnduceerde immuunsuppressie is een gewenst effect, omdat anders iedere blootstelling aan de zon (gezien als een reactie van buitenaf) zou resulteren in activering van het immuunsysteem van de huid en leiden tot een ontstekingsreactie. Een verstoring van de UV-geïnduceerde immuunsuppressie zou dus kunnen leiden tot een ontstekingsreactie, zoals CPLD.
Na eenmalige UVB belichting van de huid van een gezonde persoon worden er allerlei signalen door cellen in de bovenste lagen van de huid, de epidermis, afgegeven aan de omgeving. Deze signalen leiden tot UVB-geïnduceerde immuunsuppressie enerzijds via activatie van Langerhans cellen (LC) en anderzijds via aantrekking van cellen vanuit de bloedbaan naar de huid (onder andere neutrofielen en T-cellen). Eerder onderzoek toonde aan dat er in de huid van CPLD patiënten mogelijk een verstoring is van deze UVB-geïnduceerde cascade, wat uiteindelijk kan leiden tot de bekende huidreactie. Minder LC zouden uit de huid verdwijnen terwijl meer T-cellen de huid van CPLD patiënten binnentreden na UVB blootstelling. Tevens dringen minder neutrofielen vanuit de bloedbaan de huid binnen van CPLD patiënten na UVB belichting. Neutrofielen kunnen de immuunremmende cytokines IL-4 en IL-10 uitscheiden. Het kan zijn dat een verminderd aantal neutrofielen in UVB belichte huid van CPLD patiënten kan leiden tot verminderde immuunsuppressie. Deze verstoring in de balans zou dan juist resulteren in activering van het immuunsysteem na UVB expositie in CPLD patiënten en leiden tot huidreacties.
Verder zijn er ook stoffen in de epidermis aanwezig die het zonlicht op de huid kunnen absorberen (chromoforen). Urocaanzuur (UCA) is een dergelijke chromofoor. UCA is in de *trans*-vorm aanwezig in de huid van alle mensen. *Trans*-UCA kan als

chromofoor UVB absorberen en wordt dan omgezet in de *cis*-vorm. Onderzoek heeft uitgewezen dat *cis*-UCA, net als UVB zelf, een natuurlijk onderdrukkende werking heeft op het immuunsysteem.

Opzet van het onderzoek

Aangezien CPLD patiënten vaak geen actieve huidafwijkingen hebben ten tijde van een consult bij de dermatoloog, is de anamnese (het verhaal) van de patiënt erg belangrijk. Huidafwijkingen kunnen met behulp van lichttesten worden gereproduceerd. Verder worden deze testen gebruikt om de golflengte van het licht, welke verantwoordelijk is voor de huidafwijkingen vast te stellen en de benodigde UV dosis bepaald om afwijkingen te doen ontstaan. Met deze gegevens wordt de ernst van de CPLD geschat en wordt behandeling geadviseerd. Lichttesten nemen echter veel tijd in beslag. Idealiter zou de ernst van CPLD ook geschat kunnen worden via vragen uit de anamnese. Hiervoor hebben we bij 61 CPLD-verdachte patiënten vragen uit de anamnese gestructureerd en gecorreleerd met de uitkomsten van de lichttesten (hoofdstuk 2).

Lichttesten worden uitgevoerd volgens een bepaald schema of protocol. Deze protocollen verschillen tussen verschillende instituten, leidend tot variatie in reproduceerbaarheid van CPLD huidafwijkingen. Op de lichtafdeling in het UMC Utrecht wordt gebruik gemaakt van een protocol waarin een CPLD patiënt maximaal zes keer dagelijks wordt belicht. Dit is vrij tijdrovend. In een groep van totaal 134 CPLD patiënten is onderzocht of het huidige 6-daagse protocol verkort kan worden (hoofdstuk 3).

Zoals al eerder gemeld kan UVB de werking van het immuunsysteem in de huid onderdrukken. Deze onderdrukkende capaciteit is vooral onderzocht vanaf 24 uur na UVB expositie. Veel CPLD patiënten ontwikkelen echter al klachten binnen enkele uren na zonlichtblootstelling. Daarom is deze studie gericht op de vroege veranderingen na UVB belichting in de huid van CPLD patiënten en gezonde controles. Voor de studie is een model gebruikt waarin er een eenmalige belichting met UVB is gegeven op een plek op de bilhuid van CPLD patiënten en gezonde controles. Na 3, 6 en 18 uur is een huidbiopsie genomen van deze belichte plek, samen met een huidbiopsie van een onbelicht deel van de bilhuid. In deze biopten is gekeken naar stoffen en signalen die cellen in de huid na UVB belichting kunnen maken en naar de type cellen die op de verschillende tijdstippen na UVB expositie in de huid aanwezig zijn (hoofdstukken 4 en 5). Tevens is de hoeveelheid *trans*- en *cis*-UCA bepaald op de huid na UVB expositie. Hiervoor hebben we op de bilhuid van CPLD patiënten en gezonde controles, voor en na verschillende doses UVB, filters gedrenkt in kaliloog (KOH) op de huid geplakt. Zoals eerder besproken, wordt onder invloed van UVB belichting *trans*-UCA omgezet in *cis*-UCA in de bovenste laag in de huid. KOH in de filters neemt het aanwezige *trans*- en *cis*-UCA op. Na 24 uur worden de filters van de

huid verwijderd en kan de hoeveelheid UCA opgenomen in de filters gemeten worden. Wederom zijn hierbij de resultaten tussen CPLD patiënten en gezonde controles vergeleken (hoofdstuk 6).

Resultaten

In hoofdstuk 2 tonen we aan dat inschatting van ernst van ziekte (CPLD) uitgaande van de anamnese van CPLD patiënten niet mogelijk is. De inschatting van ernst via de anamnese correleert niet met de inschatting van ernst via de lichttesten. Lichttesten blijven dus nodig om objectief de ernst van CPLD te kunnen schatten.
In hoofdstuk 3 is gebleken dat het huidige testprotocol van 6 dagelijkse belichtingen kan worden teruggebracht tot 4 dagelijkse belichtingen, zonder dat dit afdoet aan de kwaliteit en de opbrengst van de lichttest. Langer dan 4 dagen testen heeft geen toegevoegde waarde, echter korter testen dan 4 dagen resulteert in een substantiële afname van huidreacties bij CPLD patiënten. Verder bleek dat als het verhaal van CPLD heel klassiek is (dus jeukende bultjes en blaasjes enkele uren na zonexpositie in voorjaar/zomer op zonlicht blootgestelde delen), de huidafwijkingen met behulp van lichttesten in meer dan 85% van de CPLD patiënten gereproduceerd kunnen worden. Daarom zijn lichttesten dus niet nodig om de diagnose van klassieke CPLD te stellen. Wel dienen patiënten met een niet-typische presentatie van CPLD klachten te worden getest om andere diagnosen uit te sluiten. In aansluiting hierop hebben we een praktische richtlijn ontwikkeld hoe om te gaan met een patiënt die verdacht wordt van CPLD.
In hoofdstuk 4 tonen we aan dat na eenmalige UVB belichting op de bilhuid significant minder neutrofielen vanuit de bloedbaan de huid binnengaan in CPLD patiënten vergeleken met gezonde controles. Dit verschil is 6 uur na UVB expositie al zichtbaar, maar pas significant verschillend na 18 uur. Deze bevinding is een bevestiging van eerder gedaan onderzoek, echter nu met een lagere UVB dosis en op een vroeger tijdstip. We hebben geprobeerd te bepalen wat de oorzaak is van deze verminderde binnenkomst van neutrofielen in de huid van CPLD patiënten na UVB expositie. Onderzocht is of de neutrofielen in het bloed van CPLD patiënten andere of minder signaalstoffen of hun receptoren hiervoor hebben dan neutrofielen van gezonde controles (CD11b, CD15s, CD18, CD62L, P-selectin-ligand, CXCR1 en CXCR2). Dit bleek niet het geval. Daarnaast zijn de hechtingseiwitten op de bloedvaten van CPLD patiënten en gezonde controles bepaald om te onderzoeken of daar verschillen zijn die verklaren dat minder neutrofielen vanuit de bloedvaten de huid in komen (ICAM-1, E-selectin, VCAM-1). Ook hierin lijkt op het eerste gezicht geen verschil te bestaan tussen CPLD patiënten en controles. Als laatste vonden we ook geen verschillende reactie van neutrofielen in CPLD patiënten en gezonde controles op stoffen die neutrofielen naar zich toe kunnen trekken (chemokines IL-8 en C5a),

In hoofdstuk 5 hebben we uit de huidbiopten een scala aan mRNA signaalstoffen geïsoleerd uit de huid van CPLD patiënten en gezonde controles. Door verschillende mRNA signalen naast elkaar te meten in een tijdreeks (onbelichte huid en 3, 6 en 18 uur na UVB belichting), hebben we een overzicht kunnen maken van mRNA signalen die betrokken zijn bij de reactie van van de huid op UVB. Hoewel er tijdspecifieke stijging en daling van verschillende mRNA signalen konden worden gemeten na UVB belichting in de huid, waren er in de eerste 6 uur na UVB expositie geen verschillen waarneembaar in mRNA expressieniveaus tussen gezonde controles en CPLD patiënten. Deze resultaten kunnen dus ook niet het verminderde aantal neutrofielen in de huid van CPLD patiënten na UVB blootstelling verklaren.
Wel werd een significant verschil in mRNA signaal gevonden tussen huid van controles en patiënten op 18 uur na UVB belichting. In huid van patiënten vonden we onder andere meer CD25 en FOXP3 mRNA signaal. Deze signalen worden geproduceerd door specifieke cellen in de huid, de regulatoire T-cellen (Tregs). Met deze bevinding suggereren wij een rol voor deze Tregs in de pathogenese van CPLD. In het geteste model met eenmalige UVB belichting, kan het zijn dat activering van Tregs in de huid van CPLD patiënten er voor zorgt dat de situatie niet uit de hand loopt. Dit kan de verklaring zijn waarom er geen afwijkende huidreacties gezien zijn op de bilhuid van CPLD patiënten na eenmalige UVB expositie. Misschien zijn herhaalde belichtingen nodig om te leiden tot een disbalans in het evenwicht tussen IL-4 en IL-10 van neutrofielen en Tregs, resulterend in CPLD huidafwijkingen.
Naast verschillen in UVB belichte huid tussen CPLD patiënten en gezonde controles, hebben we ook gekeken naar verschillen in mRNA expressie in onbelichte huid. De IL-1β mRNA expressie was significant minder in onbelichte huid van CPLD patiënten in vergelijking met huid van gezonde controles. De exacte betekenis hiervan dient verder uitgezocht te worden.
In hoofdstuk 6 hebben we de de hoeveelheid *trans*- en *cis*-UCA bepaald in de huid van gezonde controles en CPLD patiënten voor en na UVB belichting. Er is gebleken dat in onbelichte huid van CPLD patiënten significant meer *trans*-UCA aanwezig is vergeleken met onbelichte huid van gezonde controles. Na UVB belichting wordt in patiënten en controles even effectief *trans*- in *cis*-UCA omgezet. Echter, de concentratie *trans*-UCA blijft na UVB belichting hoger in CPLD patiënten vergeleken met gezonde controles en de concentratie *cis*-UCA is ook significant meer. *Cis*-UCA heeft een remmende werking op het immuunsysteem van de huid. We hadden daarom verwacht dat CPLD patiënten minder *cis*-UCA zouden hebben, wat een verminderde immuunsuppressie zou opleveren. We kunnen niet direct verklaren waarom CPLD patiënten juist meer *trans*- en *cis*-UCA hebben. Wel is beschreven dat *cis*-UCA invloed kan hebben op diverse cellen in de huid, waaronder neutrofielen. Verder onderzoek moet uitwijzen of de toename in *cis*-UCA concentratie in huid van CPLD patiënten na

UVB expositie kan leiden tot verminderde binnenkomst van neutrofielen in de huid van CPLD patiënten.

Conclusies

1. Inschatting van ernst van CPLD is niet mogelijk op basis van anamnese alleen. Lichttesten blijven hiervoor de meest objectieve methode.
2. Het meest optimale protocol voor lichttesten bij CPLD patiënten is die van 4 dagelijkse belichtingen. Met dit testprotocol kan meer dan 85% van de huidafwijkingen worden gereproduceerd. Gezien dit hoge reproductiepercentage kunnen we concluderen dat lichttesten niet nodig zijn om de diagnose van klassieke CPLD te stellen.
3. Achttien uur na UVB belichting komen er significant minder neutrofielen de huid binnen vanuit de bloedbaan in eenmalig belichte huid van CPLD patiënten in vergelijking met huid van gezonde controles.
4. Achttien uur na UVB belichting is er in huid van eenmalig belichte CPLD patiënten onder andere significant meer CD25 en FOXP3 mRNA expressie vergeleken met huid van gezonde controles. Tevens hebben CPLD patiënten in onbelichte huid significant minder IL-1β mRNA in vergelijking met huid van gezonde controles.
5. CPLD patiënten hebben in onbelichte en UVB belichte huid een significant hogere concentratie *trans*-UCA vergeleken met onbelichte en UVB belichte huid van gezonde controles. Na UVB belichting is verder de concentratie *cis*-UCA significant hoger in de huid van CPLD patiënten vergeleken met gezonde controles.

Onze studie laten zien dat huid van CPLD patiënten na eenmalige UVB expositie al in een vroeg stadium afwijkend reageert. Deze afwijkende reactie wordt waarschijnlijk in het gebruikte model gecompenseerd, aangezien er geen duidelijke klinische huidafwijkingen ontstaan. Herhaalde belichtingen zullen waarschijnlijk leiden tot tekort schieten van dit compensatiemechanisme en resulteren in huidafwijkingen die passen bij CPLD.

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CURRICULUM VITAE

Ines Johanna Schornagel werd geboren op 20 maart 1970 te Haarlem. Het gymnasium-β diploma behaalde zij in 1989 aan het Sancta Maria Lyceum in Haarlem. Na het propedeutisch examen HLO aan de Hoge School Alkmaar in 1990 werd de overstap gemaakt naar de studie Medische Biologie aan de Universiteit van Utrecht. Wetenschappelijke stages werden gelopen bij het Eijckman Winkler Laboratoratorium voor Medische Microbiologie in het Academisch Ziekenhuis Utrecht ("nosocomiale infecties in de neonatale intensive care") en bij het Tornblad Instituut, Universiteit van Lund, Zweden ("het effect van tijdsperiode tussen opeenvolgende zwangerschappen op laag geboortegewicht, intra-uteriene groeivertraging en perinatale mortaliteit"). Tijdens deze studie was zij lid van de Interfacultaire Commissie Medische Biologie en het Faculteitsbestuur Geneeskunde. Het doctoraalexamen Medische Biologie haalde zij in 1995.
Aansluitend werd in 1995 gestart met de studie Geneeskunde aan de Vrije Universiteit te Amsterdam, alwaar het artsexamen werd behaald in 2000. Hierna was zij kortdurend werkzaam als AGNIO Interne Geneeskunde in het Kennemer Gasthuis, locatie EG, in Haarlem. In mei 2001 begon zij als arts-onderzoeker op de afdeling Dermatologie en Allergologie van het Universitair Medisch Centrum Utrecht onder leiding van Dr. Edward Knol, Dr. Vigfús Sigurdsson en Prof. Dr. Carla Bruijnzeel-Koomen. De resultaten van het onderzoek naar patiënten met zonneallergie zijn beschreven in dit proefschrift. Op 1 oktober 2003 is zij begonnen met de opleiding Dermatologie in het UMC Utrecht met als opleider Prof. Dr. Carla Bruijnzeel-Koomen.

ABBREVIATIONS

AD	atopic dermatitis
CAD	chronic actinic dermatitis
CD	cluster of differentiation
CHS	contact hypersensitivity
CI	confidence interval
DC	dendritic cell
DLQI	dermatology life quality index
DTH	delayed-type of hypersensitivity
EC	endothelial cell
FACS	fluorescence activated cell sorting
Fig	figure
FOXP3	forkhead box P3
Gro	growth related oncogene
HPLC	high performance liquid chromatography
ICAM	intercellular adhesion molecule
IFN	interferon
IL	interleukin
J	joule
KC	keratinocyte
LC	Langerhans cell
LE	lupus erythematosus
MED	minimal erythema dose
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
PLE	polymorphous light eruption
PLE-SAS	PLE-severity-assessment-score
PLESI	PLE severity index
QOL	quality of life
ROS	reactive oxygen species
RT-PCR	real time polymerase chain reaction
SEM	standard error of mean
SPSS	statistical package for the social sciences
Th	Thelper lymphocyte
TNF	tumor necrosis factor
Treg	regulatory type T-lymphocyte
UCA	urocanic acid
UV	ultraviolet
VCAM	vascular cell adhesion molecule
y	year