Unraveling the pathogenesis of polymorphous light eruption

A comparative study in patients with polymorphous light eruption and healthy individuals

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Unraveling the pathogenesis of polymorphous light eruption / Wendy Kölgen.

Thesis Utrecht University, Medical Faculty
-with a summary in Dutch

ISBN 90-393-3305-X

Cover design: Jeroen Kölgen
Printed by: Labor Grafimedia B.V., Utrecht

Unraveling the pathogenesis of polymorphous light eruption

A comparative study in patients with polymorphous light eruption and healthy individuals

Het ontrafelen van de pathogenese van chronische polymorfe lichtdermatose

Een onderzoek waarbij patiënten met chronische polymorfe lichtdermatose worden vergeleken met gezonde personen

(met een samenvatting in het Nederlands)

Proefschrift ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de Rector Magnificus, Prof dr. W.H. Gispen, ingevolge het besluit van het College voor Promoties in het openbaar te verdedigen op dinsdag 1 april 2003 des middags te 14.30 uur.

door

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Geboren op 17 juni 1975, te Heerlen

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Publication of this thesis was financially supported by: Schering-Plough BV, Philips DAP/Suncare, 3M Pharma Nederland BV, Yamanouchi Pharma BV, Laboratoires Vichy, Novartis Pharma BV, Department of Dermatology (UMCU), Medical Faculty University of Utrecht.

Ter leering ende vermaak

Voor pap en mam

Voor Erik

Contents

Abbreviations

AP	actinic prurigo
AP	alkaline phosphatase (in immunohistochemistry)
AEC	3-amino-ethyl-carbazole
AR	actinic reticuloid
C3	complement component 3
CAD	chronic actinic dermatitis
CD	cluster of differentiation
CS	cockayne syndrom
CHS	contact hypersensitivity
CPD	cyclobutane pyrimidine dimer
CGRP	calcitonin gene-related peptide
DCP	diphenylcyclopropenone
DNCB	2,4-dinitrochlorobenzene
FITC	fluorescein isothiocyanate
GGR	global genome repair
HLA	human leukocyte antigen
HRP	horseradish peroxidase
ICAM	intracellular adhesion molecule
ICE	interleukin-1-beta-converting enzyme
IL	interleukin
IL-1-Ra	interleukin 1 receptor antagonist
IFN-γ	interferon
JSE	juvenile spring eruption
LC	Langerhans cell
MED	minimal erythema dose
MHC	major histocompatibility complex
NER	nucleotide excision repair
6-4PP	6-4 pyrimidine-pyrimidones photoproducts
PBS	phosphate buffered saline
PG	prostaglandin
PLE	polymorphous light eruption
TCR	transcription-coupled repair
TGF	tumor growth factor
Th	T helper

T4N5	T4 endonuclease V
TNF-α	tumor necrosis factor-alpha
UV	ultraviolet
wt	wildtype
XP	xeroderma pigmentosum

Introduction

Introduction

EFFECTS OF ULTRAVIOLET RADIATION ON THE SKIN

The optical radiation spectrum of the sun can be divided into three major parts: ultraviolet radiation (100 – 400 nm), visible light (400 – 760 nm) and, at the other end of the spectrum, infrared (760 nm and more). The most biological harmful radiation is ultraviolet radiation (UV), which can be subdivided in UV-A (315 – 400 nm), UV-B (280 – 315 nm) and UV-C (200 – 280 nm). Earth's atmosphere, specifically the oxygen and ozone in the stratosphere, blocks most of the short-wave UV radiation, but UV-A and partly UV-B radiation can still reach the earth surface (Webb, 1997). UV-B radiation makes up only about 0.2% of the radiant energy of the sun at ground level but it is the most photochemicallly active wavelength band. UV radiation, especially UV-B, can cause photochemical smog and degradation of plastics and other compounds. It can also be absorbed by organic molecules such as proteins and DNA (de Gruijl, 1997; Jung and Bohnert, 1995).

Keeping these effects in mind, it is *a priori* very likely that UV radiation can cause a broad range of effects in the skin. The skin needs to be very well adapted to this continuous photochemical stress. During evolution the skin has adapted itself to UV radiation *inter alia* by the presence of hair (animals), the relatively fast turnover of epidermal cells and the repair of damaged cells. Nevertheless, UV radiation is able to penetrate the skin and cause substantial damage.

The acute effects of UV radiation on the skin are erythema and pigmentation, but also the formation of vitamin D3. Other effects of UV radiation are photo-aging of the skin, tumor induction, together with a suppression of tumor rejection (Gensler *et al*, 1995), and reduced immunization against microbial pathogens (Halliday and Norval, 1997; Jeevan *et al*, 1995). The latter two effects are due to immunosuppression brought about by UV-B and UV-A II (315 - 340 nm).

All of these effects, except for the formation of vitamin D3 and perhaps pigmentation, are considered adverse effects of UV radiation. When considered more closely, one can argue that UV-induced suppression of cellular immunity can be thought of as a primarily sound physiological reaction. First of all, we are continuously exposed to UV radiation and all of us display this immunosuppression at sufficiently high doses (Cooper *et al*, 1992). Consequently, this immunosuppression could well prevent us from mounting a disruptive (auto)immune response against (neo)antigens that are created by UV-induced modification of proteins and other organic molecules in the skin (Natali and Tan, 1973; Norris *et al*, 1989b).

Diminishing the UV-induced immunosuppression could be beneficial for tumor rejection and immunization but one should be aware of the possible harmful side effects. An inadequate immunosuppression could allegedly develop into a UV-trigged sun allergy, e.g. polymorphous light eruption (PLE).

POLYMORPHOUS LIGHT ERUPTION

PLE, an idiopathic photodermatosis, was first described by Robert Willan in 1798 (Stratigos *et al*, 2002), who called it 'eczema solare'. Since then several names have been used to describe this skin disorder. Names such as summer prurigo, summer itch, solar dermatitis, benign summer eruption were used or are still used. However, the most commonly used name today is 'polymorphous light eruption', which was first introduced by the Dane Rasch in 1900 (Stratigos *et al*, 2002).

PLE is most common in temperate climate zones, with increasing prevalences towards the poles. In (sub)tropical areas with a more or less constant amount of sunlight throughout the seasons, PLE is hardly seen. PLE does not appear to be restricted to any race, skin color or gender and can occur at any stage of life. However, women are more frequently affected than men and the mean age of onset is usually lower in women (mean of 28 years) than in men (mean of 46 years) (Boonstra *et al*, 2000; Lecha, 2001). Twin studies show that there is a genetic component involved in PLE but candidate genes are not yet discovered (McGregor *et al*, 2000; Millard *et al*, 2001).

PLE patients can be sensitive to UV-A and/or UV-B radiation or sometimes even to visible light. The majority of the PLE patients have a normal sunburn sensitivity, however, 10-20% of the patients has a sunburn sensitivity that can be ten times as high as in healthy individuals (Diffey, 1986; Holzle *et al*, 1982; Jansen, 1979). The clinical symptoms of PLE often start in the spring or early summer, decrease in the course of the summer, and are often absent during winter, indicative of a seasonal dependence. The symptoms consist of itching papules and/ or vesicles on sun-exposed areas of the skin (Epstein, 1997; Grabczyska and Hawk, 1997; Salomon *et al*, 1997). There is some discussion whether eczematous lesions belong to the diagnosis of PLE (Stratigos *et al*, 2002) or whether they are symptoms of photosensitive eczema or chronic actinic dermatitis (CAD, see below). Clinical symptoms often appear within hours or days after the actual sun exposure. Because of this delay between exposure and the appearance of skin lesions, PLE is often suggested to be a delayed type hypersensitivity reaction (type IV) (Epstein, 1997; Norris *et al*, 1989b; Verheyen *et al*, 1995). Skin lesions can disappear within one day to three weeks without scaring. Sometimes symptoms like fever, headache or general malaise occur.

The histology of the skin lesions is variable but shows dense infiltrates of lymphocytic cells. Characteristic of an early lesion is a normal epidermis with edema in the stratum papillare and lymphocytic infiltrates in the upper and mid-dermis. CD4+ T-cells dominate these infiltrates while older lesions contain more CD8+ T-cells (Holze, 1995; Norris *et al*, 1989b). Skin infiltrates also contain eosinophils and neutrophils. Furthermore, presence of spongiosis, vesicle formation as well as necrotic keratinocytes are described. Verheyen *et al* (1995) and Norris *et al* (1992) showed that most keratinocytes in provoked PLE lesions express HLA-DR and ICAM-1, which is comparable to other delayed type hypersensitivity reactions, such as allergic contact dermatitis. In PLE lesions CD45RO+ memory T-cells were predominantly present (Hasan *et al*, 1999; Lecha, 2001). An increase in Langerhans cells in epidermis and upper-dermis is also reported (Norris *et al*, 1989a). A delay between exposure and occurrence of skin lesions, the presence of papules or vesicles, a flare-up of previous lesions when distant skin areas are exposed and a histological resemblance to a delayed type hypersensitivity reaction, all these phenomena together indicate that a cellular-mediated immune reactivity is involved in PLE.

DNA damage is the initiating event of many skin reactions caused by UV exposure, but DNA excision repair is normal in the (lesional) skin of PLE patients (Horkay *et al*, 1978; Ichihashi and Ramsay, 1976).

The diagnosis of PLE is based on patient's history and/or histology of skin lesions and sometimes on provocation by daily exposures to UV-A and UV-B radiation and visible light on restricted areas of the arm or other previously affected skin areas (Gonzalez and Gonzalez, 1996; Jansen, 1982). At the department of Dermatology of the University Medical Center Utrecht (the Netherlands) the diagnosis of PLE is based on the combination of a patient's history, phototesting and histology of lesions to exclude other photosensitive disorders such as lupus erythematodes. The phototesting is performed with UV-B radiation from Philips TL12 lamps and with UV-A radiation from a Mutzhas UVASUN 3000. Phototesting with visible light is performed with a high pressure mercury arc with special glass lenses and filters (Boonstra *et al*, 2000). The occurrence of lesions is dependent on the size of the exposed skin area, the site of phototesting and on the number of (repeated) exposures (Hönigsmann, 1993). PLE lesions occur more easily on a relatively large area of skin (10*10 cm) which has previously been affected and which is exposed to an average of three (maximum 6) daily doses of UV radiation (or visible light). Phototesting is ceased as soon as skin lesions occur. In our hands, the majority of the patients (men and women together) react to UV-B as well as UV-A radiation (about 64%), while only minorities react to either UV-A I (340 - 400 nm) or UV-B radiation (Boonstra *et al*, 2000). Other groups report less (Honigsmann and Ortel, 1988; Lindmaier and Neumann, 1991) or no abnormal reactions to UV-B radiation (Bergner *et al*, 1993) and/or visible light (Holzle *et al*, 1982).

Prevention of PLE lesions by controlled exposure to the sun, the use of sunscreens and protective clothing are the most common advices given to PLE patients. If these measures are not sufficient, patients can receive photo(chemo)therapy (Boonstra *et al*, 2000; Gschnait *et al*, 1983; Man *et al*, 1999; Murphy *et al*, 1987b) with initially low and then gradually increasing doses of UV radiation. When lesions are already present, patients can be treated with immunosuppressive drugs such as corticosteroids (Ferguson and Ibbotson, 1999) or azathioprine (Imuran) (Norris and Hawk, 1989) or with hydroxychloroquine (Murphy *et al*, 1987a). Imuran may reduce Langerhans cell numbers (Bergfelt, 1993). Experimental treatment with fish oil capsules for 3 months resulted in moderate protection against a UV-A-induced papular response and in a decreased erythemal sensitivity to UV-B radiation (Rhodes *et al*, 1994; Rhodes *et al*, 1995). Systemic treatment of PLE patients with vitamin C and E for 8 days had no protective effects (Eberlein-Konig *et al*, 2000).
An excellent review was recently published by Stratigos *et al* (2002) describing *inter alia* the epidemiological, pathogenetic and clinical aspects of PLE.

DIFFERENTIAL DIAGNOSES

Juvenile spring eruption

The term " juvenile spring eruption of the ears" (JSE) was used by Andersen in 1954 to define symptoms of erythema, itching papules, vesicles and even blisters on the ears of a group of London school children on summer camp. JSE appears in early spring and can disappear spontaneously but often develops later in life into a classical PLE (Toonstra and van Weelden, 1994). Histology of the lesions cannot distinguish JSE from PLE. Because of similarities of clinical symptoms and histology, JSE can be seen as a variant of PLE (Berth-Jones *et al*, 1989; Berth-Jones *et al*, 1991; Hawk, 1996). Treatment with topical corticosteroids or application of sunscreens before sun exposure is often sufficient to prevent or control JSE.

Actinic prurigo

Actinic prurigo (AP) is most common in the indigenous population of the Americas, especially among Indians (Lane, 1997). AP belongs to the ten most common skin diseases in Mexico. AP occurs in most patients in early childhood.
A genetic HLA-DR-associated component is often described in 50 – 75% of the patients (Hojyo Tomoka *et al*, 1997; Wiseman *et al*, 2001). Twice as many women as men have AP. A familial background of atopy has also been reported for AP patients (Ferguson and Ibbotson, 1999).
Clinical symptoms comprise of very itchy papules, vesicles, scratch-effects and sometimes plaque-type lesions. The itch lasts even if sun exposure is limited; there is no clear seasonal

dependence although the symptoms are more severe during summer. Scratching often leads to erosion, ulceration and finally scaring. Lesions on covered skin areas may also occur (Lecha, 2001).
Histological characteristics are almost similar to PLE and consist of spongiosis, hyper- and parakeratosis, sub-epidermal edema and a moderate number of lymphocytes. Prurigo lesions show acanthosis, hyperkeratosis, moderate perivascular lymphocytic infiltrates, fibrosis and thickening of capillary vessels in the stratum papillare. After 48 hours the lesional infiltrate contains CD4+ T-cells. Some research groups show a high expression of TNF-α (Arrese *et al*, 2001), TGF-β and interleukin 13 (Santos-Martinez *et al*, 1997) in AP lesions. One group of investigators showed that Langerhans cells in AP patients did not disappear after overexposure of the skin to UV (Torres-Alvarez *et al*, 1998).
AP is therapy resistant; corticosteroids, sunscreens or phototherapy have little or no effect.

Chronic actinic dermatitis / Actinic reticuloid

Chronic actinic dermatitis (CAD) is a collective term for persistent light reaction and actinic reticuloid (AR). The majority of the CAD patients are males over 50 years of age (for review see Menage and Hawk (1993) or Roelandts (1993)). CAD can start as an a-specific eczema on the hands or the face (photosensitive eczema) or as a PLE-like skin reaction with papules and vesicles which then increases in severity and light sensitivity. Many patients are found to react to (photo)contact allergens, but avoidance of the allergens does not lead to improvement of the symptoms. Etiology is unknown, but is probably multifactorial involving photoallergic, phototoxic, immunologic and metabolic factors (Vandermaesen *et al*, 1986).
Clinical symptoms include a strong itching eczema, sometimes with papules and confluent plaques. In the most sever cases erythroderma, lasting for years, can occur. The majority of CAD patients has no obvious seasonal dependency of the disease. Patients often have a high sunburn sensitivity to UV-B and/or UV-A radiation and sometimes even to visible light (Lecha, 2001).
Histology of the lesions is variable, depending on the duration and severity of the disease. In less severe cases, photosensitive eczema, an eczema with or without eosinophils and mostly an excess of CD4+ T-cells is observed. When persistence and/or severity increases, spongiosis and vesiculation become less, while the epidermis shows more acanthosis, parakeratosis and infiltration of mostly CD4+ lymphocytes. At the department of Dermatology of the University Medical Center Utrecht the term ‘actinic reticuloid’ is used when three criteria are met: presence of a largely infiltrated (sun-exposed) skin or persistent erythroderma, sensitivity to UV-B as well as UV-A radiation and visible light and skin lesions containing dermal infiltrates with a-typical lymphocytes (Toonstra, 1991). The abundant presence of a-typical lymphocytes makes it difficult to distinguish this skin disease from mycosis fungoides

and Sézary syndrome. The infiltrate can be present in the complete upper-dermis, but can also spread more scatteredly into the sub-cutis. Most of the a-typical lymphocytes in the AR lesion, but also in the blood, are CD8+ T-cells. A reversed CD4/CD8 ratio is the most important diagnostic criterion for differentiation from Sézary syndrome.
Advice and treatment are the same, but more strict, for CAD patients than for PLE patients. Sunscreens, however, are usually not effective and can even increase the severity of the disease as they may contain (photo)contact allergens. For patients with severe AR cyclosporin (Gardeazabal *et al*, 1992) or azathioprine (Murphy *et al*, 1989) can be prescribed to suppress immune reactivity.
Because of the resemblance in symptoms, histology and treatment but a difference in severity, one could arrange these idiopathic photodermatoses in a spectrum starting with JSE and ending with AR (figure 1). The exception to this is AP which seems to be a different entity because of a strong genetic component and therapy resistance. However, some investigators report that AP and PLE share some common genetic background (McGregor *et al*, 2000). Furthermore a photosensitive eczema (not part of this spectrum) may also eventually progress into CAD or AR.

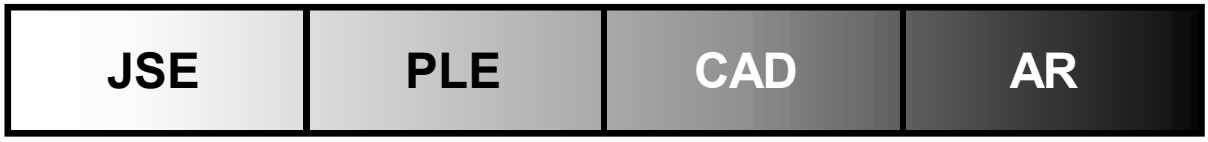

Figure 1. Spectrum of idiopathic photodermatoses increasing in severity from left to right.

ULTRAVIOLET-B-INDUCED IMMUNOSUPPRESSION

One of the effects of UV radiation, especially UV-B, is immunosuppression. This first came to light as an adverse effect of UV exposure (allowing tumor growth and worsening of infections). However, as already mentioned in the first paragraph of this introduction, this immunosuppression could also be regarded as a primarily sound physiological reaction, because an inadequate UV-induced immunosuppression may possibly provoke a skin disease such as PLE. To investigate this potential pathogenic mechanism of PLE more closely, we can focus on cells and soluble factors that are involved in UV-B-induced immunosuppression.
UV-B radiation can induce local as well as systemic immunosuppression (Morison, 1989). Application of a hapten on an irradiated area of the skin, induces local immunosuppression, probably because of a direct effect of UV-B radiation on the Langerhans cells, inhibiting their antigen-presenting capacity. A systemic or distant immunosuppression is induced when

immunization is performed on an unirradiated skin area (e.g. back) of an animal/human who is irradiated on a distant skin area (e.g. abdomen). A release of soluble factors like, urocanic acid (Noonan and De Fabo, 1992), neuropeptides (Garssen *et al*, 1998; Gillardon *et al*, 1995), nerve growth factor (Townley *et al*, 2002), neurohormones (Misery, 2000) and cytokines (e.g. prostaglandin E_2) is most likely responsible for the induction of systemic immunosuppression.
In this introduction I will only focus on cells and soluble factors, which are thought to be pivotal in UV-induced immunosuppression and consequently in the present research project. Moreover, UV-induced DNA-damage, DNA repair and apoptosis are important in primary cellular reactions and will therefore also be briefly discussed.

Langerhans cells

Langerhans cells were first identified by Paul Langerhans in 1868, who thought that these cells were neural cells (Langerhans, 1868). Later on, Langerhans cells were considered antigen-presenting cells and participants of a skin immune system (Silberberg *et al*, 1976). Langerhans cells are located in the suprabasal layer of human and also murine skin. They have long dendrites (pseudo-podia) and can be distinguished from dendritic cells by the presence of specific cytoplasmatic organelles with unknown function, the Birbeck granules. Following exposure of the skin to various chemical or biological factors (irritants or immunizing haptens), Langerhans cells round-up, retracting their dendrites, and migrate via the afferent lymph vessels to the skin draining lymph nodes. During migration Langerhans cells mature and lose their antigen capturing and processing capacity and become more effective in antigen presentation for induction of a proper immune response.
It is known from animal models (Dandie *et al*, 1998; Dandie *et al*, 2001; Sontag *et al*, 1995), but also from human models using lymph drainage (Yawalkar *et al*, 1998), that Langerhans cells migrate to the draining lymph nodes after exposure of the skin to UV. The putative antigen(s) that trigger(s) this Langerhans cell migration are still not known. It is speculated that proteins or other organic molecules modified by UV, could be recognized by the skin immune system as foreign antigens. These ‘neo-antigens’ are most likely taken up by Langerhans cells and after migration presented to the T-cells in the draining lymph nodes, leading to an immune response against this antigen. At the same time, UV radiation can be absorbed by chromophores, which initiate a signaling cascade leading to immunosuppression. Prominent chromophores are DNA and urocanic acid. The latter can be isomerized by UV radiation from the trans form to the cis form. Uptake of antigen and presentation in an immunosuppressive context leads to induction of antigen-specific tolerance (Cooper *et al*, 1992; Steinbrink *et al*, 1997).
Besides creating an immunosuppressive environment in the skin, UV-B radiation is also able to impair the antigen-presenting capacity of Langerhans cells, by affecting antigen processing

and production of mediators produced by keratinocytes which are needed for optimal T-cell responses (Stingl *et al*, 1983). Langerhans cell function can be influenced by cytokines like interleukin 10 (IL-10), which can inhibit migration and maturation of Langerhans cells (Steinbrink *et al*, 1997). Other UV-induced soluble mediators, such as the neuropeptide CGRP (calcitonin gene-related peptide) and nitric oxide can inhibit antigen presentation by dendritic cells *in vitro* (Gillardon *et al*, 1995).

Expression of costimulatory molecules on dendritic cells is needed for proper antigen presentation and stimulation of T-cells. Some investigators report that costimulatory molecules, such as CD80 (B7-1), CD86 (B7-2) and HLA-DR are downregulated by UV (Rattis *et al*, 1998; Weiss *et al*, 1995). Conversely, Laihia and Jansen (Laihia and Jansen, 1997; Laihia and Jansen, 2000) show that these molecules are upregulated after *in vivo* UV (over)exposure of the skin. The costimulatory and adhesion molecule ICAM-1 can be downregulated by UV radiation leading to an impairment of clustering of Langerhans cells and T-cells (Tang and Udey, 1991). Inhibition of costimulatory signals can lead to abrogation of Th1 stimulation (Simon *et al*, 1991), while stimulation of Th2 cells is still possible (Simon *et al*, 1990).

Langerhans cells themselves can also contribute to the immunosuppressive milieu by releasing neurohormones, such as alpha-melanocyte stimulating hormone, which can downregulate expression of costimulatory molecules on Langerhans cell and induce IL-10 production (Luger *et al*, 1997; Luger *et al*, 1998).

Another way to prevent Th1 stimulation is by apoptosis of Langerhans cells, which will prevent them from reaching the T-cells in the lymph nodes or which may induce aberrant T-cell activation signals leading to T-cell unresponsiveness (Kitajima *et al*, 1996). *In vitro* and *in vivo* (mice) experiments revealed that UV-exposed Langerhans cells undergo apoptosis, for instance via the production of reactive oxygen species. Whether this also occurs in human skin *in vivo* still had to be demonstrated (Meunier, 1999). UV-induced apoptosis of human Langerhans cells can be prevented, among others, by interaction between the costimulatory molecule CD40, present on Langerhans cells, and CD40 ligand (CD40L), present on T-cells *in vitro* (Rattis *et al*, 1998). Whether CD40 or CD40L is downregulated after UV exposure *in vivo* is still unknown.

CD11b+ monocytic/ macrophagic cells

Besides inducing an efflux of Langerhans cells, exposure of the skin to UV also results concomitantly in an influx and expansion of CD11b+ cells in the (epi)dermis (Meunier *et al*, 1995). There are several indications that these CD11b+ macrophage-like cells are involved in UV-B-induced immunosuppression: Kang *et al* (1994 and 1998) reported that human CD11b+ cells produce and secrete IL-10 *in vivo* and had a low expression of interleukin 12 (IL-12). Hammerberg *et al* (1996a, 1996b and 1998) showed that a local state of *in vivo* tolerance in

the UV-exposed murine skin is closely associated with the influx of CD11b+ MHCII+ cells and that anti-CD11b treatment can even reverse UV-induced immunosuppression. Furthermore, UV radiation can activate complement component 3 (C3) in the skin. Experiments with C3-deficient mice and prevention of the formation of iC3b, the ligand for the CD11b receptor, resulted in a reduction of the number of CD11b+ MHCII+ cells and a reversion of the failure to induce a contact hypersensitivity response in the UV-B-exposed murine skin (Hammerberg *et al*, 1998). The macrophages in UV-exposed skin appear to expand preferentially suppressor-inducer CD4+ T-cells which activate CD8+ T-cells, thereby also contributing to UV-B-induced tolerance (Baadsgaard *et al*, 1988; Baadsgaard *et al*, 1990).

Cytokines

Cytokines are cellular proteins that are often pleiotropic; they can induce many cell functions and act on many cell types. The biological activity of cytokines depends on their binding to specific cytokine receptors. Cytokines can act in an autocrine, paracrine or endocrine manner having their effects on the cells of origin, the local environment or via the periphery respectively. Some cytokines can act in a juxtacrine manner by binding of a membrane-bound cytokine to a receptor on an adjacent cell. Furthermore, identical cytokines can be produced by different cell types. Cytokine release and activity can be regulated via several mechanisms: regulation of gene transcription, regulation of conversion from a pro-cytokine to an active cytokine, regulation of cytokine receptor expression, expression of cytokine receptors with different characteristics and effects, or via the release of soluble cytokine receptors (Sundy *et al*, 1999).

Cytokines are important in the regulation of immune responses, they can either initiate or stimulate inflammatory processes (pro-inflammatory cytokines) or downregulate inflammation (anti-inflammatory cytokines). Interleukin (IL)-1, IL-6, IL-12, IL-18, tumour necrosis factor (TNF)-α, interferon (IFN)-γ are cytokines exhibiting pro-inflammatory activities. IL-4 and IL-10 are examples of cytokines involved in Th2 skewing and downregulation of inflammation in the skin. UV-B radiation induces the release of both pro-inflammatory and anti-inflammatory cytokines eventually shifting the balance towards immunosuppression.

Interleukin 1 (IL-1). IL-1 has two forms, IL-1-α and IL-1-β, with mostly indistinguishable biological activity. IL-1-α is fully active as a precursor but remains mostly intracellular. When cells are physically disrupted, pro-IL-1-α is released and cleaved by extracellular proteases or by membrane-associated calpains (Kobayashi *et al*, 1990; Watanabe and Kobayashi, 1994). IL-1-β precursor is not fully active and is activated and secreted in large amounts after cleavage by interleukin-1-β-converting enzyme (ICE, caspase 1). IL-1 can bind to two specific IL-1 receptors: IL-1 type 1 receptor, that induces signal transduction after binding of the

cytokine, and IL-1 type 2 receptor, that can bind IL-1 but does not transduce a signal and can thus act as a decoy receptor for IL-1-β. IL-1 receptor antagonist (IL-1-Ra) (Hannum *et al*, 1990) can bind to IL-1 type 1 receptor nearly irreversible preventing or disrupting the binding of IL-1 without triggering a signal (Dripps *et al*, 1991) and leading to the inhibition of IL-1 activity.
The epidermis contains a large amount of IL-1-α in keratinocytes. IL-1-α expression is upregulated by UV radiation and stimulates prostaglandin synthesis and increases TNF-α and IL-6 levels. UV radiation also induces the expression of IL-1-β by keratinocytes and Langerhans cells (Norval, 2001). IL-1-β, but also IL-1-α, is involved in the migration of Langerhans cells (Cumberbatch *et al*, 1997a; Cumberbatch *et al*, 1997b). Langerhans cell migration induced by antigen application was found to be dependent on IL-1-β and independent of IL-1-α while the converse was true for application of an irritant (Cumberbatch *et al*, 2002). Together with an increase in IL-1, IL-1-Ra expression was also elevated in the stratum corneum after UV exposure. The ratio IL-1-Ra:IL-1-α was over 100 in the UV-exposed skin compared to 8 in the unexposed skin (Hirao *et al*, 1996). The UV-induced rise in IL-1, with the concomitantly greater rise in IL-1-Ra may actually inhibit the activities of IL-1 and suppress an inflammatory response (Duthie *et al*, 1999).

Tumour necrosis factor-α (TNF-α). The pro-inflammatory cytokine TNF-α is produced as a biologically active membrane protein which is secreted only after cleavage by TNF-α-converting enzyme. TNF-α mediates its effects by binding to TNF receptor-p55 or TNF receptor-p75. Human keratinocytes only express TNF receptor-p55. Soluble forms of both TNF receptors can neutralize the effects of TNF-α (Olsson *et al*, 1993).
TNF-α is increased after UV irradiation and can be produced by keratinocytes (Kock *et al*, 1990), mast cells (Walsh, 1995), dermal fibroblasts and Langerhans cells (Norval, 2001). UV-induced TNF-α expression contributes to the apoptosis of keratinocytes (Schwarz *et al*, 1995) and the migration (Cumberbatch *et al*, 1997a; Cumberbatch *et al*, 1997b) and maturation of Langerhans cells (Streilein, 1993). Yokishawa and Streilein (1990) showed that TNF-α is involved in the UV-induced impairment of contact hypersensitivity responses by reversing the impairment with anti-TNF-α antibodies.

Interleukin 4 (IL-4). IL-4 is a cytokine with intrinsic anti-inflammatory capacities in the skin. IL-4 can be produced by activated T helper 2 (Th2) cells, basophils and neutrophils (Sundy *et al*, 1999; Teunissen *et al*, 2002). Takayama *et al* (1999) showed, by using an *in vitro* migration model, that IL-4 inhibited Langerhans cell migration by downregulation of TNF receptor-p75 on Langerhans cells. Mice overexpressing IL-4 had increased epidermal Langerhans cell numbers which can be explained by a reduction in Langerhans cell emigration from the skin

(Elbe-Burger *et al*, 2002). Experiments performed in IL-4 knockout mice showed that IL-4 was important in UV-induced suppression of a delayed type hypersensitivity reaction (el Ghorr and Norval, 1997). Contact hypersensitivity reactions were only suppressed in IL-4 knockout mice when the sensitizer (oxazolone) was applied on irradiated skin (local model)(el Ghorr and Norval, 1997) but not on unirradiated skin (systemic model), showing that IL-4 is only important in UV-induced systemic immunosuppression of contact hypersensitivity reactions (Hart *et al*, 2000). UV irradiation induces an infiltration of numerous IL-4+ neutrophils into the dermis and even the epidermis. These IL-4+ neutrophils support the development of a Th2 milieu in the UV-exposed skin, as depletion of these CD15+ neutrophils from dermal cell cultures from UV-exposed skin abolished the Th2 response (Teunissen *et al*, 2002).

Interleukin 6 (IL-6). IL-1 is a potent inducer of IL-6. IL-6 can be produced by keratinocytes and Langerhans cells and is detected in the serum after UV exposure (Urbanski *et al*, 1990). IL-6 is increased after UV exposure and has a wide range of pro-inflammatory effects including fever and synthesis of acute phase proteins. On the other hand, IL-6 has some anti-inflammatory capacities. It can induce the production of adrenocorticotrophic hormone from the central nervous system, increasing the synthesis of glucocorticoids (Tilg *et al*, 1997) which, in turn, can suppress IL-1 and TNF-α synthesis. Furthermore, IL-6 induces soluble TNF receptor-p55 and promotes the synthesis of IL-1-Ra by increasing acute phase C-reactive protein (Tilg *et al*, 1997; Urbanski *et al*, 1990).

Interleukin 10 (IL-10). IL-10 has similar activities in the induction of anti-inflammatory processes as IL-4. It can be produced by keratinocytes, macrophages, melanocytes and activated T-cells, especially Th2 cells. IL-10 is increased after UV irradiation and is involved in the decrease of pro-inflammatory cytokines, like IL-1, TNF-α, IL-12 and IFN-γ (Norval, 2001), and the increase of IL-1-Ra (Cassatella *et al*, 1994). UV-induced upregulation of IL-10 in keratinocytes (Enk *et al*, 1995; Grewe *et al*, 1995), probably triggered by DNA damage (Nishigori *et al*, 1996), may induce systemic immunosuppression (Rivas and Ullrich, 1992) by itself or perhaps with the help of prostaglandin E_2 or IL-4 (Shreedhar *et al*, 1998). Furthermore, IL-10 is shown to inhibit Langerhans cell migration, maturation (Wang *et al*, 1999) and antigen presentation (Peguet-Navarro *et al*, 1995), and convert them into tolerogenic antigen presenting cells (Steinbrink *et al*, 1997). In contrast Halliday and Le (2001) showed that IL-10 from regressor tumors enhanced Langerhans cell migration from the skin. An IL-10-induced decrease of IL-12 and an inhibition of Langerhans cell activation of Th1 cells, but not Th2 cells (Enk *et al*, 1993), may skew the T helper population towards a Th2 phenotype. Kang *et al* (1994 and 1998) reported that CD11b+ macrophages are the major source of IL-10 production and secretion in the UV-exposed skin. Depletion of these cells by anti-CD11b treatment

reversed UV-induced immunosuppression of contact hypersensitivity responses (Hammerberg *et al*, 1996a).

Interleukin 12 (IL-12) and interferon (IFN)-γ. The bioactive form of IL-12, IL-12p70, consists of the subunits p40 and p35. Monomers and homodimers of p40 are known to inhibit IL-12 activity by blocking the receptor (Mattner *et al*, 1993). IL-12 is important in preferentially inducing Th1 immune responses and promoting the production of IFN-γ by T-cells and natural killer cells (Trinchieri, 1995). IL-12 can be produced by macrophages, dendritic cells (Kang *et al*, 1996), Th1 cells and keratinocytes (Aragane *et al*, 1994; Muller *et al*, 1994). Upon UV radiation the production of IL-12 by monocytes/macrophages is reduced (Kang *et al*, 1998; Yoshida *et al*, 1998). Antigen presentation by UV-irradiated monocytes *in vitro* resulted in reduced IFN-γ and IL-12 production by T-cells, limiting Th1 responses (Kremer *et al*, 1996). UV irradiation of keratinocytes *in vivo* or *in vitro*, however, induced the expression of IL-12p40 (Enk *et al*, 1996; Kondo and Jimbow, 1998). A UV-induced increase in the production of biologically inactive IL-12p40 by keratinocytes or dendritic cells might result in a decrease of Th1 responses and an impairment of antigen presentation (Norval, 2001). IL-12 treatment can overcome UV-B-mediated local and systemic immunosuppression and tolerance by preventing the induction of suppressor T-cells (Schmitt *et al*, 1995; Schwarz *et al*, 1996), restoring the UV-induced suppression of IFN-γ by T-cells (Ando *et al*, 2000) and inhibiting the UV-induced release of IL-10 (Schmitt *et al*, 2000). Furthermore, treatment of keratinocytes *in vitro* or *in vivo* with IL-12 resulted in suppression of UV-induced apoptosis by enhancing DNA repair (Schwarz *et al*, 2002).
IFN-γ is reported to increase IL-12 production of macrophages and dendritic cells (Trinchieri, 1995) and to inhibit the proliferation of Th2 cells, but not Th1 cells. A UV-induced reduction of IFN-γ favors Th2 development and is an important mechanism by which UV irradiation prevents tumor rejection (Gensler *et al*, 1995). Reeve *et al* (1999) reported that IFN-γ plays a role in UV-A-induced photoprotection from the suppressive effects of UV-B radiation.

Interleukin 18 (IL-18). IL-18 was first discovered in 1989 by Nakamura *et al* (1989) as IFN-γ inducing factor (IGIF). Bazan *et al* (1996) showed that the amino acid sequence structure of the protein resembled very much IL-1. IL-18, like IL-1-β, needs to be cleaved by caspase 1 to generate an active protein (Gu *et al*, 1997). IL-18 binding protein can bind to IL-18 and block its activity, which resembles the mechanism of the IL-1 receptor 2 (Novick *et al*, 1999).
IL-18 can be expressed by many cell types, e.g. dendritic cells, macrophages and epithelial cells. Keratinocytes express predominantly the unprocessed biologically inactive form of IL-18 (Companjen *et al*, 2000a; Companjen *et al*, 2000b). Biologically active IL-18 is secreted by

keratinocytes after treatment with pro-inflammatory agents, such as PMA and LPS (Naik *et al*, 1999). IL-18 is dependent on and synergizes with IL-12 to induce IFN-γ production by T-cells (Chang *et al*, 2000; Tominaga *et al*, 2000) and to skew the response towards Th1. IL-18 is also involved in IFN-γ production and activation of natural killer cells (Hunter *et al*, 1997; Lauwerys *et al*, 1999). Langerhans cell derived IL-18 mRNA and protein is upregulated in migrating hapten-modified Langerhans cells and contributes to the initiation of contact hypersensitivity (Wang *et al*, 2002). Intradermal administration of IL-18 resulted in Langerhans cell migration and accumulation of Langerhans cells in the draining lymph nodes. This migration was dependent on availability of TNF-α and the integrity of the IL-1 receptor 1 signaling (Cumberbatch *et al*, 2001). Contact hypersensitivity and Langerhans cell migration were inhibited in mice deficient for caspase 1, the cleavage enzyme for IL-18 (Antonopoulos *et al*, 2001). Experiments with caspase 1 knockout mice revealed that IL-18 induced Langerhans cell migration required IL-1-β (Cumberbatch *et al*, 2001). Another biological property of IL-18 is its enhancing effect on apoptosis through Fas-Fas ligand interactions (Kodama *et al*, 2000), which is, at least in part, responsible for the anti-tumor effects of IL-18 (Hashimoto *et al*, 1999). There is only limited information on the effects of UV radiation on IL-18 expression. Nakagawa *et al* (1999) reported that ex vivo UV-B irradiation of epidermal sheets had no effect on IL-18 production.

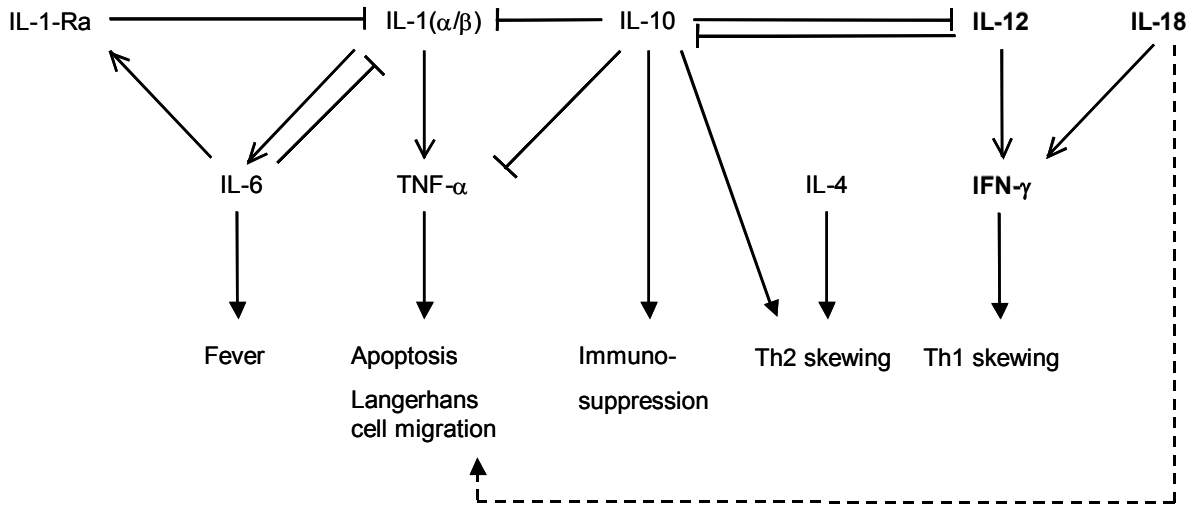

Figure 2. Simplified scheme of the effects of UV radiation on the cytokine cascade. Most cytokinesin this scheme are upregulated by UV exposure. Cytokines that are downregulated are indicated in bold. The dotted line illustrates a potential, but yet unknown, effect of IL-18 on UV-induced Langerhans cell migration. ⟶ = stimulation, ⟙ = suppression.

DNA DAMAGE, DNA REPAIR, AND APOPTOSIS

The energy of UV radiation, especially of wavelengths in the UV-B band, can be absorbed by organic molecules like DNA. Upon UV-B exposure the DNA of cells is damaged. UV-induced DNA damage consists of DNA adducts (95%) and strand breaks (5%). Neighboring pyrimidines (C, T) are more sensitive to UV-induced dimerisation than purines (G, A). UV-B radiation induces cyclobutane pyrimidine dimers (CPD) and 6-4 pyrimidine-pyrimidones photoproducts (6-4PP). CPDs are more frequently formed and disturb the DNA helix less than 6-4PP (Bykov and Hemminki, 1996; de Gruijl, 1997; Mitchell *et al*, 1992).
The predominant DNA repair mechanism for UV-induced DNA damage is Nucleotide Excision Repair (NER). NER entails the action of many proteins which are involved in damage recognition, opening of the DNA-helix, excision of the damage-containing oligonucleotide followed by gap filling and strand ligation. NER consist of two pathways: one is involved in the excision of DNA damage throughout the entire genome, called Global-Genome Repair (GGR), and the other pathway, called Transcription-Coupled Repair (TCR) preferentially repairs DNA damage in the transcribed strands of actively transcribed genes. The two pathways are essentially the same except for the damage recognition part which is performed by different proteins. An enhancement or defect of DNA repair can have dramatic effects on cytokines produced in the skin. For instance, removal of CPDs by topical treatment with T4 endonuclease V (T4N5) resulted in a decrease in UV-induced levels of IL-10 and TNF-α (Nishigori *et al*, 1996; Wolf *et al*, 2000). Furthermore, a defect in TCR gave rise to an increased production of TNF-α and IL-10 after UV exposure while a defect in GGR resulted in a decreased production of IFN-γ by lymph node cells (Boonstra *et al*, 2001).
The consequences of a defect in either one of the pathways of the NER system are evident in patients suffering from rare recessive photosensitive disorders: Xeroderma Pigmentosum (XP) and Cockayne Syndrome (CS). Eight different subgroups of XP (XPA-XPG and XP variant) and two different subgroups of CS (CSA and CSB) have been identified, reflecting a defect in a distinct gene. Most of the XP patients, of which XPA is the most severe subgroup, are extremely sensitive to sunlight and are at a more than 1000-fold risk of developing skin cancer, mainly on sun-exposed skin areas. However, patients with only a defect in the GGR arm of the NER (XPC patients) are as susceptible to sunburn as healthy individuals (Berg *et al*, 1998; Kondo *et al*, 1992). CS patients exhibit an extreme photosensitivity but are apparently not predisposed to develop skin cancer. CS patients suffer from physical and mental retardation and often die at an early age because of a variety of complications (e.g. infections). Many of the clinical symptoms of CS are not displayed by XPA patients who are completely NER deficient, making it difficult to explain them via a partial NER defect. As CS proteins are involved in transcription, a transcription deficiency, perhaps aggrevated by DNA damage,

might also contribute to the clinical symptoms. A defect in repair of oxidative DNA damage was detected in CS cells but not XPA cells (Leadon and Cooper, 1993) (for review of the above see de Boer and Hoeijmakers (2000)).
Gene-targeted mice with defects in both GGR and TCR (XPA), or either GGR (XPC) or TCR (CSB) have been generated to study the effects of UV radiation and other DNA damaging agents on the induction of DNA damage, DNA repair, apoptosis and immune reactivity.
Another mechanism, besides DNA repair, to clear the UV-induced DNA damage is by the induction of apoptosis. This mechanism protects severely damaged cells from transforming into malignant cells. After UV-B exposure apoptotic keratinocytes, called sunburn cells, can be detected in the epidermis (Daniels *et al*, 1968; Murphy *et al*, 2001). Defects in TCR (as in CSB) are associated with a dramatic increase in sensitivity to UV-induced apoptosis (Van Oosten *et al*, 2000).
UV-induced apoptosis is a multifactoral event that can be initiated from many cellular targets (e.g. cell membrane, mitochrondia). UV radiation can lead to apoptosis via de induction of reactive oxygen species (especially by UV-A I), cross linking and activation of death receptors, like Fas (CD95) (especially by UV-B) (Aragane *et al*, 1998; Kulms *et al*, 1999), the production of apoptosis-inducing cytokines, like TNF-α (Schwarz *et al*, 1995), or the activation of transcription factor AP-1 or p53, which in their turn can increase the expression of FasL (CD95Ligand) or the pro-apoptotic protein Bax respectively (Sheehan and Young, 2002). Execution of apoptosis occurs via depolarization of the mitochrondial membrane potential, release of cytochrome C and/or activation of several caspases. Caspases can be divided into two subgroups: the initiator caspases and the effector caspases. Upon induction of apoptosis the pro-caspases are proteolytically cleaved to form active caspases. Activation of effector caspases, like caspase 3, is irreversible and once activated can no further be regulated (for review see Sheehan and Young (2002)).

OUTLINE OF THE THESIS

As described in the preceding paragraphs, UV radiation, especially UV-B, has various effects on the skin immune system. All-together this leads to the induction of a local and/or systemic immunosuppression. This immunosuppression was first considered as a harmful effect of UV radiation, leading for instance to the suppression of tumor rejection. However, it can also be thought of as a perfectly sound physiological reaction: if we do not display this immunosuppression we might develop a disruptive immune reaction when we are exposed to UV. This seems to be exactly what is happening in the skin of patients with polymorphous light eruption (PLE) or sun allergy. Therefore we hypothesized that the UV-induced

immunosuppression is disturbed in PLE patients. In this thesis I will describe how we try to unravel the pathogenesis of PLE step by step, based on this hypothesis.

As described previously, CD11b+ macrophage-like cells infiltrate the UV-B-exposed skin and produce and secrete the immunosuppressive cytokine IL-10. Treatment of mice with anti-CD11b resulted in a reversion of the UV-B-induced immunosuppression. These data led us to the speculation that these CD11b+ cells must be absent or non-functional in the skin of PLE patients. To test this hypothesis we first established an experimental model in healthy individuals to reproduce the data on CD11b+ cells described by other investigators. A nearly complete UV-B-induced depletion of CD1a+ Langerhans cells, as reported by these investigators, was used as a guideline for our irradiation protocol. We then exposed the skin of PLE patients to UV-B radiation, according to our irradiation protocol, and investigated the presence of CD11b+ cells in the (un)exposed skin. These data are presented in **Chapter 2**. As we were investigating our model in PLE patients, we found a remarkable difference in the behavior of CD1a+ Langerhans cells: they did not disappear after UV (over)exposure!

With this striking difference in mind and knowing that Langerhans cells, the antigen-presenting cells of the skin, are reported to be associated with immunosuppression, we then focused on these cells.

We hypothesized that in PLE patients there is a defect in the mechanism(s) responsible for a normal UV-B-induced depletion of Langerhans cells. But before we could investigate this defect in PLE patients we first had to establish the mechanism(s) that are at the root of this UV-induced Langerhans cells depletion in healthy individuals. The two potential mechanisms that are described in literature, namely apoptosis or migration, were examined in healthy volunteers (**Chapter 3**). A novel method was developed to detect Langerhans cell migration *in vivo*.

The main mechanism responsible for UV-B-induced Langerhans cell depletion in healthy individuals turned out to be migration. With this knowledge we could now specify our former hypothesis: Langerhans cells in the skin of PLE patients show an impaired migration to UV-B radiation. Furthermore, we speculated that the Langerhans cells that remain in the epidermis after UV-B overexposure of the skin of PLE patients are activated and/or matured. This active/mature state of the Langerhans cells might result in antigen presentation at a wrong location, namely in the skin instead of the lymph nodes. The outcomes of these experiments are presented in **Chapter 4**.

Several cytokines are involved in UV-induced Langerhans cell migration and immunosuppression. The data we had collected from our experiments so far led us to two hypotheses: first, cytokines involved in UV-B-induced Langerhans cell migration (e.g. IL-1, TNF-α) are produced in lesser amounts in PLE patients compared to healthy individuals, and secondly, UV-B radiation induces an imbalance between pro-inflammatory and Th1-skewing

cytokines (IL-6, IL-12, IL-18, IFN-γ) and cytokines involved in Th2-skewing and immunosuppression (IL-4, IL-10) leading to a Th1 milieu in the skin of PLE patients. Because of a scarcity of material and the number of cytokines we wanted to investigate and because we wanted to localize cytokine-expressing cells, we used immunohistochemistry on microscopic sections for an exploratory study. The results for a series of cytokines in (un)exposed skin of PLE patients and healthy individuals are presented in **Chapter 5**.

When we investigated the UV-induced migration of Langerhans cells in healthy individuals and PLE patients we found that only a minority of the migrating Langerhans cells had UV-B-induced DNA damage (pyrimidine dimers). Therefore, we speculated that DNA damage and DNA repair might be involved in determining whether and when Langerhans cells migrate. To test this hypothesis a mouse model with DNA repair-deficient mice (XPC, XPA, CSB), was used. In this model we were also able to link data on DNA repair and Langerhans cell migration with immunosuppression. These experiments are described in **Chapter 6**.

In **Chapter 7** the studies described in this thesis will be integrated and discussed, giving an overview of the present knowledge on the pathogenesis of PLE.

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CD11b+ cells and ultraviolet-B-resistant CD1a+ cells in skin of patients with polymorphous light eruption

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Journal of Investigative Dermatology 113:4-10, 1999

Color printings: see addendum

ABSTRACT

After ultraviolet (UV) overexposure the Langerhans cells (epidermal CD1a+ cells) disappear from the healthy skin, and CD11b+ macrophage-like cells, which are reported to produce IL-10, appear in a matter of days. These phenomena are related to the UV-induced local suppression of contact hypersensitivity reactions. A defect in this suppression might allow inadvertent immune reactions to develop after UV (over)exposure; i.e., it could cause UV-B-induced polymorphous light eruption (PLE). In order to test this we first exposed buttock skin of 8 healthy volunteers to 6 MED (minimal erythema dose) from Philips TL12 lamps, and indeed observed a dramatic disappearance of CD1a+ cells 48 and 72 hours later, at which time the number of CD11b+ cells increased in the dermis, and some occurred in the epidermis. The epidermis thickened and showed large defects, with CD11b+ cells lodged into them, just below the stratum corneum. In 10 patients with PLE (5 with a normal MED and 5 with a low MED) CD1a+ cells were present epidermally as well as dermally before exposure. Strikingly, these cells were still present in considerable number at 48 and 72 hours after exposure to 6 MED. A substantial number of CD11b+ cells was already present dermally before UV exposure, and this number increased further after UV overexposure, and subsequently also invaded the epidermis. Despite the 6 MED, there were no apparent defects in the epidermis of the PLE patients. This deviant early response to UV radiation is likely to be of direct relevance to the PLE and is perhaps useful as a diagnostic criterion.

INTRODUCTION

Solar UV radiation, especially UV-B (280-315 nm), is a very potent activator of photochemical reactions: we are all familiar with resulting effects like ‘photochemical smog’ and ‘photodegradation’ of plastics and other compounds. Organic molecules such as proteins and DNA can react to UV-B and be damaged (de Gruijl, 1997; Jung and Bohnert, 1995). It is therefore likely that UV radiation induces a broad range of effects and physiological reactions in the skin. Obviously, the skin needs to be very well adapted to this natural photochemical stress. The skin has, for example, efficient mechanisms to repair or replace photo-damaged cells. Another plausible adaptive mechanism of the skin to UV-B radiation lies in the modulation of the immune system: Exposure to UV-B causes suppression of cellular immunity (Cooper, 1996; Yoshikawa *et al*., 1990). In previous research this came to light as an adverse effect on health. Tumor rejection and the immunization against microbial pathogens are markedly reduced in animal models after exposure to UV radiation (Gensler *et al*., 1995; Halliday and Norval, 1997; Jeevan *et al*., 1995). However, on reconsideration this UV-induced

immunosuppression can be thought of as a primarily sound physiological reaction. First of all it should be noted that all healthy humans consistently display this UV-induced immune suppression at sufficiently high dosages (Cooper *et al*., 1992). Because of its photochemical activity, UV radiation can modify proteins and other molecules, and thus create neo-antigens that could provoke (auto-) immune reactivity (Natali and Tan, 1973; Norris *et al*., 1989). From this perspective UV-induced immunosuppression is a very desirable mechanism to prevent a persistent, disruptive immune reactivity against sun-exposed skin. What if this immunosuppression is not adequate? What kind of pathology would evolve? A UV-induced 'sun allergy' would be most plausible. UV-B-triggered polymorphous light eruption (PLE) would therefore seem a likely candidate for exploring this possible pathogenic mechanism.

Patients with PLE are sensitive to UV-B, UV-A and sometimes even to visible light. These patients develop papules and eczema, mostly on areas that are exposed to sunlight (Epstein, 1997; Grabczyska and Hawk, 1997; Salomon *et al*., 1997). It is often suggested that PLE is a delayed type hypersensitivity reaction (type IV) because there is usually a clear delay (hours to days) between the exposure to UV and the appearance of skin lesions (Epstein, 1997; Norris *et al*., 1989; Verheyen *et al*., 1995). The histology of lesional PLE skin shows dense lymphocytic infiltrates. These infiltrates are initially dominated by CD4+ lymphocytes, but at 72 hours after the UV provocation CD8+ T-cells outnumber CD4+ T-cells (Holze, 1995; Norris *et al*., 1989). These phenomena indicate that a cellular-mediated immune reactivity is involved in PLE.

UV-B can inhibit the antigen-presenting capacity of e.g. Langerhans cells (Bacci *et al*., 1996; Beissert and Granstein, 1996; Goettsch *et al*., 1993; Krutmann *et al*., 1994; Ullrich, 1995b). After UV radiation, Langerhans cells with UV-damaged DNA migrate from the epidermis and become detectable in the draining lymph nodes (Sontag *et al*., 1995) but UV-B exposure can also kill epidermal Langerhans cells in the epidermis (Mommaas *et al*., 1993). Furthermore, immunomodulatory compounds such as IL-10, PGE_2 and TNF-α are released in the UV-exposed skin (Boonstra and Savelkoul, 1997; Cooper, 1996; Halliday and Norval, 1997). CD11b+ macrophage-like cells infiltrate the dermis and epidermis after UV-B exposure and were found to be potent IL-10 producers (Kang *et al*., 1994; Kang *et al*., 1998). UV-B-modified antigen presentation can render Th1 cells, which are involved in delayed type hypersensitivity reactions, anergic (for reviews see Ullrich (1996), Boonstra and Savelkoul (1997), Halliday and Norval (1997), Ullrich (1995a)).

Hence, in the epidermis of healthy individuals Langerhans cells disappear after UV-B (over) exposure and within a few days CD11b+ macrophage-like cells appear, which can suppress cellular immunity. As was also suggested by Dr. Cooper (personal communication), we hypothesized that a defect in this influx of CD11b+ macrophage-like cells would lead to illicit

immune reactivity. This immune reactivity could be involved in the pathogenesis of a UV-B-induced PLE.

To investigate this possible pathogenic mechanism, a group of PLE patients was studied. All patients reacted pathologically to UV-B radiation, developing vesicles, papules or eczema. For comparison a control group consisting of healthy volunteers was studied. A small area of unaffected buttock skin of all participants was overexposed to UV-B and skin biopsies were taken at successive time points following the exposure. In order to assess whether immunocellular responses were altered in the unaffected skin of PLE patients, immunohistochemical stainings were done to study shifts in CD1a+ (Langerhans cells) and CD11b+ (macrophage-like cells) cell populations. These cells were further phenotyped by double staining.

MATERIALS AND METHODS

Subjects

Five patients with PLE and normal sunburn sensitivity (2 males and 3 females, ages between 28 and 49 years), five patients with PLE and extreme sunburn sensitivity (5 males, ages between 45 and 82 years) and eight healthy individuals (4 males and 4 females, ages between 21 and 43 years) were studied. The diagnosis PLE was based on the patient's history and on provocation with daily exposures of UV-B, UV-A I and visible light on restricted areas of the upper arms, a standard procedure in our dermatological clinic. In all patients who were included, papular or eczematous lesions occurred in the UV-B exposed skin either with or without a similar reaction in the UV-A exposed skin. In some patients, who were extremely sensitive to sunburn a pathological reaction to visible light was also observed. With the exception of two extremely sunburn sensitive patients, most included patients had a season-dependent exacerbation of the disease. Patients who had received photo-therapy or who were under medication (e.g. corticosteroids) and patients and volunteers whose buttock skin was exposed to sunlight less than two months ago, were excluded. Informed consent was obtained from patients and volunteers. This study was approved by the medical ethical committee of the University Hospital in Utrecht.

Phototesting procedures

The minimal erythema dose (MED) of the buttock skin of the participants in this study was determined using a Philips TL12 lamp (57.5% of UV output in UV-B (280-315 nm)) and a testing device with nine windows (2*9 mm each) which open and close sequentially to expose the underlying skin for different periods of time (in a geometrical series e.g. from 12.5 to 200 sec). The small areas of irradiated skin were examined at 6 and 24 hours and the area showing minimal perceptible redness, not necessarily with discernable borders, was taken to correspond to the MED. MED ranged from 350 to 750 J/m^2 UV in healthy individuals, from 245 to 490 J/m^2 UV in PLE patients with a normal MED, and from 8.8 to 70 J/m^2 UV in PLE patients with a low MED. Subsequently, the (unaffected) buttock skin was exposed to 6

MED UV (again with Philips TL12). 3-mm punch biopsies were obtained 24 hours and 48 hours or 48 hours and 72 hours after UV exposure, together with one additional control biopsy from the unirradiated skin. Specimens were snap frozen in liquid nitrogen, embedded in O.C.T-compound (Tissue-Tek, Sakura, Zoeterwoude, the Netherlands) and stored at - 40^{o}C until further processing.

Antibodies

As primary antibodies, monoclonal antibodies CD1a (DAKO A/S, Glostrup, Denmark, diluted 1:60), CD11b (Immunotech, Marseille, France, diluted 1:50), and CD68 (DAKO A/S, diluted 1:50) were used. The fluorescein isothiocyanate (FITC)-conjugated primary antibodies CD1a and CD11b were purchased from Ortho Diagnostic systems Inc. (Beerse, Belgium, diluted 1:20) and Immunotech (diluted 1:60) respectively. The murine monoclonal antibody Lag was a kind gift from Prof. Imamura (Dept. Dermatology, Kyoto University, Kyoto, Japan). Biotinylated horse anti-mouse immunoglobulin (Vector, Burlingame, CA, diluted 1:800) was used as a secondary antibody. Alkaline phosphatase (AP)-labeled F(ab) fragments of sheep anti-FITC (Boehringer Mannheim GmbH, Mannheim, Germany, diluted 1:400) or horseradish peroxidase (HRP)-conjugated avidine-biotin complex (DAKO A/S, diluted 1:50) were used as detecting reagents.

Immunohistochemistry

Frozen skin sections (6 μm) on 3-aminopropyltriethoxysilane-coated glass slides were fixed for 10 minutes in 100 ml dry acetone containing 50 μl 30% H_2O_2 at room temperature (RT) and incubated for 20 minutes with a blocking reagent (PBS containing 10% normal human serum, 10% normal horse serum and 10% normal mouse serum) to prevent non-specific binding. The skin sections were incubated with the primary antibody (diluted in blocking reagent) for 1 hour. The glass slides were then washed three times with PBS, containing 0.05% Tween 20.

Subsequently, skin sections were incubated for 1 hour with a biotinylated horse anti-mouse antibody (diluted in blocking reagent) following incubation with an HRP-conjugated avidine-biotin complex (diluted in PBS/0.05% Tween) for 30 minutes. Antibody binding was visualized by incubating the sections in 0.1 M acetate buffer (pH=5) containing 20 mg 3-amino-ethyl-carbazole (AEC)(Sigma Chemical Co., St. Louis, MO) and 100 μl 30% H_2O_2 per 100 ml. The skin sections were counter-stained with Mayer's hematoxylin. Double staining reactions were done to further characterize the CD1a+ and CD11b+ cells.

For the double staining (Lag/CD1a-FITC and CD68/CD11b-FITC) the skin sections were fixed and preincubated as described above. The skin sections were then incubated with the unconjugated antibody (Lag or CD68; diluted in blocking reagent) for 1 hour and with a biotinylated horse anti-mouse antibody (diluted in blocking reagent) also for 1 hour. The sections were incubated for 20 minutes with a blocking reagent containing PBS and 10% normal mouse serum and subsequently incubated for 1 hour with the FITC-conjugated antibody CD1a or CD11b (diluted in 10% normal mouse serum). Then the sections were incubated simultaneously with an AP-labeled sheep anti-FITC antibody and an HRP-conjugated avidine-biotin complex (both diluted in PBS/0.05% Tween 20) for 1 hour. Antibody binding was visualized by incubating the skin sections first in a Tris-HCl buffer (pH=8.5), containing 25 mg Fast Blue BB salt, 12.5 mg naphtol AS-MX phosphate and 35 mg levamisol per 100 ml, all purchased from Sigma Chemical Co.. The slides were subsequently incubated with a 0.1 M acetate buffer (pH=5)

containing 20 mg AEC and 100 μl 30% H_2O_2 per 100 ml. Negative staining controls were used in single and double staining experiments in which the first antibody was omitted or was replaced by an irrelevant antibody of the same isotype. All antibody incubations were performed in a humidified chamber at room temperature. After each antibody incubation the slides were rinsed three times in PBS containing 0.05% Tween 20.

The skin sections were evaluated using a light microscope at 250 times magnification with a standard eyepiece. The number of positive cells in the skin sections was assessed independently by two investigators. The prevalence of cells was graded as: no or hardly any positive cells present (-), presence of scattered positive cells (±), clear abundant presence of positive cells (+), closed maze of positive cells (++).

The staining for epidermal CD1a+ cells and epidermal CD11b+ cells was also determined quantitatively by image processing: The percentage of epidermal cross sectional area that was stained CD1a+ or CD11b+ was measured, using a camera mounted on a light microscope together with the Optimas 6.1 and Microsoft Excel software (the targeted interfollicular epidermal area was demarcated manually).

Statistical analysis

After logarithmic transformation (as the distribution of the percentages of stained cross sectional areas is skewed to the right), a Student t-test was performed on the CD1a+ log-% values to compare PLE outcomes with those of healthy volunteers. Median values were estimated as means of log-% values. A Wilcoxon rank sum test was performed on the quantitative data of the epidermal CD11b+ area. The same test was also used on the semiquantitative data (scored -, ±, +, ++) to compare the number of CD1a and CD11b positive cells either in the epidermis or the dermis, e.g. comparing PLE patients with healthy volunteers at 48h after 6 MED (**fig 2** and **4** and **table 1** and **2**).

RESULTS

Phototesting assay

In making a reproducible assay for analyzing the reactions of CD1a+ Langerhans cells and CD11b+ macrophage-like cells, we varied the UV-B dose (3 - 6 MED) and location of exposure in a group of healthy individuals. It appeared that buttock skin, which had not been exposed to sunlight for at least two months, was the location that showed the most consistent effects of UV-B on the CD1a+ and CD11b+ cell populations. In our hands, a UV-B dose of 6 MED turned out to be adequate for the virtually complete disappearance of CD1a+ Langerhans cells from the epidermis and a clear influx of CD11b+ cells in all healthy volunteers tested. These results are in accordance with earlier reports that Langerhans cells disappeared and CD11b+ cells infiltrated the skin of healthy individuals 48 hours after UV-B (Aberer *et al*., 1981; Cooper *et al*., 1986; Meunier *et al*., 1995). After establishing this assay in healthy volunteers, PLE patients and more healthy volunteers were included in the study. PLE patients were divided into two subgroups: one with normal MEDs and one with extremely low MEDs.

UV radiation induces disappearance of Langerhans cells in healthy individuals but not in PLE patients

The buttock skin of PLE patients looked normal and unaffected before UV-B exposure. After 6 MED UV-B exposure the skin was red and a little swollen, but no pathology could be observed. In the unaffected and unexposed skin of PLE patients CD1a+ cells were present in vast amounts in the epidermis (**fig 1B**), especially in the low MED group (**fig 1C**). All PLE patients demonstrated CD1a+ cells in the upper-dermis (**table 1).** The number of epidermal CD1a+ cells in the unexposed skin of healthy volunteers (example in **fig 1A**) varied widely. Forty-eight hours after UV-B irradiation CD1a+ Langerhans cells had disappeared from the epidermis of the healthy volunteers (**fig 1D**). In the dermis there were hardly any CD1a+ cells present. A considerable number of CD1a+ cells was still present in the epidermis of PLE patients (**fig 1E**) 48 hours after the overexposure, especially in the epidermis of PLE patients with a low MED (**fig 1F**). Counting of Langerhans cells in microscopic cross-sections is cumbersome owing to scattered presence of dendrites. To quantify objectively whether the number of Langerhans cells in patients was significantly different from the numbers seen in healthy volunteers, we compared percentages of epidermal cross-sectional areas that stained CD1a+. The cross sectional area that stained CD1a+ in the epidermis of PLE patients (median of 7% for PLE patients with a normal MED and 13% for PLE patients with a low MED) was significantly larger ($p<0.01$) than in healthy individuals (median of 0.55%) 48 hours after 6 MED UV-B exposure (**fig 2**). No correlation could be found between the absolute UV dose and the % change in CD1a+ area after UV irradiation among healthy volunteers or among PLE patients with a normal MED or among healthy individuals and PLE patients with a normal MED combined.

The semiquantitative scoring of the CD1a staining, analyzed by the Wilcoxon rank sum test (**table 1**), also confirmed this significant difference ($p<0.01$) between the number of epidermal CD1a+ cells in healthy volunteers and PLE patients 48 hours after UV-B radiation. The number of CD1a+ cells in the dermis of the UV-B-exposed skin of PLE patients was significantly larger compared to healthy volunteers ($p<0.05$). The number of epidermal and dermal CD1a+ cells in the unexposed buttock skin did not differ significantly ($p>0.05$) between healthy volunteers and PLE patients with a normal MED. In PLE patients with a low MED, however, the number of dermal CD1a+ cells in the unexposed skin was significantly larger than in healthy individuals ($p<0.05$). The number of epidermal CD1a+ cells did not differ significantly between PLE patients with low MED and healthy volunteers ($p>0.05$). The changes in CD1a+ cells after UV-B exposure are only significant for epidermal CD1a+ cells in healthy individuals ($p<0.01$) and PLE patients with a normal MED ($p<0.05$).

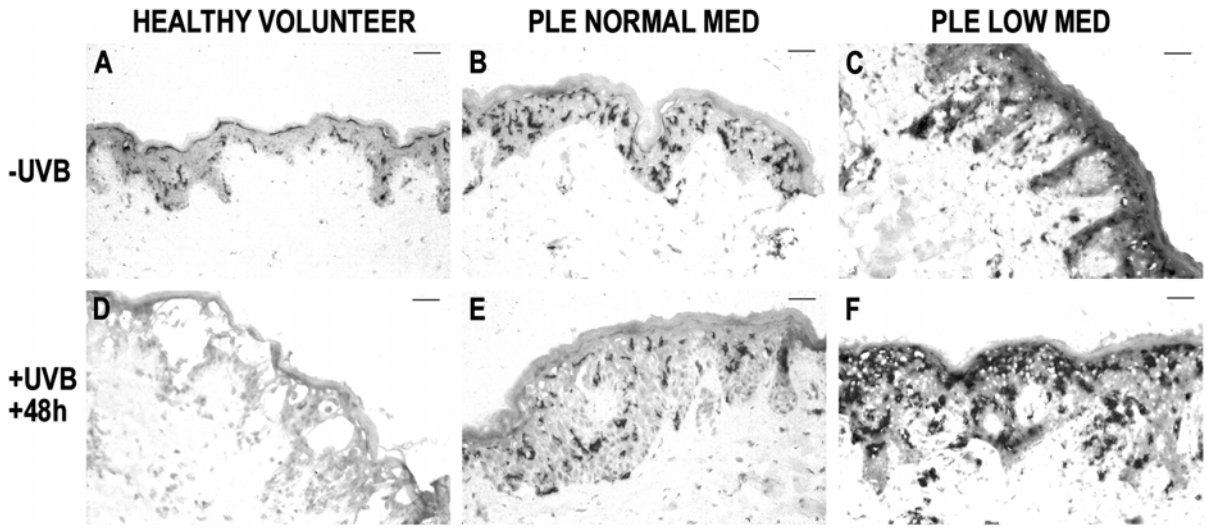

Figure 1. CD1a staining of buttock skin, unexposed (- UV) and 48 hours after UV-B exposure (+ UV + 48h). Scale bar = 50 μm.

Table I. Semi-quantitative analysis of CD1a+ cells (median) in the unexposed and UV-B irradiated buttock skin of healthy volunteers and PLE patients.

			PLE patients			
	Healthy volunteers (n=8)		normal MED (n=5)		low MED (n=5)	
	epidermis	dermis	epidermis	dermis	epidermis	dermis
- UV-B	+ / ++	- / ±	++	-	++	± (p<0.05)
+ UV-B + 48h	-	-	+ (p<0.01)	± (p<0.05)	++ (p<0.01)	+ (p<0.05)

Statistical significance (p-value) is given for PLE patients compared to healthy individuals. Where no p-value is given, no significant difference was observed.

++, closed maze of positive cells; +, clear abundant presence of positive cells; ±, presence of scattered positive cells; -, no or hardly any positive cells present

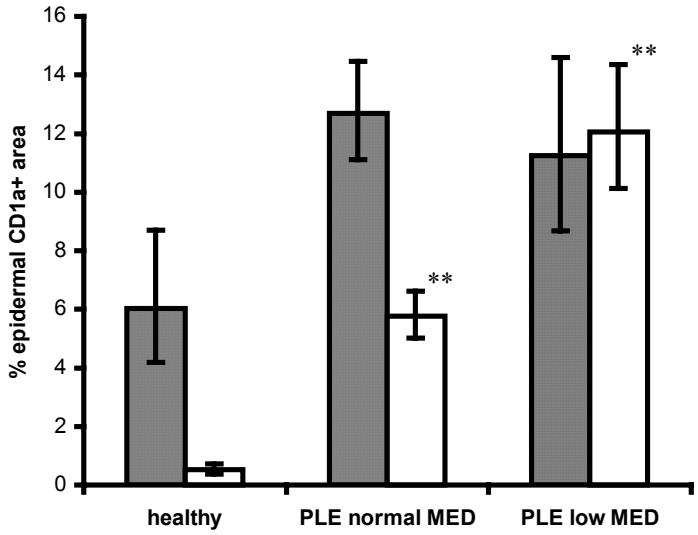

*Figure 2. Percentage epidermal CD1a+ cross sectional area of buttock skin before (gray bars) and 48 hours after UV-B irradiation (white bars) and comparisons of PLE patients with healthy volunteers (statistical significance: ** = p<0.01). Medians are given together with the standard errors.*

Characterization of the persistent CD1a+ cells

To characterize the persistent CD1a+ cells in the epidermis, the Birbeck granules, characteristic of Langerhans cells, were visualized by immunohistochemical staining with the Lag antibody (Kashihara *et al*., 1986). It appeared that all epidermal CD1a+ cells in healthy volunteers as well as in PLE patients had Birbeck granules. The CD1a+ cells that persisted in the epidermis of PLE patients after 6 MED UV-B radiation also stained for the presence of Birbeck granules. The dermal CD1a+ cells were always Lag negative (data not shown).

UV radiation induces infiltration of CD11b+ cells in the epidermis, but to a lesser extent in PLE patients with normal MEDs

In the unexposed and unaffected buttock skin of healthy volunteers and PLE patients, CD11b+ cells were already present in the dermis, not in the epidermis (**fig 3A-C** and **table 2**) The number of these cells did not vary significantly ($p>0.05$) between the volunteers and the PLE patients, when the quantitative and semiquantitative data were analyzed with the Wilcoxon rank sum test (**table 2**). The number of CD11b+ macrophage-like cells in the dermis increased 48 hours after UV-B exposure, but no significant difference could be observed between the healthy volunteers and the PLE patients ($p>0.05$)(**fig 3D-F**). An influx of CD11b+ cells in the epidermis of the volunteers (median of 3.3% areal staining) and the PLE patients (median of 0.66% for PLE patients with a normal MED and 3.36% for PLE patients with a low MED)(**fig 3E-F**) occurred 48 hours after UV-B irradiation. The influx of CD11b+ cells in the epidermis was significantly smaller in PLE patients with a normal MED than in healthy volunteers ($p<0.05$). No significant difference could be observed when the CD11b+ log-%-values of PLE patients with a low MED were compared to healthy volunteers (**fig 4**). All changes in CD11b

after UV-B exposure were significant, except for those in epidermis and dermis of PLE patients with a normal MED.

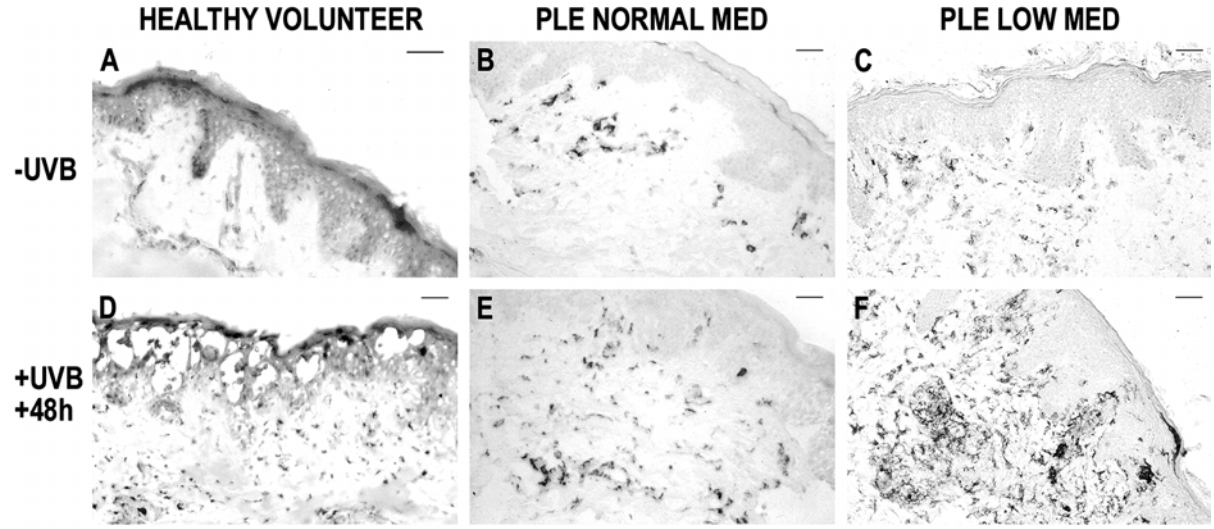

Figure 3. CD11b staining of buttock skin, unexposed (- UV) and 48 hours after UV-B exposure (+ UV + 48h). Scale bar = 50 μm.

Table II. Semi-quantitative analysis of CD11b+ macrophage-like cells (median) in the unexposed and UV-B irradiated buttock skin of healthy volunteers and PLE patients.

			PLE patients			
	Healthy volunteers (n=8)		normal MED (n=5)		low MED (n=5)	
	epidermis	dermis	epidermis	dermis	epidermis	dermis
- UV-B	-	+	-	±	-	+
+ UV-B + 48h	+	++	± (p<0.05)	+	± (p<0.05)	++

Statistical significance (p-value) is given for PLE patients compared to healthy individuals. Where no p-value is given, no significant difference was observed.

++, closed maze of positive cells; +, clear abundant presence of positive cells; ±, presence of scattered positive cells; -, no or hardly any positive cells present

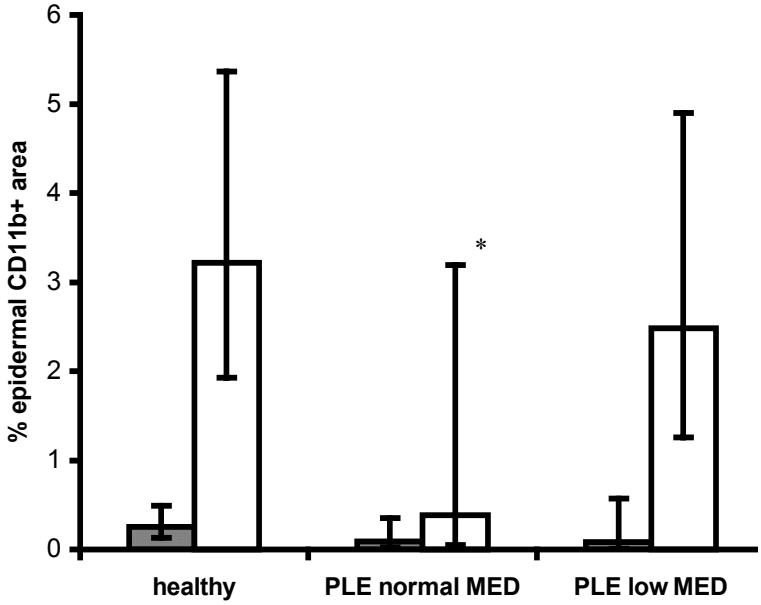

*Figure 4. Percentage epidermal CD11b+ cross sectional area of buttock skin before (gray bars) and 48 hours after UV-B irradiation (white bars) and comparisons of PLE patients with healthy individuals (statistical significance: * = p<0.05). Medians are given together with the standard errors.*

Differences in epidermal morphology

When comparing the epidermis of healthy volunteers and PLE patients 48 hours after UV-B irradiation, a clear difference in the condition of the epidermis was observed. In healthy volunteers thickening of the epidermis occurred and the epidermis showed gross defects just below the stratum corneum (**fig 1D** and **3D**). In PLE patients there was also a thickening of the epidermis, but the epidermis appeared completely intact (**fig 1E-F** and **3E-F**). Near and in the defects that appear in the epidermis of the healthy volunteers great numbers of CD11b+ cells could be observed (**fig 3**). Hardly any CD3+ cells could be observed in the UV-exposed epidermis (data not shown).

Characterization of the CD11b+ cells

To test if the CD11b+ cell was indeed belonging to the macrophage cell lineage, an immunohistochemical double staining was performed with the macrophage marker CD68 (Azzawi *et al*., 1997; Davidson *et al*., 1997). In healthy volunteers and PLE patients CD11b single positive, CD68 single positive and CD11b/CD68 double positive cells could be observed in the unexposed and UV exposed buttock skin (**fig 5**).

In the unexposed buttock skin of healthy volunteers CD11b/CD68 double positive cells were present in the upper- and mid-dermis. CD68 single positive cells were mostly present in the deeper part of the dermis. There were hardly any CD11b single positive cells present in the dermis of healthy volunteers (**fig 5A**). No single- or double-stained cells could be observed in the epidermis.

Forty-eight hours after UV-B exposure the number of CD11b single positive cells increased markedly in the upper-dermis of healthy volunteers, while the number of CD11b/CD68 double

positive cells and CD68 single positive cells did not change or only increased modestly (**fig 5C**). The CD11b+ cells that infiltrated the epidermis of healthy volunteers after UV-B overexposure were nearly all CD11b single positive (**fig 5**), some were CD68 single positive or CD11b/CD68 double positive.

In contrast, nearly all CD11b+ cells in the unexposed skin of PLE patients were also CD68 positive (**fig 5B**). A small number of CD68 single positive cells could be observed in the mid-dermis and in the deeper parts of the dermis. After UV-B exposure the number of dermal CD11b/CD68 double positive cells increased markedly (**fig 5D**). In some PLE patients the number of CD68 single positive cells also increased. Hardly any CD11b single positive cells could be observed. The few cells that infiltrate the epidermis after UV-B irradiation were also all CD11b/CD68 double positives, very much in contrast with the influx of CD11b single positive cells in the epidermis of healthy individuals.

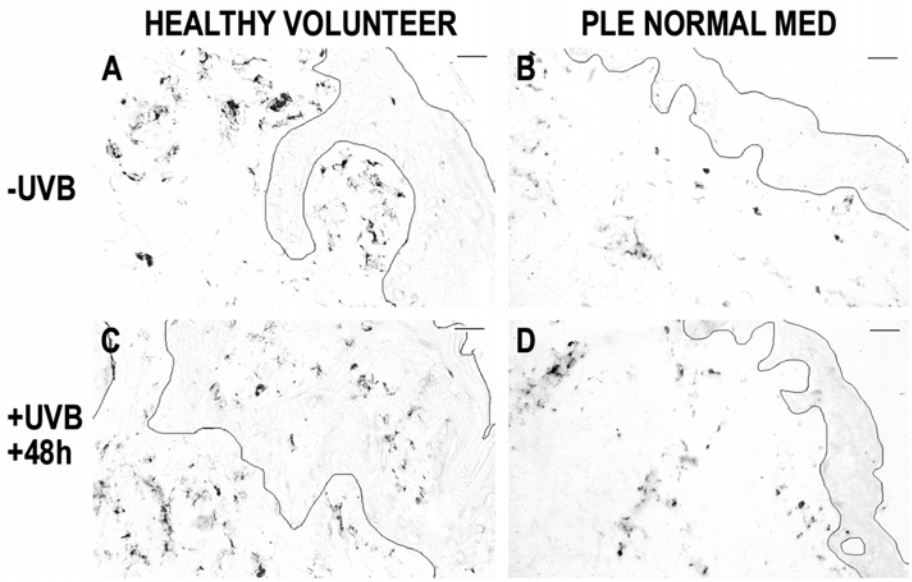

Figure 5. Double staining of CD11b (blue) and CD68 (red) of buttock skin, unexposed (- UV) and 48 hours after UV-B exposure (+ UV + 48h). The epidermis was demarcated manually. Scale bar = 50 μm.

DISCUSSION

The present study was based on the supposition that a pathological skin reaction may be evoked by UV-B exposure if a defect occurs in the normally induced suppression of cellular immunity. We hypothesized that the influx of immunosuppressive, IL-10 producing CD11b+ cells (Kang *et al*., 1994; Kang *et al*., 1998) could be absent or dysfunctional in some way in PLE patients, in whom pathological reactions could be provoked by UV-B radiation. CD11b+ cells were present in the dermis of the unexposed and UV-exposed buttock skin of PLE patients. The number of dermal CD11b+ cells did, however, increase in the uninvolved buttock skin of these patients after UV-B irradiation, and was not significantly different from that in healthy individuals. No CD11b+ cells could be observed in the epidermis of the unexposed

buttock skin of healthy volunteers and PLE patients. Remarkably, after UV exposure the influx of CD11b+ cells in the epidermis of PLE patients with normal MED was indeed significantly less than in healthy volunteers, but in PLE patients with low MEDs the influx of CD11b+ cells was comparable to that in healthy volunteers.

A far more striking difference between healthy volunteers and PLE patients occurred, however, in the response of CD1a+ Langerhans cells to UV-B overexposure. Persistent CD1a+ cells could be observed in the epidermis of PLE patients 48 hours after 6 MED UV-B exposure, while in healthy individuals the Langerhans cells completely disappeared from the epidermis. Because the UV dose varied between healthy volunteers and PLE patients, the question arose whether the disappearance of CD1a+ cells was correlated to the absolute UV dose. There was, however, no correlation between UV dose and % change in CD1a+ area: not among healthy individuals nor among PLE patients with normal MED, and not even among these two groups combined. The image processing which was used to quantify CD1a+ cells did not distinguish between lesser number of cells and a loss of dendrites by rounding up of the cells. In healthy individuals it is obvious that the effect of UV on Langerhans cells is a near complete depletion (well over 80% decrease in stained area in 7 out of 8, one with 64% reduction). In PLE patients the effect of UV on Langerhans cells is due to decreases in cell number and partially due to changes in cell morphology (all, n=10, decreases in stained area well below 70%). This means that the differences between CD1a+ cells of healthy volunteers and PLE patients after UV irradiation are even underestimated. Immunohistochemical double staining of the CD1a+ cells with the Lag antibody, directed against Birbeck granules, confirmed that the persistent epidermal CD1a+ cells were indeed Langerhans cells. CD1a+ cells in the dermis were mostly Lag negative.

The CD11b+ cells were further characterized by double staining with the pan-macrophage marker CD68 (Azzawi *et al*., 1997; Davidson *et al*., 1997). The CD11b+ cells that infiltrate the UV-irradiated epidermis in healthy volunteers are mostly CD68 negative, whereas the few CD11b+ cells that infiltrate the UV-exposed epidermis of PLE patients are all CD68 positive. The dermal CD11b+ cells in the unexposed and UV-exposed buttock skin of PLE patients and healthy volunteers were mostly CD68 positive. The CD11b+ cell population of PLE patients is therefore at least partially different from the CD11b+ cell population of healthy volunteers: different cells or cells in a different state of activation.

Another interesting difference between PLE patients and healthy individuals was observed: PLE patients appeared to have no epidermal defects after UV-B exposure, while all healthy individuals clearly did. In a study of Hammerberg *et al*. (1996) anti-CD11b treatment, of mice, partially protected against epidermal UV injury: the epidermal structure was better preserved and keratinocytes appeared less damaged. Knowing that the UV irradiated epidermis of the PLE patients showed a low influx of CD11b+ cells and that the CD11b+ cells are lodged into

the defects in healthy volunteers, it is tempting to speculate that these CD11b+ cells are, at least partially, responsible for the development of the epidermal defects. In this regard, it is also noteworthy that the epidermal CD11b+ cells of PLE patients are of a different phenotype than those in healthy volunteers: CD68+ *versus* CD68- respectively. Our hypothesis is that the CD11b+CD68- cell population in skin of the healthy volunteers consists to a large extent of granulocytes, which by their release of tissue destructing enzymes, can be responsible for the epidermal defects.

In mice UV-exposed Langerhans cells migrate from the epidermis to present antigen to the T-cells in the paracortex of the lymph nodes (Halliday and Norval, 1997). The cytokine TNF-α, which is released in the skin after UV exposure, is involved in this migration of Langerhans cells (Boonstra and Savelkoul, 1997; Cumberbatch and Kimber, 1992; Moodycliffe et al., 1994). Persistence of UV-irradiated Langerhans cells in the epidermis of PLE patients might thus be the result of a lack of TNF-α secretion. Another potential effect of TNF-α is induction of apoptosis (Norris et al., 1997; Romano et al., 1998). Apoptosis could also contribute to the depletion of Langerhans cells in the skin of healthy individuals after UV irradiation (Mommaas et al., 1993). Furthermore, a massive apoptosis induced by high levels of TNF-α might be responsible for the epidermal defects observed in healthy volunteers, and a lack of TNF-α in PLE would then explain the absence of epidermal defects.

One patient, who was initially included in the PLE group with a normal MED, was later diagnosed with subacute cutane lupus erythematodes (LE) (Nyberg *et al*., 1997) and excluded from the study. The Langerhans cells in the epidermis of this person did also not disappear 48 hours after 6 MED UV-B (data not shown). Thus, UV resistance of Langerhans cells might be a prerequisite for UV-B induced photodermatoses in general, and not specific for PLE. The differences in UV-B-triggered photodermatoses may then lie in the differences in the antigenic stimuli that evoke the pathological reaction, e.g. UV-damaged DNA in case of LE.

In an initially extremely sun-sensitive PLE patient who received a successful UV hardening therapy, we found that the Langerhans cells disappeared from the epidermis 48 hours after 6 MED overexposure. This preliminary first observation on re-adaptation would appear to confirm and emphasize the importance of persisting Langerhans cell in a photoreactive disease like PLE. These first results need to be confirmed in our follow-up studies. The importance of UV adaptation is also indicated by the observation that PLE increases in prevalence and severity towards higher Northern latitudes, where the relative differences in UV-B between summer and winter are bigger. Loss of adaptation of the skin to UV radiation (UV-B) in winter would therefore appear to be of paramount importance to the disease (van der Leun and de Gruijl, 1993).

UV-resistant Langerhans cells were also observed by Torres-Alavarez *et al*. (1998) in actinic prurigo patients, but the difference with healthy controls after 20 MED (!) UV irradiation were

considerately smaller than in our study. These researchers used an unfiltered high-pressure quartz mercury lamp, a UV-C/ UV-B source, for skin irradiation, whereas we used TL-12 lamps which have their main output in the UV-B. Overexposure to UV-C is better tolerated than overexposure to UV-B (UV-B erythema shows a steeper dose-gradient), which could explain the marginal differences that were observed by Torres-Alavarez *et al*. (1998).

PLE is often provoked with UV-A sources (Holze, 1995): e.g. with fluorescent lamps like the Philips TL09. But these broad band sources often do contain significant traces of UV-B. The UV-A I region (340-400 nm) is more distinct from UV-B (280-315 nm) than the UV-A II (315-340 nm) region in the type of biological response it evokes. Most PLE patients react pathologically to UV-A II as well as to UV-B[1]. Some patients also react to visible light. In our study only PLE patients who reacted pathologically to UV-B irradiation alone, or to UV-B and UV-A, were included. The patients who were included reacted stronger to UV-B than to UV-A. As UV immunosuppression by UV-A I or visible light is not commonly observed in healthy individuals, it is more likely that PLE reactions evoked by pure UV-A I or visible light are predominantly due to the generation of a neo-antigen to which these individuals are sensitized. UV-A I in absence of UV-B will not induce the appropriate immunosuppressive response. UV-A I may actually enhance cellular responses by inducing IFN-γ[2].

The observed deviations in the epidermal infiltration of CD11b+ cells after UV-B exposure may well play a role in the initial immune reactivity leading to PLE skin lesions, because the CD11b+ cells appear to mediate immunosuppression in healthy individuals (Hammerberg *et al*., 1996; Kang *et al*., 1994; Kang *et al*., 1998). UV-exposed Langerhans cells can also contribute to immunosuppression, as demonstrated in mice (Simon *et al*., 1991). A consequence of the abnormal persistence of Langerhans cells in the overexposed epidermis of PLE patients could well be the presentation of UV-induced neo-antigens at the wrong location, namely in the skin instead of in the draining lymph nodes, thus resulting in an illicit immune reaction in the skin instead of an immunosuppressive response in the lymph nodes. This persistence of CD1a+ cells after UV-B overexposure may serve as an important diagnostic criterion for UV-B related photodermatoses.

Clearly, the precise mechanisms for photodermatoses and PLE in particular need further elucidation, but the aberrant reaction of Langerhans cells to UV irradiation appears to be a prerequisite.

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ACKNOWLEDGEMENT

We would like to thank Jan van der Linden from the department of Pulmonary Diseases, University Hospital Utrecht for his support in image processing of the CD1a+ and CD11b+ cells.

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Epidermal Langerhans cell depletion after artificial UV-B irradiation of human skin *in vivo*: apoptosis *versus* migration

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Journal of Investigative Dermatology 118:812-817, 2002

Color printings: see addendum

ABSTRACT

UV-B radiation can suppress cellular immunity. One of the mechanisms related to this immunosuppression is the disappearance of Langerhans cells from the epidermis. The aim of the present study was to establish the mechanism of UV-B-induced Langerhans cell disappearance in healthy individuals. The two most likely mechanisms for Langerhans cell disappearance are apoptosis and migration. Apoptosis was assessed *in vivo* by exposing buttock skin of ten healthy volunteers to six minimal erythema doses UV-B. Only very few apoptotic Langerhans cells could be observed in sections from the UV-B-exposed skin. Migration of Langerhans cells cannot be established in skin sections and we therefore raised suction blisters in an attempt to trap migrating Langerhans cells in the sub-basal membrane blister fluid. Blisters were raised on the flexor side of the lower arm of thirty healthy volunteers at several time points after exposure of the skin to six minimal erythema doses UV-B. Blister fluid was collected and blister roofs were removed to check for Langerhans cell disappearance. Langerhans cells were detected in the blister fluid of the UV-B-exposed skin and not of the unexposed skin. The number of Langerhans cells in the blister fluid peaked around 18h after UV exposure, which coincided with the largest depletion of Langerhans cells in the blister roof. A fraction (20-30%) of the Langerhans cells in the blister fluid stained positive for DNA damage (cyclobutane pyrimidine dimers), showing that they originated form the epidermis. UV-B-induced Langerhans cell disappearance appears to be mainly attributable to migration.

INTRODUCTION

Ultraviolet (UV) radiation, especially UV-B radiation (280-315 nm) has several effects on the skin immune system. There are several primary photochemical reactions that could trigger these events. The most studied are: isomerization of urocanic acid and DNA damage (cyclobutane pyrimidine dimers and 6-4 photoproducts) in epidermal cells. Subsequently, the production of cytokines is induced, such as IL1-β, TNF-α, IL-10 and PGE_2. Cellular functions are altered e.g. natural killer cell activity is inhibited, antigen presentation is changed and the microenvironment becomes favorable to the development of T-helper 2-like immune responses (for review Duthie *et al.,* 1999). (Over)exposure of the skin to UV-B leads to an influx of IL-10 producing macrophagic cells (Kang *et al.,* 1994;Kang *et al.,* 1998) and a depletion of Langerhans cells. These effects are associated with suppression of cellular immune reactions.
A disturbance in these immunosuppressive mechanisms may lead to the development of a UV-induced “sun allergy” (polymorphous light eruption (PLE)). Patients with PLE are sensitive

to UV-B (as clinically tested by challenges with a Philips TL12 lamp), UV-A and sometimes even to visible light. These patients develop papules, vesicles or eczema on sun-exposed areas of the skin (Boonstra *et al.*, 2000; Grabczyska and Hawk, 1997 ;Epstein, 1997; Salomon *et al.*, 1997). PLE is suggested to be a delayed-type hypersensitivity reaction (Epstein, 1997; Norris *et al.*, 1988; Verheyen *et al.*, 1995). In earlier experiments we found that Langerhans cells in the skin of PLE patients persisted after overexposure to UV-B in contrast to what was observed in healthy volunteers (Kölgen *et al.*, 1999). We hypothesize that this persistence of Langerhans cells in the epidermis may contribute to the pathogenesis of the disease. To understand this defect in PLE patients, the main mechanism that is responsible for the UV-induced Langerhans cell disappearance in healthy individuals needs to be established.

The paramount mechanism underlying Langerhans cell depletion, especially in humans, is still unknown. Some authors suggest that a deficiency of growth factors for Langerhans cells or a downregulation of growth factor receptors is responsible for Langerhans cell depletion (Takashima, 1995). The best studied and two most likely mechanisms for epidermal Langerhans cell depletion are apoptosis and/or migration.

In vitro experiments revealed that irradiated human Langerhans cells undergo apoptosis (Rattis *et al.*,1998). Experiments with mice showed that apoptosis of Langerhans cells is induced by UV-B radiation through, among others, the production of reactive oxygen species (Takashima, 1995). Whether this also occurs in humans *in vivo* has still not been demonstrated (Meunier, 1999).

We know from animal models that Langerhans cells migrate to the lymph nodes after exposure of the skin to UV-B (Sontag *et al.*, 1995; Dandie *et al.*, 1998). UV-induced migration of Langerhans cells in humans has been described by Yawalker et al (1998). But these last experiments involve lymph drainage, and are very encumbering and difficult to perform; moreover they were not approved of by the ethical committee of our institute. We therefore had to resort to a novel technique.

To investigate the relative importance of apoptosis versus migration in UV-induced Langerhans cell depletion *in vivo* we studied a group of healthy volunteers. A small area of the buttock skin or of the flexor side of the arm was overexposed to UV-B. Biopsies were taken from buttock skin at successive time points after the exposure. Furthermore, we developed a novel method to detect migrating Langerhans cells *in vivo* by catching them in blister fluid: suction blisters were raised on (un)exposed areas of the skin of the flexor lower arm and blister roofs and blister fluid were collected at several time points after UV-B exposure. Immunohistochemical stainings for CD1a (Langerhans cells), cyclobutane pyrimidine dimers (DNA damage), and for active caspase 3 and TUNEL (both markers for apoptosis) were performed on frozen skin sections, blister roofs and cytospins of blister fluid.

MATERIALS AND METHODS

Subjects

30 healthy individuals (9 males and 21 females, ages between 19 and 55 years) were studied for Langerhans cell migration (see below) while 10 healthy individuals (3 males and 7 females, ages between 22 and 31 years) were studied for apoptosis. Volunteers whose buttock skin or skin of the lower arm was exposed to sunlight less than 2 months ago were excluded. Informed consent was obtained from all volunteers. This study was approved by the Medical Ethical Committee of the University Medical Center Utrecht.

Phototesting procedure

The UV dose required to cause a just perceptible redness, the minimal erythema dose (MED), was determined on the skin area to be experimented on i.e., the buttock skin or the flexor side of the lower arm. For MED determination a test device with 9 windows (3 x 10 mm each) was used. Through these windows the underlying skin was exposed to a Philips TL12 lamps for different periods of time (a geometrical series, e.g. from 12.4 to 200 s). The TL12 lamp emitted 58% of the UV output in UV-B (280-315nm) and 5% of the UV output below 290 nm. Erythemally weighted, 98% stemmed from the UV-B band (18% below 290 nm). The erythema was assessed 24h later. The MED for the buttock skin ranged from 212 to 750 J/m^2 UV-B (median of 410 J/m^2, 95% C.I. 180 - 940 J/m^2). The MED for the flexor side of the arm was 4 to 5 times higher and ranged from 352 to 4840 J/m^2 UV-B (median of 1650 J/m^2, 95% C.I. 470 - 5820 J/m^2). After determination of the MED the test areas of skin on the buttock or lower arm were exposed to 6 MED UV (Philips TL12). According to Barr *et al.* (1999) this dose is about equal to 4 MED for an erythema defined by clear borders, as used by Cooper *et al.* (1992) to suppress contact hypersensitivity in humans.

Biopsies

Three-millimeter punch biopsies were taken from the irradiated buttock skin 24h and 48h after exposure to 6 MED UV, together with one control biopsy from the unexposed buttock skin. Biopsies were snap frozen in liquid nitrogen, embedded in OCT compound (Tissue-Tek, Sakura, Zoeterwoude, the Netherlands) and stored at –30^{o}C until further processing.

Suction blisters

Suction blisters were raised (Black *et al.*, 1977; van der Leun *et al.*, 1974) for operational convenience on the irradiated skin of the flexor side of the lower arm at several time points after the exposure to 6 MED UV (18h, 24h, 30h, 42h, 48h) and on an unexposed side (2 blisters per volunteer). Blisters were raised over a period of around 2h, using airtight cups with circular ports exposing skin areas of 10mm in diameter to an atmospheric pressure of minus 200 mmHg. Suction blister formation was further stimulated by gently warming the skin via an electric heating coil in each suction cup. Skin temperature was measured with a copper-constantan thermocouple touching the skin, and did not exceed 40^{o}C. Blisters occurred at a dermal-epidermal junction. Blister fluid was collected using a microneedle with a diameter of 0.36 mm. Blister roofs were removed with a sterilized skin surgery set. Each volunteer was

sampled at only 1 time point after irradiation. The yield of blister fluid varied among individuals, but slightly more blister fluid was consistently recovered from UV irradiated blisters when compared to controls, as found earlier by Barr *et al.* (1999). Cytospin preparations were made of the blister fluid by centrifugation for 10 min at 55g onto 3-amino-propyltriethoxysilane coated glass slides. The slides were stored at –30^{o}C until further processing. The removed blister roofs were used immediately for immunohistochemistry.

Antibodies

As primary antibodies monoclonal antibodies CD1a (DAKO A/S, Glostrup, Denmark, diluted 1:60), fluorescein isothiocyanate (FITC)-conjugated CD1a (DAKO A/S, diluted 1:60) and biotinylated H3 antibody (directed against cyclobutane pyrimidine dimers, a kind gift from Dr. L. Roza; T.N.O., Zeist, the Netherlands), diluted 1:20) and the polyclonal antibody rabbit anti-active caspase 3 (Becton Dickinson, San Diego, CA, diluted 1:100) were used. Biotinylated horse anti-mouse immunglobulin (Vector, Burlingame, CA, diluted 1:800) and biotinylated goat anti-rabbit antibody (Vector, diluted 1:300) were used as secondary antibodies. Alkaline phosphatase (AP)-labeled F(ab) fragments of sheep anti-FITC (Boehringer Mannheim GmbH, Mannheim, Germany, diluted 1:400), AP-labeled avidin-biotin complex (DAKO A/S, diluted 1:50) horseradish peroxidase (HRP)-conjugated avidin-biotin complex (DAKO A/S, diluted 1:50) were used as detecting reagents.

Immunohistochemistry

Single staining of CD1a and anti-active caspase 3. Blister roofs and frozen skin sections (6 μm) on 3-amino-propyltriethoxysilane-coated glass slides were fixed for 10 min at room temperature in dry acetone containing 100 μl 30% H_2O_2 per 100 ml. The slides were incubated for 20 min with a blocking reagent [phosphate-buffered saline (PBS) containing 10% normal human serum/ 10% normal horse serum or 10% normal goat serum (depending on the secondary antibody species)] to prevent aspecific binding. The skin sections were subsequently incubated with the primary antibody (CD1a or rabbit-anti active caspase 3) for 1h and with a secondary antibody (biotinylated horse anti-mouse immunoglobulin or biotinylated goat anti-rabbit immunoglobulin respectively) for 1h. Subsequently, the blister roofs and skin sections were incubated for 30 min with HRP-labeled avidin-biotin complex (CD1a) or AP-labeled avidin-biotin complex (active caspase 3). Peroxidase activity was visualized by incubation in 0.1 M acetate buffer (pH=5) containing 20 mg 3-amino-9-ethyl-carbazole (AEC; Sigma, ST. Louis, MO) and 100 μl H_2O_2 per 100 ml, resulting in a red staining. Rabbit anti-active caspase 3 binding was visualized by incubating the sections in Tris-HCl (pH=8.5) containing 25 mg levamisol, 250 μl New Fuchsin, 250 μl 0.6 M sodium nitrate solution and 50 mg naphthol AS-BI phosphate, resulting in a pink staining.

Double staining of CD1a-FITC and H3-biotin. CD1a-FITC/H3. Double staining was performed as described previously (Sontag et al., 1995) without modifications. In short, blister roofs, cytospins of blister fluid and frozen skin sections were fixed, preincubated with blocking reagent (PBS containing 10% normal human serum) and incubated with the primary antibody CD1a-FITC, as described above. After incubation for 1h with AP-labeled sheep anti-FITC F(ab) the AP activity was visualized by

incubation in a BCIP/NBT solution of 5-bromo-4-chloro-3-indolyl phosphate/4-nitro blue tetrazolium chloride in 0.1 M Tris-HCL (pH=9.5), 0.1 M NaCl, 0.05M $MgCl_2$ and 2.5 mg levamisole per 10 ml. The bluish-black reaction product is water and ethanol insoluble and withstands the subsequent harsh treatment. The preparations were fixed in 25% acetic acid (vol/vol) containing 100 μl 30% H_2O_2 per 100 ml. After washing two times for 10 min in PBS the slides were incubated for exactly 2 min in 0.07 N NaOH in 70% ethanol. After washing with PBS the preparations were incubated for 20 min with PBS containing 10% normal human serum and 10% normal mouse serum, followed by a 1h incubation with H3-biotin.The preparations were incubated with HRP-labeled avidin-biotin complex for 30 min and developed in an AEC substrate solution, as described above.

*Double staining of CD1a-FITC and anti-active caspase 3***.** CD1a-FITC/active caspase 3 double staining was performed on blister roofs and frozen skin sections. Fixation and preincubation of the skin sections was performed as described above. The sections were then incubated simultaneously with CD1a-FITC and anti-active caspase 3 antibody for 1h. The preparations were subsequently incubated simultaneously with biotinylated goat-anti-rabbit and AP-labeled sheep anti-FITC for 1h, followed by incubation for 30 min with HRP-labeled avidin-biotin complex. Alkaline phosphatase activity was visualized by incubating the sections first in a Tris-HCl buffer (pH=8.5), containing 25 mg Fast Blue BB Salt, 12.5 mg naphthol AS-MX phosphate, and 35 mg levamisol per 100 ml, all purchased from Sigma, resulting in a blue staining. For visualization of the peroxidase activity the sections were then incubated with an AEC solution, as described above. Negative staining controls were used in single and double staining experiments in which the first antibody was omitted or replaced by an irrelevant antibody of the same isotype. All antibodies were diluted in the suitable blocking reagent except for the AP- and HRP-labeled antibodies, which were diluted in PBS/0.05% Tween 20. All antibody incubations were performed in a humidified chamber at room temperature. After each antibody incubation the slides were washed three times with PBS containing 0.05% Tween 20.

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL). To detect DNA breaks in situ, TUNEL staining was performed using a commercially available detection kit (Boehringer Mannheim GmbH). Frozen skin sections (6 μm) on 3-amino-propyltriethoxysilane-coated glass slides were stained according to manufacturer's instruction. Incorporation of FITC labeled nucleotides was checked using a Zeiss microscope equipped for epifluorescence. Converter-AP (anti-FITC antibody supplied in the kit) was then added and antibody binding was visualized by incubation in a Fast Blue BB Salt substrate solution, as described above. To detect whether the apoptotic cells were Langerhans cells the TUNEL assay was combined with a CD1a staining. CD1a antibody was incubated simultaneously with the TdT reaction mixture. The sections were then incubated simultaneously with biotinylated horse anti-mouse immunoglobulin and converter-AP followed by incubation with HRP-labeled avidin-biotin complex. Antibody binding was visualized by incubation with Fast Blue BB Salt substrate solution (TUNEL) and AEC solution (CD1a), as described above.

Statistical analysis

After logarithmic transformation (to correct for the right skewed distribution) a Student T-test was performed to ascertain the significance of the observed differences (significance level $p< 0.05$). The numbers of CD1a single positive and CD1a/H3 double positive cells in the blister fluid after UV exposure were compared to the numbers of unexposed, control skin.

RESULTS

Exposure to 6 MED causes apoptosis in the epidermis, but not Langerhans cells

Caspase 3 is an effector caspase which is cleaved during apoptosis in a wide variety of cells and commits a cell to apoptosis (for review see Harvey and Kumar (1998), Thornberry and Lazebnik (1998)).

Active caspase 3-positive cells are present in the epidermis 24h and 48h after UV-B irradiation and are abundantly present in blister roofs within 18h after 6 MED UV-B irradiation. The number of caspase 3-positive cells 24h after UV-B exposure varies greatly among volunteers. Active caspase 3-positive cells are found scattered throughout the epidermis 24h after irradiation, while after 48h the cells are mainly located in the upper part of the epidermis just below the stratum corneum (**fig 1**). These apoptotic cells are mainly found in conjunction with epidermal defects. Active caspase 3-positive cells were not found in unirradiated skin. Similar results were obtained with the TUNEL staining, but the TUNEL assay had a higher background staining (data not shown).

To test whether the caspase 3-positive cells found in the UV-B-irradiated skin were Langerhans cells, a double staining was performed with the dendritic cell marker CD1a. No double positive cells could be detected in the UV-B-irradiated skin (**fig 1**). The Langerhans cells tended to be low in the epidermis or in the dermis whereas the apoptotic cells were higher up in the epidermis. A few (<1% of the total number of Langerhans cells from unirradiated skin and <3% of the total number of Langerhans cells from UV-B-exposed skin) caspase 3-positive or TUNEL positive Langerhans cells could occasionally be observed in the blister roofs of the UV-B-irradiated skin.

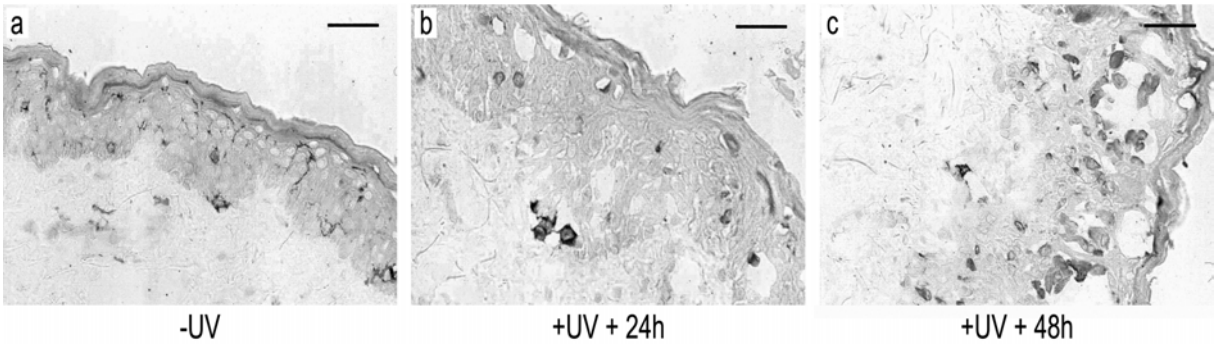

Figure 1. Double staining of CD1a (blue) and active-caspase 3 (red) of buttock skin, unexposed (- UV), 24h (+ UV +24h) and 48h after UV-B exposure (+ UV +48h). Scale bar =50 μm

CD1a+ Langerhans cells can be detected in the blister fluid of the UV-B-exposed skin, but not of the unexposed skin

To test whether UV-B-induced Langerhans cell depletion was caused by migration, suction blisters were raised on UV-B-irradiated skin and unirradiated skin. Blister roofs and blister fluid were sampled at several time points after irradiation. CD1a staining of blister roofs showed a significant decline ($p<0.0001$) in the number of Langerhans cells after UV-B irradiation (median of 259 cells/mm^2, 95% C.I. 146 - 462 cells/ mm^2) when compared to the unirradiated control (median of 720 cells/mm^2, 95% C.I. 520 - 996 cells/ mm^2) (**fig 2**). The largest decrease appeared to occur over the first 18-h period (60%), while between 18h and 48h there was a further decrease of only 10%.

Blister fluid was sampled to collect migrating Langerhans cells. Very few other cells were detected in the fluid (except for a minor admixture of about 50 cells in 3 out of 30 cases in which blisters developed more quickly around 1.5 hours). CD1a staining performed on cytospin preparations of blister fluid showed a significant difference ($p <0.0001$) between UV-B-irradiated and unirradiated skin. CD1a+ Langerhans cells could be detected in blister fluid of the UV-B-exposed skin, but not in the blister fluid of the unirradiated skin. The number of CD1a+ cells varied strongly among the healthy individuals (median of 115 cells / 100 μl sample, 95% C.I. 17 - 796 cells / 100 μl sample) (**fig 2**). The blister fluid from the earliest time point (18h) showed the largest number of Langerhans cells (median of 268 cells/ 100 μl sample, 95% C.I. 44 –1623 cells/_100 μl sample). However, the number of Langerhans cells that were found in the blister fluid of an individual did not correlate with the number of Langerhans cells that disappeared from the blister roofs of the UV-B-exposed skin (rho = -0.355, p =0.089, Spearman rank order correlation test). The individual UV-B doses (in J/m^2) showed a negative correlation with the numbers of Langerhans cells in the blister fluid (rho = -0.421, p = 0.04, Spearman rank order correlation test) while the UV-B doses showed positive correlation with the decrease of Langerhans cells in the blister roofs (rho = 0.565, p =0.001,

Spearman rank order correlation test). The maximum in the number of Langerhans cells in the blister fluid at 18h coincided with the largest decrease in Langerhans cells in the blister roof.

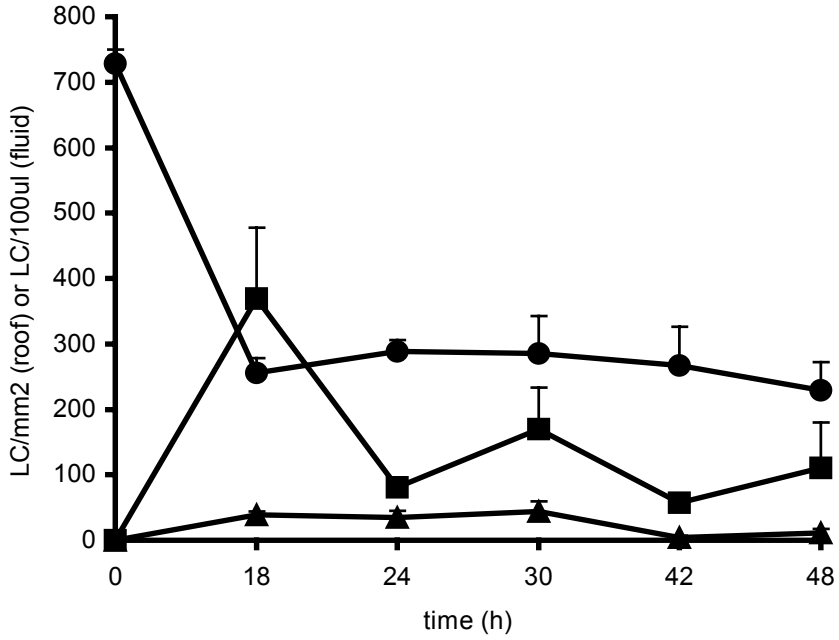

Figure 2. Number of CD1a+ Langerhans cells (mean+SEM) in blister roofs (bullets) and blister fluid (CD1a+ cells (squares) and CD1a+/H3+ cells (triangles) of arm skin, unexposed (0h) and UV-B-exposed (18h, 24h, 30h, 42h, 48h). Average n per time-point = 5

Cyclobutane thymidine dimers are detectable 18h after 6 MED UV-B in epidermal cells but only in a fraction of the migrating Langerhans cells

Skin biopsies, blister roofs and blister fluid were stained with H3 to detect UV-induced DNA damage, containing thymidine dimers. After a single dose of 6 MED UV-B most cells in blister roofs and skin biopsies are H3-positive. Blister roofs or skin biopsies from unexposed skin did not contain H3-positive cells (**fig 3**). H3 staining was performed on cytospin preparations of blister fluid to confirm that the CD1a+ cells originated from the UV-exposed epidermis. CD1a/H3 double positive cells could be observed in the blister fluid at different time points (18h – 48h) after UV irradiation (**fig 4**). But only a minority of the CD1a+ cells was H3-positive (mean of 27%). The percentage CD1a/H3 double positive cells of the total amount of CD1a+ cells in the blister fluid was the least at 42h (15.3%) and the greatest at 24h (38.8%) (**fig 2**). Because of layering of cells and excessive H3 staining it was difficult to interpret the CD1a/H3 staining in skin sections and whole blister roofs. Therefore, single cell suspensions of blister roofs and sections of skin biopsies were double stained for CD1a and H3 to check whether Langerhans cells in the UV-exposed skin also contained UV-damaged DNA (**fig 3**). The majority of the CD1a+ cells in single cell suspensions of blister roofs were H3-positive.

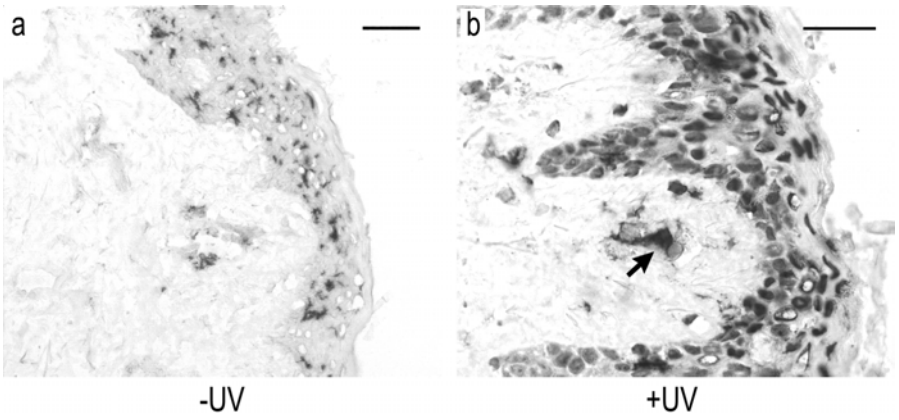

Figure 3. Double staining of CD1a (blue) and H3 (red) of buttock skin, unexposed (- UV) and 24h after UV-B exposure (+ UV) with an example of a doubled stained cell, migrating out of the epidermis (black arrow). Scale bar = 50 μm

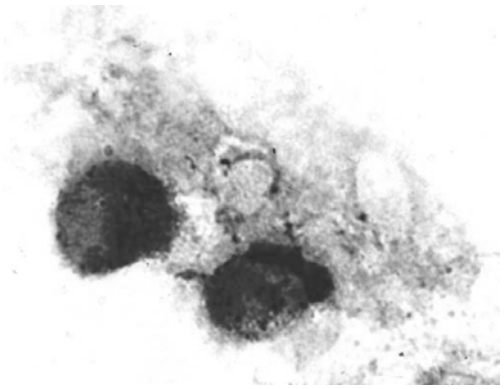

Figure 4. Double staining of CD1a (blue) and H3 (red) in blister fluid of the UV-B-exposed skin.

DISCUSSION

It is well known that UV-B can induce immunosuppression in the skin. One of the important processes associated with immunosuppression is the disappearance of the primary antigen presenting cells of the skin, the Langerhans cells. Here we investigated whether apoptosis or migration dominated this prominent effect on the epidermal antigen presenting cells. The disruption of Langerhans cell function can be fundamental to UV-induced immunosuppression either by incorrect, inappropriate or inefficient antigen presentation (Duthie *et al.*, 1999). Alternatively, depletion of Langerhans cells could lead to modified antigen presentation by another cell type, e.g., infiltrating monocytoid cells (Meunier *et al.*, 1995).

In our study we first focussed on the role of apoptosis in UV-B-induced Langerhans cell depletion, as the occurrence of this phenomenon in humans *in vivo* still has not convincingly been demonstrated (Meunier, 1999). We show that only a few epidermal CD1a+ Langerhans cells in skin biopsies or blister roofs undergo apoptosis after UV-B exposure, as detected by active-caspase 3 or TUNEL. These results are in line with experiments of Okamoto *et al.*(1999), who showed in mice that after UV radiation only 1.5 cell per 900 Langerhans cells/

mm^2 were apoptotic, which was far too few to contribute substantially to the observed Langerhans cell depletion. Hollis and Scheibner (1988) found that Langerhans cells in UV-irradiated skin of Aboriginals were apoptotic. The evidence is, however, circumstantial as the number of apoptotic Langerhans cells was very low and the time frame used was not optimal (2h or 5d). *In vitro* experiments demonstrated that Langerhans cells did go into apoptosis after UV-B irradiation and 2 days of culture (Rattis *et al.*, 1998). Tang and Udey (1992a) found that death of cultured murine Langerhans cells is augmented by UV-B irradiation. A population of HLADR+ cells migrating from cultured, *ex vivo* irradiated skin explants are shown to be apoptotic (Nakagawa *et al.*, 1999). Mommaas *et al.* (1993) showed by electron microscopy that some human Langerhans cells become necrotic when irradiated with UV-B *in vitro*. However, necrotic cells also become TUNEL positive, but were not detected as Langerhans cells in our study. Furthermore, lack of swollen, disintegrated Langerhans cells (detected by CD1a, Lag or HLADR)(data not shown) indicates that necrosis is negligible for UV-induced Langerhans cell depletion.

A possible explanation for the difference in UV-induced death of Langerhans cells between the *in vitro* or *ex vivo* and the *in vivo* situation could be the difference in environment. In human skin, which consists of several layers of epidermal cells, Langerhans cells might be protected by the surrounding cells. This might be due to protective shielding, but also by cell-cell contacts, cell surface molecules and cytokines that are lacking or different *in vitro* (Tang and Udey, 1992b; Laihia and Jansen, 2000). One could argue that the CD1a marker may be lost during apoptosis and that this is the reason why we could hardly detect any apoptotic CD1a+ cells. But Nakagawa *et al.* (1999) showed in their *in vitro* experiments that annexin V positive apoptotic cells from UV-B-irradiated skin exhibited a high expression of CD1a, which would imply that apoptotic Langerhans cells retain their CD1a surface marker. Taken together, our data show that apoptosis occurs abundantly in human epidermis after overexposure to UV-B. However, Langerhans cells apparently do not go into apoptosis and this mechanism can therefore not explain the UV-B-induced depletion of Langerhans cells in the epidermis.

The next step in unravelling the mechanism of UV-B-induced Langerhans cell depletion was investigating Langerhans cell migration. Pilot experiments had shown that it is feasible to catch migrating Langerhans cells *in vivo* in the fluid of suction blisters. Using this method we were indeed able to detect Langerhans cells with pyrimidine dimers reproducibly in the blister fluid of the UV-B-exposed skin, and not in the blister fluid of the unexposed skin. Blocking the emission of the Philips TL12 lamps below 290 nm (using a WG305/3 and a WG295/1 filter simultaneously) to simulate a solar-simulting irradiation source resulted in an equal number of Langerhans cells in the in blister roofs and blister fluid of the UV-exposed skin as compared to the unfiltered TL12 lamps (tested on 2 persons, data not shown). The decrease in Langerhans cell number in the blister roof after UV-B radiation did not correlate with the number of

Langerhans cells found in the blister fluid of the UV-B-exposed skin, although the largest decrease in the blister roof coincided with the largest increase in Langerhans cell number in the blister fluid. The correlation is, however, likely to be affected by factors like variations between individuals in time over which blisters were raised, differences in adherence of Langerhans cells to the dermal blister floor, amount of blister fluid and blister cross-section.
The presence of cyclobutane pyrimidine dimers in the CD1a+ cells in the blister fluid confirmed that the cells originated from the UV-B-exposed epidermis. Overall 27% of Langerhans cells in the blister fluid, but most of the epidermal cells (mainly keratinocytes), had detectable levels of pyrimidine dimers. One could think of several explanations for this low percentage of Langerhans cells with detectable pyrimidine dimers. First of all, one could speculate that the CD1a single positive cells do not originate from the epidermis but are new dendritic cells coming from the blood stream or hair follicles (Gilliam *et al.*, 1998), or entering the blister fluid as a result of the suction blister technique. It is, however, unlikely that the undamaged Langerhans cells come from the dermis as their dominant occurrence in blister fluid does not coincide with a repopulation of the epidermis (Kölgen *et al.*, 1999). Furthermore, it does not appear to be an artefact of the method used, no influx of Langerhans cells could be observed in the blister fluid of the unexposed skin. Moreover, a cytokeratin 14 staining on basal keratinocytes in the blister roof of the UV-B-irradiated skin showed that the integrity of the blister roof was not affected by the method used nor by UV-B irradiation (data not shown). Hence, Langerhans cells are not likely to be released from the epidermis through defects caused by the blister formation. Alternatively, it could be that the Langerhans cells had a low initial damage level (Vink *et al.*, 1994) or a rapid DNA repair. The undamaged Langerhans cells may show selective responsiveness to migratory signals, such as TNF-α. By staining single cell suspensions of blister roofs (18h time point) we were indeed able to see a clear enrichment of DNA-damaged Langerhans cells (approx. 80-90%) in blister roofs compared with blister fluid. Of note is the difference in percentage of double positive cells between 18h (20.1% of Langerhans cells) and 24h (38.8% of Langerhans cells). These percentages are mainly influenced by the number of CD1a single positive cells because the absolute number of damaged Langerhans cells in the blister fluid is almost the same between 18h and 30h. This also suggests that undamaged Langerhans cells are quicker to migrate.
In this study, we have shown that raising of suction blisters is an easy-to-use method to catch migrating Langerhans cells in human beings *in vivo*. Our data show that UV-B-induced Langerhans cell depletion in human skin is mainly caused by migration and not by apoptosis.

ACKNOWLEDGEMENT

We are very grateful to Ina Sybesma from the Department of Dermatology, University Medical Hospital Utrecht for irradiation of healthy volunteers. We would like to thank Yvonne Sontag for her contribution to the initial development of the suction blister method for catching migrating Langerhans cells.

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Impaired ultraviolet-B-induced Langerhans cell migration in patients with polymorphous light eruption

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ABSTRACT

Ultraviolet-B radiation induces a local immunosuppression which is associated with a migration of Langerhans cells from healthy skin. An inadequate immunosuppression might lead to the development of a ultraviolet-B-induced sun allergy (polymorphous light eruption). After (over)exposure of the skin of patients with polymorphous light eruption to ultraviolet-B a considerable number of Langerhans cells still remained in the epidermis. The aim of this study was to investigate whether Langerhans cell migration was impaired in patients with polymorphous light eruption. In addition, we examined if the Langerhans cells that remained in the ultraviolet-B-exposed skin of patients with polymorphous light eruption were activated and/or matured. In order to study these phenomena we exposed buttock skin of patients with polymorphous light eruption and healthy controls to six minimal erythema doses of ultraviolet-B. Suction blisters were raised 18h after ultraviolet exposure. The decrease of Langerhans cells in blister roofs and the number of migrating Langerhans cells in the blister fluid was significantly less in patients with polymorphous light eruption compared to healthy controls. No activated (CD86, CD40, CD54) or matured (CD83) epidermal Langerhans cells could be observed in skin biopsies from ultraviolet-B-exposed skin of patients with polymorphous light eruption. However, the number of epidermal HLA-DR+ Langerhans cells and dermal activated/matured Langerhans cells was larger in the ultraviolet-B-exposed skin of patients with polymorphous light eruption than in healthy controls. A defective Langerhans cell migration, and an accumulation of dermal activated/matured Langerhans cells, might be involved in the pathogenesis of polymorphous light eruption.

INTRODUCTION

Ultraviolet radiation (UV), especially UV-B (280-315 nm) is able to modulate the immune system. Exposure of the skin to UV-B can lead to a cascade of events starting with the formation of urocanic acid and DNA damage leading to the production of cytokines, such as interleukin (IL)-1-β, tumor necrosis factor (TNF)-α and IL-10 and the disappearance of Langerhans cells and influx of IL-10 producing macrophage-like cells (for review see Duthie et al, 1999). These events together may ultimately lead to the local and systemic immunosuppression that can be observed after exposure of the skin to UV-B radiation (Cooper, 1996; Morison, 1989; Yoshikawa et al, 1990).

UV-B irradiation is able to alter organic molecules and proteins which may act as 'neo-antigens' in evoking an immune reaction (Natali and Tan, 1973; Norris et al, 1988). UV-B-induced immunosuppression could therefore be a very desirable mechanism to prevent a

disruptive immune reactivity against sun-exposed skin. A disturbance of this immunosuppression may lead to the development of a UV-induced “sun allergy” (polymorphous light eruption (PLE)). PLE patients are sensitive to UV-B and/or UV-A radiation and sometimes even to visible light. They develop papules and/or vesicles on sun-exposed areas of the skin (Epstein, 1997; Grabczyska and Hawk, 1997; Salomon et al, 1997). PLE is suggested to be a delayed type hypersensitivity reaction (type IV) because of a delay (hours-days) between the UV exposure and the appearance of the skin lesions (Epstein, 1997; Norris et al, 1988; Verheyen et al, 1995).

In previous experiments we showed that Langerhans cells still persisted in the epidermis of PLE patients after exposure of the skin to UV-B in contrast to healthy volunteers (Kölgen et al, 1999). As the predominant mechanism underlying UV-B-induced Langerhans cell depletion in healthy individuals *in vivo* was found to be migration instead of apoptosis (Kölgen et al, 2002), we hypothesize that Langerhans cell persistence in PLE patients is caused by a defect in migration. Furthermore, we speculate that the Langerhans cells that remain in the UV-B-exposed epidermis are either activated and/or matured, leading potentially to antigen presentation in the skin instead of the lymph nodes. These phenomena might then contribute to the pathogenesis of PLE.

To investigate these hypotheses we studied a group of PLE patients and for comparison a control group of healthy controls. All PLE patients reacted pathologically to UV-B exposure. A small area of unaffected buttock skin of all participants was exposed to UV-B radiation. Suction blisters were raised on the (un)exposed area of the skin to study Langerhans cell migration. Immunohistochemical staining was performed for CD1a (Langerhans cells) and cyclobutane pyrimidine dimers (DNA damage) on blister roofs and cytospins of blister fluid. Skin biopsies were obtained from UV-B-irradiated and unirradiated skin of PLE patients and healthy volunteers to study Langerhans cell activation and maturation. Frozen skin sections were stained immunohistochemically for CD1a together with a Langerhans cell activation marker (CD86, CD40, CD54, HLA-DR) or the Langerhans cell maturation marker CD83.

MATERIALS AND METHODS

Subjects

Six patients with PLE and normal sunburn sensitivity (3 males and 3 females, ages between 30 and 52 years) and five healthy individuals (5 females, ages between 21 and 27 years) were studied for Langerhans cell migration while four PLE patients (2 males and 2 females, ages between 31 and 49 years) and four healthy volunteers (1 male and 3 females, ages between 21 and 35 years) were studied for activation of Langerhans cells. The diagnosis PLE was based on the patient’s history and on a provocation with cumulative UV-B, UV-A I, and visible light exposure on restricted areas of the upper

arms, a standard procedure in our dermatological clinic. All patients developed papules and/or vesicles after exposure to UV-B radiation, sometimes accompanied by a similar reaction to UV-A radiation. All patients included had a season-dependent exacerbation of the disease. Patients receiving phototherapy or medication (e.g. topical or oral corticosteroids) and patients and volunteers whose buttock skin was exposed to sunlight less then 2 months ago were excluded. Informed consent was obtained from patients and volunteers. This study was approved by the Medical Ethics Committee of the University Medical Center Utrecht.

Phototesting procedure

The dose required to cause a just perceptible redness, the minimal erythema dose (MED), was determined on the buttock skin. For MED determination a test device with 9 windows (2 x 9 mm each) was used. The underlying skin was exposed through these windows to a Philips TL12 lamp for different periods of time (a geometrical series, e.g. from 12.4 to 200 s). The TL12 lamp emitted 58% of its UV output in UV-B (280-315nm) and 5% of its UV output below 290 nm. Erythemally weighted, 98% of the effectivity stemmed from the UV-B band (18% below 290 nm). The erythema was examined 24h later. The MED ranged from 245 to 750 J/m^2 in PLE patients (median of 473.4 J/m^2, 95% C.I. 206.4-1085.7 J/m^2) and from 650 to 1500 J/m^2 in healthy controls (median of 1022.5 J/m^2, 95% C.I. 540.2-1935.3 J/m^2) and differed significantly (p=0.009) between PLE patients and healthy controls. After determination of the MED the (unaffected) buttock skin was exposed to 6 MED UV (Philips TL12). After UV-B exposure the skin of PLE patients and healthy volunteers was red and a little swollen but no pathology could be observed.

Suction blisters

Suction blisters were raised (Black et al, 1977; van der Leun et al, 1974) 16-18h after exposure to 6 MED on the irradiated buttock skin and on an unexposed side (2 blisters per individual). The optimal time point (18h) for raising suction blisters to catch migrating Langerhans cells in the blister fluid was determined in a previous study (Kölgen et al, 2002). Blister fluid was collected using a microneedle with a diameter of 0.36 mm. Blister roofs were removed with a sterilized skin surgery set. Cytospin preparations were made of the blister fluid by centrifugation for 10 min at 55g onto 3-amino-propyltriethoxysilane-coated glass slides. The slides were stored at –30^{o}C until further processing. The removed blister roofs were used immediately for immunohistochemistry.

Biopsies

After local anesthesia (xylocaine/adrenaline) three millimeter punch biopsies were obtained from the irradiated buttock skin 24h and 48h after UV-B exposure, together with one control biopsy from unirradiated skin. Biopsies were snap-frozen in liquid nitrogen, embedded in OCT compound (Tissue-Tek, Sakura, Zoeterwoude, the Netherlands) and stored at –30^{o}C until further processing.

Antibodies

As primary antibodies monoclonal antibodies CD1a (DAKO A/S, Glostrup, Denmark, diluted 1:60), fluorescein isothiocyanate (FITC)-conjugated CD1a (DAKO A/S, diluted 1:60), biotinylated H3 antibody

(directed against cyclobutane thymine dimers, a kind gift from Dr. L. Roza; T.N.O., Zeist, The Netherlands, diluted 1:20), CD86 (diluted1:10), CD40 (diluted 1:900), CD54 (diluted 1:10), CD83 (diluted 1:20)(all purchased from Serotec, Oxford, United Kingdom) and HLA-DR (Becton Dickinson, Heidelberg, Germany, diluted 1:100) were used. Biotinylated horse anti-mouse immunoglobulin (Vector, Burlingame, CA, diluted 1:800) was used as a secondary antibody. Alkaline phosphatase (AP)-labeled F(ab) fragments of sheep anti-FITC (Boehringer Mannheim GmbH, Mannheim, Germany, diluted 1:400) and horseradish peroxidase (HRP)-conjugated avidin-biotin complex (DAKO A/S, diluted 1:50) were used as detecting reagents.

Immunohistochemistry

Single staining of CD1a. Blister roofs were fixed for 10 min at room temperature in dry acetone containing 100 μl 30% H_2O_2 per 100 ml. The slides were incubated for 20 min with a blocking reagent (phosphate-buffered saline (PBS) containing 10% normal human serum and 10% normal horse serum) to prevent aspecific binding. The blister roofs were subsequently incubated with primary antibody (CD1a) for 1h and with a secondary antibody (biotinylated horse anti-mouse immunoglobulin) for 1h. Subsequently, the blister roofs were incubated for 30 min with HRP-conjugated avidin-biotin complex. Peroxidase activity was visualized by incubation in 0.1 M acetate buffer (pH=5) containing 20 mg 3-amino-9-ethyl-carbazole (AEC; Sigma, ST. Louis, MO) and 100 μl H_2O_2 per 100 ml, resulting in a red staining.

Double staining of CD1a-FITC and H3-biotin. CD1a-FITC/H3 double staining was performed as described previously (Sontag et al, 1995). Blister roofs and cytospins of blister fluid were fixed in dry acetone, preincubated with blocking reagent (PBS containing 10% normal human serum) and incubated for 1h with the primary antibody CD1a-FITC. After incubation for 1h with AP-labeled sheep anti-FITC F(ab) the AP activity was visualized by incubation in a BCIP/NBT solution containing 37.5 μl 5-bromo-4-chloro-3-indolyl phosphate, 50 μl 4-nitro blue tetrazolium chloride and 2.5 mg levamisole per 10 ml 0.1 M Tris-HCL (pH=9.5), 0.1 M NaCl, 0.05M $MgCl_2$. The bluish-black reaction product is water and ethanol insoluble and withstands the subsequent harsh treatment. The preparations were fixed in 25% acetic acid (vol/vol) containing 100 μl 30% H_2O_2 per 100 ml. After washing two times for 10 min in PBS the slides were incubated for exactly 2 min in 0.07 N NaOH in 70% ethanol. After washing with PBS the preparations were incubated for 20 min with PBS containing 10% normal human serum and 10% normal mouse serum, followed by a 1h incubation with H3-biotin.The preparations were incubated with HRP-conjugated avidin-biotin complex for 30 min and developed in an AEC substrate solution, as described above.

Double staining of CD1a-FITC and CD86, CD40, CD54, HLA-DR or CD83. Frozen skin sections (6 μm thickness) on 3-aminopropyltriethoxysylane-coated glass slides were fixed and preincubated with blocking reagent (PBS containing 10% normal human serum and 10% normal horse serum). The sections were then incubated with the primary antibody CD1a-FITC for 1h and subsequently for 1h with AP-labeled sheep-anti-FITC. AP activity was visualized by incubation in a 0.1M Tris-HCL buffer

(pH=8.5), containing 25 mg Fast Blue BB salt, 12.5 mg naphthol AS-MX phosphate, and 35 mg levamisole per 100 ml (all purchased from Sigma, St. Louis, MO). After washing the slides in PBS, the sections were incubated for 20 min with 10% normal mouse serum. Subsequently the sections were incubated for 1h with the primary antibody CD86, CD40, CD54, HLA-DR or CD83 and for 1h with biotinylated horse-anti-mouse immunoglobulin, followed by incubation for 30 min with HRP-conjugated avidin-biotin complex. Peroxidase activity was visualized by incubation with an AEC substrate solution, as described above. The skin sections were evaluated using a light microscope at 250 times magnification. The density of active/mature Langerhans cells as well as the percentage of active/mature Langerhans cells with regard to the total number of Langerhans cells were determined (mean of 2 or 3 microscopic fields). The density of double positive cells was graded as: no active/mature Langerhans cells (0), a few active/mature Langerhans cells (1), presence of scattered active/mature Langerhans cells (2), clear abundant presence of active/mature Langerhans cells (3), a closed maze of active/mature Langerhans cells (4). The percentage of active/mature Langerhans cells were scored by assignment to one of the following categories: 0%, 0-10%, 10-50%, 50-80%, 80-100%.
Negative staining controls were used in which the first antibody was omitted or replaced by an irrelevant antibody of the same isotype. All antibodies were diluted in the suitable blocking reagent except for the AP- and HRP-conjugated antibodies, which were diluted in PBS containing 0.05% Tween 20. All antibody incubations were performed in a humidified chamber at room temperature. After each antibody incubations the slides were rinsed three times with PBS/0.05% Tween 20.
CD1a staining in the upper dermis was quantitatively determined by image processing: The percentage of a standardized dermal cross-sectional area that was CD1a positive was measured using a camera mounted onto a light microscope together with the Optimas 6.1 and Microsoft Excel software. CD1a staining in hair follicles or glands was not included.

Statistical analysis

After logarithmic transformation (to correct for the right-skewed distribution) a Students T-test was performed to ascertain the significance of the observed differences (significance level $p< 0.05$). The number of CD1a positive cells in the blister roofs and the number of CD1a positive and CD1a/H3 double positive cells in the blister fluid after UV exposure were compared to the numbers in unexposed, control skin. Furthermore, cell numbers from blister roofs and blister fluid and from skin biopsies of (un)exposed skin of PLE patients were compared to healthy controls. A Pearson correlation test (significance level $p< 0.05$) was performed on the log-transformed data to test whether a correlation existed between MED and UV-induced Langerhans cell depletion in blister roofs and Langerhans cell increases in blister fluid. A Wilcoxon ranksum test was performed on the density of the epidermal HLADR+ CD1a+ Langerhans cells.

RESULTS

Migrating CD1a positive Langerhans cells can be detected in the blister fluid of the UV-B-irradiated skin of PLE patients but in smaller numbers than in healthy controls

Previous experiments showed that Langerhans cells still remain in the epidermis after exposure of buttock skin of PLE patients to 6 MED in contrast to healthy controls (Kölgen et al, 1999). To test whether this deviant Langerhans cell behavior in PLE is caused by a defect in Langerhans cell migration, we raised suction blister on UV-B-irradiated and unirradiated buttock skin of PLE patients and healthy controls.

Blister roofs (epidermis) were removed to check for Langerhans cell disappearance. CD1a staining of blister roofs from UV-B-exposed skin of PLE patients (median of 408 cells/mm^2, 95% C.I. 303-548 cells/mm^2) and healthy controls (median of 209 cells/mm^2, 95% C.I. 77-562 cells/mm^2) showed a significant decrease (both $p<0.001$) in the number of Langerhans cells when compared to the respective unirradiated controls (median of 590 cells/mm^2, 95% C.I. 364-955 cells/mm^2 and median of 646 cells/mm^2, 95% C.I. 540-771 cells/mm^2 resp.). The number of Langerhans cells disappearing from the blister roof after UV-B exposure was significantly larger in healthy volunteers (mean of 65.15%) than in PLE patients (mean of 30.4%) ($p=0.018$). No difference could be observed in the number of Langerhans cells in the unexposed skin of PLE patients compared to healthy controls ($p=0.44$). Conversely, the number of Langerhans cells that were still present in the blister roofs of the UV-exposed skin was significantly higher in PLE patients than healthy controls ($p=0.037$) (**fig 1**). No correlation could be observed between the individual UV doses (MED) and the UV-induced depletion of Langerhans cells in the blister roofs ($p=0.14$).

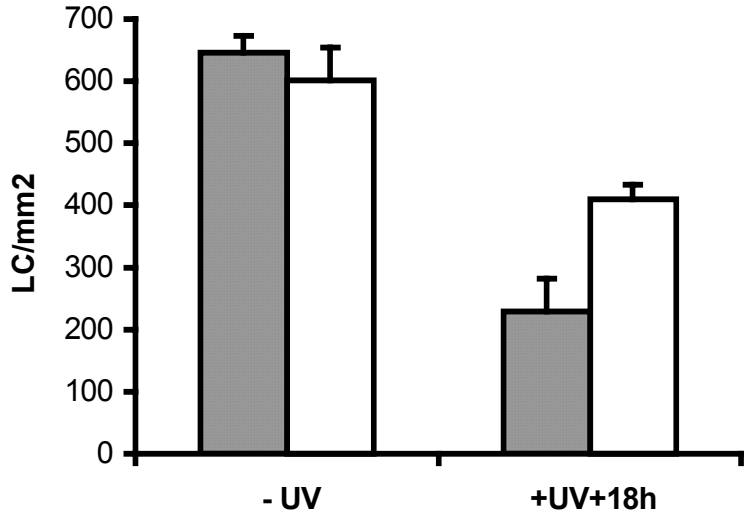

Figure 1, Number of CD1a+ Langerhans cells (LC) (mean ± SEM) in blister roofs of the unexposed (- UV) and UV-B-exposed (+UV+18h) skin of PLE patients (white bars) and healthy controls (gray bars).

Blister fluid was sampled to collect migrating Langerhans cells. The number of CD1a+ Langerhans cells found in the blister fluid of the UV-B-exposed skin of PLE patients (median of 103 cells/ 100 μl sample, 95% C.I. 21-491.cells/ 100 μl sample) and healthy controls (median of 534 cells/ 100 μl sample, 95% C.I. 77-3692 cells/ 100 μl sample) was significantly higher (both $p<0.001$) than in the respective unexposed controls, where no Langerhans cells could be detected. The number of Langerhans cells in the blister fluid of the UV-B-exposed skin of PLE patients was significantly less ($p=0.012$) than in healthy controls (**fig 2**). Neither the individual UV dose (MED) ($p=0.34$) nor the decreases in Langerhans cell number in the blister roofs ($p=0.078$) correlated with the amount of Langerhans cells in the blister fluid.

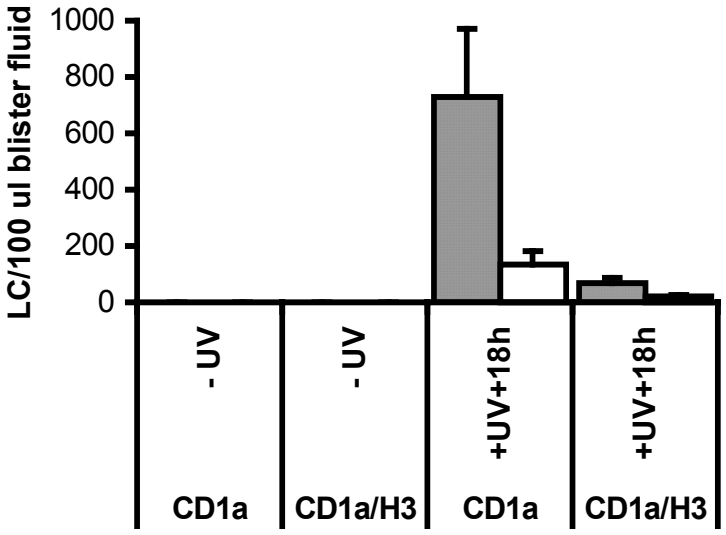

Figure 2, Number of CD1a+ and CD1a/H3+ Langerhans cells (LC) per 100 μl blister fluid (mean ± SEM) from the unexposed (- UV) and UV-B exposed (+UV+18h) skin of PLE patients (white bars) and healthy controls (gray bars).

The percentage of migrating Langerhans cells with cyclobutane pyrimidine dimers does not differ between PLE patients and healthy controls

Blister roofs and cytospin preparations of blister fluid were stained biotinylated H3 antibody to detect UV-B-induced DNA damage and to confirm that the CD1a+ Langerhans cells originated from the epidermis. After a single exposure of 6 MED UV-B most cells in blister roofs are H3 positive, while no H3 positive cells could be observed in blister roofs of the unexposed skin (data not shown; Kölgen et al, 2002). Single cell suspensions of blister roofs from UV-B-exposed skin showed that the majority of the CD1a+ Langerhans cells were H3-positive (data not shown).

CD1a/H3 double positive cells could be detected in the blister fluid of the UV-B-exposed skin of PLE patients and healthy controls. However, only a fraction of the Langerhans cells in the blister fluid of the UV-B-exposed skin of PLE patients and healthy controls was H3 positive (**fig 2**). This subset of Langerhans cells with pyrimidine dimers did not differ between PLE patients (mean of 15.9%) and healthy controls (mean of 16.8%) ($p=0.6$).

Epidermal Langerhans cells in the UV-B-irradiated skin of PLE patients are not activated or matured, but do have an increased expression of HLA-DR compared to unexposed skin

To investigate whether the persistent Langerhans cells in the UV-B-exposed skin of PLE patients are activated or matured, we stained frozen skin sections from UV-B-exposed and unexposed buttock skin of PLE patients and healthy controls with CD1a and several activation markers (CD86, CD40, CD54, HLA-DR) or the maturation marker CD83. Due to the dendrites it is difficult to determine the number of Langerhans cells quantitatively in frozen skin sections. Furthermore, the image processing equipment was unable to distinguish between the blue reaction product (CD1a) and the red reaction product (activation/maturation markers). Therefore we had to asses the number of activated/matured Langerhans cells semi-quantitatively, as described in the Materials en Methods section.

Similar to previous experiments (suction blisters and Kölgen *et al*, 1999) the number of Langerhans cells decreased after UV exposure but a considerable number of Langerhans cells was still present in the UV-B-exposed skin of PLE patients in contrast to healthy controls. The majority of the epidermal Langerhans cells of PLE patients and healthy controls had no detectable expression of the activation markers CD86, CD40 or CD54 or the maturation marker CD83. No active/mature epidermal Langerhans cells could be discovered in the unexposed skin of any of the participants.

An exception to this is the expression of the activation marker HLA-DR, which was detected at 0-10% of the epidermal Langerhans cells in the unexposed buttock skin and increased up to 80-100% in the UV-B exposed skin of PLE patients as well as healthy controls (**fig 3**). However, the density of epidermal HLA-DR+ Langerhans cells was significantly larger ($p<0.05$) in the UV-exposed skin (at 24h) of PLE patients compared to healthy controls (**fig 4**).

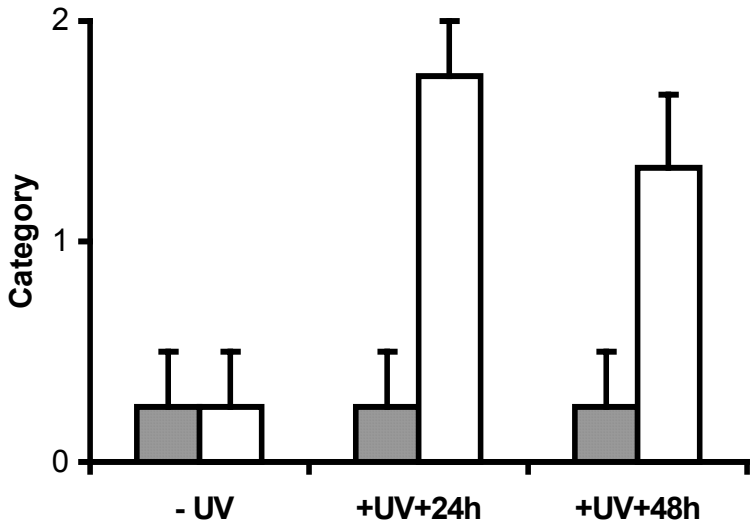

Figure 3, Number (n) of PLE patients (white bars) and healthy controls (gray bars) with HLA-DR+ Langerhans cells (LC) (%) in the unexposed skin (- UV) or 24h (+UV+24h) or 48h (+UV+48h) after UV exposure.

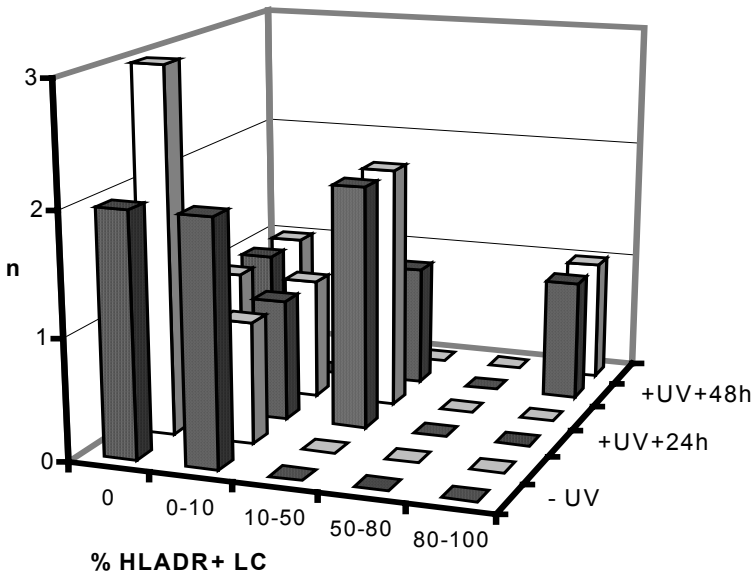

Figure 4, Density score of HLA-DR+ Langerhans cells (categories (0-4); see materials and methods section) in the epidermis of the unexposed skin (- UV) and 24h (+UV+24h) or 48h (+UV+48h) after UV-exposure of PLE patients (white bars) and healthy controls (gray bars).

The density of dermal activated/matured Langerhans cells increased after UV exposure in PLE patients and healthy controls. Slightly more CD54, CD83, CD40 and HLA-DR positive (the latter two only 48h after irradiation) dermal Langerhans cells were detected in the UV-exposed skin of PLE patients compared to healthy controls. The density of dermal dendritic cells (% CD1a positive area) was significantly larger ($p=0.005$) in the UV-exposed skin (48h) of PLE patients (mean of 0.91%) compared to healthy controls (mean of 0.39%). The percentage of dermal Langerhans cells that are activated or matured increased after UV exposure but was comparable for PLE patients and healthy controls.

DISCUSSION

Previous experiments showed that after exposure of the skin of PLE patients to UV-B Langerhans cells still remain in the epidermis in contrast to healthy controls (Kölgen et al, 1999). In healthy individuals UV-B-induced Langerhans cell depletion was caused by migration out of the skin and not apoptosis (Kölgen et al, 2002). Therefore, in this present study, we investigated whether Langerhans cell migration was impaired in PLE patients. We observed a decrease in the number of Langerhans cells in the blister roofs of the UV-B-exposed skin of PLE patients (30.4%), but the decrease was significantly less than in healthy controls (65.15%). In correspondence to this we found an increase in the number of migrating Langerhans cells in the blister fluid of the UV-exposed skin of PLE patients which was significantly less than in healthy controls. No correlation, however, was found between the individual decrease in the number of Langerhans cells in the blister roof and the number of

Langerhans cells in the blister fluid of the UV-exposed skin. But this correlation could be influenced by factors such as individual time over which blisters were raised, adherence of Langerhans cells to the dermal blister floor or blister cross sectional area.

The number of Langerhans cells with UV-induced pyrimidine dimers varied per individual but did not differ significantly between PLE patients (15.9%) and healthy controls (16.8%). This low percentage of damaged Langerhans cells might be explained by a rapid DNA repair and a selective responsiveness of undamaged cells to migratory signals, as also discussed in Kölgen *et al.* (2002).

One could speculate that the UV dose (MED), which was on average less in PLE patients, was responsible for the lower number of migrating Langerhans cells. However, no correlation could be detected between the MED and UV-induced Langerhans cell depletion in blister roofs or Langerhans cells increases in blister fluid. Moreover, a PLE patient with a MED similar to the MED of a healthy individual still had significantly less Langerhans cells disappearing from the skin.

A possible explanation for the impairment of Langerhans cell migration in PLE patients might be an intrinsic inability (insensitivity) of the cells to migrate. Nevertheless, when the skin of PLE patients (n=2) was exposed to an even higher UV-B dose, namely 8.4 MED, an additional depletion of Langerhans cells occurred (data not shown). This indicates that the Langerhans cells were capable of migration. It is therefore most likely that signals required for Langerhans cell migration, such as the cytokines IL-1 and TNF-α, are present in lesser amounts in PLE patients compared to healthy controls. A significantly lower MED in PLE patients and the observation that Langerhans cells from PLE patients are able to migrate when given an higher UV dose point to an imbalance between erythema and immune responses. Increasing the MED by e.g. supplementation with dietary fish oil (Rhodes et al, 1995) or by phototherapy might therefore be therapeutical in bringing erythemal versus immunosuppressive responses back to normal, i.e. in balance.

Another possible mechanism that might be involved in the pathogenesis of PLE is the activation and/or maturation of the Langerhans cells that remain in the epidermis of PLE patients after UV-exposure. During migration Langerhans cells mature and increase their expression of costimulatory molecules such as CD80, CD86 and CD40 as well as the proportion of MHCII molecules (HLA-DR) on their membrane. These transformations, among others, turn them into potent antigen presenting cells, capable of stimulating T-cells which reside in the draining lymph nodes (Cumberbatch et al, 2000; Flores-Romo, 2001). Presence of active or mature cells in the skin might lead to antigen presentation in the skin instead of the lymph nodes. Antigen presentation at this aberrant location might then lead to a disruptive immune reaction.

We were not able to detect active or mature Langerhans cells in the epidermis of the UV-B exposed skin of PLE patients. However, the expression of HLA-DR was upregulated in the UV-B exposed skin of both PLE patients and healthy controls. The density of HLA-DR+ Langerhans cells in the epidermis was larger in PLE patients than healthy controls. An enhanced expression of HLA-DR as well as costimulatory molecules and the presence of T-cells are required for proper antigen presentation. As no costimulatory molecules were detected and hardly any epidermal T-cells were observed (data not shown) antigen presentation by Langerhans cells in the epidermis of PLE patients will probably not occur. On the other hand, detection of costimulatory molecules by other, more sensitive techniques than immunohistochemistry (e.g. by FACS analysis), might still reveal an enhanced expression of these molecules.

Some investigators report that costimulatory molecules like CD80, CD86 and CD54, and HLA-DR are downregulated after UV exposure (Dittmar et al, 1999; Rattis et al, 1998; Weiss et al, 1995). Others show that CD80, CD86, CD40, CD54 and CD83 (Nakagawa et al, 1999) or CD86 and HLA-DR (Laihia and Jansen, 2000) are upregulated after respectively *ex vivo* or *in vivo* irradiation of the skin. We showed that the number of dermal dendritic cells with costimulatory molecules or HLA-DR on their membranes increased after UV exposure in healthy controls as well as PLE patients. The density but not the percentage of dermal dendritic cells that express CD40, CD54, CD83 or HLA-DR was larger in the skin of PLE patients compared to healthy controls. A possible reason for this difference is that the majority of the Langerhans cells have already migrated out of the skin of healthy controls in contrast to PLE patients. The simultaneous presence of activated Langerhans cells and T-cells in the dermis might lead to antigen presentation in the skin instead of the lymph nodes. Presentation of (neo)-antigens to dermal T-cells (possibly Th1 cells) might contribute to the pathogenesis of PLE.

Taken together, we showed that Langerhans cells migration was impaired in PLE patients. Furthermore, we were not able to detect any expression of activation or maturation molecules on the membranes of epidermal Langerhans cells in the UV-B exposed skin of PLE patients, except for the expression of HLA-DR. Therefore, we conclude that an impaired Langerhans cell migration and perhaps a higher number of activated/matured Langerhans cells in the dermis might contribute to the pathogenesis of PLE. Future experiments should focus on the expression of migration-inducing factors, such as the cytokines IL-1 and TNF-α, and their role in the pathogenesis of PLE.

ACKNOWLEDGEMENT

We are very grateful to Ina Sybesma from the Department of Dermatology, University Medical Center Utrecht for irradiation of PLE patients and healthy controls.

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Differential expression of cytokines in ultraviolet-B-exposed skin of patients with polymorphous light eruption: correlation with Langerhans cell migration and immunosuppression

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Submitted

Color printings: see addendum

ABSTRACT

Objective: Establish whether ultraviolet-B (UV-B) exposure induces aberrant cytokine expression in uninvolved skin of patients with polymorphous light eruption (PLE). Disturbances in normally induced Langerhans cell migration and skewing toward T helper (Th) 2 responses could be early steps in the pathogenesis of PLE.

Design: Immunohistochemical staining of microscopic sections from UV-B-irradiated and unirradiated skin.

Setting: University Hospital; Dutch National Center for Photodermatoses.

Patients: PLE patients (n=6) with clinically proven pathological responses to repeated UV-B exposures, and with normal erythemal sensitivity. Healthy volunteers (n=5) were recruited from students and hospital staff.

Main Outcome Measures: Expression of cytokines related to **a)** Langerhans cell migration (Interleukin (IL)-1-α, IL-1-β, IL-18 and Tumor Necrosis Factor (TNF)-α), **b)** Th2 responses (IL-4, IL-10) and **c)** Th1 responses (IL-6, IL-12 and Interferon (IFN)-γ). Double staining was done for elastase (neutrophils), tryptase (mast cells) and CD36 (macrophages).

Results: The number of cells expressing IL-1-β and TNF-α was reduced in the UV-B-exposed skin of PLE patients in comparison with healthy individuals ($p<0.05$ for TNF-α). No differences were observed in the expression of Th1-related cytokines but the expression of Th2-related cytokines was less in PLE patients: significantly fewer IL-4-expressing cells infiltrated the epidermis of PLE patients 24 hours post irradiation ($p= 0.02$). TNF-α, IL-4 and partly IL-10 were predominantly expressed by neutrophils after UV exposure.

Conclusions: The reduced expression of TNF-α, IL-4 and IL-10 in UV-B-irradiated skin of PLE patients appears largely attributable to a lack of neutrophils, and is indicative of less Langerhans cell migration and less Th2 skewing.

INTRODUCTION

Ultraviolet radiation (UV), especially UV-B (280-315 nm), can modify proteins and other organic molecules which may act as neo-antigens to provoke an (auto)immune reaction (Natali and Tan, 1973; Norris *et al*, 1989). This adverse immune reaction can be suppressed by another effect of UV radiation, i.e. the induction of cellular immunosuppression (Cooper, 1996; Yoshikawa *et al*, 1990). A defect in this immunosuppression might therefore allow an illicit immune reaction to UV radiation to occur i.e. polymorphous light eruption (PLE) could evolve.

Key players in the effects of UV radiation on the skin immune system are cytokines. UV-B radiation can (in)directly induce cytokine release in the skin resulting in an efflux and influx of

several cell types. Interleukin (IL)-1-β and tumour necrosis factor (TNF)-α release by e.g. keratinocytes can induce Langerhans cell migration out of the skin (Cumberbatch *et al*, 1997; Cumberbatch *et al*, 1999). The pro-inflammatory cytokine IL-18, which can be produced by keratinocytes and Langerhans cells, is reported to be involved in Langerhans cells migration out of the skin, and is dependent upon IL-1-β and TNF-α (Cumberbatch *et al*, 2001). There is little data on the effect of UV radiation on IL-18 expression in the skin. Nakagawa *et al* (1999) showed that UV radiation had no effect on IL-18 production in an ex vivo irradiated epidermal sheet in culture. IL-18, together with IL-12, stimulates the production of interferon (IFN)-γ by T-cells (Chang *et al*, 2000; Tominaga *et al*, 2000). When Langerhans cells are depleted after UV exposure macrophages concomitantly expand in the dermis and infiltrate the epidermis (Meunier *et al*, 1995). Kang *et al* (1994 and 1998) showed that these CD11b+ cells produce the immunosuppressive cytokine IL-10. There are some conflicting data on the effect of IL-10 in Langerhans cell migration: Wang *et al* (1999) showed that Langerhans cell migration was enhanced in IL-10 knockout mice, which also have an increased TNF-α and IL-1 production, while Halliday *et al* (2001) observed an enhancement of Langerhans cell migration by IL-10 from regressor tumors.

Besides IL-10 from macrophages, UV-induced IL-10 from keratinocytes is also contributing to an immunosuppressive environment in the skin (Enk *et al*, 1995; Rivas and Ullrich, 1992). Furthermore, IL-10 counteracts IL-12 activity and inhibits the activation of Th1 cells but allows activation of Th2 cells (Enk *et al*, 1993). TNF-α release by dermal mast cells is involved in UV-induced systemic immunosuppression (Walsh, 1995). Teunissen *et al* (2002) showed that IL-4-producing neutrophils infiltrate the skin after UV exposure, supporting the development of a Th2 milieu in the skin. IL-4 is shown to be involved in the UV-induced suppression of delayed type hypersensitivity (el Ghorr and Norval, 1997) and contact hypersensitivity (Hart *et al*, 2000). The production of IL-12 by monocytes / macrophages is decreased after UV-B irradiation (Kang *et al*, 1998; Kremer *et al*, 1996) as is the production of IFN-γ by T-cells. Conversely, the production of IL-12p40 homodimers, the natural antagonist of IL-12, by keratinocytes and Langerhans cells is increased after UV exposure (Norval, 2001; Schmitt and Ullrich, 2000). Most importantly a decrease in IL-12 activity and an increase in IL-4 favours the differentiation of Th0 cells into Th2 cells.

All these cytokines have complex effects on each other and on cells of the skin immune system. The cytokine balance in the UV-exposed skin is ultimately shifted towards an immunosuppressive reaction in healthy individuals.

One of the cellular mechanisms related to this immunosuppression is the disappearance of Langerhans cells. In previous experiments, we showed that in the skin of PLE patients Langerhans cells still persisted after overexposure of the skin to UV-B radiation, in contrast to what was observed in healthy individuals (Kölgen *et al*, 1999). The main mechanism

responsible for Langerhans cell depletion in healthy individuals, migration, was found to be impaired in PLE patients[1]. Additionally, when the skin of PLE patients was exposed to an even larger UV dose, Langerhans cells did disappear from the epidermis, thus revealing a larger gap between erythema and Langerhans cell migration-inducing UV doses in PLE patients.
In the context of the possibility of a defective UV-induced immunosuppression PLE patients, these observations lead us to two hypotheses. Firstly, cytokines which are involved in Langerhans cell migration (e.g. IL-1, TNF-α) are induced less in the skin of PLE patients when compared to healthy volunteers. Secondly, we speculate that in PLE patients an imbalance exists between pro-inflammatory and Th1-skewing cytokines (e.g. IL-12, IFN-γ), and cytokines involved in Th2-skewing and Th1 suppression (e.g. IL-4, IL-10), ultimately shifting the balance to pro-inflammatory and Th1-skewed immune responses.
To test these hypotheses six PLE patients and five healthy volunteers were studied. A small area of the (unaffected) buttock skin of all participants was overexposed to UV-B. Skin biopsies were obtained from all participants from the UV-B-exposed skin 24h and 48h after irradiation and one biopsy from the unexposed buttock skin as a control. As a first exploration of the cytokine patterns in the UV-B-exposed and unexposed skin of PLE patients and healthy volunteers we performed immunohistochemical stainings on frozen skin sections for a series of cytokines to locate and identify cytokine-expressing cells in the skin.

MATERIALS AND METHODS

Subjects

Six patients with PLE and normal sunburn sensitivity (1 male and 5 females, ages between 32 and 64 y) and 5 healthy volunteers (2 males and 3 females, ages between 19 and 21 y) were included in the study. The diagnosis PLE was based on patient's history and photoprovocation testing with daily exposures of UV-A I, UV-B and visible light on restricted areas on the upper arms. In all patients, papules or vesicles occurred on the UV-B-irradiated skin area with or without a reaction on the UV-A I-exposed skin. Patients who were under medication (e.g. corticosteroids) or who received phototherapy were excluded from the study. Patients and healthy volunteers whose buttock skin was exposed to sunlight less than 2 month ago were also excluded. The Medical Ethics Committee of the University Medical Center Utrecht, the Netherlands, approved of this study.

Phototesting procedures

The UV-B dose giving a minimal perceptible redness of the skin (MED) was determined on the buttock skin, using a Philips TL12 lamp. The TL12 lamp emitted 58% of the UV output in UV-B (280-315 nm) and 5% below 290 nm. Erythemally weighted, 98% of the effectivity stemmed from the UV-B band (18%

[1] Kölgen *et al, submitted*

below 290 nm). For MED determination a test device with nine windows (3*10 mm) was used. These windows opened and closed sequentially exposing the underlying skin to UV-B for different periods of time (12.4 - 200 s). The erythema was assessed 24h after irradiation. MED ranged from 483 to 945 J/m^2 in PLE patients (median of 654 J/m^2, 95% C.I. 396-1080 J/m^2) and from 490 to 980 J/m^2 in healthy controls (median of 734 J/m^2, 95% C.I. 421-1279 J/m^2). Subsequently, the (uninvolved) buttock skin was exposed to 6 MED UV-B (Philips TL12), resulting in a sunburn reaction, but no pathology. Three-millimeter punch biopsies were obtained from UV-exposed skin 24h and 48h after irradiation, together with one control biopsy from the unexposed buttock skin. Biopsies were snap frozen in liquid nitrogen, embedded in OCT compound (Tissue-Tek, Sakura, Zoeterwoude, the Netherlands) and stored at -80^{o}C until further processing.

Antibodies

As primary antibodies, monoclonal antibodies against IL-1-α and IL-1-β conjugated with biotine (both a gift from Dr. P. Ghiara, IRIS, Sienna, Italy), IL-4, IL-6, (both purchased from Genzyme, Cambridge, MA, diluted 1:100 and 1:50 resp.) IL-10 (Instruchemie, Delfzijl, the Netherlands, diluted 1: 400), IL-12 p40/p70 (BD Biosciences Pharmingen, Erembodegem, Belgium, diluted 1: 150), IL-18 (MBL, Nagoya, Japan, diluted 1: 200), IFN-γ (U-Cytech, Utrecht, the Netherlands, diluted 1: 100), TNF-α (Biosource, Nivelles, Belgium, diluted 1:50), horseradish peroxidase (HRP)-conjugated elastase (a neutrophil marker; DAKO A/S Glostrup, Denmark, diluted 1:10), alkaline phosphatase (AP)-conjugated AA1 (against tryptase; Chemicon, Temecula, CA, diluted 1:50 and fluorescein isothyocyanate (FITC) labelled CD36 (Immunotech, Marseille, France, diluted 1:80) were used. Biotinylated rabbit-anti-mouse immunoglobulin (Ig)(DAKO A/S, diluted 1:400) or biotinylated horse anti-mouse Ig (Vector, Burlingame, CA, diluted 1:800) were used as secondary antibodies. AP- or HRP-conjugated avidine-biotine complex (DAKO A/S, diluted 1:50), HRP-conjugated rabbit anti-mouse Ig (IL-10, DAKO A/S, diluted 1:100) or AP-conjugated F(ab) fragments of sheep-anti-FITC (Boehringer Mannheim GmbH, Mannheim, Germany) were used as detecting reagents.

Immunohistochemistry

*Single staining of cytokines***.** The technique to detect cytokines by immunohistochemistry in the cytoplasm of cells in microscopic sections has been described in detail by Hoefakker et al (1995). Frozen skin sections (6 μm thickness) on 3-aminopropyltriethoxysilane-coated glass slides were fixed for 10 min in 65 ml acetone containing 100 μl 30% H_2O_2. Endogenous peroxidase activity was additionally blocked by incubating the slides for 15 min in 65 ml 0.05 M Tris-HCl (pH 7.6) containing 26 mg 4-Cl-1 naphthol diluted in absolute ethanol.

Primary antibodies for cytokine detection were all diluted in PBS / 0.1% BSA and incubated overnight. Subsequently, slides were incubated for 30 min with biotinylated rabbit anti-mouse Ig (diluted in PBS / 1% BSA / 1% normal human serum (nHus)) or for 60 min with HRP-conjugated rabbit anti-mouse Ig (for IL-10 staining; diluted in PBS/ 1%BSA). After incubation with biotinylated rabbit anti-mouse Ig, slides were incubated for 60 min with HRP-conjugated avidin-biotin complex diluted in PBS/1%BSA. Antibody binding was visualized by incubating the skin sections simultaneously in 65 ml 0.05 M acetate buffer

(pH=5.0) containing 26 mg 3-amino-9-ethyl-carbazole (AEC; Sigma, St Louis, MO) and 32,5 μl 30% H_2O_2, resulting in a red staining. The sections were counter-stained with Mayer's hematoxylin.
The skin sections were evaluated using a light microscope at 250 times magnification. The number of cytokine-expressing cells (TNF-α and IL-4) were assessed per mm^2 epidermis or dermis.

Double staining of TNF-α, IL-4 or IL-10 with elastase. After 10 min fixation in 65 ml acetone containing 100 μl 30% H_2O_2 and 20 min preincubation with 10% nHus and 10% normal horse serum (nHos), the skin sections were incubated overnight with antibodies against TNF-α, IL-4 or IL-10 diluted in PBS / 0.1% BSA. Subsequently, the slides were incubated for 60 min with biotinylated horse-anti mouse Ig (diluted in PBS/1% nHus, /1% nHos), followed by 30 min incubation with AP-conjugated avidin-biotin complex. After incubation of the slides with 10% normal mouse serum (nMs), the slides were incubated for 60 min with HRP-conjugated anti-elastase. AP activity was visualized by incubating the skin sections in a Tris-HCL buffer (pH=8.5) containing 25 mg Fast Blue BB salt, 12,5 mg naphtol AS-MX phosphate, and 35 mg levamisole (all purchased from Sigma), resulting in a blue staining. For visualization of the peroxidase activity the slides were then incubated with an AEC solution, as described above.

Double staining of TNF-α with tryptase and IL-10 with CD36. Immunohistochemical staining for TNF-α and IL-10 was performed as explained above in the double staining except that HRP-conjugated instead of AP-conjugated avidin-biotin complex was now used as a detecting reagent. The slides were then incubated for 20 min with 10% nMs. Subsequently the skin sections were incubated for 60 min with AP-labeled anti-AA1 (against tryptase)(for TNF-α double staining) or FITC-labelled CD36 (for IL-10 double staining). Incubation of the slides with CD36-FITC was followed by 60 min incubation with AP-conjugated F(ab) fragments of sheep anti-FITC. AP en HRP activity were visualized as describe above.
The antibodies used in this study were priory extensively tested for their suitability on microscopic (skin) sections. Negative staining controls were used by which the primary antibody was omitted or replaced by an irrelevant antibody of the same isotype. Sections of human tonsil were used as positive staining control. All antibody incubations were performed in a humidified chamber at room temperature except for incubation with the cytokine antibody, which was carried out at 4°C. After each antibody incubation, slides were rinsed in PBS containing 0.05% Tween 20.

Statistical analysis

A Student's t test was performed to ascertain the observed differences (significance level of $p<0.05$). The numbers of TNF-α-expressing cells and IL-4-expressing cells in the UV-exposed skin were compared to the unexposed control skin. Furthermore, the number of cytokine-expressing cells (TNF-α and IL-4) in the (un)exposed skin of PLE patients were compared to healthy controls. A Spearman correlation test was carried out to reveal a possible correlation between the number of cells expressing TNF-α or IL-4 and the individual UV-dose (MED).

RESULTS

The expression of cytokines involved in Langerhans cell migration is reduced in PLE patients compared to healthy controls

Previous experiments showed that after overexposure of the skin to UV-B, Langerhans cells still persist in the epidermis of PLE patients and only a small number migrates out of the skin, in contrast to healthy individuals (Kölgen *et al*, 1999). Therefore we studied the expression of the pro-inflammatory cytokines IL-1-α, IL-1-β, TNF-α and IL-18, which are involved in the migration of Langerhans cells out of the skin.

Cells expressing IL-1-β were found in the epidermis of the unexposed skin of PLE patients and healthy controls. After exposure of the skin to 6 MED UV-B an increase in the number of dermal cells expressing IL-1-β was observed in PLE patients and healthy controls. This increase, however, was far less in PLE patients when compared to healthy controls (**fig 1**). No appreciable change in the number of epidermal cells expressing IL-1-β was detected in the UV-B-exposed skin. Unfortunately, the variable and at times strong background staining hampered any good overall quantification of the IL-1-β expression. A semi-quantitative scoring (from ++ to --) was too crude to objectify this difference in any measure of statistical significance.

In the unexposed skin of PLE patients and healthy controls, a limited number of cells expressing TNF-α (weak positive staining) were observed scattered throughout the dermis (mean±SEM= 29±9 cells/mm^2 and 42±19 cells/mm^2 resp.)(**fig 2**). We did not find any expression of TNF-α in the epidermis. However, 24h after UV-B exposure we observed a significant increase in the number of dermal cells (mean±SEM= 157±17 cells/mm^2)(p=0.02) as well as an influx of epidermal cells expressing TNF-α (mean±SEM= 34±6 cells/mm^2)(p=0.004) in the skin of healthy controls. Forty-eight hours post irradiation the number of TNF-α-expressing cells in the dermis or epidermis of healthy controls decreased slightly again but not significantly (mean±SEM= 125±28 cells/mm^2 and 24±12 cells/mm^2 resp.). In the skin of PLE patients we observed a small, but significant, increase in the number of dermal TNF-α-expressing cells 24h and 48h post irradiation (mean±SEM= 64±13 cells/mm^2 and 68±16 cells/mm^2 resp.)(p=0.024 and p=0.037 resp.). However, no difference could be observed in the number of TNF-α-expressing in the UV-exposed epidermis 24h and 48h after irradiation (mean±SEM= 12±5 cells/mm^2 and 7±3 cells/mm^2 resp.) compared to the unexposed skin. The number of dermal and epidermal cells expressing TNF-α was significantly less (p=0.001 and p=0.014 resp.) in the UV-exposed skin (24h post irradiation) of PLE patients when compared to healthy controls. To summarize, the number of TNF-α-expressing cells in the dermis and epidermis of PLE patients and healthy controls increased after UV exposure but was

significantly less in the skin of PLE patients 24h after UV exposure in comparison with healthy controls. No correlation could be observed between the number of TNF-α expressing cells and the individual UV-dose (MED).

Cells expressing IL-18 were found in the basal layer of the epidermis of healthy controls and PLE patients. The intensity of the expression was highly variable among all participants. Some IL-18-expressing cells could also be observed in the dermis. The number of dermal cells expressing IL-18 did not change after exposure of the skin to UV-B. Forty-eight hours after UV-B exposure we noticed that the expression of IL-18 was no longer restricted to the basal layer of the epidermis but was now more diffuse and spread out into the suprabasal cell layers. Although the kinetics were mostly the same in PLE patients, the number of cells and intensity of the IL-18 expression in the UV-B-exposed skin was lower than in healthy controls, but the present technique is not suitable for quantification (data not shown).

A constitutive, diffuse expression of IL-1-α was observed in the epidermis and a considerable number of dermal cells expressing IL-1-α were observed in the unexposed skin of PLE patients and healthy controls. Forty-eight hours after UV irradiation the intensity of the epidermal IL-1-α expression appeared to decrease slightly in the skin of most participants but the present technique is not suitable for quantification. The number of dermal cells expressing IL-1-α did not change after UV-irradiation (data not shown).

To summarize, the number of dermal cells expressing IL-1-β or TNF-α increased after UV exposure, but less in PLE patients when compared to healthy controls. Furthermore, the influx of TNF-α-expressing cells into the UV-B-exposed epidermis was significantly less in PLE patients when compared to healthy controls. The expression of IL-18 and IL-1-α decreased slightly 48h post irradiation in both PLE patients and healthy controls.

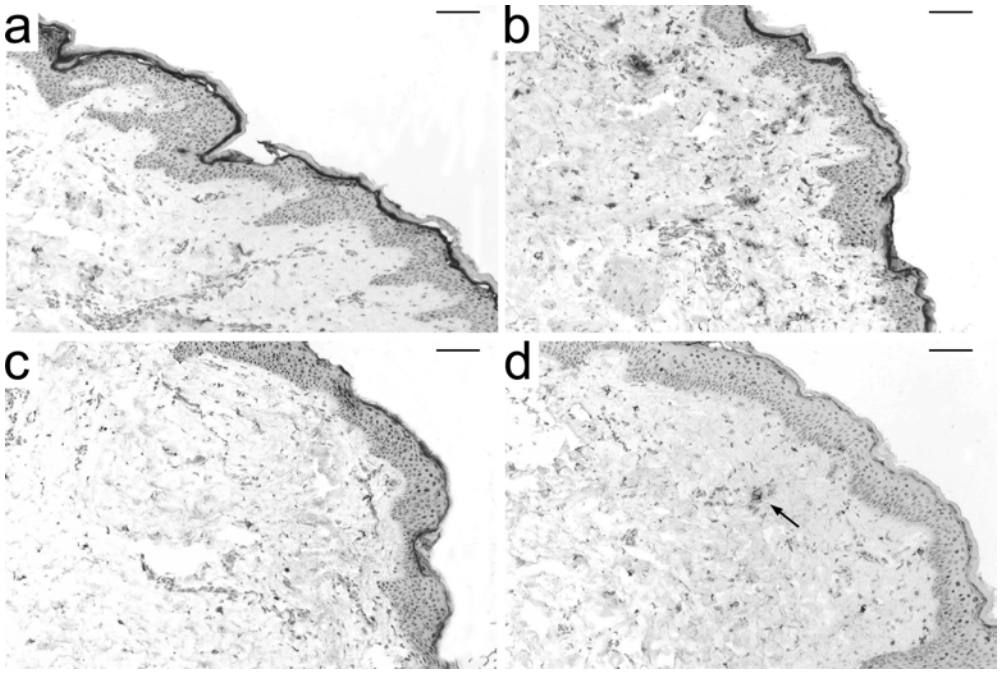

Figure 1. IL-1-β expression in unexposed skin (a,c) and 24h after UV exposure (b,d) of a PLE patient (c,d) and a healthy control (a,b), with an example of a rare cell expressing IL-1-β in the UV-exposed skin of a PLE patient (black arrow). Scale bar = 100 μm.

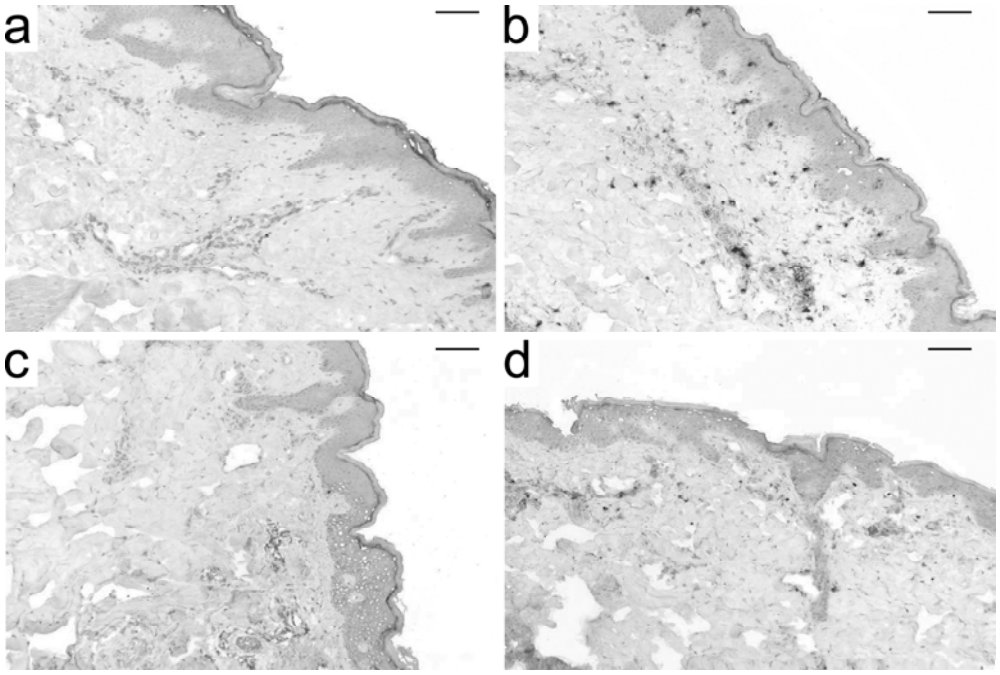

Figure 2. TNF-α expression in unexposed skin (a,c) and 24h after UV exposure (b,d) of a PLE patient (c,d) and a healthy control (a,b). Scale bar = 100 μm.

No differences in Th1-skewing cytokines (IL-6, IL-12, IFN-γ) are observed between PLE patients and healthy controls

IL-6, which can be induced by IL-1, is upregulated after UV exposure (Urbanski *et al*, 1990). IL-12 and IFN-γ are involved in cellular immune responses and skew T-cell responses towards a type 1 response (Kremer *et al*, 1996).

Only very few epidermal and dermal cells expressing IL-6 were detected in the unexposed skin of PLE patients and healthy controls. We did not observe any difference in the number of cells expressing IL-6 between the unexposed and UV-B-exposed skin of all participants (data not shown).

IL-12 was constitutively expressed by all epidermal cells although some cells showed a more pronounced IL-12 expression (**fig 3**). After UV exposure the epidermal IL-12 expression became less diffuse and more focal. A few brightly cells expressing IL-12 could be observed in the upper epidermis. The number of dermal cells expressing IL-12 did not differ between unexposed and UV-B-exposed skin. The above observations were made in both PLE patients and healthy controls.

The number of epidermal and dermal cells expressing IFN-γ was very low in the unexposed and UV-B-exposed skin of PLE patients and healthy controls. We did not detect any difference in IFN-γ expression in the unexposed *versus* the UV-B-exposed skin or in PLE patients *versus* healthy controls (data not shown).

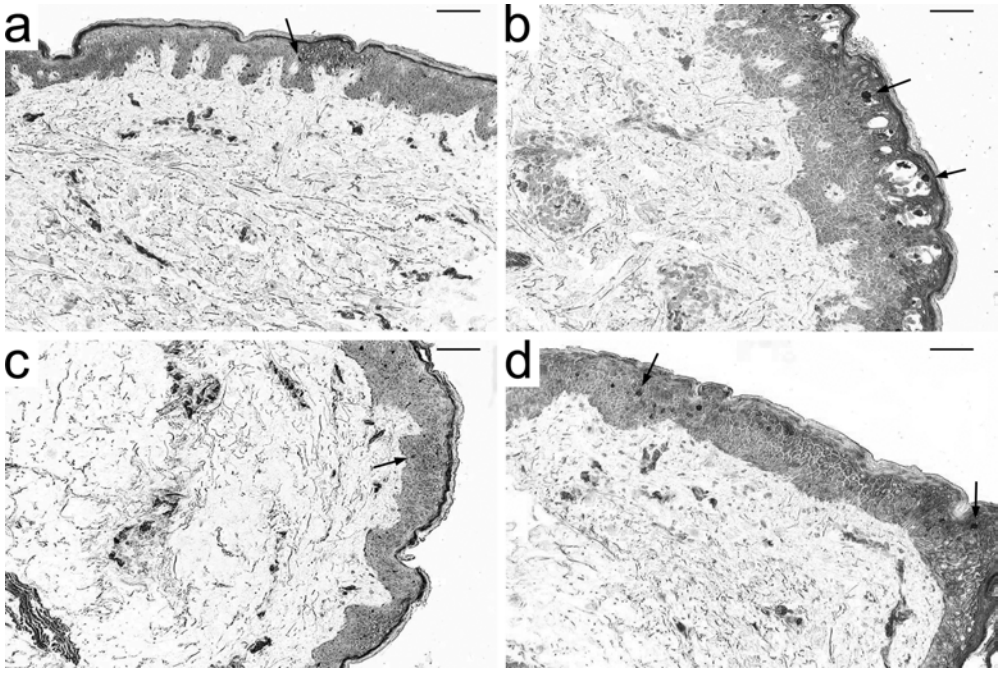

Figure 3. IL-12 expression in unexposed skin (a,c) and 48h after UV exposure (b,d) of a PLE patient (c,d) and a healthy control (a,b). Black arrows indicate brightly IL-12-expressing cells. Scale bar = 100 μm.

The expression of Th2-skewing and immunosuppressive cytokines (IL-4, IL-10) is reduced in PLE patients compared to healthy controls

To investigate the hypothesis that a UV-induced suppression of cellular immunity is disturbed in PLE patients we determined the expression of IL-4, which is involved in UV-induced type 2 T-cells responses (Teunissen et al, 2002), and IL-10, which is involved in UV-B-induced immunosuppression (Enk et al, 1995; Hammerberg et al, 1996; Kang et al, 1994), in the skin of PLE patients.

The number of dermal cells expressing IL-4 in the unexposed skin of PLE patients and healthy controls was very low (mean±SEM= 4±2 cells/mm^2 and 1±0,5 cells/mm^2 resp.)(**fig 4**). No IL-4-expressing cells could be detected in the epidermis of the unexposed skin of PLE patients and healthy controls. The number of dermal IL-4-expressing cells increased significantly 24h post irradiation in the skin of PLE patients and healthy controls (mean±SEM= 208±49 cells/mm^2 and 246±20 cells/mm^2 resp.)($p=0.008$ and $p<0.001$ resp.). Subsequently, 48h after UV exposure the number of dermal cells expressing IL-4 decreased slightly, but not significantly, in the skin of PLE patients and healthy controls (mean±SEM= 103±28 cells/mm^2 and 196±46 cells/mm^2 resp.). Twenty-four hours and 48h after UV exposure cells expressing IL-4 infiltrated the epidermis of healthy controls and to a lesser extent of PLE patients (mean±SEM= 47±13 cells/mm^2 and 55±26 cells/mm^2 and mean±SEM= 11±3 cells/mm^2 and 9±4 cells/mm^2 resp.). The number of epidermal IL-4-expressing cells 24h post irradiation differed significantly from the unexposed skin of PLE patients and healthy controls ($p=0.018$ and $p=0.024$ resp.). The number of IL-4-expressing cells that infiltrated the epidermis of PLE patients 24h after UV exposure was significantly less when compared to healthy controls ($p=0.02$). To summarize, the number of IL-4 expressing cells increased after UV exposure in the dermis and epidermis of PLE patients and healthy controls and was significantly less in the epidermis of PLE patients 24h post irradiation in comparison with healthy controls. No correlation could be observed between the number of IL-4-expressing cells and the individual UV dose (MED).

Cells expressing IL-10 were present in the basal layer of the epidermis and in the dermis of the unexposed skin (**fig 5**) of PLE patients and healthy controls. The intensity of the IL-10 expression of these cells differed among all individuals. After irradiation the expression of IL-10 in the basal layer appeared to increase at 24h and decreased again at 48h in the majority of the participants. A few brightly IL-10-expressing cells appeared in the epidermis within 24h (majority of healthy controls) or 48h (majority of PLE patients) after UV exposure. The number of dermal cells expressing IL-10 did not differ between the unexposed and UV-exposed skin of all participants. The diffuse staining of IL-10 in the epidermis hampered a good quantification of this expression, moreover, the difference seemed to be more in a time delay in the occurrence of densely stained cells in the PLE patients than in the absolute number of these cells (compare epidermises at 24h and 48h in **fig 5**).

Taken together, the number of dermal IL-4-expressing cells in the UV-exposed skin was reduced in PLE patients compared to healthy controls. The influx of IL-4-expressing cells in the UV-exposed skin of PLE patients was significantly less when compared to healthy controls. The level of IL-10 expression increased post irradiation but did not differ between PLE patients and healthy controls.

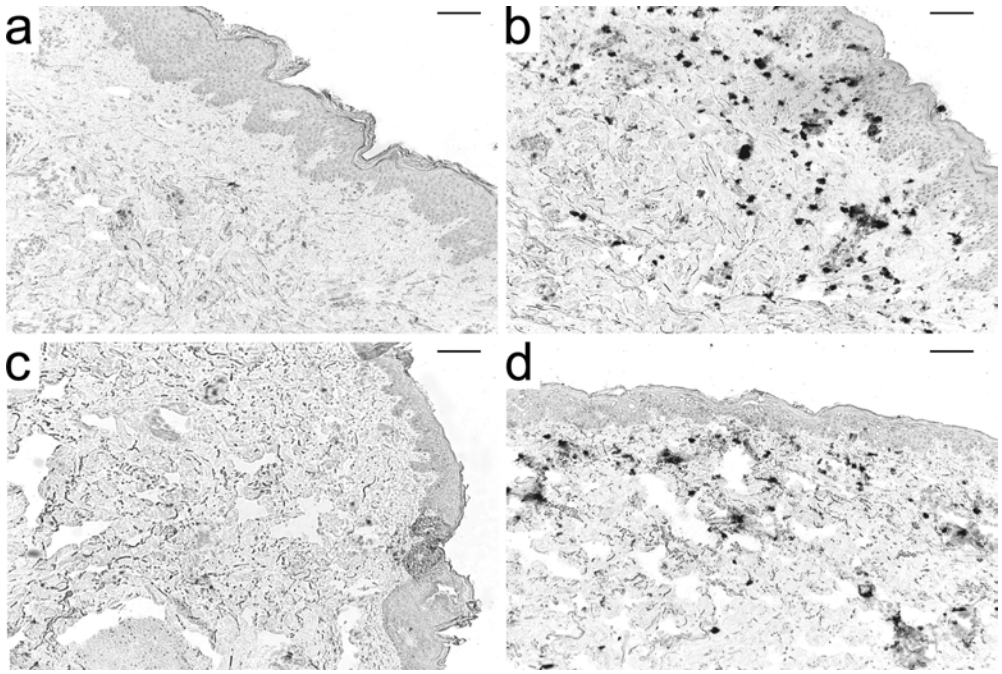

Figure 4. IL-4 expression in unexposed skin (a,c) and 24h after UV exposure (b,d) of a PLE patient (c,d) and a healthy control (a,b). Scale bar = 100 μm.

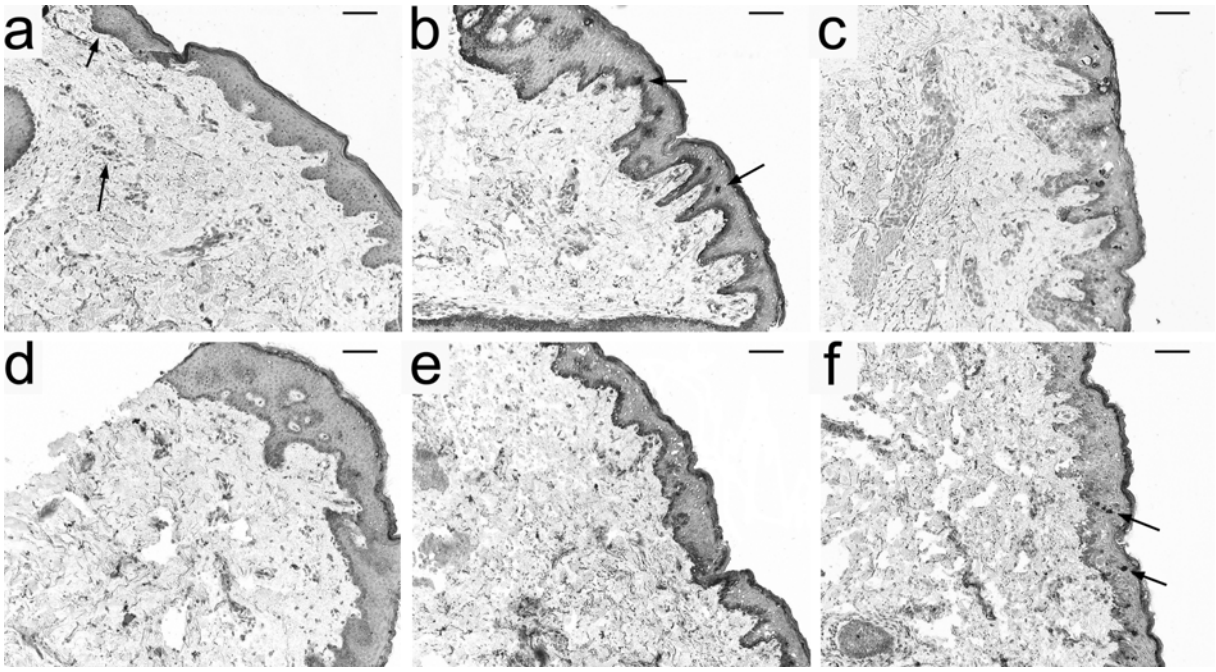

Figure 5. IL-10 expression in unexposed skin (a,d) and 24h (b,e) or 48h (c,f) after UV exposure of a PLE patient (d-f) and a healthy control (a-c). Black arrows indicate IL-10-expressing cells (a) or brightly IL-10-expressing cells (b,f). Scale bar = 100 μm.

IL-10 is predominantly expressed by keratinocytes while TNF-α, and IL-4 are expressed by neutrophils

Since Meunier *et al* (1995) reported that CD36+ CD11b+ CD1a- cells infiltrate the skin after UV exposure and that the CD11b+ CD1a- cells produce IL-10 (Kang *et al*, 1994) and are involved in UV-induced immunosuppression (Hammerberg *et al*, 1996) we determined whether the brightly IL-10-expressing cells in the epidermis of the UV-exposed skin were CD36+ macrophages. Double-stainings were performed for IL-10 with antibodies against CD36 or elastase (neutrophil marker). In the skin of healthy controls 24 hours after irradiation almost all epidermal cells expressing IL-10 were neutrophils. However, in the UV-exposed skin of PLE patients and in the skin of healthy volunteers 48h post irradiation the brightly epidermal IL-10-expressing cells did not co-express elastase (**fig 6**). Only very few cells expressing IL-10 were CD36+ macrophages. Therefore, the IL-10-expressing cells in the basal layer of the epidermis, and probably also a fraction of the brightly IL-10-expressing cells are most likely keratinocytes.

Mast cells release large amounts of TNF-α upon activation by UV-B radiation (Walsh, 1995). On the other hand, the pattern of TNF-α expression resembled the pattern of neutrophil influx after UV irradiation. Therefore, double stainings were performed for TNF-α with antibodies against tryptase (mast cell marker) or elastase (neutrophil marker) to identify the cell type expressing this cytokine. All dermal and epidermal cells expressing TNF-α in the skin of

healthy controls and PLE patients turned out to be neutrophils (**fig 6**) and not mast cells. A minority of the neutrophils did not express TNF-α.

Since Teunissen *et al* (2002) showed that neutrophils are the main source of IL-4 in the UV-B-exposed skin we determined whether the IL-4-expressing cells in the skin of the healthy individuals and PLE patients were also neutrophils. Hence a double staining was carried out for IL-4 and elastase. All cells expressing IL-4 in the skin of healthy controls and PLE patients were indeed neutrophils (**fig 6**). A minority of the neutrophils did not express IL-4.

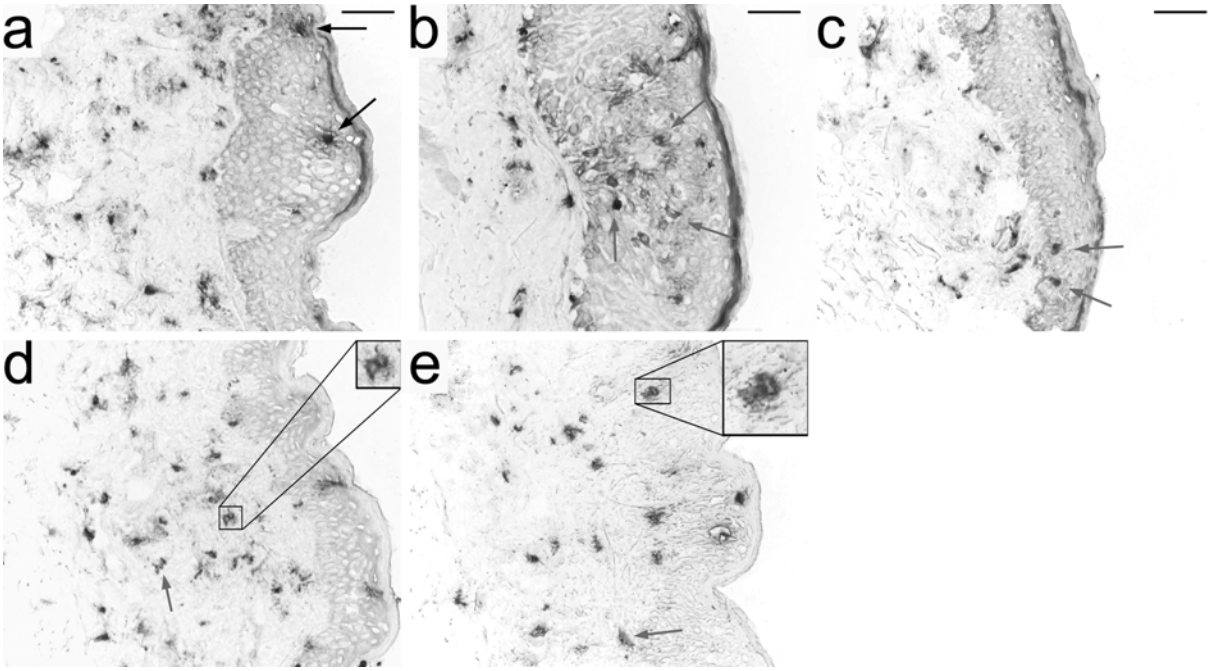

Figure 6. Co-expression for elastase (red) and IL-10 (a-c), TNF-α (d) or IL-4 (e) (blue) in the skin of a healthy control (a,b,d,e) or a PLE patient (c) 24h (a,c,d,e) or 48h (b) after UV exposure. Black arrows and magnifications indicate double positive cells. Gray arrows indicate single positive cells. Scale bar = 100 μm.

DISCUSSION

UV-induced immunosuppression is associated with the disappearance of Langerhans cells from the epidermis. In preceding experiments it was shown that Langerhans cells still persist in the UV-(over)exposed skin of PLE patients in contrast to healthy individuals (Kölgen *et al*, 1999). This Langerhans cell persistence was caused by a disturbance in Langerhans cell

migration[2]. Here we investigated whether this defect in migration could be related to a lack of cytokines involved in Langerhans cell migration, such as IL-1, TNF-α, and IL-18.

The expression of IL-1-β, but not of IL-1-α, was reduced in the UV-exposed skin of PLE patients compared to healthy controls. While the UV-induced increase in IL-1-β was mainly observed in keratinocytes and Langerhans cells (Norval, 2001), we observed an UV-induced increase of scattered dermal cells expressing IL-1-β, potentially macrophages. Although the number of cells expressing TNF-α was increased after UV exposure, it was significantly lower in PLE patients when compared to healthy controls. In contrast to healthy controls hardly any TNF-α-expressing cells infiltrated the epidermis of PLE patients. TNF-α is produced by keratinocytes (Kock *et al*, 1990) and mast cells (Bazzoni *et al*, 1994) upon UV exposure. The TNF-α-expressing cells that we observed in the (epi)dermis, however, were not mast cells but turned out to be neutrophils. Cumberbatch *et al* (2001) showed that IL-18 stimulates Langerhans cell migration after skin sensitisation. No significant differences could be seen in the number of cells expressing IL-18 between the unexposed and UV-exposed skin of PLE patients and healthy controls. The IL-18 antibody that was used was not able to discriminate between the non-active protein, which is expressed constitutively by keratinocytes and the biologically active IL-18 protein. Therefore it might still be possible that the expression of biologically active IL-18 differs between PLE patients and healthy controls. In summary, the reduced expression of IL-1-β and neutrophil-derived TNF-α support our hypothesis that migration-inducing cytokines are decreased in the UV-exposed skin of PLE patients thereby leading to an impairment in UV-induced Langerhans cell migration. The present data on migration-inducing cytokines are consistent with the data on disturbance of Langerhans cell migration in PLE patients, using the same irradiation protocol[1]. As some Langerhans cells in the skin of PLE patients are still able to migrate after UV exposure it seems reasonable to expect at least some degree of expression of migration-inducing cytokines.

Another hypothesis we wanted to investigate was whether the Th1-skewing cytokines were expressed in larger amount in the UV-exposed skin of PLE patients in comparison with healthy controls, thereby contributing to the pathogenesis of PLE.

There are very limited data on the cytokine profile in PLE patients and the only available data are on lesional skin. Norris *et al* (1999) showed an increased activity of IL-6, IL-8 and possibly IL-1 in experimentally provoked lesions of PLE patients compared to non-lesional skin. However, we did not detect any differences in the number of cells expressing IL-6 in the unexposed compared to the UV-exposed skin nor any differences in IL-6 expression between PLE patients and healthy controls. Some investigators describe an increase in secretion of the pro-inflammatory cytokine IL-6 after irradiation of cultured keratinocytes (Kirnbauer *et al*, 1991;

[2] Kölgen *et al*, *submitted*

Petit-Frere *et al*, 1998) or an increase of IL-6 in human serum after a single dose of UV (Urbanski *et al*, 1990), but secreted cytokines are difficult to detect by the immunohistochemical method that we used. There was no detectable difference between the expression of IL-12 and IFN-γ in the skin of PLE patients and healthy controls. Only very few IFN-γ-expressing cells were observed in the UV-exposed as well as the unexposed skin. IL-12 was constitutively expressed by keratinocytes, as also described by Yawalker *et al* (1996). UV radiation did not change the level of IL-12 expression but did change the expression pattern as brightly IL-12-expressing cells could now be observed in the epidermis. IL-12 expression was similar in PLE patients and healthy controls. The antibody used to detect IL-12 is not able to discriminate between the two subunits of IL-12 nor can it discriminate between heterodimers and homodimers of IL-12. Homodimers of IL12 p40 can be increased upon UV exposure (Schmitt and Ullrich, 2000) and can inhibit IL-12 activity by blocking its receptor (Mattner *et al*, 1993), resulting in a decrease of Th1 responses instead of an increase. Collectively, the present findings show that expression Th1-skewing cytokines is similar in the UV-B exposed skin of PLE patients and healthy individuals. However, the expression of homodimers of IL-12p40 still might be less in the skin of PLE patients in comparison with healthy controls.

In order to investigate the balance between Th1-skewing and Th2-skewing and/or immunosuppressive cytokines further and to examine whether PLE patients are disturbed in their UV-induced immunosuppression, we performed immunohistochemical stainings for IL-4 and IL-10. In line with the results from Teunissen *et al* (2002) we found a UV-induced increase in dermal IL-4-expressing neutrophils and an influx of these cells into the epidermis of healthy controls. The number of dermal neutrophils expressing IL-4 in the UV-exposed epidermis of PLE patients was significantly lower than in healthy controls, indicating that the balance is somewhat more shifted towards a Th1 response in PLE patients. UV radiation can induce the production of IL-10 by keratinocytes (Enk *et al*, 1995; Grewe *et al*, 1995) and macrophages (Kang *et al*, 1994). IL-12 treatment to inhibit IL-10 release (Schmitt *et al*, 1995) or depletion of macrophages by anti-CD11b treatment (Hammerberg *et al*, 1996) reversed the UV-mediated immunosuppression. Our results showed that IL-10 is expressed by keratinocytes, mainly in the basal layer. This expression is increased after UV exposure and brightly IL-10-expressing cells can be observed in the epidermis of PLE patients (mostly at 48h) and healthy controls (mostly at 24h). The number of cells expressing IL-10 did not differ between PLE patients and healthy controls. Only very few IL-10-expressing cells were CD36+ macrophages. The brightly epidermal IL-10-expressing cells in the skin of healthy controls 24h after UV exposure were neutrophils. However, 48h post irradiation and in the UV-exposed skin of PLE patients cells expressing IL-10 neither co-expressed CD36 nor elastase (neutrophil marker) and hence are most probably keratinocytes. CD11b is also expressed by neutrophils and anti-CD11b treatment (Hammerberg *et al*, 1996) will therefore also deplete neutrophils expressing IL-10

resulting in reversion of the immunosuppression. To summarize, our findings show that the expression of the Th2-skewing cytokine IL-4 was less in the epidermis of PLE patients, shifting the balance more towards a Th1 response. Furthermore, a difference in IL-10 expression, reflecting a difference in UV-induced immunosuppression, between PLE patients and healthy controls was restricted to infiltrating neutrophils in the epidermis of healthy controls.

Interestingly, exposure of the skin of PLE patients to 8.4 MED resulted in a decrease and almost complete depletion of Langerhans cells 48h post irradiation. In addition, the expression of IL-1-β, TNF-α and IL-4 was increased compared to the 6 MED UV-B-exposed skin and more TNF-α-expressing and IL-4-expressing cells infiltrated the epidermis (data not shown). These observations indicate that the balance between erythema and immune responses is disturbed in PLE. Supplementation of dietary fish oil, containing omega 3 fatty acids, resulted in a reduction of UV-B erythemal sensitivity (Rhodes *et al*, 1994), a decrease of prostaglandin E_2 levels, which is involved in erythema (Rhodes *et al*, 2001), and in a protection against UV-A provocation of a papular response in PLE patients (Rhodes *et al*, 1995). Therefore it would be interesting to investigate whether the reduction in erythema and prostaglandin E_2 levels by dietary fish oil supplementation is accompanied by a complete UV-B-induced depletion of Langerhans cells in PLE patients and a recovery of the cytokine levels of IL-1-β, TNF-α and IL-4 (tested with the 6 MED protocol), and by protection against UV-B-provoked PLE lesions.

Another remarkable observation (n=1) was made in a healthy individual, with no history of PLE or other (photosensitive) skin disorders, whose buttock skin had been exposed extensively to sunlight during a 6-weeks vacation in Australia. After exposure of the buttock skin to 6 MED UV-B two weeks after the vacation, Langerhans cells were only partially depleted and TNF-α and IL-4 expression was low and resembled the level of expression seen in PLE patients. IL-1-α expression was almost completely absent except for very rare cells expressing IL-1-α scattered in the dermis (data not shown). Exposure of the skin to UV radiation increases the production of IL-1-Ra in the stratum corneum (Hirao *et al*, 1996), which can bind nearly irreversible to the IL-1 receptor and block its activity. An extensive, long-term exposure might perhaps not only lead to inhibition of IL-1 activity but also downregulation of the level of IL-1.

Immunohistochemistry appears to be a very suitable method for localization of cytokines in cytoplasm and identification of the cells expressing certain cytokines *in vivo* (Asadullah *et al*, 2002; Hoefakker *et al*, 1995). However, we are aware of the limitations of this technique. Most of all, this technique will not detect secreted cytokines, but only cytokines bound in the cytoplasm or on the cellular membrane. A clear intracellular staining and a difficulty in detecting very low levels of cytokines by immunohistochemistry lead to a visualization of cytokine producing cells rather than receptor-bound cytokines on target cells, although this cannot be excluded completely.

A first exploration of the cytokine patterns in UV-B-exposed skin of PLE patients *versus* healthy controls demonstrated that in conjunction with a diminished Langerhans cell migration, migration-inducing cytokines are also present to a lesser extent in PLE patients compared to healthy individuals. No difference in the Th1-skewing cytokines IL-12, IFN-γ and IL-6 could be observed in PLE patients. However, we found a smaller number of cells expressing IL-4 in the epidermis of PLE patients, shifting the cytokine response somewhat more to a Th1 response. This scarce expression of TNF-α and IL-4 in the UV-exposed skin of PLE patients appears to be largely attributable to a smaller influx of neutrophils.

Based on the present cytokine data and on previous experiments we speculate that the following events occur in the skin of PLE patients upon UV exposure: Langerhans cells residing in the epidermis capture UV-induced neo-antigens. Subsequently these cells migrate out of the skin but their migration is impaired due to low levels of IL-1-β and TNF-α. The Langerhans cells that do migrate increase their expression of costimulatory molecules and HLA-DR[3]. Due to the impaired migration, activated Langerhans cells accumulate in the dermis where they stimulate T-cells. A reduced expression of IL-4 in the skin of PLE patients favours the development of a Th1-like response in contrast to a pronounced Th2 response in healthy controls. These events together might contribute to the pathogenesis of PLE.

Future experiments on cytokine profiles in PLE patients should focus on the levels of expression of cytokines *in vivo* in suction blister fluids, e.g. by ELISA cytokine detecting techniques, as has been described for healthy individuals by Barr *et al* (1999), but this technique requires many blisters for an extensive study. Alternatively, one could determine cytokine mRNA levels by quantitative PCR, however, cytokine gene expression does not necessarily lead to protein synthesis because there are still several posttranscriptional regulatory bases (Asadullah *et al*, 2002). Additionally, cytokine expression kinetics can be extended by inserting more time points. Furthermore, it would be interesting to examine the effects of successful phototherapy on the UV-induced decrease in Langerhans cells and the expression of cytokines like IL-1-β, TNF-α and IL-4.

ACKNOWLEDGEMENT

We are grateful to Ina Sybesma from the department of Dermatology, University Medical Center Utrecht for irradiation of PLE patients and healthy controls.

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Association of transcription-coupled repair but not global genome repair with ultraviolet-B-induced Langerhans cell depletion and local immunosuppression

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Journal of Investigative Dermatology, in press

ABSTRACT

Exposure to ultraviolet-B radiation impairs immune responses in mammals by inhibiting especially Th1-mediated immune responses such as contact hypersensitivity. This immunosuppression seems to be associated with Langerhans cell migration. Immunomodulation is not restricted to the exposed skin (local immunosuppression), but is also observed at distant sites (systemic immunosuppression). DNA damage appears to play a key role since enhanced nucleotide excision repair, a pathway essential for elimination of ultraviolet-B-induced DNA lesions, strongly counteracts immunosuppression. To determine the effect of DNA repair on ultraviolet-B-induced local immunosuppression and Langerhans cell disappearance three mouse strains carrying different defects in nucleotide excision repair were compared. XPC mice who were defective in global genome repair were as sensitive to ultraviolet-B-induced local suppression of contact hypersensitivity to picryl chloride as their wildtype littermates. CSB mice who were defective in transcription-coupled repair were, however, far more sensitive for immunosuppression as were XPA mice who were defective in both transcription-coupled repair and global genome repair. In XPC mice Langerhans cell migration was observed at a similar UV dose as in the wildtype littermates. Ultraviolet-B-induced Langerhans cell depletion was, however, enhanced in CSB en XPA mice compared to their wildtype littermates. Hence, the major conclusion is that local immunosuppression is only affected when transcription-coupled DNA repair is impaired. Furthermore, only a defect in transcription-coupled repair was linked to enhanced ultraviolet-B-induced Langerhans cell depletion. In combination with an earlier study by our group, it can be concluded that Langerhans cell disappearance is related to ultraviolet-B-induced local but not to systemic immunosuppression.

INTRODUCTION

Ultraviolet radiation (UV) causes a broad range of effects in the skin. One of these effects, predominantly caused by UV-B (280-315 nm), is the suppression of cellular immunity. This immunosuppression is associated with the disappearance of antigen-presenting cells, the Langerhans cells, from the epidermis. UV can be absorbed by organic molecules such as DNA, leading to the induction of cyclobutane pyrimidine dimers (CPDs) as well as 6-4 pyrimidine pyrimidone photoproducts (6-4PPs). Cells with UV-specific DNA damage, i.e. Langerhans cells, were found in murine lymph nodes (Sontag *et al*, 1995) after exposure of the skin to UV radiation. Furthermore, migrating Langerhans cells were detected in the blister fluid of UV-exposed human skin (Kölgen *et al*, 2002).

In situ action spectra suggested that DNA damage is involved in UV-induced immunosuppression of allogeneic reactions in humans (Hurks *et al*, 1997). DNA damage can trigger the production of tumor necrosis factor (TNF)-α, interleukin (IL)-10 and IL-6 and thereby modulate Langerhans cell activity and local immune responses (Nishigori *et al*, 1996; O'Connor *et al*, 1996; Petit-Frere *et al*, 1998). Treatment of mice with thymidine dinucleotides (pTpT), mimicking DNA fragments that are released when UV-induced DNA damage is repaired, inhibits contact hypersensitivity and activates the TNF-α gene (Cruz, Jr. *et al*, 2000). Furthermore, removal of CPDs by photoreactivation enzyme (Reeve *et al*, 1996) or by treatment with liposomes containing T4 endonucleose V (T4N5) (Applegate *et al*, 1989; Vink *et al*, 1997) resulted in a reversion of UV-induced local as well as systemic immunosuppression. Topical treatment with T4N5 leads to a protection against UV-induced upregulation of the immunosuppressive cytokine IL-10 (Nishigori *et al*, 1996) and TNF-α (Wolf *et al*, 2000). The products released by UV-damaged cells can be transported by the circulation throughout the whole body, which may contribute to the systemic immunosuppressive effects.

Depending on the type of DNA damage different DNA repair pathways can be activated (de Boer and Hoeijmakers, 2000). The most important mechanism for the repair of CPDs and 6-4PPs in placental mammals is Nucleotide Excision Repair (NER). NER consists of two pathways namely Global Genome Repair (GGR) and Transcription-Coupled Repair (TCR). GGR is involved in helix-distorting repair of DNA damage throughout the entire genome. TCR, on the other hand, preferentially eliminates transcription-blocking damage in actively transcribed genes. A defect in NER can lead to rare photosensitive disorders like Xeroderma Pigmentosum (XPA-XPG) and Cockayne syndrome (CSA and CSB). The majority of the XP patients, of which XPA with a deficiency in GGR as well as TCR is the most severe subgroup, are extremely sensitive to sunburn and run a more than 1000-fold increased risk of developing skin cancer. XPC patients, who are deficient in GGR alone, have a normal sensitivity to sunburn but are highly cancer-prone. Morison *et al* (1985) showed that XP patients had an impaired development of CHS, depending on the severity of the disease. Patients with CS, who have an selective impairment in TCR, are extremely photosensitive and develop neurological abnormalities. However, these CS patients do not appear to be predisposed to develop skin cancer.

Studies with NER-deficient mouse mutants, carrying defects in TCR (CSB), GGR (XPC) or both (XPA) have the advantage that confounding genetic background differences can be eliminated. Such studies have provided additional evidence for a significant role of DNA damage in UV-B-induced immunosuppression. Miyauchi-Hashimoto *et al* (1996) and Garssen *et al* (2000) demonstrated that in XPA mice UV-induced local and systemic immunosuppression were increased. However, systemic immunosuppression was not enhanced in XPC nor in CSB mice (Garssen *et al*, 2000). Furthermore, Boonstra *et al* (2001)

showed that the production of IL-10 and TNF-α was augmented in the lymph nodes of UV-exposed XPA and CSB but not XPC mice.

A decrease in Langerhans cell number could result in a disturbance in immune surveillance of UV-exposed skin. Langerhans cell densities in chronically sunlight-exposed skin of patients with XPA were remarkably reduced. In addition, the recovery of the epidermal Langerhans cell population was delayed in XPA patients compared to healthy controls (Jimbo *et al*, 1992).

The aim of the present study was to determine the influence of GGR and TCR on the UV-induced depletion of Langerhans cells and on UV-induced local immunosuppression. Subsequently, the relationship between local immunosuppression and Langerhans cell disappearance could be ascertained.

In order to investigate these different features, experiments on DNA repair-deficient mice with defects in GGR and TCR (XPA), GGR alone (XPC) or TCR alone (CSB) were performed. After UV irradiation of the skin for five consecutive days a contact allergen (picryl chloride) was applied to the irradiated site. Following antigen challenge with picryl chloride on the ears of the animals, ear swelling was determined as a measure of cellular immunity, or conversely of UV-induced local immunosuppression. Similar to this experiment a separate group of animals was subjected to the same UV exposure regimen. Two days prior to immunization and at the day of immunization these mice were sacrificed and their ears and lymph nodes were collected. Immunohistochemistry was performed for NLDC145 (a dendritic cell marker) and H3 (a CPD marker) on frozen sections of the ear skin and the lymph nodes.

MATERIALS AND METHODS

NER-deficient mouse models

XPA, CSB, and XPC mice refer to NER-deficient mice homozygous for the targeted allele in the respective genes (Cheo *et al*, 1997; de Vries *et al*, 1995; van der Horst *et al*, 1997). XPA deficiency induces a virtual complete defect in transcription-coupled repair as well as global genome repair. CSB mice have only a total impairment of transcription-coupled repair, while XPC mice have a selective inactivation of global genome repair.

Mixed 129-C57Bl/6 or pure C57Bl/6 littermates of the homozygous knockout mice were used as control animals (the background of all mutant strains used in this study). The genotype of each mouse was determined by PCR. Mice were kept at an ambient temperature of 25 $\pm$ 1 °C. The room was illuminated with yellow fluorescent tubes (Philips TL40W/16) in a 12 hour cycle (switched on and off at respectively 6.00 a.m. and 6.00 p.m.). These lamps do not emit any measurable UV radiation. No daylight entered the animal facilities. Animals were housed individually in macrolon type I cages for the entire experiment. Standard mouse chow (Hope Farms RMH-B, Woerden, the Netherlands) and tap water were available *ad libitum*. Formal permission for the animal experiments was granted by an independent

ethical committee of the National Institute of Public Health and the Environment, as required by Dutch law.

Reagents

Picryl chloride (PCl) (Chemotronix, Swannanoa, NC, USA) was used as contact sensitizer. It was recrystallized three times from methanol/H_2O before use, and protected from light during storage at 4°C.

UV exposure

The animals were shaven (on the back) one day prior to UV exposure using an electric clipper under light ether anesthesia. The animals were exposed to broadband UV-B radiation from a filtered (Schott WG305 filter) Hanovia Kromayer Lamp Model 10S (Slough, United Kingdom). This is a hand-held lamp that allows short exposures to limited skin areas by placing the circular port (approximately 2 cm^2) in close contact to the skin (Goettsch *et al*, 1999; Sontag *et al*, 1994). The dose rate was 150 J/m^2/second (280-400 nm), as measured by a Kipp E11 thermopile.

For determination of immunomodulation the animals were exposed to 5 consecutive UV doses (one exposure per day, last exposure 4 days prior to immunization). The NER-deficient mice and their respective wildtype controls were exposed in accordance to their acute UV sensitivity i.e. to 150 J/m^2 (XPA), 150 and 300 J/m^2 (CSB) or to 300 J/m^2, 600 J/m^2, or 900 J/m^2 (XPC) each day.

Contact hypersensitivity to picryl chloride

The mice were sensitized 4 days after the last day of (sham) irradiation by topical application of 150 μl of 5% PCl in ethanol/acetone (3:1) to the UV-irradiated shaved back. Control mice were sham-sensitized by topical application of 150 μl ethanol/acetone (3:1). Four days after sensitization both ears of the mice were challenged by topical application of one drop (27-gauge needle) of 0.8% PCl in olive oil. Prior to, and at 24h after challenge duplicate measurements of ear thickness were made using an engineer's micrometer in a blinded fashion. From earlier studies it is known that the maximal contact hypersensitivity response was 24h after topical ear challenge, also in UV-B pre-exposed animals (Sontag *et al*, 1994). In each experiment, the increase in ear thickness in similarly challenged, non-sensitized, control mice was measured at the same time, and subtracted from increments in ear thickness in sensitized test animals (net ear swelling). The CHS response (net ear swelling) in sensitized mice that were not exposed to UV-B radiation (minus the negative background swelling found in non-sensitized mice) was set at 100% in order to compare the effects of UV between the different mouse strains that were tested.

Langerhans cell depletion

Groups of mice were irradiated with different UV doses for 5 consecutive days on their ears as described above. One control group per mouse strain was not irradiated. Forty-eight hours before immunization (2 days post irradiation) or at the day of immunization (4 days post irradiation) the animals were sacrificed and the ears and the skin draining lymph nodes were removed. No immunization was performed on these animals. Ears and lymph nodes were snap-frozen in liquid nitrogen, embedded in

OCT compound (Tissue-Tek, Sakura, Zoeterwoude, the Netherlands) and stored at –80^{o}C until further processing.

Immunohistochemical staining of NLDC145 and pyrimidine dimers

Frozen sections (6 μm thickness) of the ears and lymph nodes were mounted onto 3-aminopropyl triethoxysilane (Sigma, St Louis, MO, USA)-coated slides. A double staining was performed for NLDC145 (a dendritic cell marker; a kind gift from Prof. Dr. G. Kraal, department of Cell Biology, Free University, Amsterdam, the Netherlands) and biotinylated H3 (directed against thymidine-containing cyclobutane pyrimidine dimers; a kind gift from Dr. L. Roza, T.N.O., Zeist, the Netherlands) as described previously (Sontag *et al*, 1995). The sections were fixed in dry acetone for 10 min, preincubated with blocking reagent (PBS containing 10% normal rabbit serum) and incubated with the primary antibody NLDC145 overnight at 4^{o}C. After incubation for 1h with biotinylated rabbit anti-rat immunoglobulins (DAKO A/S, Glostrup, Denmark) the sections were subsequently incubated for 30 min. with alkaline phosphatase (AP)-conjugated avidin-biotin complex (DAKO A/S). AP activity was visualized by incubation in a BCIP/NBT solution of 37.5 μl 5-bromo-4-chloro-3-indolyl phosphate, 50 μl 4-nitro blue tetrazolium chloride and 2.5 mg levamisole per 10 ml 0.1 M Tris-HCL (pH=9.5)/ 0.1 M NaCl/ 0.05M $MgCl_2$. The bluish-black reaction product is water and ethanol insoluble and withstands the subsequent harsh treatment. The preparations were fixed in 25% acetic acid (vol/vol) containing 100 μl 30% H_2O_2 per 100 ml. After washing in PBS the slides were incubated for exactly 2 min in 0.07 N NaOH in 70% ethanol. The slides were rinsed in PBS and incubated for 1h with biotinylated H3 antibody. Subsequently, the sections were incubated with horseradish peroxidase-conjugated avidin-biotin complex (DAKO A/S) for 30 min. Peroxidase activity was visualized by incubation in 0.1 M acetate buffer (pH=5) containing 20 mg 3-amino-9-ethyl-carbazole (AEC; Sigma) and 100 μl 30% H_2O_2 per 100 ml, resulting in a red staining.

After each antibody incubation the slides were rinsed in PBS containing 0.05% Tween 20. Antibody incubations were performed in a humidified chamber at room temperature except for incubation with NLDC145 which was performed at 4^{o}C. Negative staining controls were used in which the primary antibody was omitted.

The sections were evaluated using a light microscope at 250 times magnification. The NLDC145 staining of the ears was determined quantitatively by image processing: the percentage of epidermal cross-sectional area that stained for NLDC145 was measured, using a camera mounted onto a light microscope together with the Optimas 6.1 and Microsoft Excel software (the targeted interfollicular epidermal area was demarcated manually).

Statistical analysis

CHS experiments:

The CHS responses were statistically evaluated using an independent two-tailed T-test. $P<0.05$ indicated a significant difference compared to the unexposed sensitized positive control.

Immunohistochemical studies:
After logarithmic transformation (to correct for the right skewed distribution) an independent two-tailed T-test was performed to ascertain the significance of the observed differences (significance level $p< 0.05$). The percentage of epidermal NLDC145+ area in the crosssection of UV-exposed ears of mutant mice and wildtype littermates were compared to their respective unexposed, control skin. Furthermore, the percentages of epidermal NLDC145+ area in the ears were compared between the mutant mice and their wildtype littermates for the different UV doses.

RESULTS

A defect in nucleotide excision repair does not affect contact hypersensitivity to picryl chloride

Contact hypersensitivity (CHS) in XPA, CSB, and XPC mice and their respective wildtype littermates was measured 24h after ear challenge with PCl in olive oil. Significant CHS (ear swelling) responses ($p<0.05$) to PCl were observed in each strain of mice as compared to the background swelling responses found in the non-sensitized control animals from the same strain. The background ear swelling in non-sensitized animals of each strain was always less than $1*10^{-3}$ cm. CHS responses in the mutant mice (XPA, CSB and XPC) were not different from the CHS responses in the respective wildtype littermates. Thus, the various NER defects did not affect the normal T-cell dependent CHS response as compared to the repair-competent wildtype littermates (data not shown).

Ultraviolet-B-induced suppression of contact hypersensitivity occurs at a lower dose in XPA and CSB but not XPC mice

To compare the suppressive effects of UV exposure, the control response in unirradiated animals was set at 100%. In each study the mutant mice were compared to their wildtype littermates. The findings are depicted in figures 1-3. Each bar represents the percentual CHS response compared with the control response in the respective unirradiated animals (set at 100%). A UV dose of 150 J/m^2 was already sufficient for a significant immunosuppression in XPA mice ($p<0.001$) (**fig 1**) and CSB mice ($p<0.001$) (**fig 2**). In the respective wildtype littermates, however, CHS was not suppressed after exposure to 150 J/m^2 or 300 J/m^2. The CHS response of XPC mice was significantly suppressed only after irradiation with 900 J/m^2 ($p<0.001$)(**fig 3**). A minimal UV dose of 900 J/m^2 was also required for a significant suppression of CHS in the respective wildtype littermates ($p<0.01$).

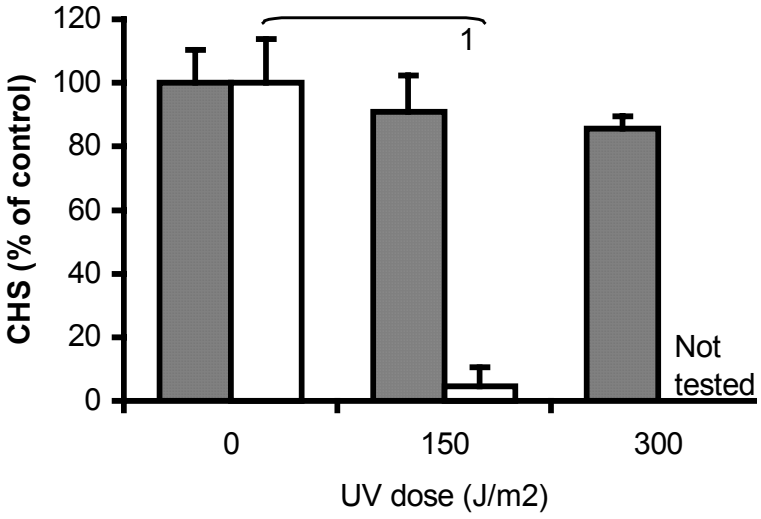

Figure 1, UV-induced local immunosuppression of CHS in XPA mice (white bars) and wildtype littermates (gray bars). Each bar represents net ear swelling (mean ± SEM). The ear swelling in sensitized unirradiated mice was set at 100%. p<0.001 (1).

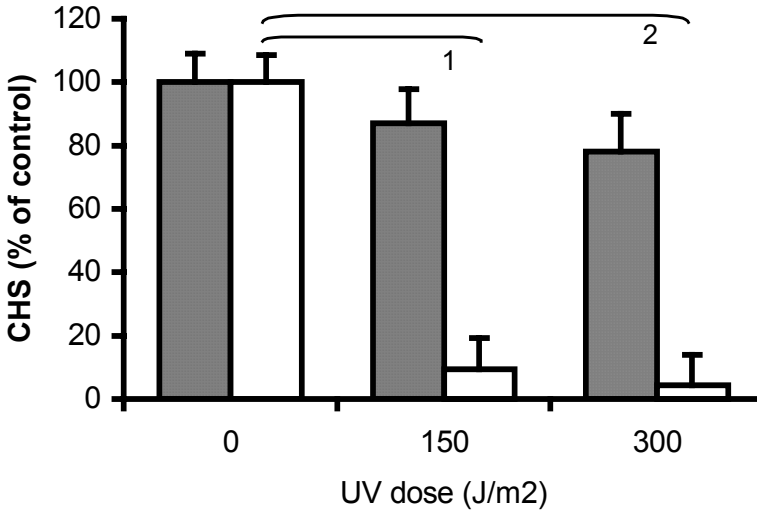

Figure 2, UV-induced local immunosuppression of CHS in CSB mice (white bars) and wildtype littermates (gray bars). Each bar represents net ear swelling (mean ± SEM). The ear swelling in sensitized unirradiated mice was set at 100%. p<0.001 (1+2).

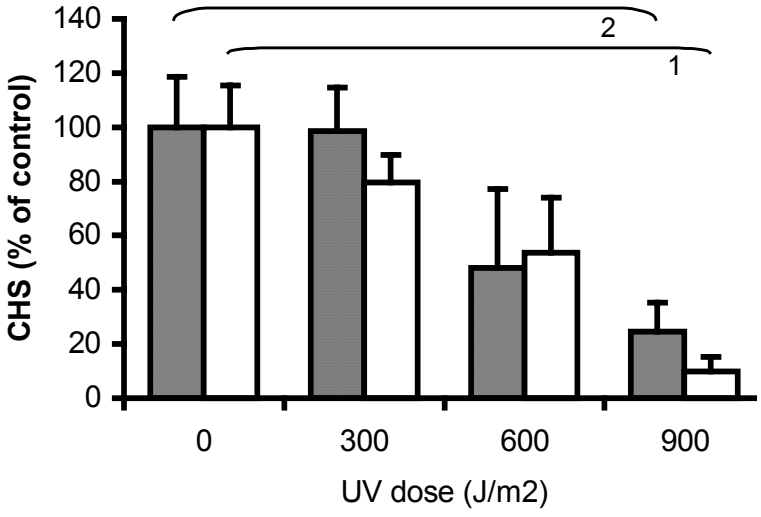

Figure 3, UV-induced local immunosuppression of CHS in XPC mice (white bars) and wildtype littermates (gray bars). Each bar represents net ear swelling (mean ± SEM). The ear swelling in sensitized unirradiated mice was set at 100%. p<0.001 (1); p<0.01 (2).

Ultraviolet-B-induced Langerhans cell depletion is enhanced in XPA and CSB but not XPC mice

In order to examine the influence of GGR and TCR on UV-induced Langerhans cell depletion we exposed NER deficient mice and their wildtype littermates to different UV doses and determined the density of Langerhans cells in a crosssection of the (un)exposed skin of the ears. The density of epidermal NLDC145+ Langerhans cells in the unexposed skin of XPA, CSB and XPC mice did not differ significantly from their wildtype littermates.

A UV dose of 150 J/m^2 was sufficient for a significant decrease in epidermal Langerhans cell numbers in XPA (p=0.011) and CSB mice (p<0.001)(**fig 4** and **5** res.). Wildtype littermates of CSB mice showed a significant decline in the density of epidermal Langerhans cells after irradiation with 300 J/m^2 (p=0.009). A minimal UV dose of 900 J/m^2 was required for a significant decrease in epidermal Langerhans cell density in the skin of wildtype littermates (p=0.006), but remarkably at this high dose the density of Langerhans cells in the skin of XPC mice was still not significantly reduced (**fig 6**).

The density of epidermal Langerhans cells was significantly less in the UV-exposed skin (150 and 300 J/m^2) of XPA mice (p=0.012) and CSB mice (p=0.008 and p=0.017 resp.) compared to the respective wildtype littermates (**fig 4** and **5** resp.). The density of epidermal NLDC145+ Langerhans cells in the UV-B-exposed skin (300 and 600 J/m^2) did not differ between XPC mice and their wildtype littermates. The density of epidermal Langerhans cells was even significantly larger in the UV-B-exposed skin (900 J/m^2) of XPC mice than in that of their wildtype littermates (p=0.015) (**fig 6**).

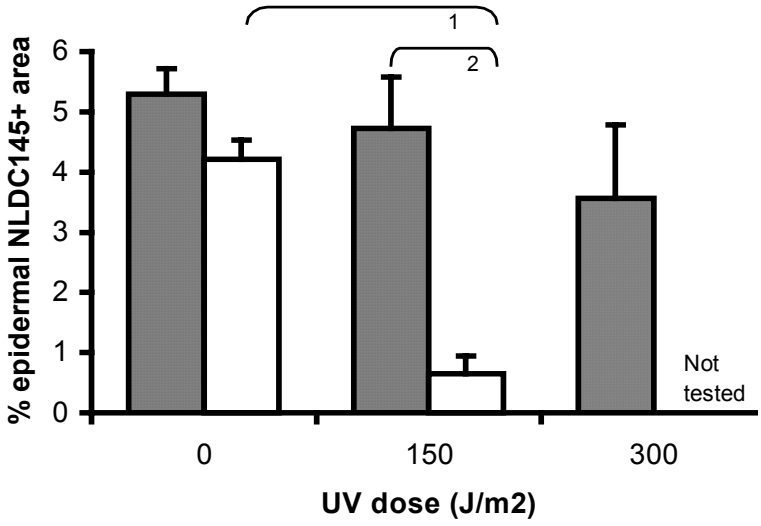

Figure 4, Percentage of epidermal NLDC145+ area (mean ± SEM) of the (un)exposed ear skin of XPA mice (white bars) and wildtype littermates (gray bars). p=0.011 (1); p=0.012 (2).

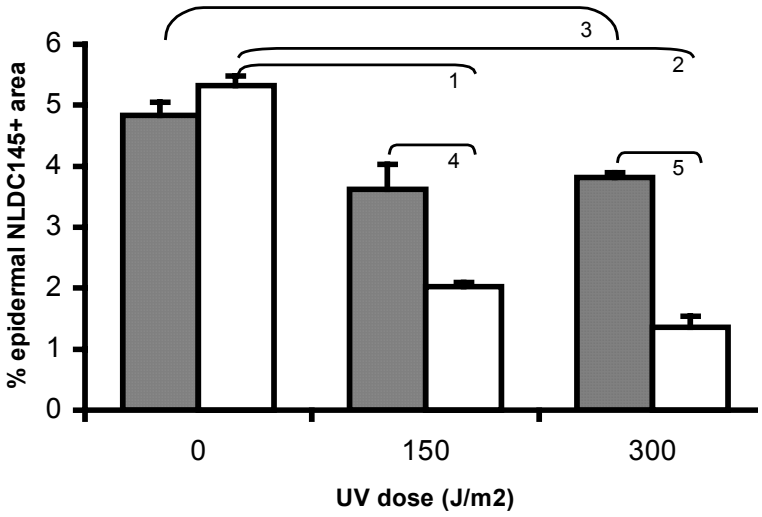

Figure 5, Percentage of epidermal NLDC145+ area (mean ± SEM) of the (un)exposed ear skin of CSB mice (white bars) and wildtype littermates (gray bars). p<0.001 (1); p=0.001 (2), p=0.009 (3), p=0.008 (4), p=0.017 (5)

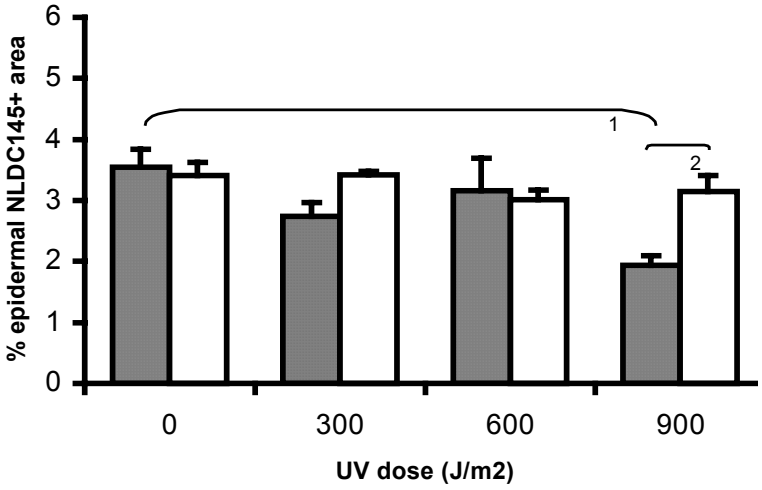

Figure 6, Percentage of epidermal NLDC145+ area (mean ± SEM) of the (un)exposed ear skin of XPC mice (white bars) and wildtype littermates (gray bars). p=0.006 (1); p=0.015 (2)

Skin draining lymph nodes of the (un)exposed skin of all mouse strains were removed to check for the presence of dendritic cells with CPDs. NLDC145+ cells with CPDs were detected in the draining lymph nodes of the UV exposed skin, but not in those of the unexposed skin (checked in an a-select group of samples)(data not shown).

DISCUSSION

In this study we investigated whether TCR and/or GGR were involved in UV-induced local immunosuppression using genetically defined mouse mutants with specific defects in one or both of these DNA repair systems. An immunization with the contact allergen picryl chloride on an irradiated site and subsequent challenge of the ears was used as a tool to determine the susceptibility to UV-induced local immunosuppression. Previously, we found that CHS

responses to picryl chloride could be inhibited by low doses of UV-B radiation (Sontag *et al*, 1994). This correlated with decreased levels of interferon (IFN)-γ and IL-12, which are important in the initiation and effector phase of Th1-mediated immunity (Garssen *et al*, 1999). Furthermore, it was shown that a complete defect in NER, as in XPA mice, increased the susceptibility to UV-induced systemic immunosuppression (Garssen *et al*, 2000; Miyauchi-Hashimoto *et al*, 1996), while IFN-γ levels were decreased (Boonstra *et al*, 2001). Systemic immunosuppression was not observed in XPC and CSB mice with a defect in GGR or TCR respectively (Garssen *et al*, 2000).

In the present study it was demonstrated that UV-induced local immunosuppression was enhanced in XPA and CSB mice but not in XPC mice or wildtype littermates. These observations are in line with and extend the findings by Miyaushi-Hashimoto *et al* (1996) who found that local immunosuppression was increased in XPA mice. However, importantly the present experiments give evidence that this increased susceptibility to local immunosuppression is not restricted to XPA mice but also applies for CSB mice and not to XPC mice. This implies that UV-B-induced local immunosuppression is influenced by TCR and not GGR, while systemic immunosuppression is only affected by a complete NER defect.

Moreover, this research demonstrated that together with an increased local immunosuppression, Langerhans cell depletion was also enhanced in TCR-deficient mice (XPA and CSB) but not in GGR-deficient mice (XPC) in comparison with the respective wildtype littermates. This is in agreement with data from Jimbo *et al* (1992), who demonstrated that UV-induced Langerhans cell depletion was enhanced and Langerhans cell recovery was delayed in XPA patients compared to healthy controls. Boonstra *et al* (2001) showed that TNF-α production was augmented in the draining lymph nodes of the UV-exposed skin of XPA and CSB but not XPC mice. TNF-α is involved in the induction of Langerhans cell migration from the skin to the draining lymph nodes (Cumberbatch *et al*, 1997; Cumberbatch *et al*, 1999). This suggests that the enhanced Langerhans cell depletion, induced by a defect in TCR, is the result of an increased expression of TNF-α. This needs to be confirmed further by determination of TNF-α expression in the skin where Langerhans cells reside and which is the site of UV-exposure.

Dendritic cells with UV-specific DNA damage could be observed in variable numbers in the skin draining lymph nodes of UV-exposed XPA, CSB and XPC mice. The presence of dendritic cells with CPDs in the lymph nodes indicated that the observed Langerhans cell depletion in the skin is at least in part caused by migration. Other investigators showed that UV-induced Langerhans cell depletion in mice *in vivo* could be caused by migration (Sontag *et al*, 1995). It was found that UV-irradiated murine Langerhans cells show delayed apoptosis *in vitro* (Tang and Udey, 1992), but *in vivo* hardly any apoptotic Langerhans cells were found in the epidermis among the UV-induced apoptotic ‘sunburn cells’ (Okamoto *et al*, 1999). Van Oosten

et al (2000) reported that apoptosis was increased in the UV-B-exposed skin of XPA and CSB but not XPC mice. An enhanced apoptosis could not only affect keratinocytes but also Langerhans cells. The experiments by Okamoto *et al* (1999), which showed a lack of Langerhans cells among the UV-induced apoptotic epidermal cells and the observed presence of UV-damaged Langerhans cells in the draining lymph nodes of NER-deficient mice indicate that UV-induced Langerhans cell depletion in the various mouse strains is most likely mainly caused by migration. This is in line with the results of a recent study from our group on healthy human volunteers (Kölgen *et al*, 2002). However, it cannot be excluded that the enhanced Langerhans cell depletion in XPA and CSB mice is (in part) caused by apoptosis.

The present data together with experimental data from other researchers can give more insight into the pathogenesis of XP en CS and the influence of the different NER mechanisms on the skin immune system. In summary, TCR plays a crucial role in sunburn-sensitivity (Berg *et al*, 1998; Garssen *et al*, 2000), UV-B-induced Langerhans cell depletion, production of TNF-α in the lymph nodes, UV-B-induced local immunosuppression and UV-B-induced apoptosis. In contrast both TCR and GGR play a significant role in systemic immunosuppression and inhibition of IFN-γ production by lymph node cells (Boonstra *et al*, 2001). These findings indicate that local and systemic immunosuppression have a different origin. Since XPA and XPC patients are very cancer-prone in contrast to CS patients, GGR seems to be involved in cancer-susceptibility, particularly in man.

One should be careful in extrapolation of these observations from mice to man. One of the differences between mice and man is the repair of CPDs. The only CPDs that are removed are eliminated by TCR in mice, while being ‘overlooked’ by GGR. This difference in CPD repair between mice and man is predominantly caused by difference in the expression of p48 in the skin. As a consequence CPDs are hardly removed from the non-transcribed strand in mice (Ruven *et al*, 1993; Tan and Chu, 2002; Tang *et al*, 2000). However, this does not seem to have any consequences for UV-survival since this survival is equal for wildtype mice and human fibroblasts (for review see Hanawalt (2001)).

Taken together, this work demonstrates that DNA damage is critical for depletion of epidermal Langerhans cells and local immunosuppression. In particular, the minor fraction of UV-induced lesions in the transcribed strand of active genes appears to be the culprit class of lesions, whereas the bulk of DNA injury in the remainder of the genome is of minor importance. Transcription-coupled repair, the DNA repair process that enables recovery of gene expression after DNA damage, is found to be crucial in order to prevent UV-induced depletion of epidermal Langerhans cells and local immunosuppression. Thus, restoration of gene expression is an important prerequisite for preventing this type of immunomodulation. Furthermore, evidence was given for a relationship between UV-B-induced Langerhans cell depletion and local but not systemic immunosuppression. Future studies should investigate in

more detail the impact of the different NER pathways on cytokine production in the skin *in vivo* and the possible correlation with Langerhans cell migration, immunosuppression, sunburn-sensitivity and susceptibility to skin cancer.

ACKNOWLEDGEMENT

We are grateful to Coen Molenbeek, Diane Kegler, Piet van Schaaik and Hans Strootman from the animal facility of the National Institute of Public Health and the Environment in Bilthoven for performing many of the described experiments. We thank Ilse Bihari (Department of Dermatology, University Medical Center Utrecht) and Marjan Jongsma (Department of Dermatology, Leiden University Medical Center) for technical assistance. We are grateful also to Jan van der Linden from the department of Pulmonary Diseases, University Medical Center Utrecht for his support in image processing of the NLDC145+ cells. The work of J.H. and G.H. was supported by the Dutch Cancer Society (projects 98-1774, 1999-2004, and 2002-2701), the Research Institute for Diseases in the Elderly, funded by the ministry of Health, Welfare and Sports, through the Netherlands Organization for Scientific Research and an NIH program grant (AG 17242-02).

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General discussion

General discussion

The aim of our study was to unravel the pathogenesis of PLE by investigating the supposition that PLE is caused by an impairment in UV-B-induced immunosuppression. This hypothesis runs as a continuous thread through this thesis. The results obtained show that a defect in certain UV-induced cellular responses in the skin, i.e. an impaired Langerhans cell migration and a lack of influx of neutrophils into the epidermis of the uninvolved skin of PLE patients. Our data provide evidence for a disturbance of the relationship between erythema and immunosuppression in PLE. In this chapter the data described in the previous sections will be integrated and discussed in an overall synthesis of a model of the early pathogenic steps in PLE.

EFFECT OF UV-B RADIATION ON CD11b+ CELLS

Cooper and co-workers (Hammerberg *et al*, 1996b; Kang *et al*, 1994; Meunier *et al*, 1995) concluded from their experiments that interleukin (IL)-10 producing CD11b+ macrophage-like cells were involved in UV-B-induced immunosuppression. Treatment with anti-CD11b antibodies resulted in a reversion of this immunosuppression (Hammerberg *et al*, 1996a). Therefore we hypothesized that these cells were absent or non-functional in PLE patients.
Initially we tested several UV exposure regimens on healthy individuals to reproduce the data found by Cooper and co-workers. Their observation of a nearly complete depletion of Langerhans cells and an influx of CD11b+ cells into the epidermis after exposure of the skin to 4 MED (Minimal Erythema Dose) UV-B, was used as a guideline. At first, dorsal skin of healthy volunteers was exposed to 4 MED (Philips TL12 lamps). However, the skin area and the UV dose gave variable results on Langerhans cell depletion and influx of CD11b+ cells. A UV dose of 6 MED on buttock skin instead of dorsal skin gave more consistent results, comparable to that reported by the other investigators (**chapter 2**). Cooper and co-workers defined the MED as the UV dose causing a clear erythema with defined borders, while we defined the MED as the UV dose causing a just perceptible redness of the skin. Barr *et al* (1999) calculated that a dose of 4 MED according to Cooper and co-workers was comparable to 5.6 MED, according to our definition.
Consecutively, buttock skin of PLE patients (with normal and extreme sunburn-sensitivity) and healthy individuals was exposed to 6 MED. CD11b+ cells were observed in the unexposed and UV-exposed skin of healthy individuals as well as PLE patients. The number of CD11b+ cells was even larger in PLE patients with an extreme sunburn sensitivity (low MED). However,

hardly any CD11b+ cells infiltrated the UV-exposed epidermis of PLE patients compared to healthy controls. Judging by the large number of CD11b+ cells in the dermis of PLE patients, the hypothesis that a mere lack of these cells would cause the suspected failure in immunosuppression in these patients proved wrong. However, the lack of these cells migrating into the epidermis was highly significant. The CD11b+ population in the skin of PLE patients consisted predominantly of macrophages (CD68+) (**chapter 2**). In healthy individuals the dermal and epidermal CD11b+ cells in the UV-exposed skin were primarily neutrophils (elastase+) (in agreement with Strickland *et al* (1997) and Teunissen *et al* (2002)).
The question remained whether the macrophages in the UV-exposed skin of PLE patients were functional in producing the immunosuppressive cytokine IL-10. A small number of dermal neutrophils and CD36+ macrophages in the skin of healthy individuals and PLE patients expressed the immunosuppressive cytokine IL-10 in their cytoplasm (as detected by immunohistochemistry on microscopic sections). In contrast, infiltrating neutrophils in the epidermis of healthy controls expressed IL-10. Although the CD11b+ population in PLE patients is distinct from healthy controls, the difference in IL-10 expression is restricted to infiltrating (epidermal) neutrophils. This suggests that a lack of IL-10-expressing epidermal neutrophils is an aberrant UV response that contributes to the pathogenesis of PLE (**chapter 5**).

UV-B-INDUCED DEPLETION OF LANGERHANS CELLS

An immunohistochemical staining for Langerhans cells (CD1a) was performed on skin biopsies from (un)exposed buttock skin to check the reproducibility of our irradiation protocol and to compare it with the protocol used by Cooper and co-workers. Unexpectedly, we noticed a striking difference in the UV-induced depletion of Langerhans cells between PLE patients and healthy controls. Whereas the Langerhans cell depletion was nearly complete after 6 MED UV-B in healthy individuals, a considerable number of epidermal Langerhans cells was still present in the skin of PLE patients. Initially, we referred to these cells as ‘UV-resistant’. However, when PLE patients were exposed to an even larger UV dose (8.4 MED) a greater number of Langerhans cells disappeared from the epidermis. This indicated that these cells were not UV-resistant but relatively less sensitive to UV-B radiation with regard to their migratory response (**chapter 2 and 5**).
The MED of PLE patients with a normal MED was in the lower 50 percentile of the MED range of healthy controls. Therefore one might assume that the impaired Langerhans cell depletion in PLE patients is mainly caused by an on average lower UV dose. However, no correlation could be found between the decrease in Langerhans cell number and the absolute UV exposure.

Furthermore, a PLE patient receiving the same absolute UV dose as a healthy control still had a considerably smaller decrease in the number of epidermal Langerhans cells.
The next step was to establish the mechanism underlying the impaired Langerhans cell depletion. To this end we first determined what mechanism was involved in UV-induced Langerhans cell disappearance in healthy individuals. The best studied and most likely mechanisms involved in this depletion were apoptosis and migration. Apoptosis was determined by using the TUNEL assay (identifying late apoptosis by labeling of DNA strand breaks) and by staining of active-caspase 3, a protease involved in the apoptotic cascade. A substantial number of apoptotic cells was detected in the UV-exposed skin of healthy volunteers, however no apoptotic Langerhans cells could be found. Migration of Langerhans cells was confirmed using a newly developed method to trap migrating Langerhans cells in the fluid of a blister raised on the UV-exposed skin (**chapter 3**). Migrating Langerhans cells could also be detected in the blister fluid of the UV-exposed skin of PLE patients, although the number of cells was significantly smaller than in healthy controls (**chapter 4**). PLE patients do have apoptotic cells in their UV-exposed skin but again no apoptotic Langerhans cells could be observed (data not shown). The number of apoptotic cells, predominantly keratinocytes (sunburn cells), in the UV-exposed skin of PLE patients is variable but less than in healthy controls. It seems, though, that the number of apoptotic cells is related to the UV-dose. However, this has to be confirmed in a follow-up study. In conclusion, the impaired UV-B-induced Langerhans cell depletion in PLE patients is completely attributable to an impairment in migration.
Cytokines such as IL-1-α and IL-1-β, tumour necrosis factor (TNF)-α and also IL-18 have been described to be involved in Langerhans cell migration (Cumberbatch *et al*, 1997a; Cumberbatch *et al*, 1997b; Cumberbatch *et al*, 2001). For that reason we investigated the expression of these cytokines in (un)exposed skin of PLE patients and healthy controls (**chapter 5**). The number of cells expressing IL-1-β and TNF-α was less in the UV-exposed skin of PLE patients compared to healthy controls. In contrast to healthy individuals, PLE patients had no TNF-α-expressing neutrophils in their UV-exposed epidermis. The impaired Langerhans cell migration in PLE patients appears, therefore, to be related to a diminished expression of the migration-inducing cytokines IL-1-β and TNF-α.

LANGERHANS CELL MIGRATION AND IMMUNOSUPPRESSION

How can a deficiency in Langerhans cell migration be linked to an impairment in immunosuppression? Although UV-induced Langerhans cell migration has been found to be

associated with immunosuppression, it had not been proven that this constitutes a causal relationship.
In experiments with mice deficient in nucleotide excision repair (NER) we demonstrated that a defect in transcription-coupled repair (TCR) resulted in an enhanced local, but not systemic immunosuppression as well as in an enhanced UV-induced Langerhans cell depletion (**chapter 6**). These observations are indicative of a relationship between UV-B-induced Langerhans cell depletion and local immunosuppression. Hence, our finding of an impairment in UV-induced Langerhans cell migration in PLE patients would suggest an impaired local immunosuppression, as confirmed by a recent study (van de Pas *et al*, *in press (Journal of Investigative Dermatology)*).

MED AND IMMUNOSUPPRESSION

A relationship between MED and immunosuppression was confirmed in humans by Kelly *et al* (2000), who showed that a sensitivity to sunburn is associated with a susceptibility to UV-induced local suppression of contact hypersensitivity (CHS). A lower MED correlated with an increased susceptibility to UV-induced immunosuppression. This correlation between erythema and immunosuppression appears to be disturbed in PLE patients. As mentioned before, the MED of most PLE patients is in the lower range of the MED of healthy individuals. Furthermore, the immune responses in the UV-exposed skin of PLE patients e.g. immunosuppression, Langerhans cells migration and the expression of migration-inducing as well as Th2-skewing cytokines (epidermal IL-4 and IL-10 expression), are reduced compared to healthy individuals. In addition, irradiation of the skin of PLE patients with 8.4 MED instead of 6 MED increased Langerhans cell depletion and the expression of IL-1-β, TNF-α and IL-4 (**chapter 5**). Taken together, these observations suggest that the erythema response in PLE is too strong in comparison to the immunosuppressive response. Thus, the relationship between UV-B-induced erythema and immunosuppression is disturbed in PLE. Another indication for a disturbed relationship is given by the observation that supplementation of dietary fish oil or phototherapy given to PLE patients to prevent a pathological skin reaction to UV exposure, results in a decreased UV-erythemal sensitivity (increase of the MED) (Boonstra *et al*, 2000; Rhodes *et al*, 1994; Rhodes *et al*, 1995). Apparently, an elevation of the threshold for the erythema response normalizes the balance with respect to the immunosuppressive response.

DNA DAMAGE AND LANGERHANS CELL MIGRATION

We demonstrated that only a minority of migrating Langerhans cells in the blister fluid of the UV-B-exposed skin had UV-specific DNA damage (**chapter 3 and 4**). The predominance of Langerhans cells without detectable DNA damage could not be explained by an artifact of the suction blister technique or by an influx of new dendritic cells from the bloodstream or hair follicles, because these cells were not found in blister fluid of the unirradiated skin. Moreover, we observed an enrichment of UV-damaged Langerhans cells in the blister roofs of the UV-B exposed skin. Hence, it appeared that undamaged or DNA-repaired Langerhans cells exhibited a selective responsiveness to migratoin signals. Repair of UV-B-induced DNA damage, e.g. cyclobutane pyrimidine dimers (CPDs), could then be a prerequisite for Langerhans cell migration. To investigate the relationship between DNA repair and Langerhans cell migration more closely we exposed transgenic mice with defects in nucleotide excision repair (NER) to different doses of UV-B radiation and studied the epidermal depletion of NLDC145+ Langerhans cells (**chapter 6**). CSB mice deficient in transcription-coupled repair (TCR) and XPA mice deficient in TCR and global genome repair (GGR) showed an enhanced UV-induced depletion of Langerhans cells compared to GGR-deficient XPC mice and the respective wildtype (wt) littermates. Correspondingly, the CSB and the XPA mice proved to be much more sensitive to the induction of local immunosuppression by UV exposure than the XPC and wt mice. The sunburn sensitivity of XPA and CSB is also much higher than XPC and wt mice. At very low UV doses, where XPC and wt show no detectable reaction at all, the CSB and XPA mice already show Langerhans cell migration and local immunosuppression. This suggests that the persistence of a low level of damage in active genes triggers migratory signals (presumably from keratinocytes) to which the Langerhans cells react. The low level of DNA damage in the Langerhans cells does apparently not obstruct their migration. The fact that Langerhans cells react even at higher UV doses in XPC mice, indicates that a persistence of higher levels of DNA damage in non-transcribed strands does not hinder migration either. This indicates that, at least in mice, DNA repair does not seem to be a prerequisite for UV-induced Langerhans cell depletion. However, a persistence of a high level of DNA damage in transcribed strands could perhaps hinder the migration of Langerhans cells, but a suitable model to test this in transgenic mice is not available.

LANGERHANS CELL ACTIVATION AND MATURATION

Langerhans cells persisting in the epidermis of PLE patients after UV-B exposure might present antigen there instead of in the lymph nodes. This antigen presentation at an aberrant location might contribute to the pathogenesis of PLE. In order to present antigen efficiently

Langerhans cells need to upregulate their expression of costimulatory molecules as well as the number of antigen-HLA-DR complexes on their membrane. Furthermore, responding cells, e.g. T-cells, need to be present to carry out a specific immune response. No epidermal Langerhans cells in the UV-exposed skin of PLE patients expressed costimulatory molecules (**chapter 4**). However, the number of epidermal HLA-DR+ Langerhans cell was increased after UV exposure. Additionally, hardly any CD3+ T-cells could be detected in the epidermis of the UV-exposed skin of PLE patients. Taken together, these data demonstrate that antigen presentation in the epidermis of the UV-B-exposed skin of PLE patients most likely does not occur.
Langerhans cells expressing costimulatory molecules and HLA-DR as well as T-cells could be observed in the dermis of the UV-exposed skin of PLE patients and healthy individuals. The absolute number of activated and matured (CD83+) dermal Langerhans cells was substantially larger in PLE patients compared to healthy controls. Illicit antigen presentation could therefore occur in the dermis, with a greater likelihood in PLE patients.

SKEWING TOWARDS A TH1 OR TH2 RESPONSE

Cytokines are important in determining the outcome of an immune response, e.g. a type 1 (Th1) or a type 2 (Th2) response, and most likely as a result, influence the induction of PLE. In healthy individuals UV-B radiation provokes the development of Th2 cells while suppressing Th1 cells (Simon *et al*, 1990; Simon *et al*, 1991). A first immunohistochemical exploration of the cytokine profiles in the skin of PLE patients revealed that the Th2-skewing cytokine IL-4 was expressed in fewer cells in the UV-exposed epidermis of PLE patients when compared to healthy controls (**chapter 5**). The number of cells expressing Th1-skewing cytokines IL-12, interferon (IFN)-γ and IL-6 did not differ between PLE patients and healthy individuals. Overall, the cytokine profile in the UV-exposed skin of PLE patients appeared to be shifted more towards Th1 in comparison to healthy individuals.

FUNCTIONAL TESTING

An impairment in immunosuppression can be tested functionally by immunization or challenge with a contact allergen on an irradiated site (local immunosuppression) or on an unirradiated site after irradiation of another skin area (systemic immunosuppression). The severity of the contact hypersensitivity reaction (CHS) in terms of erythema, edema and the appearance of papules or vesicles can be used as a measure for immune reactivity. Antigens, not yet encountered by the immune system (causing immunization), as well as recall antigens

(causing a challenge reaction) can be used as contact allergens. Application of immunizing contact allergens like DNCB or DCP not only encountered ethical objections but also carried the risk of sometimes provoking severe, traumatic skin reactions. Therefore, we used different concentrations (0.5% and 2%) of the recall antigen nickelsulphate ($NiSO_4$) in petrolatum, which is also used in the clinic (at a concentration of 5%) for assessment of CHS. Before testing (nickel allergic) PLE patients we first wanted to establish a consistent standardized reaction in healthy individuals who had a positive skin reaction to nickel. In accordance with the UV exposure protocol in this project we exposed buttock skin of seven healthy individuals to 6 MED UV-B (Philips TL12 lamps) and applied different concentrations of nickelsulphate to the UV-B-exposed and unexposed skin 48 hours post irradiation. Following the classical CHS protocol the allergen remained on the skin for 48h and the reaction was measured 24h after removal of the allergen. Assessment of CHS was done by eye and with a chromameter. We did not observe a consistent reaction in healthy individuals (n = 7). CHS reactions of the UV-exposed skin fluctuated from an immunosuppressive reaction to an induction of immune reactivity compared to the unexposed skin.

The increased skin reaction to $NiSO_4$ after UV exposure was not anticipated as UV radiation is known to suppress delayed type (type IV) hypersensitivity reactions like these (Damian *et al*, 1999). However, not only a type IV hypersensitivity reaction has been described for nickel but also a type I IgE-mediated hypersensitivity reaction (Estlander *et al*, 1993). An additional assessment of the skin reaction at 2h after application of the contact allergen might therefore have given a more reliable definition of the reaction in some individuals. Another parameter that had not been taken into account was the menstrual cycle of the participants (all were female). The menstrual cycle might be of influence on skin reactivity (Agner *et al*, 1991; Agner, 1992). Damian *et al* (1999) were able to suppress a CHS on nickelsulphate with UV radiation. However, they used a different UV exposure regimen and applied the antigen immediately after UV exposure.

The inconsistent and sometimes unpleasant reactions made us decide to discontinue these experiments. In the meantime van de Pas *et al* (*in press (Journal of Investigative Dermatology)*) were performing experiments in the United Kingdom on the suppression of immunization against DNCB. PLE patients and healthy volunteers were exposed to solar-simulating radiation prior to application of DNCB onto the buttock skin. They showed that UV-induced local immunosuppression was impaired in PLE patients compared to healthy individuals after exposure to 0.6 MED and 1 MED, but not after exposure to 2 MED.

These results imply that there is a dose range in PLE patients over which the immunosuppression is lacking in comparison to the inflammatory (erythemal) response. Although these experiments have not measured the allergic response to UV radiation itself, one could envisage that there is a corresponding "UV dose-window" over which the PLE

patients can develop an allergic reaction to UV exposure. It could then be speculated that the allergic response would not develop if the UV exposure were high enough, resulting in a severe sunburn instead.
Although the efflux of Langerhans cells and influx of neutrophils we observed at 6 MED in the PLE patients were low in comparison to the healthy volunteers, they may have been strong enough to attain to an adequate immunosuppression, as measured by van de Pas *et al* (*in press (Journal of Investigative Dermatology)*).

EARLY EVENTS IN THE PATHOGENESIS OF PLE

A model of the pathogenesis of PLE based on the aberrant responses in the skin to UV-B radiation, as described in this thesis, is presented in Figure 1. In this figure the effect of UV-B radiation on the skin of PLE patients is therefore compared to that in healthy controls.

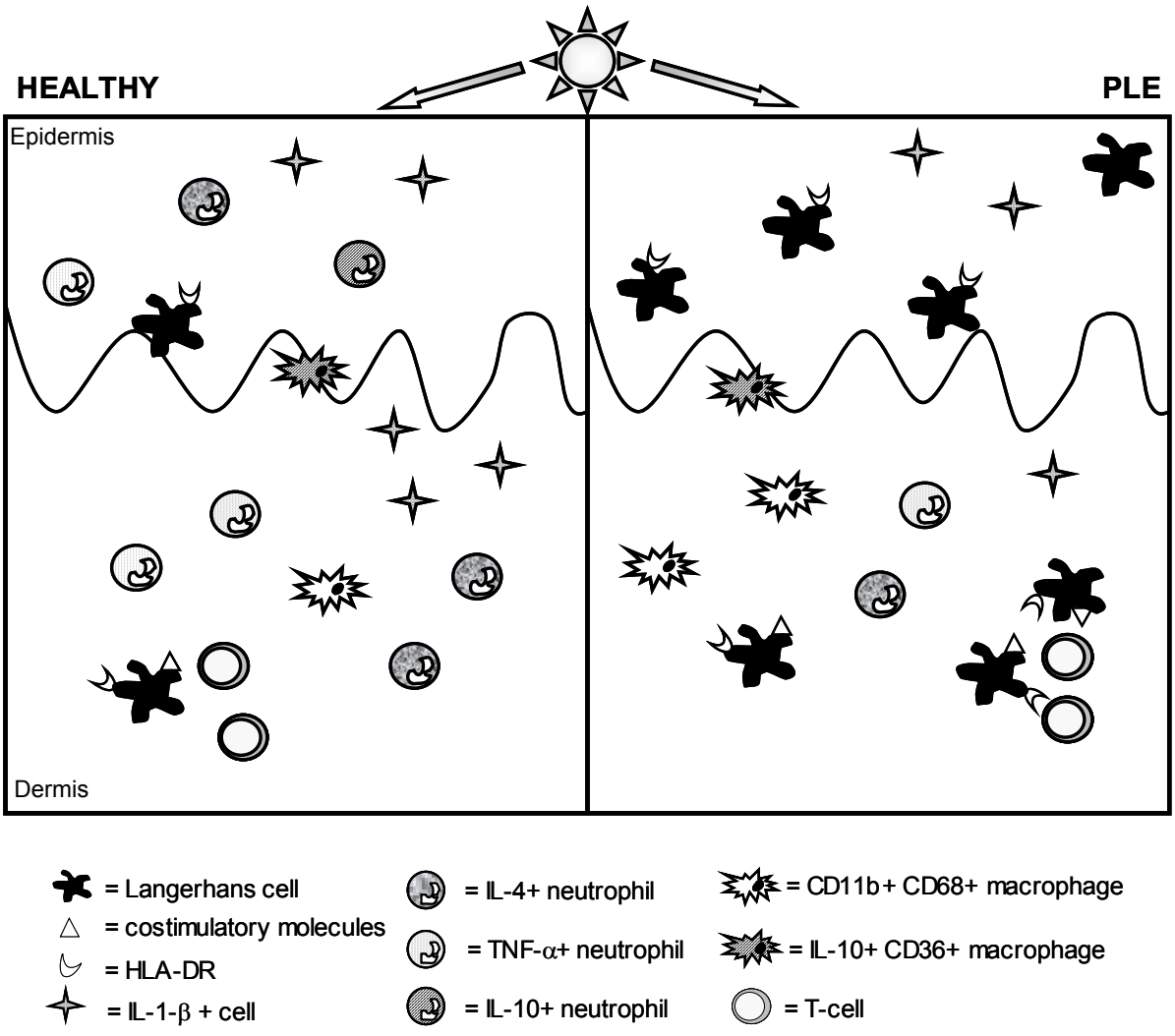

Figure 1, Model of the early steps in the pathogenesis of PLE. After UV exposure [ ] Langerhans cells [ ] still persist in the skin of PLE patients (right panel) in contrast to healthy individuals (left panel). Fewer dermal cells expressing IL-1-β [ ] together with a decreased number of TNF-α-expressing neutrophils [ ] in the dermis and an absence of TNF-α-expressing cells in the epidermis (above the curved line) lie at the root of this impaired Langerhans cell migration in PLE patients. This deficient Langerhans cell migration correlates with an impairment in UV-induced local immunosuppression in PLE patients. Langerhans cells in the epidermis of PLE patients do not express the activation markers CD86, CD40, CD54 or the maturation marker CD83 but these cells do express HLA-DR [ ] after UV exposure. Dermal Langerhans cells express costimulatory molecules [ ] as well as HLA-DR and are therefore capable of presenting (neo-)antigens to dermal T-cells [ ], thus activating these T-cells. Activated dermal Langerhans cells are present in larger numbers in PLE patients compared to healthy individuals. A decreased number of IL-4-expressing neutrophils [ ] (without a difference in the expression of Th1-skewing and pro-inflammatory cytokines) skews the balance to a type 1 T-cell response in PLE patients. The CD11b+ cell population in PLE patients consists predominantly of macrophages [ ], while in healthy individuals this population is dominated by neutrophils. The immunosuppressive cytokine IL-10 is expressed by infiltrating neutrophils [ ] and by a minority of the macrophages [ ]. The difference in IL-10 expression between PLE patients and healthy individuals is restricted to infiltrating neutrophils. Taken together, it appears that the normal, healthy immunosuppressive response to UV radiation is reduced in PLE patients while the erythemal response is increased (possibly by an increased expression of prostaglandin E_2). This shift in immune responses together with the possibility of an activation of Th1 cells (instead of Th2 cells) in the dermis by slowly migrating Langerhans cells may underlie the pathogenesis of PLE.

PLE DEMARCATION FROM OTHER PHOTODERMATOSES

In our first study on the pathogenesis of PLE we included PLE patients with a normal MED as well as a low MED. Later on we focussed on PLE patients with a normal MED because there are no healthy individuals with a low MED who can be included as proper controls for PLE patients with a low MED. At first, we defined a pathological skin reaction in PLE patients clinically as papules, vesicles and eczema on sun-exposed areas of the skin. It appeared that an eczematous reaction to UV radiation in the PLE patients we included, was restricted to PLE patients with a low MED. Furthermore, a skin reaction to UV radiation in patients with photosensitive eczema at an early stage often resembles a PLE reaction with papules and vesicles which later develops into an eczematous lesion. To circumvent possible confounding from eczematous reactions we restricted the definition of PLE to papules and vesicles, excluding any history of (non-)photosensitive eczema. All PLE patients with a normal MED that were included fitted this definition.

These issues on MED and eczematous reactions highlight controversies on the definition of PLE. PLE may well be a diagnosis that entails different forms of photodermatoses. Some structure might be created by a division according to susceptibility to sunburn, sensitivity to different wavelengths, type of reaction (papules and vesicles versus eczematous), extent of the disease (seasonally dependent or not) and maybe even gender. Thus, one could construct the diagram in figure 2 to delineate the relationships between various forms of photodermatoses. The proposed demarcations and relationships between the separate photodermatoses are still very much open to debate, and need to be substantiated by well targeted future research.

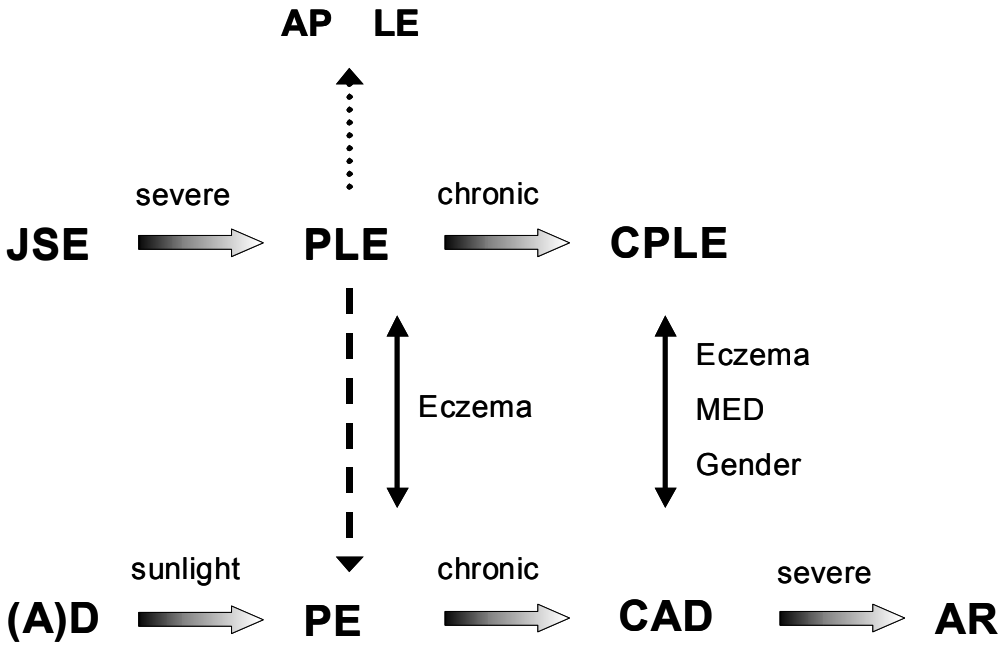

Figure 2, A hypothetical diagnostic diagram (constructed by Soe Janssens, Frank de Gruijl and Wendy Kölgen). This diagram consists of two main branches. The first branch represents a sunlight-induced pathological skin reaction dominated by papules and vesicles. The course of the condition in this branch could start with juvenile spring eruption (JSE). In this diagnosis lesions are commonly restricted to the sun-exposed ears. If severity increases this disorder passes in adulthood into a PLE with a seasonal dependent (predominantly spring and summer) appearance of lesions on sun-exposed areas of the body. Skin disorders like actinic prurigo (AP) and lupus erythematodes (LE) need to be excluded from this diagnosis, although PLE has been reported to precede LE in some patients. When seasonal dependence is lost and skin lesions persist, this can be diagnosed as chronic PLE (CPLE). The other branch in the diagram is dominated by the appearance of eczematous lesions. A photo-provoked eczema could arise de novo or (atopic) dermatitis ((A)D) may evolve into a photosensitive eczema (PE) when the eczema is aggravated by sunlight. PE lesions often first appear as papules and vesicles and

might therefore initially be (mis)diagnosed as PLE (⇢). If these photosensitive eczematous lesions persist a chronic actinic dermatitis (CAD) develops. An actinic reticuloid (AR), characterized by three diagnostic criteria (see introduction), will develop when CAD increases in severity. The major difference between PLE and PE (⟷) is most likely the clinical appearance of lesions: PLE lesions are characterized by papules and vesicles while PE lesions are characterized by eczematous plaques. A distinction between CPLE and CAD (⟷) may be characterized by a difference in type of lesions (papular and vesiculous versus eczematous resp.), a difference in MED (a normal MED versus an extremely low MED resp.) and possibly by a difference in gender (predominantly women >30 years versus predominantly men >50 years, resp.).

Langerhans cell persistence in the UV-B-exposed skin does not seem to discriminate between different PLE subsets as all PLE patients and even a patient with sub-acute cutaneous lupus erythematoses display this persistence (**chapter 2**). Furthermore, Torres-Alvarez *et al* (1998) demonstrated that UV-resistant Langerhans cells were still present in the skin of patients with actinic prurigo after exposure to 20 MED from a UV-C/UV-B light source. An impairment in UV-induced Langerhans cell depletion might, on the other hand, be used as a diagnostic criterion for photosensitivity of skin disorders because no persistence was observed in healthy volunteers and patients with (non-photosensitive) psoriasis.

FUTURE RESEARCH

Many investigations have been performed on immune parameters in lesional versus non-lesional skin of PLE patients. However, the experiments presented in this thesis give information on the initial phase, not the lesional phase, of a UV-induced skin reaction in PLE patients. Knowledge of this early phase of the pathological skin reaction can give more insight into the pathogenesis of PLE as well as provide some potential tools for early preventive or therapeutic interventions. Therefore, it is important that investigations on the initial effect of UV on the skin of PLE patients are continued. To this end some suggestions will be made for future research.

Besides the studied cytokines, chemokines and chemokine-receptors are important in the trafficking of Langerhans cells. Chemokines, like for instance MIP3-α and MIP3-β, are involved in the influx and efflux respectively of Langerhans cells from the skin. Immature dendritic cells respond to MIP3-α via chemokine-receptor CCR6. Mature dendritic cells, on the other hand, have lost responsiveness to MIP3-α but acquire a responsiveness to MIP3-β via upregulation of the chemokine-receptor CCR7 (Dieu-Nosjean *et al*, 1999). Thus, during migration and maturation Langerhans cells increase the expression of CCR7. In a pilot study we examined

the expression of CCR7 in the (un)exposed skin of PLE patients and healthy individuals. Curiously, as the Langerhans cells disappeared the expression of CCR7 in the epidermis increased and was at a maximum 48h post irradiation. No CCR7 expression was observed in the unexposed skin. The expression was slightly less in the UV-B-exposed skin of PLE patients (data not shown). These observations indicate that CCR7 in the UV-B-exposed skin is not expressed by Langerhans cells and most likely neither by lymphocytes, as hardly any epidermal T-cells could be detected in the UV-B-exposed skin. Which epidermal cells do express CCR7 after UV exposure needs to be determined. Future investigations on the expression of chemokines and chemokine-receptors could perhaps solve this problem and could give more information on the causes of an impaired Langerhans cell migration in PLE patients.

The impairment in UV-B-induced Langerhans cell depletion may not be restricted to PLE patients but could be involved in other photosensitive skin disorders as well. An extensive evaluation of UV-induced Langerhans cell depletion in photosensitive skin disease (i.e. lupus erythematodes and photosenstive eczema) and non-photosensitive skin disorders (i.e. atopic dermatitis and psoriasis) could provide more information on similarities in the pathogenesis. In addition, this could demonstrate whether an impairment in UV-induced Langerhans cell depletion could be used as a diagnostic tool for photosensitivity.

From this perspective it would be interesting to know whether this defect in UV-B-induced Langerhans cell depletion is also observed in PLE patients who only develop a pathological skin reaction to UV-A. If not, this would provide some evidence for a different pathogenesis in UV-A-induced PLE.

It was shown that antigen presentation by Langerhans cells could not take place in the epidermis of the UV-exposed skin of PLE patients. However, an increased number of activated and matured dermal Langerhans cells was observed in the UV-B-exposed skin of PLE patients compared to healthy controls. It needs to be determined whether this is the result of an delayed reaction in PLE patients and whether this higher number of cells may also be found in healthy individuals earlier after UV exposure. A follow-up study identifying Th1 and Th2 cells by expression of specific chemokine-receptors (CCR5, CXCR3 and CCR3, CRTH2 respectively) could give more information on the type of response (type 1 or 2) that evolves in the skin of PLE patients after UV exposure. Furthermore, the expression of activation markers on T-cells, e.g. CD25, should be evaluated to reveal whether these cells are indeed activated.

Regulatory T-cells, which are recognized by the expression of CTLA-4 and the secretion of high levels of IL-10 and TGF-β and low levels of IFN-γ are probably involved in UV-induced immunosuppression (Schwarz *et al*, 2000). It would be interesting to investigate the presence of these regulatory T-cells in the skin of PLE patients in relation to the (impaired)

immunosuppression, as shown by van de Pas *et al* (*in press (Journal of Investigative Dermatology)*).

In our experiments we looked at the cytokine expression in cytoplasm of cells in the skin *in vivo*. Measuring cytokine levels in a skin suspension by ELISA or in blister fluid of (un)exposed skin could give more quantitative data on the cytokine profile in the skin of PLE patients *versus* healthy controls.

The induction of neo-antigens of unknown identity by UV radiation are probably responsible for immune reactivity in the skin. One could isolate T-cells from the skin and stimulate these cells with a suspension of UV-B-exposed skin (containing the putative neo-antigens). A comparison of T-cell proliferation, T-cell activation and cytokine production in T-cell clones of PLE patients and healthy controls could also give more information on the type of response. An ambitious follow-up study could focus on the identification of these neo-antigen(s) that are specifically responsible for the UV-induced immune reaction in PLE patients.

Because we wanted to reproduce the data observed by Cooper and co-workers and because we could easily detect a difference between PLE patients and healthy controls we irradiated the skin with a single dose of 6 MED. However, when reproducing a pathological skin reaction by photoprovocation the skin is repeatedly exposed to UV-B doses of about 2 MED, depending on the size of the tested skin area (Boonstra *et al*, 2000). A follow-up study using a UV-B exposure regimen comparable to the photo-provocation protocol could give some more insight into the ultimate development of the pathological skin reaction in PLE. One should be aware though that these repeated exposures may give rise to a full-blown pathological reaction. In this case, skin biopsies will be taken from lesional skin which may make it hard to distinguish early events leading up to the pathological reaction from later non-specific inflammatory reactions.

UV radiation, especially UV-A, can induce oxidative damage in the skin (Kielbassa *et al*, 1997). This oxidative damage is involved in the formation of erythema (Dreher and Maibach, 2001; Halliday *et al*, 1999). Future research on mediators involved in erythema, like nitric oxid and prostaglandin E_2, could perhaps give an explanation for the moderately increased susceptibility to sunburn in most PLE patients. This could possibly clarify why the erythema response in PLE patients is enhanced compared to the immunosuppressive response and how this erythema response is dampened after e.g. phototherapy.

Supplementation of dietary fish oil or phototherapy subdues the pathological skin reaction in PLE and increases the MED. It would be interesting to known whether UV-induced immunosuppression, as well as Langerhans cell migration and cytokine expression have also returned to a normal, healthy level after therapy. Knowledge of the immunologic effects of different therapies is of importance for refinement and increasing the effectiveness of therapies.

This thesis describes the first steps in unraveling of the pathogenesis of PLE. The present considerations on possible future research clearly delineate the complexity of the pathogenic mechanisms involved in PLE. Well-targeted experiments are likely to provide us with a good understanding of these mechanisms. Ultimately, such understanding may lead to highly specific and efficient therapies for PLE and other related photodermatoses.

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Summary

Ultraviolet (UV) radiation, especially the short wavelengths in the UV-B (280-315 nm) range, has several effects on human health. UV radiation is involved in the formation of vitamin D and in pigmentation of the skin. UV radiation also has some adverse effects: it is involved in tumor initiation and growth and in worsening of infections. The latter two (undesirable) effects are predominantly caused by a UV-induced suppression of cellular immunity. This immunosuppression can, however, be regarded as a perfectly sound physiological reaction. It prevents the occurrence of a disruptive immune reaction against UV-modified organic molecules (neo-antigens; e.g. DNA, proteins) whenever the skin is exposed to UV-B radiation. An inadequate immunosuppression could therefore develop into a UV-triggered sun allergy e.g. polymorphous light eruption (PLE).

PLE patients develop papules and/or vesicles on sun-exposed areas of the skin. Whether eczematous lesions belong to the diagnosis of PLE is still under debate. Because of a delay (hours to days) between the actual exposure and the occurrence of PLE lesions, PLE is considered a delayed type (type IV) hypersensitivity reaction. PLE is independent of race, skin color or gender. However, women under the age of 50 years are more affected than men.

PLE patients included in our experiments reacted pathologically to UV-B radiation (and often also to UV-A radiation), i.e. they developed papules and/or vesicles, but had a normal sunburn sensitivity (normal MED (minimal erythema dose)). In the experiments described in chapter 2 we also included PLE patients with an extremely low MED and eczematous lesions. To study the initial reaction to UV radiation we exposed the skin of PLE patients to 6 MED UV-B and took skin biopsies from the (unaffected) UV-exposed skin 24h and 48h post irradiation and from unexposed skin as a control. The results from the experiments in PLE patients were compared to healthy controls who underwent the same regimen. In healthy individuals this regimen is known to cause a change in cell populations in the skin which is associated with UV-induced immunosuppression.

To investigate our hypothesis that PLE is caused by a disturbance in UV-B-induced immunosuppression we first determined whether immunosuppressive CD11b+ cells were present in the UV-irradiated skin of PLE patients (**chapter 2**). CD11b+ cells were present in the UV-exposed skin of PLE patients, however, at the time we were not able to determine whether they indeed produced immunosuppressive interleukin (IL)-10. The CD11b+ cell population in the UV-exposed skin of PLE patients did not infiltrate the epidermis in contrast to the CD11b+ cells in healthy controls. Because CD11b is a broad-spectrum marker for myeloid cells we specified the cell population further by immunohistochemical staining with CD68 (macrophages) and elastase (neutrophils). The CD11b+ cell population in the UV-exposed skin

of PLE patients consisted predominantly of macrophages while in healthy controls the majority of the CD11b+ cells were neutrophils.
A more striking difference could be found in the behavior of the Langerhans cells. These cells disappeared from the skin of healthy individuals after UV-B exposure but persisted in the UV-exposed epidermis of PLE patients.
We first established in healthy controls whether apoptosis or migration of Langerhans cells was the main mechanism responsible for their disappearance (**chapter 3**). Although apoptotic (active caspase-3 or TUNEL positive) keratinocytes could be detected in the UV-exposed skin of healthy individuals, no apoptotic Langerhans cells could be found. To examine the migration of Langerhans cells we developed a novel method to trap migrating Langerhans cells in the blister fluid of suction blisters raised on the (un)exposed skin to an atmospheric pressure of –200 mmHg using airtight cups. Migrating Langerhans cells with detectable levels of UV-specific DNA damage (cyclobutane pyrimidine dimers (CPDs)) could be retrieved from the blister fluid of the UV-exposed and not the unexposed skin, showing that migration was the main mechanism responsible for UV-induced Langerhans cells depletion in healthy individuals.
This novel method to detect migrating Langerhans cells in the blister fluid was subsequently used to determine whether UV-B-induced Langerhans cell migration was impaired in PLE patients (**chapter 4**). The isolated blister roofs confirmed that significantly less Langerhans cells disappeared from the skin of PLE patients in comparison with healthy individuals. Most importantly, significantly less migrating Langerhans cells could be detected in the blister fluid of the UV-exposed skin of PLE patients. This impaired Langerhans cell migration appeared to be attributable to a reduced number of IL-1-β and tumor necrosis factor (TNF)-α-producing cells in frozen sections from the UV-exposed skin of PLE patients in comparison with healthy controls (**chapter 5**).
Only a minority (about 10-15%) of the migrating Langerhans cells in PLE patients and healthy volunteers had a detectable amount of CPDs, in contrast to 80-90% of the remaining Langerhans cells in the blister roofs. Therefore, we speculated that DNA repair was a prerequisite for UV-B-induced Langerhans cell migration. To investigate this hypothesis UV-induced Langerhans cell depletion was studied in DNA repair-deficient mice with a defect in transcription-coupled repair (TCR) (CSB mice), global genome repair (GGR) (XPC mice) or in both TCR and GGR (XPA mice) (**chapter 6**). Exquisitely UV sensitive mice with a defect in TCR (XPA and CSB) showed already a UV-induced Langerhans cell disappearance at a very low UV dose in contrast to their wildtype littermates. In solely GGR-deficient mice (XPC) Langerhans cell disappearance appeared at a high UV dose comparable to their respective wildtype littermates. Together with a UV-induced Langerhans cell depletion, local immunosuppression could be observed at a low UV dose in TCR-deficient mice (XPA and CSB) and at a high UV-dose in solely GGR-deficient mice (XPC). TCR appeared to be

essential for a normal 'wildtype' response regarding Langerhans cell depletion and local immunosuppression. To summarize, these experiments showed that repair of UV-induced DNA damage is not a prerequisite for Langerhans cell disappearance in mice and that UV-induced Langerhans cell depletion is associated with local (but not systemic) immunosuppression. The latter statement is in agreement with data from van de Pas *et al*. showing that PLE patients have an impaired immunosuppression after UV exposure together with our data on an impaired Langerhans cell migration in these patients.

With regard to the persistent Langerhans cells in the UV-exposed skin of PLE patients the question rose whether these cells were activated, presenting antigen in the skin instead of the lymph nodes. Antigen presentation at an aberrant location might contribute to the pathogenesis of PLE. In contrast to what we expected, we could not find any activated (CD86, CD40, CD54) or matured (CD83) Langerhans cells in the UV-exposed epidermis of PLE patients (**chapter 4**). However, a significant higher number of activated Langerhans cells accumulated in the dermis of the UV-exposed skin of PLE patients in comparison with healthy controls. A simultaneous presence of activated, HLADR+ Langerhans cells together with (potentially type 1) T-cells in the dermis of PLE patients might lead to antigen presentation in the skin, thereby contributing to the pathogenesis of PLE.

An exploration of the cytokine profiles in the UV-B exposed skin of PLE patients revealed that cells that expressed the Th2-skewing cytokine IL-4 were present in lesser numbers in the UV-exposed skin of PLE patients in comparison with healthy individuals (**chapter 5**). This observation, together with an equal expression of the Th1-skewing and pro-inflammatory cytokines (IL-12 and interferon-γ) indicated a shift in the cytokine profile in the skin of PLE patients more towards a Th1 response in comparison with healthy individuals. Because both IL-4 and TNF-α were expressed by neutrophils, these cells appear to play a key role in the pathogenesis of PLE.

Based on all data together, a hypothetical model can be constructed for the pathogenesis of PLE (**chapter 7**). A diminished expression of IL-1-β and TNF-α in the UV-exposed skin of PLE patient leads to an impaired Langerhans cell migration. This impaired and slow Langerhans cell migration results in an accumulation of activated Langerhans cells in the dermis. These Langerhans cells can activate dermal (potentially type 1) T-cells- if present. A reduced number of IL-4 producing neutrophils without a difference in the expression of Th1-skewing cytokines leads to a shift towards a Th1 response in the skin of PLE patients. The normal, healthy immunosuppressive responses are reduced while the erythemal response is increased (slightly lower MED) in PLE patients. This shift in immune responses together with the possibility of an activation of Th1 cells in the dermis may underlie the pathogenesis of PLE.

In this thesis, the early events in the pathogenesis of PLE are described. Future research is necessary to further unravel the pathological mechanism underlying PLE and to develop highly specific and efficient therapies for this photodermatose.

Nederlandse samenvatting

Het spectrum van de zon kan opgesplitst worden in drie delen: het zichtbare licht, de infra-rode straling en aan de andere kant van het spectrum, bij golflengten van minder dan 400 nm, de ultraviolette straling. Het grootste gedeelte van de UV straling die het aardoppervlakte bereikt is UV-A straling (315-400 nm). Maar een klein gedeelte van de UV-B straling (280-315 nm) bereikt het aardoppervlakte, de rest wordt geabsorbeerd in de ozonlaag. Deze kleine hoeveelheid UV-B straling in het zonlicht heeft echter de meeste invloed op de huid.

UV straling, met name UV-B straling, is zeer fotoreactief. Het kan fotochemische smog veroorzaken, maar kan ook plastic afbreken. Voorts kan UV straling geabsorbeerd worden door organische moleculen in de huid, zoals het genetisch materiaal (DNA) en eiwitten, en deze moleculen fotochemisch veranderen. Vanuit dit oogpunt bezien, kan men zich voorstellen dat UV straling een groot aantal verschillende effecten kan hebben op de huid.

UV straling heeft een aantal positieve effecten op de huid: het is bijvoorbeeld betrokken bij de vorming van vitamine D (o.a. belangrijk voor de botopbouw) en bij de bruining van de huid (pigmentvorming). Aan de andere kant heeft UV straling een aantal schadelijke effecten: het is betrokken bij verbranding van de huid door de zon, het ontstaan en onverstoord doorgroeien van (huid)tumoren, huidveroudering en het verergeren van infecties. Een deel van deze schadelijke effecten, zoals de groei van tumoren en het verergeren van infecties, is toe te schrijven aan het feit dat UV straling het afweersysteem (immuunsysteem) onderdrukt. Een gedegen reactie van het immuunsyteem, dat betrokken is bij de afweer tegen geïnfecteerde of kwaadaardige cellen, is hierbij niet meer mogelijk.

Vanuit dit laatste perspectief gezien, zou men kunnen concluderen dat deze onderdrukking van het immuunsysteem (immuunsuppressie) een slechte zaak is en voorkomen zou moeten worden. Elke gezonde persoon vertoont echter deze door UV straling veroorzaakte immuunsuppressie. Wanneer we er dan ook wat dieper over zouden nadenken, kunnen we beargumenteren dat we deze onderdrukking juist nodig hebben. Als deze onderdrukking namelijk niet plaatsvindt, zou dat betekenen dat iedere keer wanneer onze huid aan zonlicht blootgesteld wordt, een activatie van het immuunsysteem en dus een ontstekingsreactie zal plaatsvinden.

Een verstoring van de UV-geïnduceerde onderdrukking van het immuunsysteem zou dus kunnen lijden tot een ontstekingsreactie zoals waarneembaar is bij mensen met zonneallergie oftewel chronische polymorfe lichtdermatose (CPLD). CPLD behoort tot de groep huidaandoeningen die we fotodermatosen noemen, omdat ze ontstaan en verergeren onder invloed van zonlicht. Ongeveer 10-20% van de van de bevolking in de gematigde klimaatzones heeft in meer of mindere mate last van zonneallergie. Deze CPLD patiënten krijgen jeukende

bultjes en blaasjes en soms eczeemachtige reacties op de zonbeschenen delen van hun huid. De huidreactie is meestal seizoensafhankelijk: de eerste reactie ontstaat vaak in de lente, neemt af gedurende de zomer en is meestal in de herfst en winter geheel afwezig. In zeer ernstige gevallen echter heeft de patiënt vaak het hele jaar door last van zijn aandoening en moet zijn/haar dag en nacht ritme omdraaien om een blootstelling aan zonlicht te vermijden.
De huidreactie treedt op binnen enkele uren tot dagen na de eigenlijke blootstelling aan zonlicht en verdwijnt vaak na een dag tot enkele weken zonder littekenvorming. Omdat de tijd tussen de eigenlijke blootstelling en het opkomen van de huidreactie vrij lang is (meerdere uren), wordt de reactie ook wel een vertraagd-type overgevoeligheidsreactie genoemd. In de meeste, minder ernstige gevallen is een eenmalige, kortdurende blootstelling aan zonlicht niet voldoende om de huidreactie op te wekken maar zijn daarvoor een aantal belichtingen nodig. CPLD is niet gerelateerd aan een bepaalde huidskleur of ras en kan in iedere levensfase ontstaan. De huidreactie komt meer voor bij (jonge) vrouwen dan bij mannen en meestal zijn UV-B straling en UV-A straling de veroorzakers van de reactie.
Het meeste onderzoek op het gebied van CPLD is verricht naar de kenmerken van de huidaandoening op het moment dat de huidreactie aanwezig is. Er is echter nog maar weinig bekend over de fase die vooraf gaat aan het ontwikkelen van de huidreactie en die de eigenlijke aanleiding is tot het ontstaan van de symptomen. Het onderzoek beschreven in dit proefschrift richt zich nu juist op deze aanloopfase.
In ons onderzoek hebben we gekeken naar de effecten van een eenmalige hoge dosis UV-B straling op de huid van CPLD patiënten en dat vergeleken met de reacties die optreden bij gezonde personen.
De onderzoeksgroep van de Amerikaan Kevin Cooper heeft zich bezig gehouden met het effect van UV-B straling op de huid van gezonde personen. Daarbij hebben zij in het bijzonder gekeken naar bepaalde witte bloedcellen (CD11b+ cellen). Deze cellen komen in grote getale de huid in na een eenmalige hoge blootstelling aan UV-B straling. Ze spelen een belangrijke rol bij de onderdrukking van het immuunsysteem doordat ze interleukine 10 kunnen produceren, een stofje met een immuunsuppressieve werking. Gebruikmakend van deze kennis stelden wij de volgende hypothese op: immuunsuppressieve CD11b+ cellen zijn niet aanwezig in de huid van CPLD patiënten na blootstelling aan UV straling. CPLD patiënten werden daarom op een klein huidveldje blootgesteld aan een hoge dosis UV-B straling. Na 1 dag en 2 dagen werd van de belichte huid en, ter controle, van de onbelichte huid onder lokale verdoving een klein stukje huid (biopsie) van 3 mm doorsnee afgenomen. Door middel van een biochemische kleurreactie hebben we kunnen aantonen dat deze CD11b+ cellen wel aanwezig zijn in de huid van CPLD patiënten, waarmee onze hypothese verworpen zou worden (**hoofdstuk 2**). Er waren echter duidelijke verschillen te zien in de huid van CPLD patiënten ten opzichte van gezonde controles: de CD11b+ cellen in de huid van CPLD patiënten

drongen niet door tot in de bovenste huidlaag (de opperhuid of epidermis) en het bleek ook om een ander type cellen te gaan (deze CD11b+ cellen waren voornamelijk macrofagen terwijl deze celpopulatie in gezonde personen voornamelijk uit neutrofielen bestond).
Een ander, veel verrassender verschil konden we waarnemen bij de Langerhans cellen. Deze cellen zijn in staat om lichaamsvreemde stofjes (antigenen) op te nemen, te verteren en te presenteren op hun celoppervlakte. Deze cellen spelen een belangrijke rol in de activatie en suppressie van het immuunsysteem. De Langerhans cellen bevinden zich in de epidermis en verdwijnen bij gezonde personen na belichting van de huid met UV-B straling. In de huid van CPLD patiënten zagen we daarentegen, zelfs bij een belichting met een hoge UV dosis, dat het merendeel van deze Langerhans cellen bleven zitten in de epidermis. Deze Langerhans cellen leken resistent te zijn voor UV-straling!
In een volgende reeks van experimenten wilden we daarom uitzoeken waarom deze UV-resistente Langerhans cellen niet verdwenen uit de huid van CPLD patiënten en of dat mede een oorzaak kon zijn voor het ontstaan van CPLD. Daartoe moesten we eerst achterhalen welk mechanisme een rol speelt bij het verdwijnen van de Langerhans cellen uit de huid van gezonde personen. Wanneer dat bekend is, kunnen we pas nagaan of dat mechanisme defect is bij CPLD patiënten. Daarom hebben we eerst in de UV-B-belichte huid van gezonde vrijwilligers gekeken. Twee mechanismen die ten grondslag zouden kunnen liggen aan de verdwijning van de Langerhans cellen zijn apoptose of migratie de huid uit (**hoofdstuk 3**). Wanneer een cel zoveel schade heeft dat hij die niet meer kan herstellen, zal hij een zelfmoordprogramma (apoptose) in werking zetten waardoor hij langzaam dood gaat en desintegreert zonder een ontstekingsreactie te veroorzaken. Apoptose is een beschermingsmechanisme waarbij het ontstaan van gemuteerde cellen, die later tot kankercellen kunnen uitgroeien, wordt voorkomen. Door middel van een biochemische kleurreactie in de afgenomen huidmonsters hebben we aangetoond dat de Langerhans cellen niet in apoptose gaan, maar veel van de omliggende opperhuidcellen (keratinocyten) wel.
Het is moeilijk om een dynamisch proces als migratie aan te tonen in een biopsie die een statische momentopname laat zien. Daarom hebben we eerst een nieuwe methode ontwikkeld voor het meten van migratie. Door het trekken van een blaartje met behulp van onderdruk kunnen de epidermis en de onderste huidlaag (lederhuid of dermis) pijnloos van elkaar gescheiden worden. De holte die daardoor ontstaat, wordt gevuld met blaarvocht. De Langerhans cellen die zich in de epidermis bevinden ‘vallen’ als het ware, wanneer ze de huid uit migreren, in het blaarvocht. Wanneer we nu het blaarvocht met behulp van een klein naaldje uit de blaar halen, zouden we migrerende Langerhans cellen in dit blaarvocht moeten kunnen terugvinden. Dit bleek inderdaad ook het geval: in de UV-belichte huid, maar niet in de onbelichte huid, konden we migrerende Langerhans cellen terugvinden in het blaarvocht. Ter controle werd ook het blaardak (epidermis) van de blaar gehaald om aan te tonen dat de

Langerhans cellen inderdaad verdwenen waren. Het onderliggende mechanisme voor de verdwijning van Langerhans cellen bleek dus geen apoptose maar migratie te zijn.
Met behulp van deze gegevens konden we nu nagaan of CPLD patiënten een defect hebben in de UV-geïnduceerde migratie van hun Langerhans cellen (**hoofdstuk 4**). Daartoe hebben we bij CPLD patiënten blaren getrokken op de belichte en onbelichte huid. Het bleek dat CPLD patiënten een sterk verminderde migratie hebben van hun Langerhans cellen. Een groot gedeelte van de cellen blijft gewoon zitten in de epidermis na UV-B belichting terwijl maar een heel klein gedeelte van de cellen terug te vinden is in het blaarvocht.
Waardoor is deze Langerhans cel migratie nu verstoord in CPLD patiënten? Om dat te onderzoeken hebben we gekeken naar de aanwezigheid van allerlei wateroplosbare stofjes, cytokines, die migratie van deze Langerhans cellen kunnen induceren **(hoofdstuk 5**). De twee belangrijkste migratie-inducerende cytokines, die in gezonde personen geproduceerd worden na belichting van de huid met UV-B zijn interleukine (IL)-1-β en tumor necrosis factor-α (TNF-α). Deze twee cytokines bleken door significant mindere cellen geproduceerd te worden in de belichte huid van CPLD patiënten. Deze verminderde productie vormt een plausibele verklaring voor de verminderde Langerhans cel migratie in CPLD patiënten.
UV-B straling dringt niet dieper dan epidermis de huid in, waar het schade kan veroorzaken aan het DNA (thymidine dimeren). Migrerende Langerhans cellen uit de UV-B-belichte epidermis zijn dan ook herkenbaar aan hun UV-specifieke DNA schade. Het bleek echter dat maar 10-15% van alle migrerende Langerhans cellen UV-specifieke DNA schade had. Vermoedelijk wordt de schade eerst hersteld voordat de Langerhans cellen kunnen gaan migreren. Om dit te onderzoeken hebben we experimenten gedaan in (transgene) muizen die een defect hebben in het DNA herstelmechanisme waardoor ze niet of in beperkte mate de UV-geïnduceerde DNA schade kunnen herstellen (**hoofdstuk 6**). Deze muizen zijn zeer gevoelig voor UV-B straling en verbranden snel. Door deze muizen bloot te stellen aan lage doses UV-B straling konden we nagaan of een gebrek aan DNA herstel de migratie van de Langerhans cellen beïnvloedt. Het bleek echter dat deze muizen bij deze lage UV-B doses en geringe DNA schade al Langerhans cel migratie vertoonden. Herstel van het DNA is dus geen absolute voorwaarde voor Langerhans cel migratie in deze dieren. Met behulp van deze muizen konden we ook nagaan of er een verband bestond tussen de Langerhans cel migratie en de onderdrukking van het immuunsysteem. Met andere woorden, leidt een verminderde migratie tot een verminderde immuunsuppressie? De muizen die bij een lage dosis UV-B straling al Langerhans cel migratie vertoonden, lieten ook bij deze lage dosis al een onderdrukking van het immuunsysteem zien. Samenvattend kunnen we dus zeggen dat DNA herstel niet nodig is voor Langerhans cel migratie, in tegenstelling tot wat we vermoedden bij de mens, en dat Langerhans cel migratie gerelateerd is aan immuunsuppressie in de huid. Dit laatste lijkt ook het geval te zijn bij de mens want Chantalle van de Pas en anderen hebben

recent in Londen experimenteel aangetoond dat CPLD patiënten inderdaad, zoals wij vermoedden, na UV-B straling een verminderde onderdrukking hebben van het immuunsysteem en dat ze daarom waarschijnlijk ook, naar onze waarnemingen, minder migratie van Langerhans cellen uit de opperhuid vertonen.

Terugkomend op de verstoorde Langerhans cel migratie in CPLD patiënten en het achterblijven van de Langerhans cellen in de epidermis kunnen we ons afvragen wat die achterblijvende Langerhans cellen in de epidermis doen. Kunnen ze daar het immuunsysteem activeren en zo de huidreactie veroorzaken? Om dit te onderzoeken hebben we gekeken naar de aanwezigheid van activatiemarkers op de Langerhans cellen (**hoofdstuk 4**). Wanneer de Langerhans cellen zowel hun 'verteerde' antigeen als deze activatie moleculen op hun celoppervlakte hebben, zijn ze in staat om cellen van het immuunsysteem, zoals de T-cellen, te activeren en hierdoor (eventueel) een ontstekingsreactie te veroorzaken. De Langerhans cellen die achterbleven in de epidermis van CPLD patiënten bleken, tegen onze verwachting in, niet geactiveerd te zijn (geen activatie moleculen op hun oppervlakte). Wat we wel konden waarnemen was een ophoping van geactiveerde Langerhans cellen in de onderste huidlaag (dermis). De gelijktijdige aanwezigheid in de dermis van geactiveerde Langerhans cellen samen met een bepaald type ontsteking-veroorzakende T-cellen zou mede verantwoordelijk kunnen zijn voor het ontstaan van CPLD.

Diverse cytokines spelen een belangrijke rol bij het aansturen van het immuunsysteem. Zo bestaan er cytokines die een remmende werking op een vertraagde allergische reactie hebben (IL-10, IL-4) en cytokines die een stimulerende werking hebben (IL-12, interferon-γ). Deze cytokines kunnen op verschillende wijze worden beïnvloed door UV-B straling. De productie van remmende cytokines wordt verhoogd na belichting van de huid van gezonde personen terwijl de productie van stimulerende cytokines juist wordt verlaagd. Alles bij elkaar genomen leidt dit tot een onderdrukking van het immuunsysteem bij gezonde personen. In CPLD patiënten, bij wie de UV-geïnduceerde immuunsuppressie vermoedelijk verstoord is, is de balans tussen de productie van remmende en stimulerende cytokines hoogstwaarschijnlijk doorgeslagen richting stimulerende cytokines. Om deze hypothese te onderzoeken hebben we gekeken naar de productie van deze verschillende cytokines in de belichte huid van CPLD patiënten en die vergeleken met gezonde vrijwilligers (**hoofdstuk 5**). We konden bij de UV-dosis die wij gebruikten voor de belichting geen verschil vinden in de productie van stimulerende cytokines in de huid van CPLD patiënten ten opzichte van gezonde vrijwilligers. Wel zagen we dat in de huid van CPLD patiënten, in vergelijking met gezonde personen, significant minder cellen aanwezig waren die IL-4 bevatten, een remmende cytokine. Deze kleinere hoeveelheid IL-4 draagt waarschijnlijk bij tot een verminderde onderdrukking van het immuunsysteem in CPLD patiënten. Deze verminderde productie van IL-4, maar ook van TNF-

α, bleek vooral veroorzaakt te worden door een lager aantal cytokine-producerende neutrofielen in de huid van CPLD patiënten ten opzichte van gezonde personen.

Wanneer we alle gegevens op een rijtje zetten kunnen we tot een hypothetisch model komen voor het ontstaan van CPLD (**hoofdstuk 7**). Doordat na UV-B belichting minder IL-1-β en TNF-α wordt geproduceerd in de huid van CPLD patiënten migreren minder Langerhans cellen uit de epidermis. Door een verminderde en vertraagde Langerhans cel migratie hopen de Langerhans cellen zich op in de dermis. Deze Langerhans cellen, die geactiveerd zijn, kunnen daar hun antigenen presenteren aan specifieke T-cellen. Deze T-cellen kunnen vervolgens een ontstekingsreactie veroorzaken. Het lage aantal cellen dat het remmende cytokine IL-4 produceert, speelt een belangrijke rol bij de verminderde onderdrukking van het immuunsysteem. Aangezien zowel TNF-α en IL-4 door (CD11b+) neutrofielen wordt geproduceerd, lijken deze cellen een prominente rol te spelen bij het ontstaan van CPLD.

In dit proefschrift staan de eerste schreden beschreven op weg naar een opheldering van de mechanismen die ten grondslag liggen aan het ontstaan van CPLD. Toekomstig onderzoek is nodig om het pathologisch mechanisme verder te ontrafelen en specifiekere en efficiëntere therapieën te ontwikkelen voor deze fotodermatose.

Dankwoord

Hier ligt het dan. Mijn proefschrift. Vijf jaar werk verzamelt in een boekje. Mijn proefschrift zou niet compleet zijn zonder dankwoord, want al die pagina's heb ik niet alleen gevuld. Heel wat mensen hebben een steentje bijgedragen, een voetspoor achter gelaten. Al deze mensen zou ik willen bedanken voor hun bijdrage.

Allereerst mijn copromotor en inspiratiebron, Frank de Gruijl. Frank, jij hebt me de weg gewezen in de wetenschap en me omgevormd tot de wetenschapper die ik nu ben en altijd heb willen zijn. Zonder jouw enthousiasme en hart voor het onderzoek had dit boekje er nu niet gelegen. Zelfs je onfortuinlijke verhuizing naar het LUMC heeft geen roet in het eten kunnen gooien, het heeft ons uiteindelijk alleen maar sterker gemaakt. Ik hoop dat ik voor anderen kan zijn wat jij voor mij bent geweest: een voorbeeld in de wetenschap.

Prof. Van Vloten, Willem, mijn promotor. Van begin af aan heeft u mijn onderzoek in de gaten gehouden, maar ook buiten het onderzoek om heeft u altijd een oogje in het zeil gehouden. Een prettige gedachte. Ondanks uw minder goede gezondheid die helaas uiteindelijk leidde tot uw vertrek bij de afdeling, bent u iedere keer speciaal voor mij vanuit Wageningen naar Utrecht gereisd voor onze pow-wows. Als uw laatste promovendus sluit niet alleen ik maar ook u een hoofdstuk af. Ik ben blij dat u mijn promotor hebt willen zijn.

Verder wil ik, Edward Knol, mijn copromotor bedanken. Edward, bij jou kon ik altijd terecht voor immunologische vragen. Het is jammer dat het lichtonderzoek jou niet zo heeft kunnen raken, het is echt heel leuk!

Prof. Carla Bruijnzeel-Koomen, mijn promotor, wil ik bedanken voor haar pittige bijdrage aan het onderzoek. Wat dat betreft, Carla, heb je een zware dobber aan me gehad, we zijn beiden erg eigenwijs. En je ziet, uiteindelijk kom ik toch nog bij 'T-cellen' uit.

Voor aanlevering van patiënten was ik aangewezen op jou, Ina en op jou, Huib. Hoewel de aanvoer van nieuwe, geschikte patiënten af en toe erg mager was, hebben we het toch maar gered! Bedankt voor jullie hulp.

Ilse en Kees, bedankt! Ilse, zonder jouw praktische hulp zou mijn boekje nog wel even op zich hebben laten wachten. Kees, meneer Binas, hoewel we niet al jouw inspirerende en innovatieve ideeën hebben kunnen uitvoeren, was het altijd zeer boeiend en motiverend om ze te bedenken. Ik zal ze missen…maar ik weet je te vinden!

De (oude) lichtclub (pow-wowwers): Rob, Anne(marie), Heggert, Niek, Prof. Van de Leun, Marjan, Arne, Gerdine. Het was altijd heel gezellig samen! Marjan, ondanks je overgang naar een nieuwe baan heb je me nog op het laatst uit de brand kunnen helpen. Dank je wel!

In het verlengde hiervan, Soe Janssens. We kennen elkaar nog niet zo heel lang, maar hopelijk is mijn proefschrift een inspiratiebron voor jouw onderzoek. Ik hoop dat we ook in de toekomst nog vaak contact zullen hebben. Succes!

Alle studentes die hun steentje hebben bijgedragen aan de experimenten wil ik bij deze graag bedanken. Sarah, Mandy, Jet en Hilde (leuke foto's!), bedankt!

Johan (Garssen), ondanks onze soms complexe communicatie en de geduld-op-de-proef-stellende kleuring van de muizenoortjes, hebben we het experiment toch tot een goed einde weten te brengen.
Jon Laman en Marjan van Meurs, bedankt voor jullie gastvrijheid en hulp bij het opzetten van de cytokinekleuringen.
Bert, Marjolein, Feiko en Ines, bedankt voor jullie hulp bij het afnemen van alle huidbiopten.
Ook alle patiënten en vrijwilligers die deel hebben genomen aan de proefjes wil ik bij deze graag bedanken. Mijn boekje zou leeg zijn zonder jullie.
Mijn kamergenoten door de jaren heen: Rob, Heggert, Lilian, Bert, Anoek, Annemarie, Arne, Gerdine, Ines, Rebecca, Feiko, Inge, Mayke en Chantal. Het is altijd een levendige, gezellige kamer geweest, waar buiten de boeiende wetenschappelijke discussies om, ook gelukkig tijd overbleef om te lachen!
Adrie, Annebeth, Audrey, Coraline, Dirk-Jan, Els, Inge de V., Jantine, Joke-Afke, Machteld (Houdt vol!), Marja, Marjan H., Marjolein W., Marloes, Rogier en Suzanne B., bedankt voor de gezellige tijd samen.
Verder wil ik alle arts-assistenten, stafmedewerkers en overige medewerkers van de afdeling Dermatologie bedanken voor hun interesse in mijn onderzoek.
In het bijzonder wil ik jullie bedanken: Bert, Marjolein en Rebecca. Binnen één jaar tijd hebben we met z'n 3-en een promotieonderzoek afgerond en toch nog tijd gezien om elkaar onderling ermee te helpen. We kunnen een promotiebureautje beginnen! Rebecca, bedankt voor al je hulp, de inspirerende discussies en de ritjes naar Woerden. En die kamer delen, dat hadden we eerder moeten doen! Marjolein, ondanks de zeer drukke periode voor jou ben je altijd even attent en in voor een praatje, gezellig. Bert, mijn paranimf, je hebt altijd maar een half woord nodig om te begrijpen wat ik bedoel: beda…! En die gezellige etentjes laten we niet aan onze neus voorbijgaan! Wanneer gaan we weer?
De NKT club van het Vrije Universiteit Medisch Centrum: Johan, Rik, Mary en Fons. Nu kan echt 100% van mijn hersenen zich aan de NKT cel gaan wijden.
Last but not least mijn familie:
Pap en mam, jullie hebben me altijd gestimuleerd in mijn hunker naar kennis en me het doorzettingsvermogen meegegeven dat nodig was om dit te bereiken. Bedankt!
Jeroen, mijn broer(tje) en paranimf, bedankt voor al je computerhulp en het vervaardigen van de kaft en uitnodigingen.
Cindy & Maarten, mijn schoonmoeder, Patrick & Brigitte (en Tom): jullie wil ik graag bedanken voor jullie onuitputtende interesse in mijn onderzoek.
Erik, dank je voor je luisterend oor als ik weer thuis kwam met bergen verhalen die ik kwijt moest. Jouw nuchtere, rationele kijk op dingen heeft me iedere keer weer in balans gehouden. Je bent er voor me als ik je nodig heb, dank je wel!

Wendy

Curriculum vitae

Wendy Kölgen is geboren op 17 juni 1975 te Heerlen. Zij behaalde haar Gymnasium diploma in 1993 aan het Katholiek Gymnasium Rolduc te Kerkrade. In datzelfde jaar begon zij met de studie Gezondheidswetenschappen aan de Universiteit Maastricht. Tijdens deze studie met als afstudeerrichting Biologische Gezondheidkunde werd een eerste stage gelopen aan de Universiteit Maastricht bij de afdeling Gezondheidsrisico Analyse & Toxicologie onder leiding van Drs. Vermeer en Prof. Kleinjans. Er werd onderzoek gedaan naar de vorming van nitroamines na consumptie van nitraat en amines. Een tweede onderzoeksstage werd aan Universiteit Maastricht verricht bij de afdeling Moleculaire Celbiologie & Genetica onder leiding van Dr. Schutte en Prof. Ramaekers. Tijdens deze onderzoeksstage werd een antilichaam gekarakteriseerd dat gericht bleek te zijn tegen keratine in apoptotische cellen. Voor haar afstudeerscriptie ontving zij de Nijbakker morra prijs. Haar doctoraal examen behaalde zij in augustus 1997. In september 1997 begon zij als AIO bij de afdeling Dermatologie / Allergologie van het Universitair Medisch Centrum Utrecht . Hier verrichte zij, onder supervisie van Dr. F.R. de Gruijl, Prof. dr. W.A. van Vloten, Dr. E.F. Knol en Prof. dr. C.A.F.M. Bruijnzeel-Koomen, onderzoek naar de pathogenese van chronische polymorfe lichtdermatose wat heeft geleid tot de bevindingen beschreven in dit proefschrift.

Sinds 1 oktober 2002 is zij werkzaam als post-doc bij de onderzoeksgroep Immunotherapie, een samenwerking tussen de afdelingen Geneeskundige Oncologie en Pathologie van het Vrije Universiteit medisch centrum te Amsterdam. Onder supervisie van Dr. A.J.M. van den Eertwegh, Dr. B.M.E. von Blomberg en Prof. dr. R.J. Scheper verricht zij onderzoek naar de anti-tumor activiteiten van natural killer T cellen.

Addendum

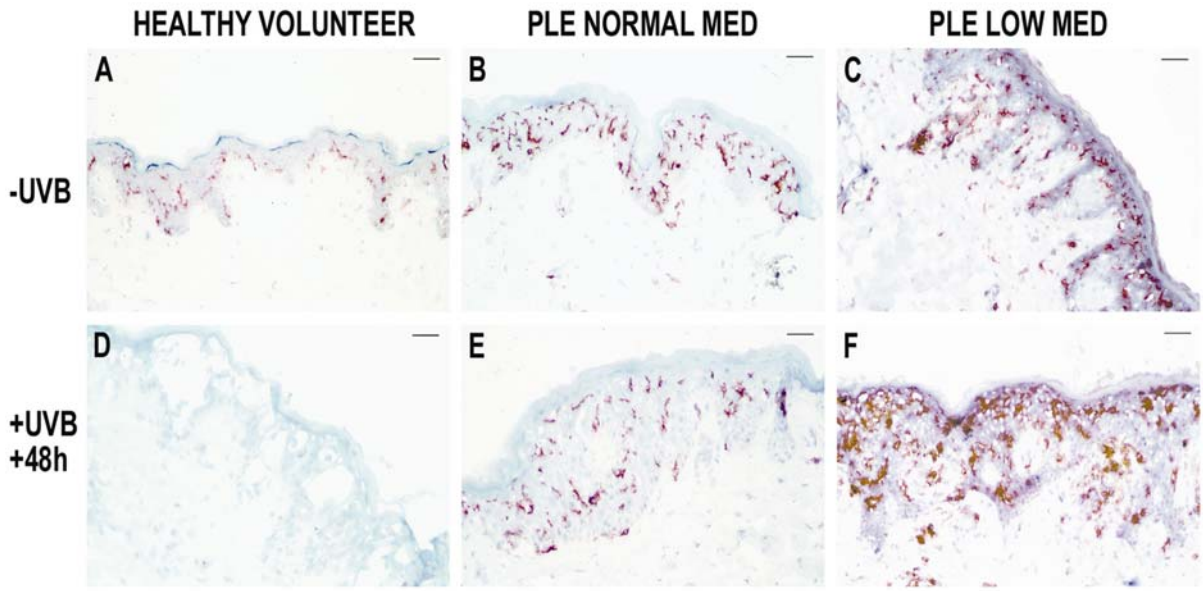

Figure 1. CD1a staining of buttock skin, unexposed (- UV) and 48 hours after UV-B exposure (+ UV + 48h). Scale bar = 50 μm. **(chapter 2)**

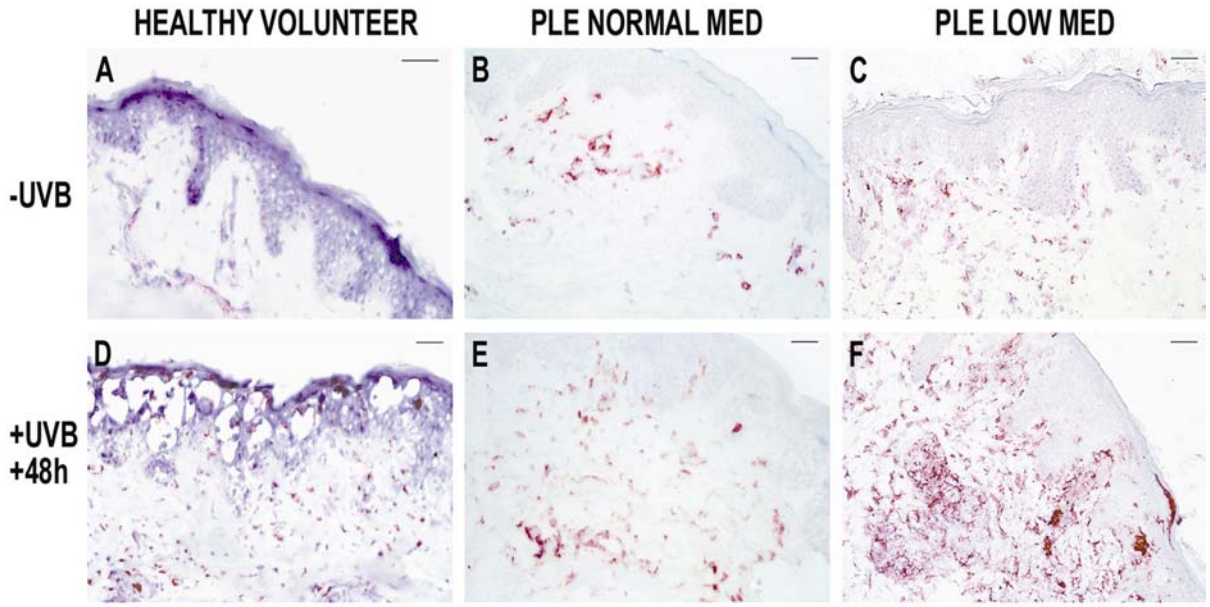

Figure 3. CD11b staining of buttock skin, unexposed (- UV) and 48 hours after UV-B exposure (+ UV + 48h). Scale bar = 50 μm. **(chapter 2)**

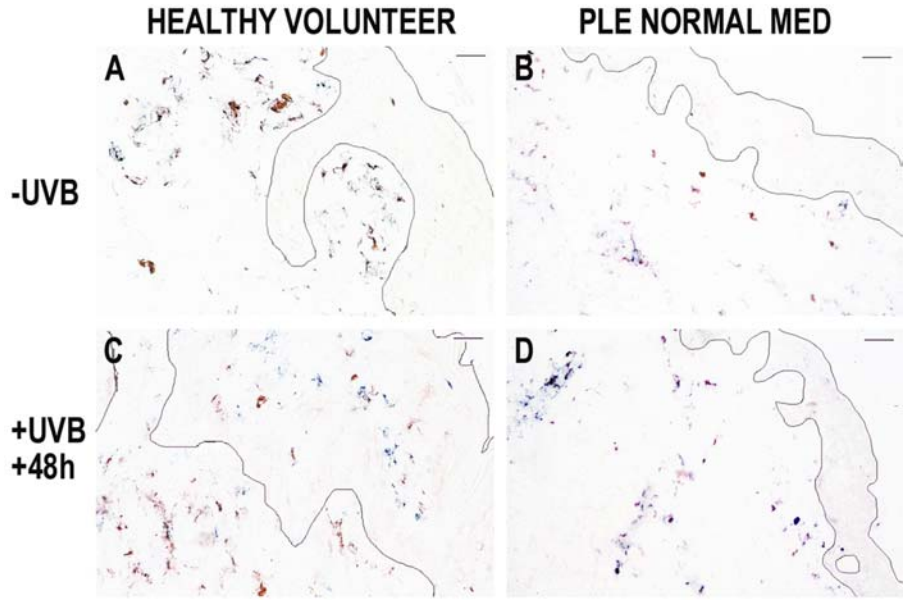

Figure 5. Double staining of CD11b (blue) and CD68 (red) of buttock skin, unexposed (- UV) and 48 hours after UV-B exposure (+ UV + 48h). The epidermis was demarcated manually. Scale bar = 50 μm. **(chapter 2)**

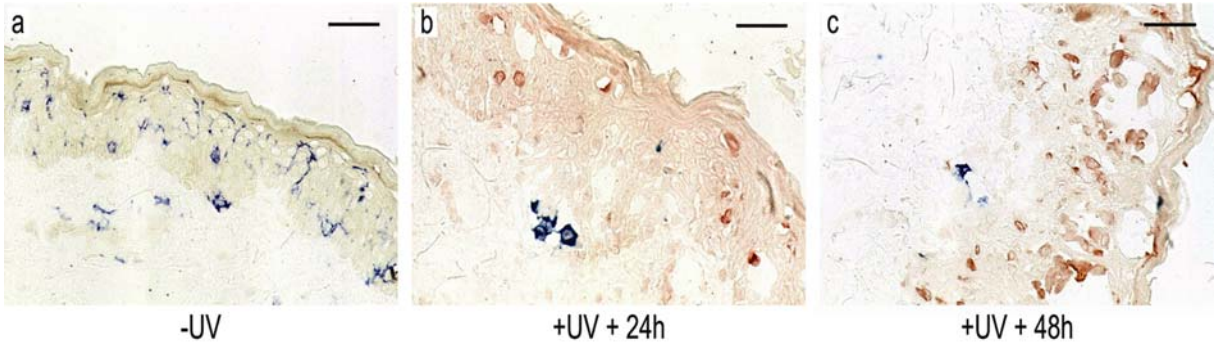

Figure 1. Double staining of CD1a (blue) and active-caspase 3 (red) of buttock skin, unexposed (- UV), 24h (+ UV +24h) and 48h after UV-B exposure (+ UV +48h). Scale bar =50 μm. **(chapter 3)**

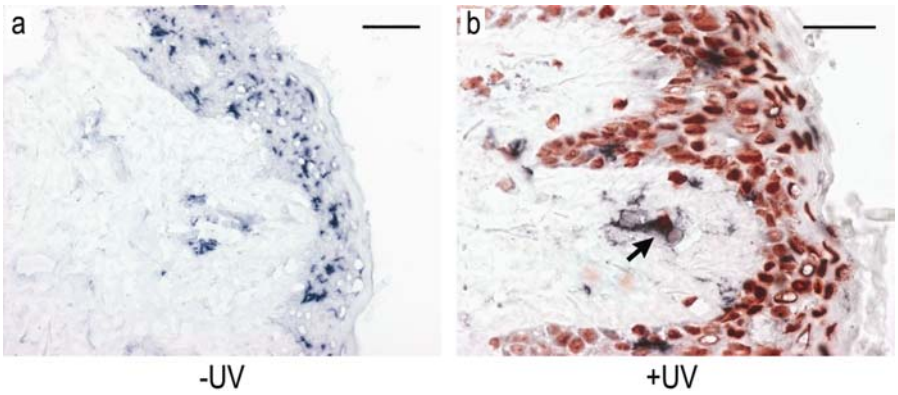

Figure 3. Double staining of CD1a (blue) and H3 (red) of buttock skin, unexposed (- UV) and 24h after UV-B exposure (+ UV) with an example of a doubled stained cell, migrating out of the epidermis (black arrow). Scale bar = 50 μm. **(Chapter 3)**

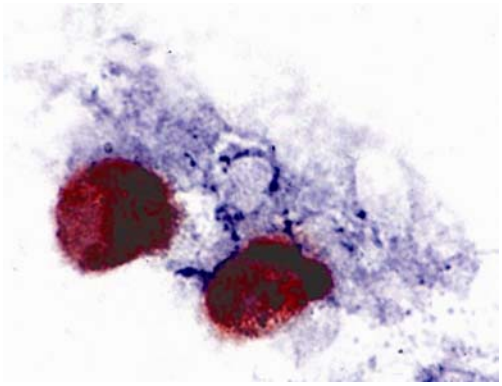

Figure 4. Double staining of CD1a (blue) and H3 (red) in blister fluid of the UV-B-exposed skin. **(Chapter 3)**

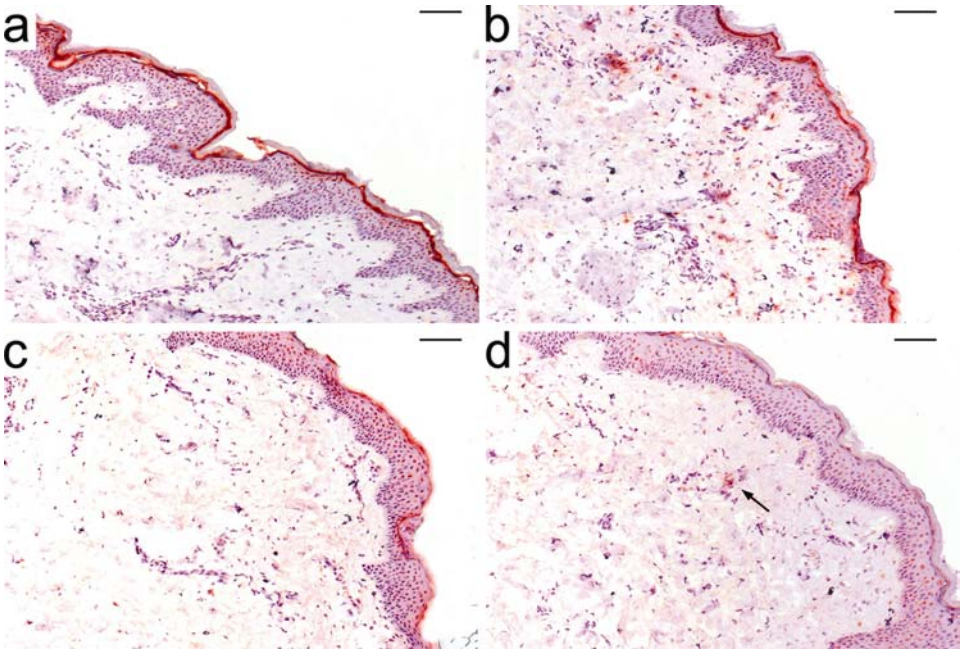

Figure 1. IL-1-β expression in unexposed skin (a,c) and 24h after UV exposure (b,d) of a PLE patient (c,d) and a healthy control (a,b), with an example of a rare cell expressing IL-1-β in the UV-exposed skin of a PLE patient (black arrow). Scale bar = 100 μm. **(Chapter 5)**

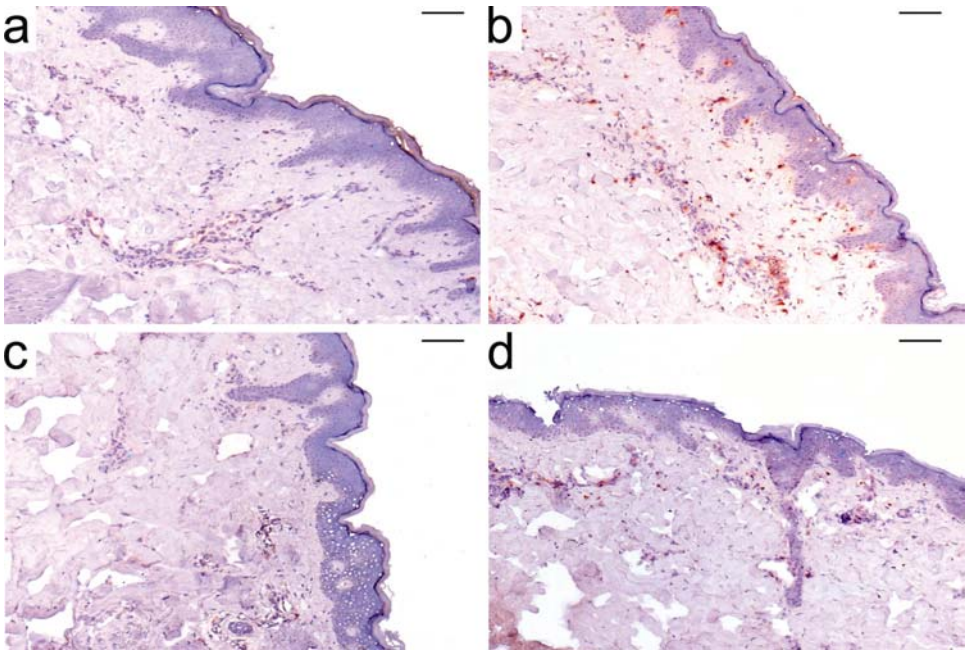

Figure 2. TNF-α expression in unexposed skin (a,c) and 24h after UV exposure (b,d) of a PLE patient (c,d) and a healthy control (a,b). Black arrows indicate examples of TNF-α-expressing cells. Scale bar = 100 μm. **(Chapter 5)**

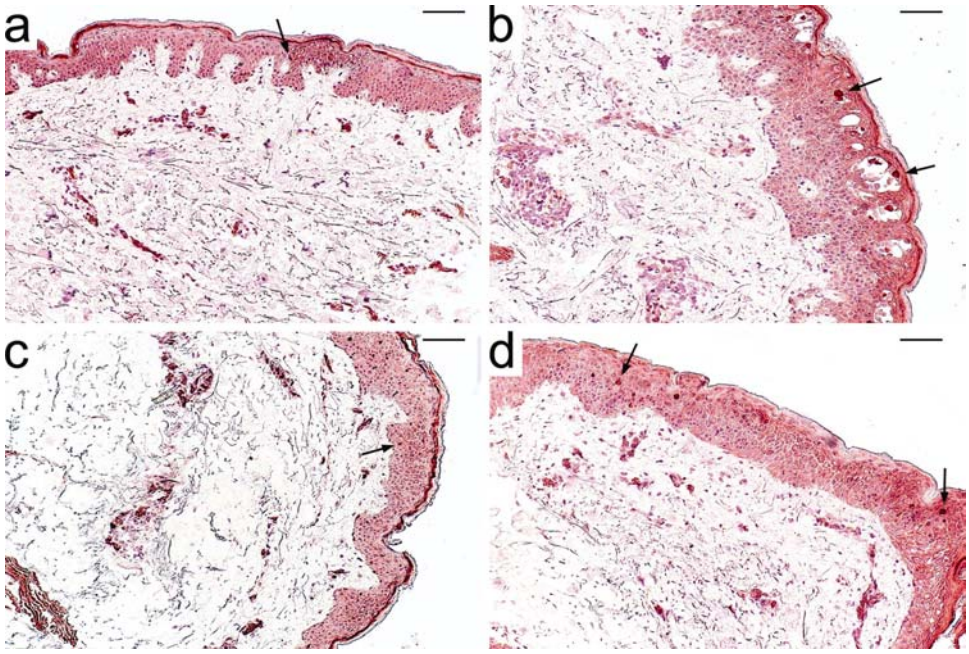

Figure 3. IL-12 expression in unexposed skin (a,c) and 48h after UV exposure (b,d) of a PLE patient (c,d) and a healthy control (a,b). Black arrows indicate brightly IL-12-expressing cells. Scale bar = 100 μm. **(Chapter 5)**

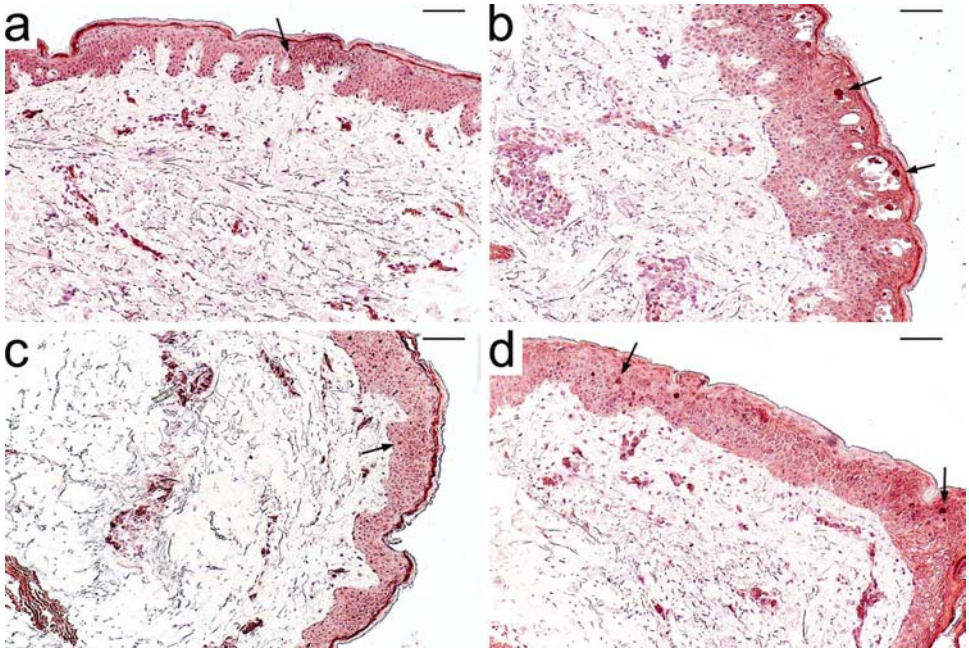

Figure 4. IL-4 expression in unexposed skin (a,c) and 24h after UV exposure (b,d) of a PLE patient (c,d) and a healthy control (a,b). Scale bar = 100 μm. **(Chapter 5)**

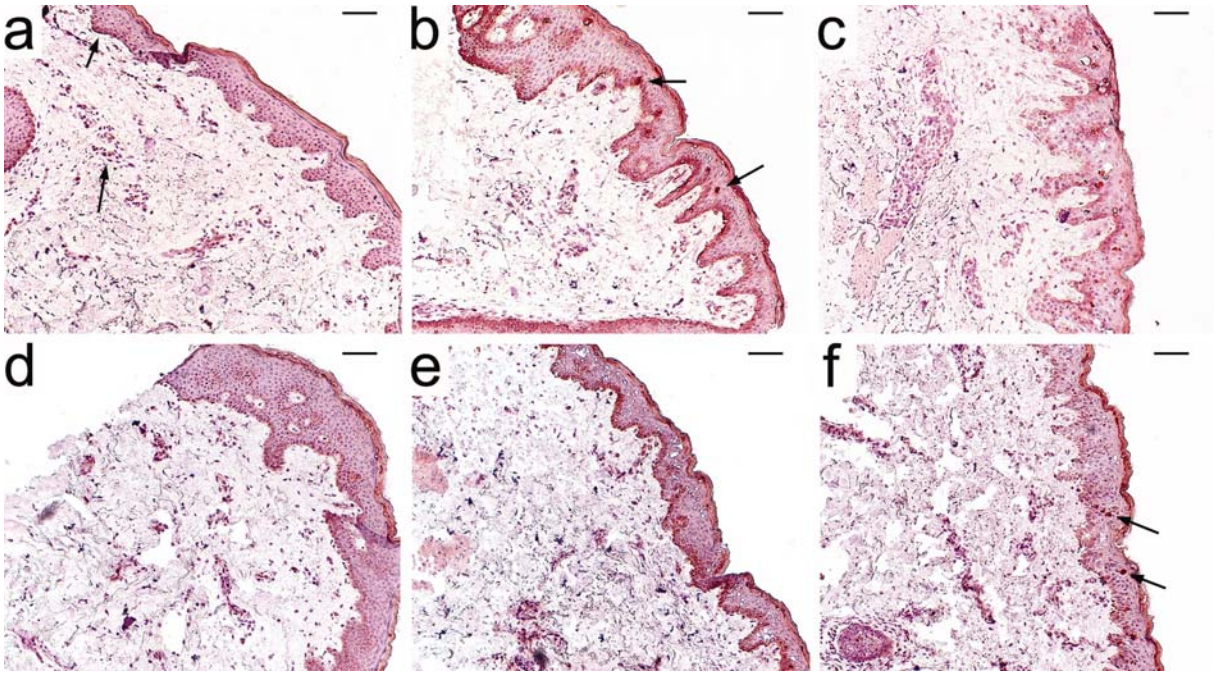

Figure 5. IL-10 expression in unexposed skin (a,d) and 24h (b,e) or 48h (c,f) after UV exposure of a PLE patient (d-f) and a healthy control (a-c). Black arrows indicate IL-10-expressing cells (a) or brightly IL-10-expressing cells (b,f). Scale bar = 100 μm. **(Chapter 5)**

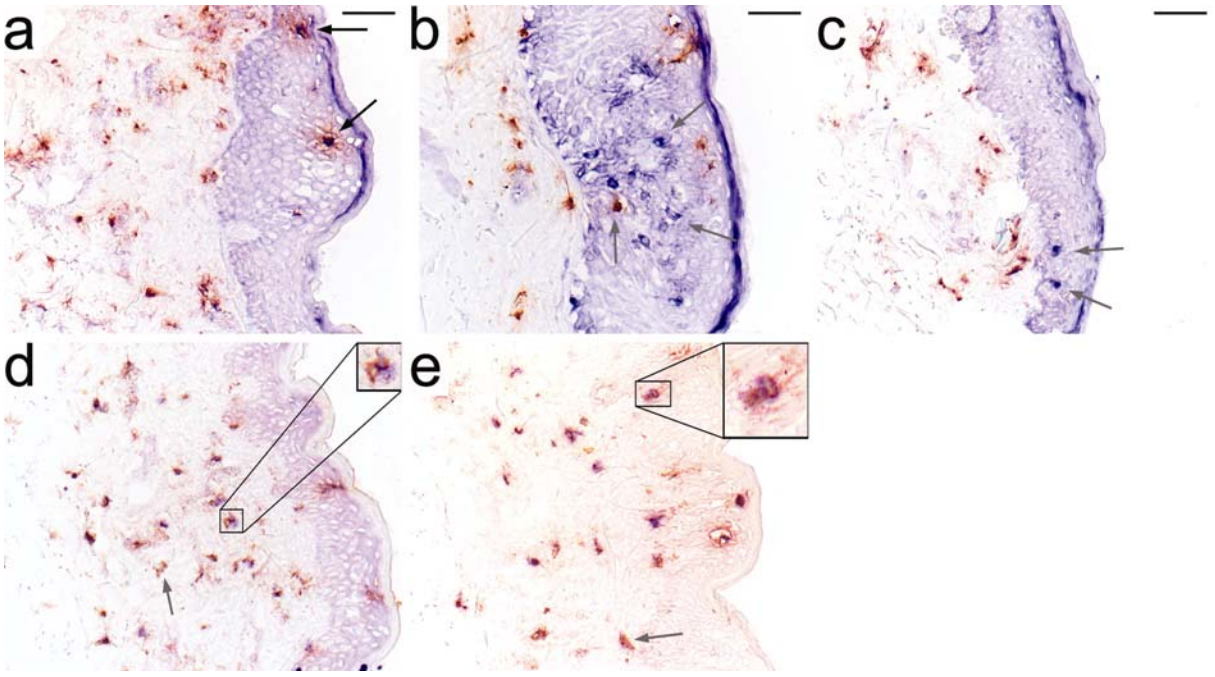

Figure 6. Co-expression for elastase (red) and IL-10 (a-c), TNF-α (d) or IL-4 (e) (blue) in the skin of a healthy control (a,b,d,e) or a PLE patient (c) 24h (a,c,d,e) or 48h (b) after UV exposure. Black arrows and magnifications indicate double positive cells. Gray arrows indicate single positive cells. Scale bar = 100 μm. ***(Chapter 5)***