UVB-induced leukocyte trafficking in the epidermis of photosensitive lupus erythematosus patients: normal depletion of Langerhans cells

Soe Janssens, Lisa Lashley, Coby Out-Luiting, Rein Willemze, Stan Pavel and Frank de Gruijl

Experimental Dermatology 2005; 14:138-142

Leiden University Medical Center, the Netherlands
Abstract

The pathogenetic mechanisms of UV-induced skin lesions of lupus erythematosus (LE) are unknown. In a recent study of pathogenetic mechanisms of polymorphic light eruption (PLE), significantly more Langerhans cells persisted in the epidermis after UVB overexposure than in healthy individuals. Interestingly, the same phenomenon was observed in one SCLE patient. It could therefore be hypothesised that both photodermatoses share a common pathogenetic mechanism of photosensitivity. In the present study, we tested this hypothesis by investigating leukocyte trafficking in the initial phase of cutaneous LE after intense UVB exposure.

In 22 photosensitive LE patients (12 CDLE, 7 SLE and 3 SCLE) and 9 age/sex-matched controls, uninvolved buttock skin was exposed to 6 MED UVB-radiation. Subsequently, biopsies were taken after 24 hrs, 48 hrs and 72 hrs, and one control biopsy from unirradiated skin. Skin sections were stained for the presence of LCs, neutrophils and macrophages. Areal percentages of positively stained cells within the epidermis were quantified and compared between the patients and controls.

A gradual decrease of epidermal Langerhans cells and a gradual increase of epidermal neutrophils and macrophages, at several timepoints after 6 MED irradiation were observed equally in both LE patients and controls.

Immunohistopathology of irradiated uninvolved skin of photosensitive LE patients did not reveal the same pathologic trafficking of Langerhans cells and neutrophils as described for PLE patients. We conclude that different mechanisms are operative in the pathogenesis of PLE and photosensitive LE.
Introduction

Subacute cutaneous lupus erythematosus (SCLE), chronic discoid lupus erythematosus (CDLE) and systemic lupus erythematosus (SLE) are auto-immune diseases involving the skin. Many of these patients react abnormally to ultraviolet radiation (UVR), i.e. by worsening or induction of lesions in sun-exposed skin. The pathomechanisms of UV-induced skin lesions of LE are largely unknown. The UV-exposed skin of LE patients contains an increased number of apoptotic keratinocytes. There is an emerging consensus that abnormalities in generation and clearance of apoptotic material may be an important source of antigens. UV radiation is one of the proapoptotic stimuli that not only alters DNA but also causes cytoskeletal changes in keratinocytes.\(^1\) The frequently targeted molecules in LE include nucleosomes, splicing ribonucleoproteins, phospholipid/phospholipid-binding protein complexes and the ribonucleoprotein particles Ro/SS-A and La/SS-B. These nuclear antigens are translocated to the surface of keratinocytes during apoptosis\(^2\) and subsequent autoantibody binding could render UV-irradiated cells more susceptible to antibody-dependent cell-mediated cytotoxicity. There is now evidence that apoptotic hydrolysis may also expose 'hidden' epitopes and hence generate neo-antigens. The UV-mediated translocation of nuclear antigens and protein neo-antigens could be mediated by a group of chaperone molecules called heat shock proteins\(^3\) that were found to be upregulated in skin of SLE patients.\(^4\) The abundance of apoptotic cells with accumulated apoptotic debris on the surface of the cells could eventually lead to the loss of tolerance and the induction of an autoimmune response.

Several investigator groups have constructed a model for the pathomechanism of UV-induced LE lesions. Most of these investigations were conducted on artificially UV-provoked lesions which arose between 24 hrs and 3 weeks after repeated UV irradiations.\(^5\)\(^-\)\(^7\) Immunohistochemistry of these lesions showed some similarities with naturally occurring LE lesions. In some cases it is difficult to distinguish clinically photosensitive LE from polymorphous light eruption (PLE), which is a very common idiopathic photosensitivity disorder. It may even be expected that PLE and LE share common photo-pathogenetic mechanisms. In a retrospective study among 85 SCLE and CDLE patients, Millard et al. found a high prevalence of PLE.\(^8\)

PLE is commonly regarded as a delayed type hypersensitivity reaction to a UV-induced neo-antigen.\(^5\)\(^-\)\(^11\) It has been surmised that Langerhans cells may play an important role in the pathogenesis of PLE. Kölgen et al. conducted a study in PLE patients in which they observed that epidermal Langerhans cells persisted in the epidermis of PLE patients after intense UVB exposure, dramatically more than in healthy individuals.\(^12\) One patient who later turned out to have SCLE (instead of PLE) was mistakenly included in this study. Interestingly, the same phenomenon of Langerhans cell persistence after UVB overexposure had been observed in this particular patient.\(^12\) It was therefore postulated that UV resistance of LCs might be
connected with UVB-induced photodermatoses in general, and not specifically with PLE. Another observation by Kölgen et al. was that epidermal neutrophil infiltration was lower after 6 MED irradiation in PLE patients than in healthy controls. This phenomenon may also play a role in early UV-induced LE-lesions.

In the present study we tested the hypothesis that epidermal Langerhans cell migration and neutrophil influx in the initial phase of cutaneous LE after intense UVB exposure are similar to those in PLE. This would lend support to the hypothesis that photodermatoses share a common pathogenetic mechanism of photosensitivity. Moreover, in the obtained skin samples from photosensitive LE patients we also examined for the presence of disturbed apoptotic responses to UVB irradiation: i.e. the number of apoptotic cells after a single high dose of UVB radiation in LE patients was compared to that in controls.

Methods

Subjects
From May until August 2002, twenty-two patients with photosensitive LE were included. The patients were selected from a patient database of the Department of Dermatology of the Leiden University Medical Centre. Nine healthy individuals were included as controls. The patient group consisted of twelve patients with photosensitive CDLE, seven patients with photosensitive SLE and three patients with SCLE (see table 1). Photosensitivity was defined as the induction or worsening of cutaneous LE lesions after sun exposure. The diagnosis of SLE was based on the presence of > 4 criteria of the American College of Rheumatology. The diagnosis of CDLE was based on clinical investigation and histology that included specific immunofluorescence for IgG and IgM deposits along the dermo-epidermal basement membrane. Besides characteristic skin lesions, two out of three SCLE patients had positive serum SSA antibodies. All patients had a history of photosensitivity with triggered or worsened skin lesions after sun exposure. All CDLE and SCLE patients and one SLE patient had active skin lesions at the time of the study, but the test sites were uninvolved. Three CDLE patients and 4 SLE patients were using hydroxychloroquine 200 mg, one CDLE patient was taking chloroquine 100 mg and 3 other SLE patients were using low dose systemic steroids. The healthy volunteers in the control group had no history of chronic dermatologic disorders or auto-immune pathology nor did they use systemic glucocorticoids. Informed consent was obtained from patients and healthy volunteers. The study was approved by the medical ethical committee of the Leiden University Medical Centre in Leiden.

Phototesting procedures
The minimal erythema dose (MED) was determined on previously unexposed and unaffected buttock skin. By testing on buttock skin we wanted to exclude all influences of previous sun exposures on cellular responses in the skin. Philips
TL12 lamps were used (irradiance 1 mW/cm², distance 22 cm). A flat rubber device with 6 small rectangular windows measuring 2.5 x 0.5 cm was placed over the buttock skin. Each opening was irradiated with an increasing dose of UVR, increasing stepwise with 25 seconds per irradiation. The range of irradiation was based on the patients' skin types, so that a person with skin type I was tested with a dose ranging from 21 to 128 mJ/cm² and a person with skin type IV with a dose between 149 and 255 mJ/cm² (the odd doses are caused by a retrospective correction). This high range was only used incidentally if a lower dose range failed to determine the MED. The areas of irradiated skin were examined 24 hrs later and the area showing sharply demarcated borders, was regarded as one MED (see table 1). Subsequently, the opposite buttock skin was exposed to 6 MED in a field of 2 x 2 cm using the same lamp. Four millimetre punch biopsies were obtained from the irradiated field after 24 hrs and 48 hrs, or after 48 hrs and 72 hrs, together with one additional control biopsy from the adjacent unirradiated skin. Specimens were embedded in OCT-compound (Tissue-Tek, Sakura, Zoeterwoude, the Netherlands), snap frozen in liquid nitrogen, and stored at -80°C until further processing.

**Immuno-histochemistry**

Frozen skin sections (6 µm) on 3-amino-propyltriaethoxysilane-coated glass slides were fixed for 10 min. in 100 ml dry acetone containing 100 µl 30% hydrogen peroxide at room temperature. Before and between incubation steps, the glass slides were washed three times with phosphate buffered saline (PBS) containing 0.05% Tween 20 for five minutes. Subsequently, the slides were incubated with a blocking reagent containing 10% normal human serum and 10% normal goat serum diluted in PBS/0.05% Tween 20 for 20 min. to prevent nonspecific binding. The skin sections were incubated overnight with the primary antibody (see below), diluted in blocking reagent with 1% human serum and 1% goat serum. After washing, skin sections were incubated with the second antibody, diluted 1/200 in blocking reagent for 45 minutes. Subsequently, the slides were incubated with a horseradish peroxidase-labeled avidin-biotin complex (diluted 1/100 in PBS/0.05% Tween) for 30 min. Antibody binding was visualized by incubating the sections in 0.1 M acetate buffer (pH=5) containing 20 mg 3-amino-ethyl-carbazole (Sigma, St. Louis, MO, USA) and 100 µl 30% hydrogen peroxide per 100 ml. The skin sections were counter-stained with 10% Mayer’s haematoxylin diluted in distilled water and rinsed with running tap water for 10 minutes. All slides were mounted with Kaiser's glycerin (Merck, Whitehouse Station, NJ, USA) and kept dry and in dark.

**Antibodies**

As primary antibodies we used monoclonal CD1a antibody, clone NA 1/34 (DAKO diagnostics BV, Amsterdam, the Netherlands) diluted 1:60 in PBS, anti-neutrophil elastase (clone NP57) diluted 1:100 in PBS, mouse anti-human monoclonal CD68 antibody (clone EBM11) diluted 1:100, purchased from DAKO and purified.
polyclonal rabbit Anti-active caspase 3 Clone 19, isotype IgG2a (BD Pharmigen, Bedford, MA, USA) diluted 1:30. Biotinylated goat anti-mouse immunoglobulin (Vector, Burlingame, CA, USA) diluted 1/200) was used as the second antibody. As detecting reagent, horseradish peroxidase (HRP)-conjugated avidin-biotin complex (DAKO, diluted 1/100) was used.

Quantification of epidermal cells
The epidermis was photographed at a magnification of 10 x 20 on a light microscope (Nikon Eclipse E-800, Tokyo, Japan) with a standard eye piece. Image-Pro Plus software (MediaCybernetics, Silver Spring, MD, USA), a digital image analysis programme was used for the detection of epidermal red stained cells. Three to five frames per biopsy were analysed. The area covered by these red cells within an area of interest (i.e. epidermis) was expressed as an area percentage and imported in a file of Excel 2000 for Windows (Microsoft, Redmond, WA, USA). Epidermal areas around follicular orifices were avoided, because they serve as epidermal reservoirs of Langerhans cells. 17 Apoptotic cells were counted in the histologic preparations stained with anti-active caspase 3. A distinction was made between cells with positive nuclear staining and cells with positive cytoplasmic staining. The cells with nuclear staining were regarded as true apoptotic (sunburn) cells, whereas the cells with cytoplasmic staining were not. The latter cells could be pre-apoptotic cells or macrophages with phagocytosed apoptotic fragments. 18 The number of cells were counted per high power field at 400x magnification.

Statistical analysis
For statistical analysis, software of SPSS 10 for Windows (SPSS Inc., Chicago, IL, USA) was used. To compare the means, a student’s T-test was performed. For comparison of the effect of UVB at different times in different patient groups, a Mann-Whitney U-test was carried out. Results were considered significant if p < 0.05.

Results
The mean MEDs of CDLE and SLE patients were slightly higher than in controls (not significantly). All 6 MED irradiated skin fields of LE patients were firmly red and oedematosus but did not display any cutaneous LE lesions nor any PLE lesions (e.g. papules, plaques or vesicles). After immunohistochemical staining of biopsies taken at different time points after 6 MED, numbers of epidermal Langerhans cells, macrophages and neutrophils were counted in the skin of CDLE, SLE and SCLE patients and compared with controls.

At several timepoints after 6 MED UVB irradiation a gradual decrease of epidermal Langerhans cells was observed (see figure 1A). At 48 and 72 hrs after 6
MED irradiation almost all LCs had disappeared from the epidermis. However, there was no difference between SLE and CDLE patients and controls.

Seventy-two hours after 6 MED irradiation, an influx of macrophages was observed in controls. In LE patients, more macrophages tended to infiltrate the epidermis, but this was not significantly different from controls (see figure 1B).
Table 1. Characteristics of the subjects

<table>
<thead>
<tr>
<th>group</th>
<th>subjects (n)</th>
<th>male ; female</th>
<th>age (yrs)</th>
<th>MED (mJ/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDLE</td>
<td>12</td>
<td>3 ; 9</td>
<td>43 (24-56)</td>
<td>121 (64-255)</td>
</tr>
<tr>
<td>SLE</td>
<td>7</td>
<td>1 ; 6</td>
<td>41 (24-58)</td>
<td>118 (64-234)</td>
</tr>
<tr>
<td>SCLE</td>
<td>3</td>
<td>2 ; 1</td>
<td>42 (32-56)</td>
<td>100 (68-128)</td>
</tr>
<tr>
<td>controls</td>
<td>9</td>
<td>4 ; 5</td>
<td>36 (18-56)</td>
<td>99 (64-149)</td>
</tr>
</tbody>
</table>

*age and MED (minimal erythema dose) as mean with range between brackets; CDLE: chronic cutaneous lupus erythematosus; SLE: systemic lupus erythematosus; SCLE: subacute cutaneous lupus erythematosus*

Table 2. Number of apoptotic epidermal cells

<table>
<thead>
<tr>
<th>group</th>
<th>nuclear stained epidermal cells²</th>
<th>cytoplasmic stained epidermal cells³</th>
<th>total stained epidermal cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDLE (n=5)</td>
<td>10 ± 5</td>
<td>13 ± 3</td>
<td>23 ± 5</td>
</tr>
<tr>
<td>SLE (n=4)</td>
<td>8 ± 8</td>
<td>11 ± 7</td>
<td>19 ± 12</td>
</tr>
<tr>
<td>controls (n=5)</td>
<td>9 ± 8⁴</td>
<td>16 ± 15⁴</td>
<td>25 ± 23⁴</td>
</tr>
</tbody>
</table>

*data as mean ± standard deviation
² expressed per high power field at 400x magnification
³ cells with positive cytoplasmic staining were considered pre-apoptotic cells or macrophages phagocytosing apoptotic fragments
⁴ not significant; a comparison was made between healthy controls and patients (CDLE and SLE vs controls)*

There was no difference in neutrophil influx between CDLE, SLE and healthy controls (see figure 1C). However, the influx of neutrophils in SCLE patients was very high at 48 and 72 hrs after 6 MED irradiation, but the difference was not statistically significant from controls, which probably relates to a small sample size. The number of apoptotic cells (active caspase 3 in nuclei), at 48 hrs after 6 MED irradiation in the skin of SLE and CDLE patients did not differ from that in healthy controls (see table 2). The number of infiltrating or migrating cells after UVB irradiation in patients who used medication did not differ from patients without medication (data not shown).

Discussion

The hypothesis that photodermatoses, PLE and LE, may share a common pathogenetic mechanism is not supported by our present results. We did not find any evidence of epidermal LC persistence at 24, 48 or 72 hrs after a single high dose of UVB radiation in a group of photosensitive LE patients. Our findings may link up with observations from other groups that found persistently lower numbers
of epidermal LCs in both
Figure 1. Epidermal area percentages of Langerhans cells (A), neutrophils (B) and macrophages (C) after 6 MED irradiation in photosensitive lupus erythematosus.

A

B

C
Biopsies were taken from 6 MED irradiated and unirradiated buttock skin of 12 CDLE, 7 SLE and 3 SCLE patients and 9 age/sex-matched controls. Immunohistochemic staining was performed for the presence of (A) epidermal Langerhans cells (LCs) with CD1a, (B) macrophages with CD68 and (C) neutrophils with elastase. (A) No differences were found in epidermal area percentages of LCs at different time points between LE patients and controls. (B) Macrophages infiltrate the epidermis in similar numbers in LE patients and controls. (C) The epidermal influx of neutrophils in CDLE and SLE did not differ from controls.

Artificially induced and spontaneous LE skin lesions than in adjacent normal skin or skin from healthy controls. Neither could we find any differences in the epidermal infiltration patterns of neutrophils and macrophages between SLE and CDLE patients and healthy controls. The slightly higher average MEDs of CDLE and SLE patients were apparently caused by one patient in each group with a very high MED (resp. 255 mJ/cm² and 234 mJ/cm²). These patients were using hydroxychloroquine and the high MED values may reflect a photoprotective action of this medication.

The exclusion of these cases would bring the average MEDs down to respectively, 108 and 99 mJ/cm² but it would not change the outcome of our study. Irradiation with a higher MED could result in more Langerhans cell migration and more neutrophil influx. We did observe the lowest number of epidermal LCs 48 hrs after 6 MED in the one CDLE patient with a high MED but not in the SLE patient. Three CDLE patients and 4 SLE patients were using hydroxychloroquine, one CDLE patient was taking chloroquine and 3 other SLE patients were using low dose systemic steroids. In CDLE patients using hydroxychloroquine and chloroquine, the numbers of epidermal LCs 48 hrs after 6 MED irradiation did not differ significantly from CDLE patients without medication (resp. mean 2.5 ± 0.6 vs 2.0 ± 0.9; p= 0.36). We did not observe extreme values of neutrophil infiltration in the patients with high MEDs. The number of apoptotic epidermal cells was equal in LE patients and controls at 48 hrs after UVB irradiation. In sum, epidermal cell migration after UVB irradiation in skin biopsies of unaffected LE skin did not differ from controls and did not confirm our initial hypothesis of a shared photo-pathogenetic mechanism between PLE and LE. As was already mentioned in the introduction, for yet unclear reasons, PLE and LE apparently concur or develop after one another in many cases. Still, in these cases PLE should be considered a distinct entity with a different clinical appearance and time course than cutaneous lesions in LE. From this perspective, we suspect that the finding by Kölgen et al. in one SCLE patient could be attributed to concurrent PLE (which may have confused the clinical diagnosis). The results of our investigation show that the Langerhans cells in photosensitive LE do not exhibit the 'sluggish' migration pattern observed in PLE after UVB overexposure. This indicates that different pathogenetic mechanisms are operative in these two photodermatoses.

Additionally, the lack of differences between LE patients and controls in the number of epidermal apoptotic cells within two days after intense UV irradiation of
unaffected LE skin, does not support the inference of impaired apoptotic clearance from LE skin.

Acknowledgements
This research was supported by the European Union Framework V Programme, project number QLK4-CT01-0015. We thank dr. M. Karperien of the Department of Endocrinology and Metabolic diseases for his support and facility to analyse digital images.

References
18. Dukers D F, Meijer C J, ten Berge R L *et al.* High numbers of active caspase 3-positive Reed-Sternberg cells in pretreatment biopsy specimens of patients with Hodgkin disease predict favorable clinical outcome. *Blood* 2002; 100: 36-42