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## The Dynamics of the Response of Normal Skin to Single and Multiple Epicutaneous Leukotriene B<sub>4</sub> Applications Analysed by Three-colour Flow Cytometry

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Leukotriene B<sub>4</sub> (LTB<sub>4</sub>) is a potent chemoattractant and a well-established stimulator of DNA-synthesis in keratinocytes. Previously, repeated applications of LTB<sub>4</sub> have been reported to induce a topically defined tachyphylaxis with respect to the extravasation of polymorphonuclear neutrophils. The aim of the present study was to quantify epidermal proliferation (% basal keratinocytes in S- and G<sub>2</sub>M phase), epidermal keratinization (% keratin 10-positive keratinocytes) and the appearance of "non-keratinocytes", including melanocytes, Langerhans' cells and infiltrate cells (% vimentin-positive cells) in order to further elucidate the effect of chronic exposition of normal skin to LTB<sub>4</sub>. Using three-colour flow cytometry, we could reconfirm that the response to one single epicutaneous application of LTB<sub>4</sub> was characterized by a marked increase of the percentage of basal keratinocytes in S- and G<sub>2</sub>M phase, and a marked increase of non-keratinocytes. Repeated applications of LTB<sub>4</sub> induced a moderate increase of the percentage of cells in S- and G<sub>2</sub>M phase and a moderate increase of the percentage of keratin 10-positive keratinocytes. Remarkably, the percentage of non-keratinocytes had decreased following repeated applications of LTB<sub>4</sub>, compared to unchallenged normal skin. The present study suggests that chronic exposure of normal skin to LTB<sub>4</sub> induces changes which differ markedly from the histological features of the chronic psoriatic lesion. Therefore, LTB<sub>4</sub> is unlikely to be responsible for the perpetuation of the psoriatic plaque. **Key words:** flow cytometry; proliferation; inflammation; psoriasis.

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The chemokine leukotriene B<sub>4</sub> (LTB<sub>4</sub>), a metabolite from the 5-lipoxygenase pathway in the transformation of arachidonic acid, is a potent chemoattractant for polymorphonuclear neutrophils (PMNs) in vitro and in vivo (1,2). PMNs, human keratinocytes in vitro (3) and human cells derived from epidermal biopsies (4) can synthesize LTB<sub>4</sub>. It is well established that epicutaneous application of LTB<sub>4</sub> to normal skin causes a dose-dependent influx of leukocytes, subsequently followed by recruitment of cycling cells resulting in epidermal hyperproliferation (5–7). As the main features of psoriasis consist of increased epidermal proliferation, cutaneous inflammation and impaired differentiation, it has been hypothesized that these changes in psoriasis might at least to some extent be induced by LTB<sub>4</sub>. Indeed, compared to normal skin, there is a moderately increased LTB<sub>4</sub> synthesis in uninvolved skin of psoriatic patients (8). In lesional psoriatic skin a massive overproduction of arachidonic acid metabolites, including LTB<sub>4</sub>, has been demonstrated (9).

Until now the in vivo effect of LTB<sub>4</sub> has been assessed by qualitative analysis such as histology, immunocytochemistry or ultrastructural studies. Single application of LTB<sub>4</sub> to normal skin results in the induction of intra-epidermal neutrophil microabscesses after 24 h, which resolve after 48 to 72 h. Using elastase as a marker enzyme for PMNs, maximum elastase activity is measured 18 h after application of LTB<sub>4</sub> (10). Not only PMNs are attracted by epicutaneous LTB<sub>4</sub> application (6). In the epidermis the number of T-lymphocytes reaches a maximum after 48 h. CD1a-positive Langerhans' cells show a moderate transition from the epidermis to the dermis after application of LTB<sub>4</sub>. Also monocytes accompany PMN invasion (11). A marked increase of cycling cells was demonstrated 72 h after application of LTB<sub>4</sub> (6). A flow cytometric quantification of DNA on unselected cell populations revealed an increase of the number of non-diploid cells from 3% to 15% 72 h after LTB<sub>4</sub> challenge (7). No quantitative data are available on the effect of LTB<sub>4</sub> upon differentiation of keratinocytes.

Only to a limited extent can the response to single applications of LTB<sub>4</sub> be regarded as a model for psoriasis. So far, a single application of LTB<sub>4</sub> has never resulted in the appearance of a psoriatic lesion. The responses to repeated applications of LTB<sub>4</sub> have been evaluated by Wong et al. (12) using standard histology. Only modest histological changes, compared to unchallenged skin, have been reported by these authors. Indeed Colditz & Movat (13) also reported on the "habituation" to LTB<sub>4</sub> after repeated applications with respect to LTB<sub>4</sub>-induced chemotaxis of PMNs.

The aim of the present study was to quantify aspects of epidermal proliferation, differentiation and inflammation in normal skin following single and repeated applications of LTB<sub>4</sub>. A three-colour flow cytometric method (14–16) with simultaneous measurement of DNA content and the expression of two intermediate filament proteins was used. The following questions were addressed: i) to what extent are the flow cytometric analyses of epidermal cell populations compatible with the histological findings in skin biopsies taken after one single application of LTB<sub>4</sub>?, and ii) what is the response of normal skin to repeated applications of LTB<sub>4</sub> compared to skin challenged with one single application of LTB<sub>4</sub> and unchallenged skin?

### MATERIALS AND METHODS

#### *Volunteers, LTB<sub>4</sub> applications and biopsy procedure*

Nineteen healthy volunteers (9 males and 10 females, age range 20–29 years) without signs or history of skin diseases participated in this study after giving their informed consent. Aliquots of 100 ng LTB<sub>4</sub> (Paesel GmbH, Frankfurt, Germany) dissolved in 10 µl ethanol were applied to the skin of the upper arm through a plastic cylinder obtained by cutting the edges from a micro test tube of 0.5 ml (Eppendorf, Netheler Hinz

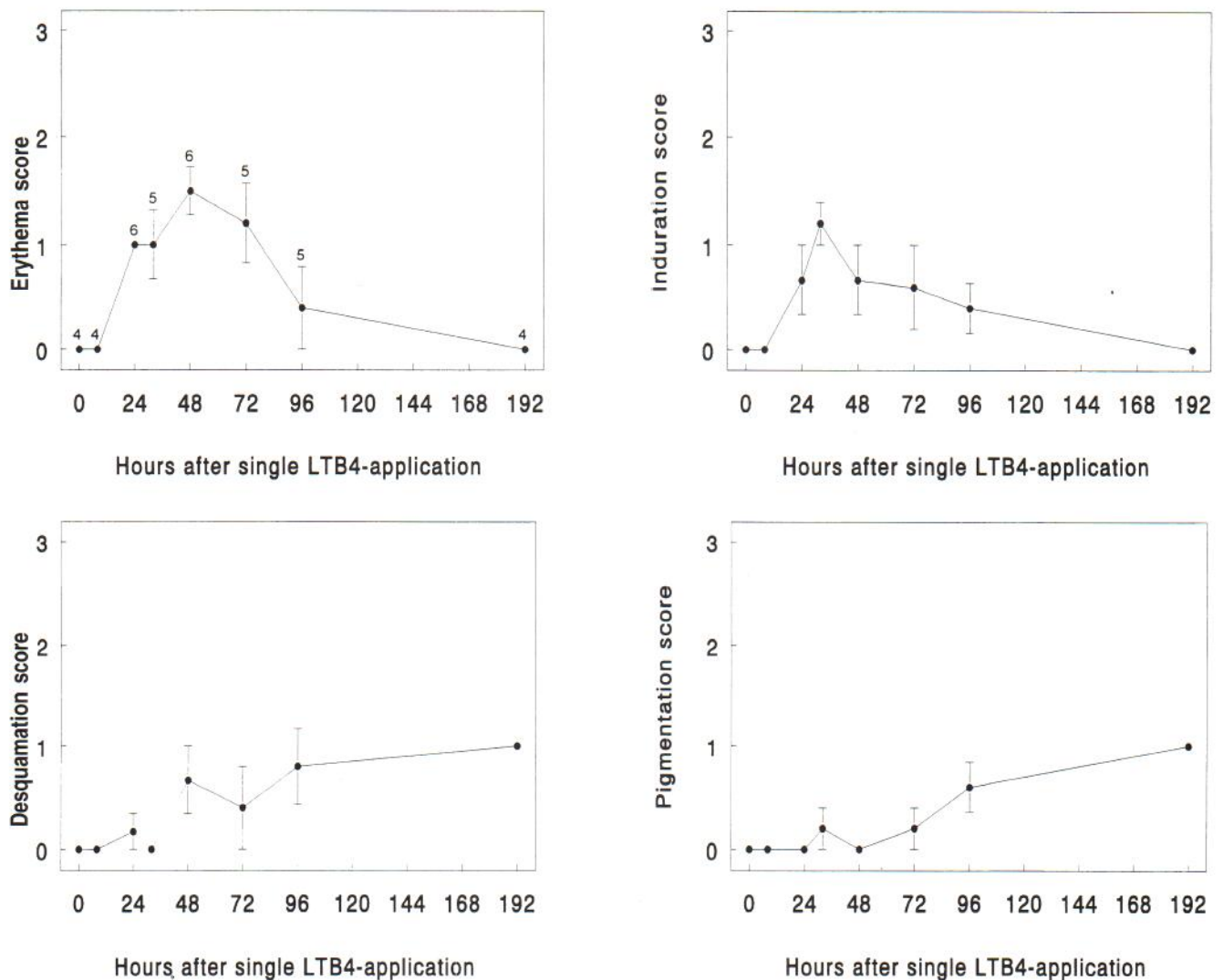


Fig. 1. Clinical scores (mean  $\pm$  SEM) at different time intervals after a single application of 100 ng LTB<sub>4</sub>. (0 = not present, 1 = slightly present, 2 = moderately present, 3 = markedly present). The number of observations is indicated above the error bars.

GmbH, Hamburg, Germany). After evaporation of the ethanol under a stream of nitrogen gas, the test sites were marked with eosin and then occluded with impermeable dressings (Silverpatch, van der Bend bv, Brielle, The Netherlands) for 6 h.

Thirteen persons (6 males and 7 females) participated in the "single application study". At 0, 8, 24, 32, 48, 72, 96 and 192 h after LTB<sub>4</sub> challenge, keratome biopsies (0.2 mm thick and 0.5 cm<sup>2</sup>) were taken using a small dermatome (Coriotome 6B333, Aesculap AG, Tuttlingen, Germany). The volunteers were divided at random with regard to the different time intervals, and three biopsies were obtained from each person. The 0-h test site was challenged with ethanol only. Before biopsies were taken, the clinical effects (erythema, induration, desquamation and pigmentation) were scored using a 4-point scale (0 = not present, 1 = slightly present, 2 = moderately present, 3 = markedly present). Histological studies were performed in four subjects at 24, 48 and 96 h after application of 100 ng LTB<sub>4</sub>. Routine haematoxylin-eosin (HE) staining was carried out on paraffin slides sectioned from 4-mm punch biopsies, taken after infiltration of the skin with 1% xylocain (1:100,000 adrenalin). At each time interval three biopsies were obtained. One biopsy was taken from non-challenged skin of one individual.

Six persons (3 males and 3 females) participated in the multiple application study. Every 24 h for 9 consecutive days (with a weekend break of 2 days after the 5th application) 100 ng LTB<sub>4</sub> was applied to the

same site of the skin of the upper arm. On day 9, 100 ng LTB<sub>4</sub> in 10  $\mu$ l ethanol and 10  $\mu$ l ethanol only were applied to a second and to a third test site, respectively. Twenty-four hours after these last applications in each volunteer three keratome biopsies were taken from these test sites, as described before. Clinical scores were assessed immediately before taking the biopsies.

#### Cell isolation procedure

Epidermal single cell suspensions were prepared as described before (16). In short, the biopsies were incubated in phosphate-buffered saline (PBS) containing 0.25 mg/ml trypsin (Sigma, St. Louis, USA) and 3.0 mg/ml dithioerythritol (Sigma) for 30 min at 37°C. Then, in PBS containing 10% heat-inactivated newborn calf serum (HINCS, Life Technologies Ltd., Paisley, UK) the dermis was separated from the epidermis with fine forceps. The remaining epidermis was gently mixed on a vortex to loosen the keratinocytes, resulting in a single cell suspension. After the horny layer had been discarded, the suspension was centrifuged, the supernatant removed and the cells fixed in 70% ice-cold ethanol. The cell suspension was stored at -20°C until staining and flow cytometric analysis.

#### Staining procedure

A triple labelling was performed, using a DNA fluorochrome combined

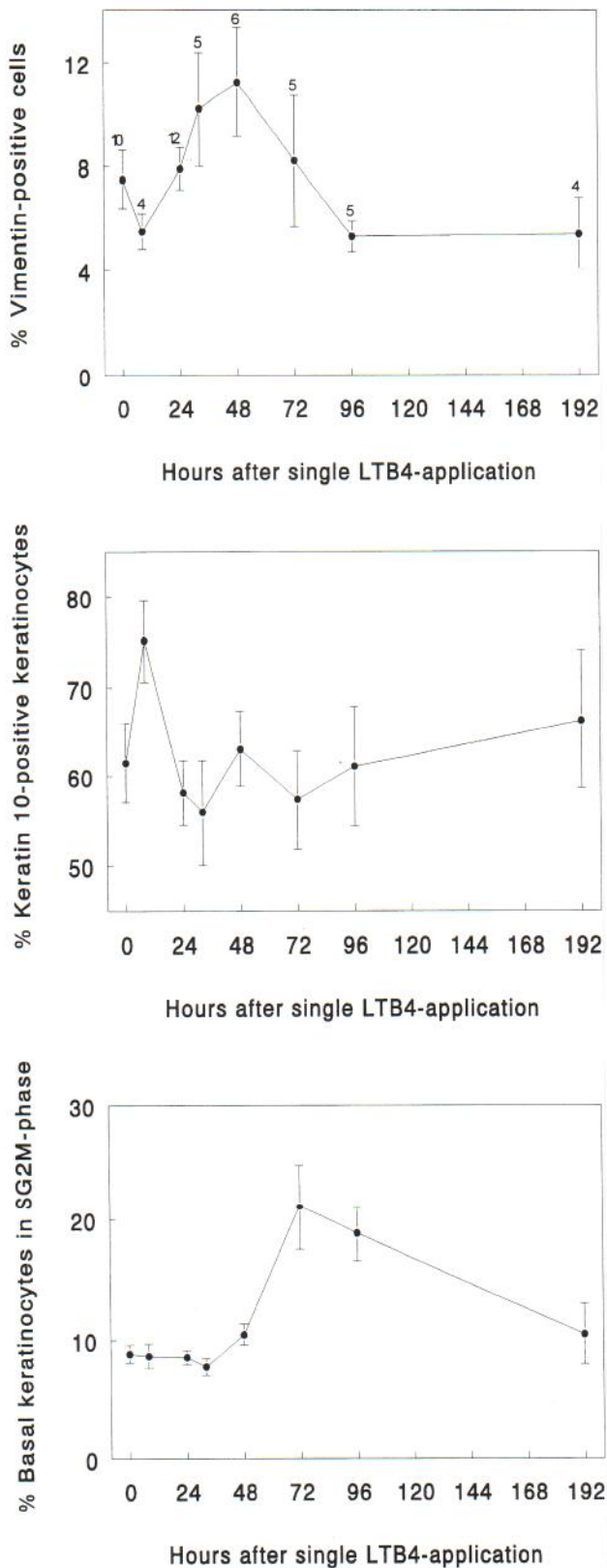


Fig. 2. Flow cytometric analysis (mean  $\pm$  SEM) of epidermal cell suspensions prepared from biopsies taken from normal skin at different time intervals after a single application of 100 ng  $LTB_4$ . The number of biopsies is indicated above the error bars.

with two antibodies against intermediate filaments. Samples of the cell suspensions, containing approximately  $1-2 \times 10^5$  cells, were stained. The procedure has been described by us in detail before (16). To assess hyperproliferation, the DNA fluorochrome TO-PRO-3 iodide (TP3, Molecular Probes, Eugene, USA) was used (15). TP3 intercalates with double-string DNA and permits measurement of the proliferative activity of cells by quantification of the percentage of cells in S- and G<sub>2</sub>M phase. As TP3 also binds to RNA to some extent, it was used in combination with RNase. To study the inflammatory response we used Vim3B4 (Novocastra Laboratories Ltd., Newcastle upon Tyne, UK). This IgG2a-type mouse monoclonal antibody stains vimentin, an intermediate filament-type protein which occurs in mesenchymal cells (17). In this study, PMNs, lymphocytes, monocytes, macrophages, melanocytes and Langerhans' cells were stained by Vim3B4. To quantify the effect of topical  $LTB_4$  application on the differentiation process, we used the IgG1-type mouse monoclonal antibody RKSE60 (Dept. of Mol. Biology, University of Maastricht, The Netherlands). RKSE60 is directed against keratin 10, an intermediate filament-type protein that is expressed in differentiating keratinocytes (18). Three-colour fluorescence was obtained with the fluorochromes fluorescein-isothiocyanate (FITC) and phycoerythrin (PE), which were conjugated to monoclonal goat antibodies against mouse IgG2a and mouse IgG1, respectively (Southern Biotechnology Associates, Birmingham, USA), in combination with TP3.

#### Flow cytometric analysis

In a dual laser system (Epics<sup>®</sup> Elite flow cytometer, Coulter, Luton, UK) the fluorochromes FITC and PE were excited at 488 nm (air-cooled argon ion laser, 15 mW) and TP3 at 633 nm (HeNe laser, 10 mW). Emission spectra were separated with bandpass filters of  $525 \pm 10$  nm,  $575 \pm 10$  nm and  $675 \pm 10$  nm, for FITC (green signal), PE (orange signal) and TP3 (red signal), respectively. The spectral overlap from PE to TP3 was 2% and therefore easily compensated electronically. Compensation for FITC in PE was 21%. After correction for clumps and cellular debris by setting appropriate gates, 5,000 cells from each sample were analysed. With Elite and Multicycle<sup>™</sup> software percentages of vimentin- and keratin 10-positive cells and percentage of cells in S- and G<sub>2</sub>M phase were calculated. As this triple labelling procedure allows simultaneous assessment of different epidermal cell populations, we were able to quantify the proliferative activity of the basal epidermal compartment exclusively.

## RESULTS

### Single application

The clinical scores at different time intervals after one single  $LTB_4$  application are shown in Fig. 1. Maximal erythema was reached after 48 h. Maximal induration occurred 32 h after  $LTB_4$  challenge. Desquamation and pigmentation appeared at 48 h and 72 h, respectively, and increased slowly, reaching a maximum at 192 h at all test sites.

The histological assessment of the response to epicutaneous application of  $LTB_4$  was in line with previous reports (5, 6). A massive infiltration with PMNs was observed 24 h after challenge. PMNs were partly grouped together as abscesses and partly scattered through the epidermis. In the dermis the infiltrate was pronounced at perivascular and subepidermal localizations. At 48 h no microabscesses were present in the epidermis, but some PMNs could be observed. Between the stratum corneum and the stratum spinosum an amorphous zone with a granular aspect was identified, and focal parakeratosis was observed. A mononuclear dermal infiltrate was seen. At 96 h the picture was comparable to the situation after 48 h. No PMNs were seen in the epidermis and the dermal infiltrate was mainly located in the perivascular areas.

Table I. Comparison of flow cytometric values (mean  $\pm$  SEM) in unchallenged normal skin, in normal skin after single (peak values) and multiple applications of 100 ng LTB<sub>4</sub>, and in psoriatic skin

	Normal skin (n=10)	Single application (n=5/6)	Multiple application (n=6)	Psoriatic skin (n=38)
%S+G <sub>2</sub> M (basal keratinocytes)	8.8 $\pm$ 0.8	21.2 $\pm$ 3.7 <sup>a</sup>	12.2 $\pm$ 1.9 <sup>b,d</sup>	18.9 $\pm$ 1.3
%vimentin-positive cells	7.5 $\pm$ 1.1	11.2 $\pm$ 2.1	4.6 $\pm$ 1.4 <sup>c,d</sup>	15.6 $\pm$ 1.7
%keratin 10-positive keratinocytes	61.5 $\pm$ 4.5	57.3 $\pm$ 5.6	68.7 $\pm$ 6.1 <sup>c</sup>	36.5 $\pm$ 2.3

<sup>a</sup>  $p < 0.05$ , versus normal skin (unequal variance *t*-test)

<sup>b</sup>  $p < 0.01$ , versus normal skin (paired *t*-test,  $n=6$ )

<sup>c</sup>  $p < 0.05$ , versus normal skin (paired *t*-test,  $n=6$ )

<sup>d</sup>  $p < 0.05$ , versus single application (unequal variance *t*-test)

Fig. 2 shows the results of the flow cytometric analysis. In unchallenged normal skin the percentage of vimentin-positive cells was 7.5%  $\pm$  1.1% (mean  $\pm$  SEM). After 48 h the maximum of 11.2% was reached. The amount of non-keratinocytes then returned to levels of  $\pm$  5.3%. In the unchallenged skin, the percentage of keratin 10-positive cells, i.e. differentiated keratinocytes, was 61.5%  $\pm$  4.5%. After a slight decrease the percentage returned to the normal level from 96 h onwards. The percentage of basal keratinocytes in S- and G<sub>2</sub>M phase in the unchallenged skin was 8.8%  $\pm$  0.8%. This percentage remained more or less stable up to 48 h. From 48 h onwards there was a statistically significant increase of basal cells in S- and G<sub>2</sub>M phase with a maximum of 21.2% at 72 h (see Table I), showing a tendency to return to normal at 192 h.

#### Multiple application

The clinical response to multiple applications was comparable to the response to one single application. Both erythema and induration were maximal at 72 h. From 72 h onwards a sharp decline of these clinical scores was observed. Both desquamation and pigmentation slowly increased until 264 h.

In Table I the flow cytometric data of normal unchallenged skin and normal skin after single and multiple applications are summarized. Statistical analysis revealed a significant increase with respect to keratin 10 expression and proliferative activity, and a significant decrease with respect to vimentin expression for multiple challenged skin compared to unchallenged skin. The mean percentage of vimentin-positive cells in the multiple application sites (4.6%) was significantly lower than the peak value at 48 h in the single application series. The percentage of basal keratinocytes with a more than diploid DNA content was significantly lower in multiple challenged sites (12.2%) than the peak value at 72 h after single application.

#### DISCUSSION

In previous flow cytometric studies, mean percentages of cells in S- and G<sub>2</sub>M phase of 2.9% and 4.0% have been measured in normal skin (7,14). These values refer to the proliferative activity of the total cell population present in the cell suspension. The value of 8.8% in unchallenged skin (present study), however, reflects the specific proliferative activity of the basal keratino-

cytes and represents a value of 3.1% for the total cell population. Percentages of 57.2 for keratin 10-positive cells and 7.6 for vimentin-positive cells were found in healthy skin (14). Correction for vimentin-positive cells reveals a percentage of 61.9% of keratin 10-positive keratinocytes. As these values correspond with the present data, the reproducibility of the flow cytometric method is warranted.

Using histological and flow cytometric analysis we reconfirmed that epicutaneous application of one single dose of 100 ng of LTB<sub>4</sub> consistently yields an inflammatory response. A single application of 100 ng LTB<sub>4</sub> to normal skin resulted in a thin layer of parakeratosis after 48 h (5). Repeated intracutaneous injection of LTB<sub>4</sub> in guinea pig ears caused epidermal hyperplasia with an unaltered keratinization pattern (19). The tendency of a temporary decrease of keratin 10-positive keratinocytes after single application of 100 ng LTB<sub>4</sub> to human skin and the appearance of parakeratosis suggest that in vivo LTB<sub>4</sub> might interfere with the process of keratinization. The present data show that there is an increase in epidermal proliferation following single application of LTB<sub>4</sub> (Fig. 2). Other studies confirm the proliferative response 72–96 h after LTB<sub>4</sub> application (6, 7). Although a direct effect of LTB<sub>4</sub> on the germinative cell population has been described in vitro (20), an indirect effect via interferences with the suprabasal compartment or via the inflammatory response is by no means ruled out.

Maximal erythema was seen 48 h after single-dose LTB<sub>4</sub> application. Interestingly, the number of vimentin-positive cells also reached a maximum 48 h after challenge. Histological studies have demonstrated that the infiltrate mainly consists of PMNs during the first 24 h. However, the maximum percentage of vimentin-positive cells at 48 h after the application of LTB<sub>4</sub> is not exclusively due to high levels of PMN at this time interval but is mainly caused by infiltrating T-lymphocytes reaching their maximum after 48 h (5, 6). Also monocytes invade the epidermis after LTB<sub>4</sub> challenge (11). It is remotely possible that not all PMNs present in the epidermis were analysed flow cytometrically. A certain quantity of PMNs might have been captured in the stratum corneum and may therefore not reach the cell suspension. Furthermore, some PMNs degenerate at the moment of extravasation, resulting in pycnotic nuclei and vacuolisation (11).

During the first 3 days, the clinical response to single and

repeated applications of LTB<sub>4</sub> proved to be similar. However, during the chronic LTB<sub>4</sub> exposure the erythema began to subside from 72 h onwards, in spite of the five following applications. This response is compatible with the "tachyphylaxis" of the LTB<sub>4</sub> induced accumulation of PMNs, as reported by Wong et al. and by Colditz & Movat (12, 13). Besides a quantification of epidermal proliferation, percentage of vimentin-positive cells and percentage of keratin 10-positive keratinocytes in unchallenged and LTB<sub>4</sub>-challenged normal skin, Table I provides these data in psoriatic skin without epicutaneous LTB<sub>4</sub> challenge (16). It can be concluded that (i) compared to unchallenged skin (intra-individual comparison), repeated applications of LTB<sub>4</sub> induce a decrease in the number of vimentin-positive cells, an increase of keratin 10-positive keratinocytes, and an increase of epidermal proliferation; (ii) compared to the maximum values reached by one single application LTB<sub>4</sub> (inter-individual comparison), a reduced responsiveness was observed in the repeatedly challenged skin with respect to the number of vimentin-positive cells and epidermal proliferation; and (iii) comparing the present data with observations in the psoriatic plaques (without epicutaneous LTB<sub>4</sub> challenge), multiple applications of LTB<sub>4</sub> induce far less hyperproliferation, far less intra-epidermal accumulation of vimentin-positive cells and not a reduction but an increase of the number of keratin 10-positive keratinocytes.

In conclusion, the present data indicate that LTB<sub>4</sub> cannot be a major factor in the perpetuation of epidermal proliferation and inflammation, and the reduction of keratin 10 expression in the chronic psoriatic lesion. However, in an early stage of the psoriatic process, changes similar to one single application might be of pathogenetic significance.

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