DYNAMICS OF EPIDERMAL GROWTH AND INFLAMMATION IN PSORIASIS

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DYNAMICS OF EPIDERMAL GROWTH AND INFLAMMATION IN PSORIASIS

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"Misschien is niets geheel waar,
en zelfs dát niet."

Multatuli.

Aan mijn ouders

CONTENTS:

CHAPTER I

INTRODUCTION

EPIDEMIOLOGY, AETIOLOGY AND GENETICS

Psoriasis is a chronic skin disease prevaling in 2% of the Dutch population. It appears to be most common in northern Europe and North America. In certain ethnic groups no psoriasis is seen, such as the American Indians, and Eskimos. Psoriasis is equally common in males and females[1]. The age of onset of psoriasis is variable, the first manifestations may occur at any age and its duration may vary from a few weeks to a whole lifetime. Triggering factors can induce psoriatic phenomena in predisposed persons. These include exogenous and endogenous systemic factors, such as psychological stress, somatic disease, drugs, and climate. In addition, local factors such as trauma to the skin can precipitate a psoriatic lesion (Koebner phenomenon)[2-4].

The genetic component in the aetiology of psoriasis is indisputable. A family history for psoriasis is indicated by about one third of the patients. However, the significance of hereditary factors seems variable in various patients. It is now clear from population surveys, family studies, twin studies and pedigree analysis that psoriasis is a polygenic and multifactorial disease[5]. This implies that the cause of the disease is due to the effect of several genes, each of small psoriatic effect, with a controlling function for some of these genes. Psoriatic patients possess a critical number of causative factors, unaffected individuals carry varying amounts of these factors, but not in sufficient quantity to cause disease.

The discovery of the HLA antigen system has provided additional support for the genetic base of psoriasis. Firstly, associations of psoriasis vulgaris with HLA-B13 and HLA-B17 were demonstrated[6-8]. Afterwards, similar associations with HLA-B37[9] and with HLA-Bw16[10] were added. The association with HLA-Bw16 is not clear in all populations. An individual with HLA-B13, HLA-B17 or HLA-B37 has about a 5-6 times higher relative risk of developing psoriasis than a person without these antigens[2]. Later studies suggested that these associations were secondary to a primary association of psoriasis vulgaris with HLA-Cw6[9] and with HLA-DR7[11].

It appears that HLA-types dictate in part the course of the disease[12]. The influence of environmental factors and the polygenic inheritance make it difficult to estimate the chance that psoriasis will become manifest in any particular person.

CLINICAL ASPECTS

Psoriasis has a chronic relapsing course. The cutaneous lesions of psoriasis are highly characteristic for the disease (Fig 1). They show prominent features: a sharp demarcation, the presence of silvery, non-coherent scales, elevation and a homogenous erythema. This indicates that both the dermal compartment (erythema), and the epidermal compartment (elevation, increased scaling) are involved. After scratching the lesions, the scales become white, like scratching a candle (signe de la tache de bougie). When scales are removed, small bleedings are seen on the surface (Auspitz sign). Often a white ring is seen around a lesion, first described by Woronoff.

A considerable variation in morphology of the lesion exists. The clinical picture can be dominated by small lesions (guttate psoriasis), by large plaques (chronique plaque psoriasis), or they may have a considerable pustular component (pustular psoriasis). Lesions may be generalized (generalized pustular psoriasis, erythrodermic psoriasis) or localized. The most common form of psoriasis is chronic plaque psoriasis or psoriasis vulgaris (Fig 2). Lesions are then distributed symmetrically on predeliction areas: elbows, knees, scalp, sacral region, and umbilicus. Topographical variations of psoriasis vulgaris are flexural psoriasis or psoriasis inversa (involvement of groins and axillae), psoriasis capitis (scalp), palmoplantar psoriasis (palms and soles) and psoriasis unguium (nails)[3,4,13,14].

Psoriasis is not exclusively a skin disease. Extracutaneous manifestations are common. Nail changes (Fig 3) are frequent in psoriasis[1,15]. The main alterations are pits within the nail plate, brownish macules (oil spots) beneath the nail plate, subungual hyperkeratosis, and distal onycholysis. Psoriatic arthropathy is a complication of psoriasis which occurs in 5-10% of the patients (Fig 4). It is also seen in patients without cutaneous lesions. Most often arthritis of the peripheral interphalangeal joints of the fingers, and a negative serological test for rheumatoid factor are

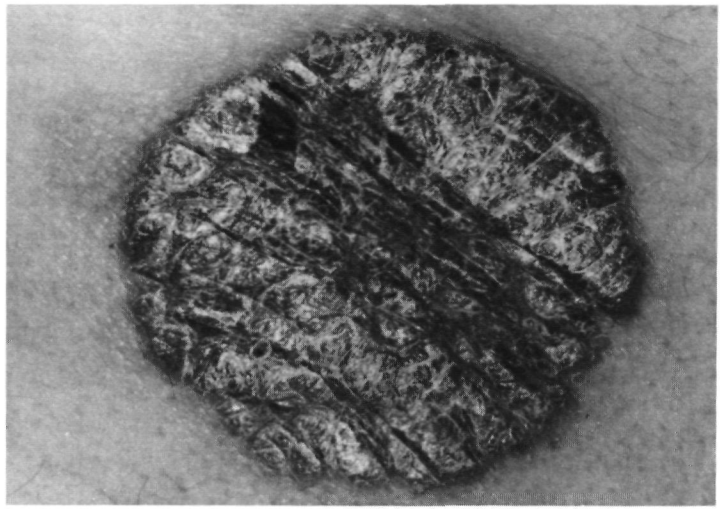

Figure 1. Sharply demarcated erythematosquamous psoriatic lesion.

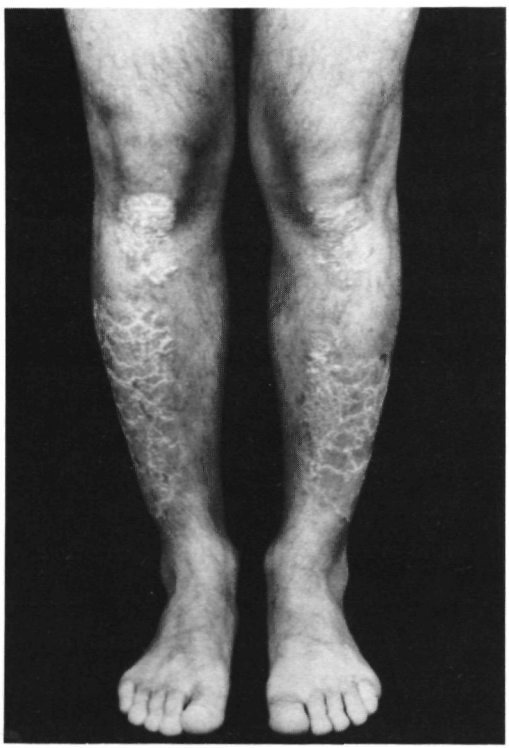

Figure 2. Chronic plaque psoriasis.

seen. In patients with psoriatic arthropathy, an increased frequency of HLA-B27[16] and HLA-Bw38[2] is found.

The typical course of psoriasis is a chronic one, with remissions and exacerbations. The prognosis of psoriasis is good. Threatening complications are psoriatic arthropathy and erythroderma. Triggering factors for exacerbation of psoriasis include systemic factors such as infections, endocrine factors, hypocalcaemia, various drugs (lithium, indomethacin, antimalarials, beta-adrenergic antagonists), and psychogenic stress. In addition, local trauma can induce psoriatic lesions (Koebner phenomenon). Psoriasis does not show a static clinical picture, lesions grow and regress. New lesions start as small pinpoint papules. In the early phase, the papules unite, become confluent, and form plaques. These plaques may enlarge by centrifugal expansion. Involution of the psoriatic lesions can proceed gradually and evenly, however sometimes involution starts in the center of the lesion. The end result is often a psoriatic leukoderma, which becomes repigmented later[13].

The differentiation of psoriasis from other skin diseases is not difficult in patients with a classical clinical picture. However sometimes it is not easy to delineate psoriasis from several other skin disorders with abnormal epidermal growth and inflammation. Seborrhoeic dermatitis can be quite similar to psoriasis. Some infections can mimic psoriatic lesions such as syphilis, dermatophytic infections and candidiasis. Mycosis fungoides in the premycotic phase can look like psoriasis. In widespread psoriasis especially the differentiation from pityriasis rubra pilaris can be difficult[13].

HISTOLOGY

The histologic picture of psoriasis vulgaris varies with the stage of the lesion and is diagnostic in early scaling papules and near the margin of advancing plaques[17,18]. Controversy exists whether the initiating events in early psoriasis are located primarily in the epidermal or in the dermal compartment[19].

In the initial psoriatic lesion, a preponderance of dermal changes is seen (capillary dilatation, dermal oedema, mononuclear infiltrate around the capillaries)[20]. Also the phenomenon of the "squirting papillae" seems to occur early in the development of

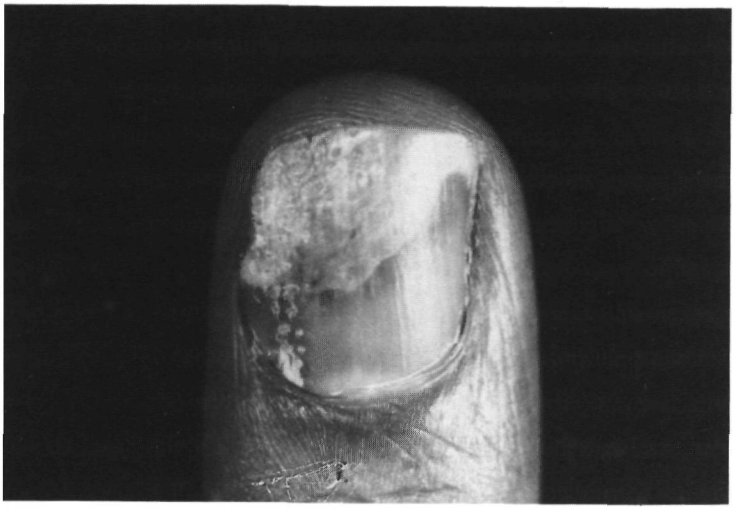

Figure 3. Nail changes in psoriasis: onycholysis, pits.

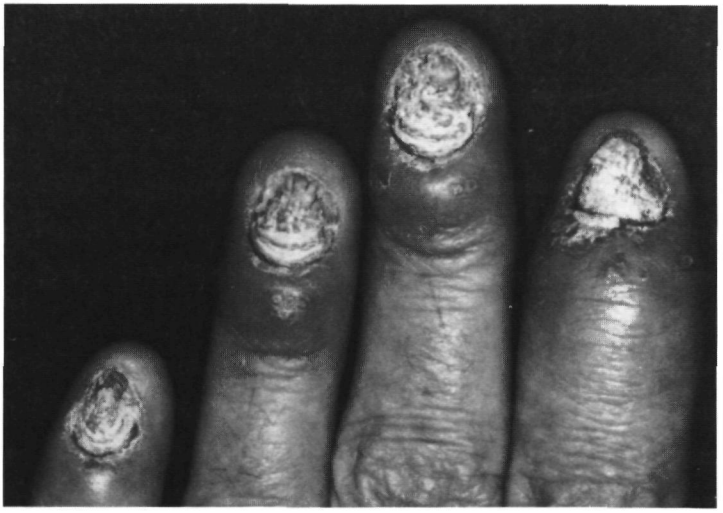

Figure 4. Arthritis psoriatica with nail changes.

the psoriatic lesion[21]. This means that neutrophil polymorphonuclear leukocytes (PMN) are moving from the tip of capillary loops in the dermal papillae, into the epidermis. Early in the evolution of a lesion, thickening of the stratum corneum and parakeratosis are seen. Whether parakeratosis appears before or after invasion of inflammatory cells in the epidermis remains controversial. PMN can often be seen trapped in the parakeratotic stratum corneum. They form micro-abscesses (Munro-abscesses) in the stratum corneum. Sometimes PMN can be seen in the stratum spinosum, where they aggregate as spongiform pustules (of Kogoj).

In the fully developed lesion of psoriasis, the histologic picture is again characterized by epidermal and dermal changes (Fig 5). Epidermal characteristics are elongation of the rete ridges, thinning of the suprapapillary epidermis, absence of the granular layer, hyperparakeratosis, and the presence of Munro microabscesses containing PMN (Fig 6). Sometimes spongiform pustules of Kogoj are seen in the epidermis. These are highly diagnostic for psoriasis[17]. Munro abscesses are easily found in early psoriatic lesions, but more difficult in old lesions[22]. In the psoriatic lesion, mitoses are not limited to the basal cell layer (as in normal skin), but are also found in two to three rows of cells above the basal layer. Due to this, in combination with the elongation of the rete ridges, the number of mitoses per unit skin surface area is increased more than twenty times compared to normal skin[23] (Fig 7). Dermal changes include elongation and oedema of the papillae, with dilated and tortuous capillaries[24]. An inflammatory infiltrate is present in the upper dermis and the papillae (Fig 8). It consists mainly of T-lymphocytes[25], but PMN are also present, especially during periods of exacerbation. It is postulated that also mast cells are increased[26]. Lymphocytes and macrophages are present in the epidermis as well[27].

In pustular psoriasis, not only microabscesses are formed within the epidermis, but also macro-abscesses. The fully developed pustule is large, unilocular and intraepidermal in location. In the dermis a variable dense lymphohistiocytic infiltrate is seen, with some neutrophils around dilated vessels in the oedematous papillae[28].

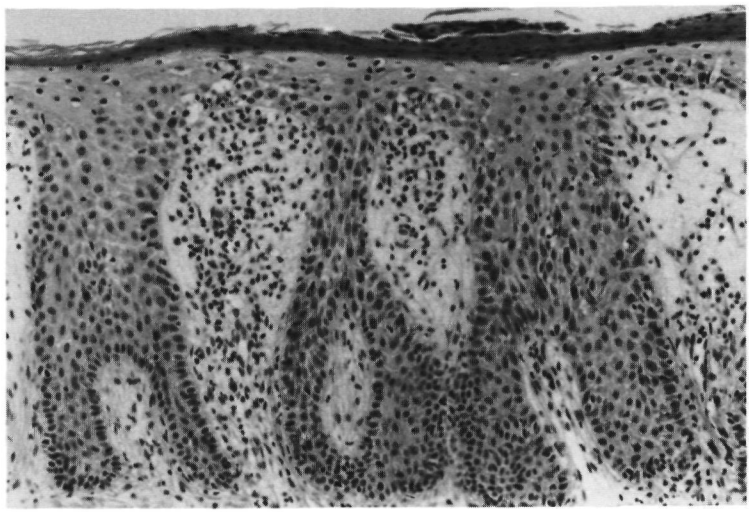

Figure 5. Psoriasis: dermal and epidermal aberrations (x 350).

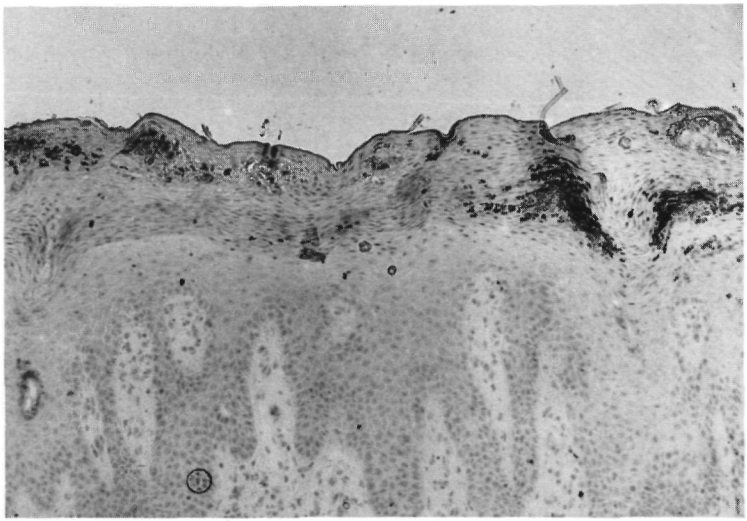

Figure 6. Polymorphonuclear leukocytes in lesional psoriatic skin (x 200).

In psoriasis, an excess of cellular proliferation and inflammation exists, that nevertheless remains controlled. There is no animal model to investigate psoriasis. Therefore it is difficult to study the events with in vivo relevance in the pathogenesis of psoriasis. An attempt to investigate the earliest changes in the development of psoriasis has been made by studying initial psoriatic lesions such as pinpoint papules[20,21,29], the margin zone of spreading psoriatic plaques[18,30-32], or relapsing psoriatic lesions after corticosteroid treatment[33]. In addition, psoriatic lesions have deliberately been induced by tape-stripping of the epidermis, thereby removing the stratum corneum[34,35]. As mentioned before, it still remains controversial whether the epidermal or the dermal compartment show the earliest changes.

After corticosteroid treatment under occlusion, the first changes in relapsing lesions were swelling and intercellular widening of endothelial cells, followed by the appearance of mast cells, showing degranulation. Afterwards, macrophages migrated into the lower epidermis. Only then lymphocytes and neutrophils were seen[33].

Various other investigations have postulated that dermal vascular changes were the first abnormalities detected in initial psoriatic lesions[20 21], and in the clinically uninvolved skin adjacent to spreading psoriatic plaques[30,31]. In other studies, the invasion of the dermis and epidermis by PMN proved to be the first event in the inition of a lesion[29].

On the other hand, various abnormalities of the keratinocytes have been described in diseased and uninvolved skin in psoriasis[36]: changes in size of keratinocytes[37], in ultrastructure[38], in plasma membrane binding sites[39], and various biochemical abnormalities such as increased levels of phospholipase A_2 activity, calmodulin levels, and activity of ornithine decarboxylase (ODC)[36,40,41].

In vivo[42] and in vitro experiments[43] showed that skin, removed from its psoriatic host, loses its psoriatic features. This implies that both epidermis and dermis are needed to produce and maintain a psoriatic lesion[44]. Although intrinsic abnormalities of the keratinocytes are present, also cellular or humoral factors are required to induce a clinical psoriatic lesion.

Alterations in various cell types of skin have been reported in psoriasis.

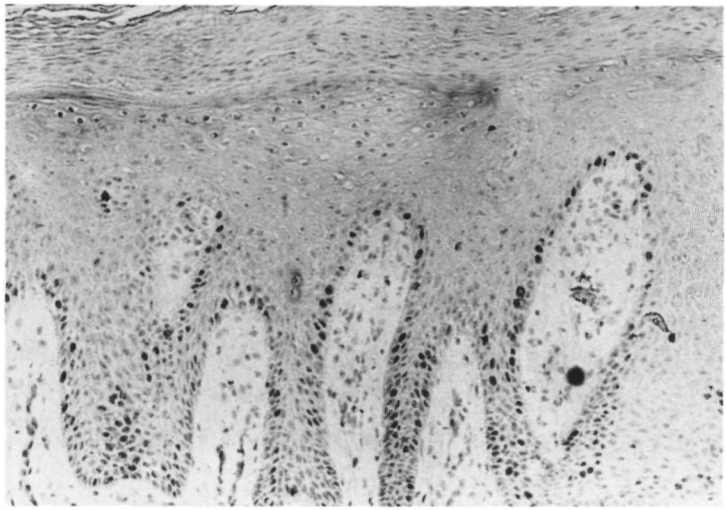

Figure 7. Cycling cells in psoriasis stained with the antibody Ki-67 (x 250).

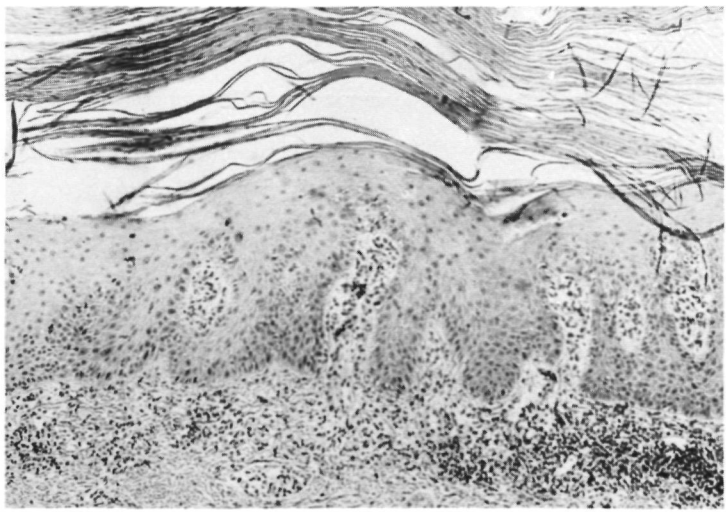

Figure 8. Inflammatory infiltrate in a psoriatic lesion (x 155).

Keratinocytes:

A characteristic feature of involved psoriatic skin is hyperproliferation, first described by Scott and Van Ekel[23]. Hyperproliferation can be concluded from the higher number of mitotic figures and the greater number of cells labelled with tritiated thymidine[45]. This increase in the proliferative cell population was suggested to be due to a shortening of the cell cycle time[46]. However, strong evidence is available that in normal human skin only a part of the germinative cells are really cycling, the others are in a resting phase (G_0). The fraction of the cells that is actively cycling is called the growth fraction. Recruitment of G_0 cells provides an explanation for the increased growth fraction in psoriasis[47]. Not only keratinocyte proliferation is changed, keratinocyte differentiation is altered as well. This is reflected in the altered expression of intermediate filaments, components of the cytoskeleton. For example, increased amounts of keratin 16, and reduced expression of keratin 10 have been reported in psoriatic epidermis[48,49]. As mentioned before, many structural and functional abnormalities have been identified in psoriatic keratinocytes[36].

Granulocytes:

Much attention has been focussed on the polymorphonuclear leukocyte (PMN)[50]. These cells accumulate in the dermis and epidermis of early and active psoriatic lesions. Many abnormalities in blood granulocytes from psoriatic patients have been reported, such as enhanced chemotaxis[51] and phagocytosis[52], increased cytotoxic activity[53] and adherence[54]. PMN are attracted by mediators of inflammation, especially leukotriene B_4. Several antipsoriatic therapies such as corticosteroids[55], ultraviolet light (UVB and PUVA)[56], dithranol[57] and retinoids[58] have proven to inhibit the intra-epidermal accumulation of PMN in normal skin after epicutaneous application of leukotriene B_4. The exact role of PMN in psoriasis is not clear. The migration of PMN into the epidermis is probably a necessary link in the pathogenesis of psoriasis. From a therapeutical point of view, the migration of PMN into the skin seems important[50].

Monocytes:

Monocytes from psoriatic patients show increased locomotion[59] and increased phagocytosis[60], as well as enhanced cytotoxic activity[53].

Langerhans cells:

Histochemical and immunological methods have demonstrated clustering of Langerhans cells in psoriatic epidermis[61-63]. It is controversial whether their numbers are decreased or increased[61,64,65].

Lymphocytes:

The dermal mononuclear infiltrate in psoriatic lesions is composed mainly of T-lymphocytes[25]. Several abnormalities of circulating T-lymphocytes have been reported[66-69]. A disequilibrium is found between suppressor-T-cells and helper-T-cells[62,66,67]. The ratio between T-helper and T-suppressor-cells is increased compared to normal skin[62,68]. T-lymphocytes are present in the epidermis as well. They belong to a "memory" subpopulation of T-cells (CD4+, CDw29+)[70]. The success of treatment with cyclosporin indicates that the T-lymphocyte plays an important role in psoriasis[71].

Dermis:

The dermal microcirculation is altered in psoriasis and has been incriminated as the primary defect[72,73]. Fibroblasts cultured from psoriatic dermis show abnormal functions such as increased proliferation and synthesis of collagen[74]. They have been reported to be less sensitive to vitamin D_3[75], but this is controversial. Whether the sensitivity to vitamin D_3 is altered in psoriatic keratinocytes is disputed[76,77]. There is also evidence that the composition of extracellular matrix proteins is changed[74,78-80].

Data to support a role for each of these components in the initiation, maintenance or resolution of psoriasis are still accumulating. Research has shifted from investigations of peripheral blood cells to studies of the inflammatory and immunological cells in the skin[81]. The mechanism which brings the inflammatory cells into the lesions of psoriasis is unknown. Proposed mechanisms include chemotactic factors

from the arachidonic acid cascade, immunological mechanisms, and metabolic abnormalities.

The arachidonic acid cascade:

Arachidonic acid is an unsaturated fatty acid and is present in phospholipids in the plasmamembrane of keratinocytes. It is the precursor of prostaglandins, thromboxanes, hydroxyeicosatetranoic acids (12-HETE, 15-HETE), and leukotrienes (Fig 9), substances which are formed after epidermal trauma and which are mediators of inflammation and proliferation[82-84]. In psoriatic epidermis, the amounts of arachidonic acid, 15-HETE, and leukotriene B_4 are increased[84-87]. Leukotriene B_4 is a potent chemo-attractant for PMN and is formed via the enzyme 5-lipoxygenase. Inhibitors of this enzyme proved to be benefitial in the treatment of psoriasis[88,89].

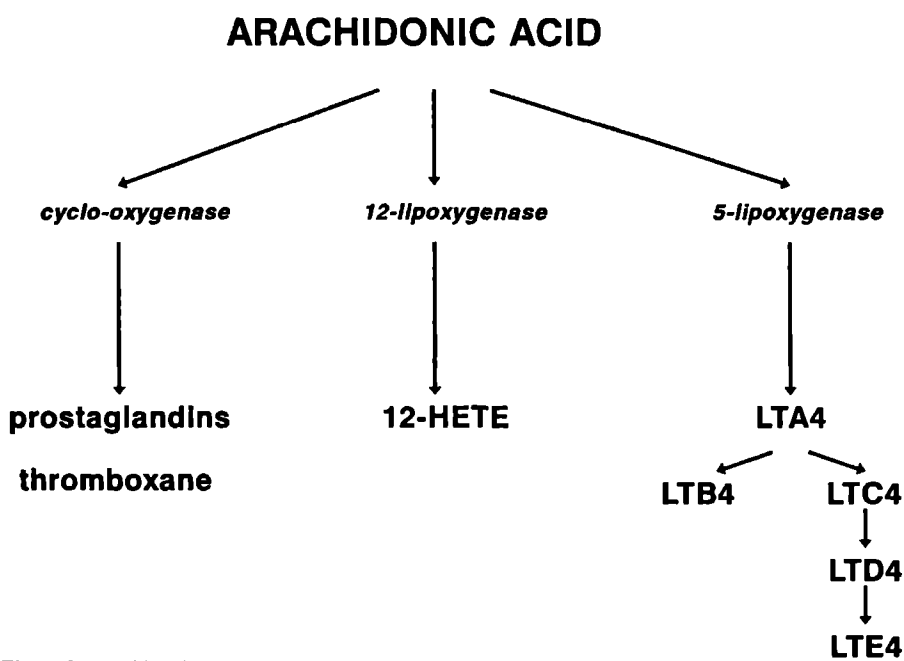

Figure 9. Arachidonic acid cascade.

Immunological factors:

After the discovery of depositions of IgG and complement in the stratum corneum of psoriatic epidermis, the hypothesis has been formulated that immunological factors play an important role in its pathogenesis[90,91]. In this hypothesis, it is assumed that in normal skin a stratum corneum antigen is present, but masked. In psoriasis, this antigen is unmasked, possibly presented by Langerhans cells, leading to an immunological answer, e.g. an influx of T-helper cells[92,93]. These T-lymphocytes are able to trigger the development of a psoriatic lesion (Fig 10). During therapeutical

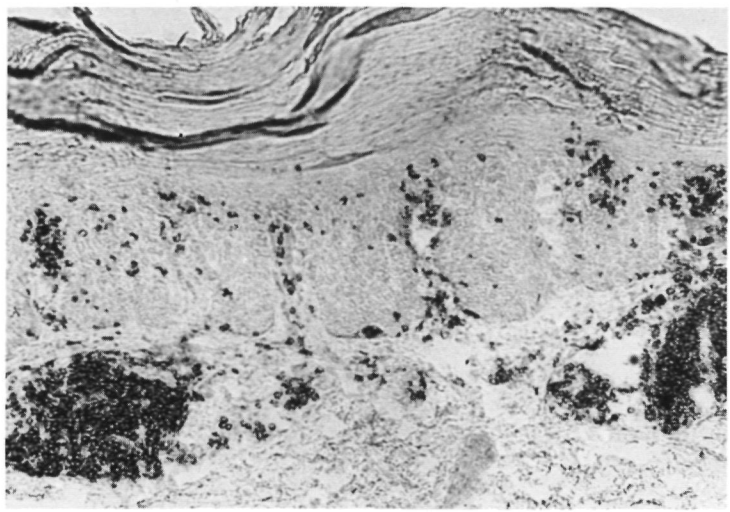

Figure 10. T-lymphocytes in an untreated psoriatic lesion (x 200).

regression of a lesion, the ratio of T-helper/T-suppressor cells changes in favour of the T-suppressor cells[71]. T-lymphocytes are able to secrete various interleukins, such as interleukin-2 (IL-2), which is a signal for activation and proliferation of other T-lymphocytes[94]. Not only T-lymphocytes, but also other epidermal cells produce cytokines[95]. The epidermis of psoriasis contains abnormal amounts of interleukin-1 (IL-1)[96], interleukin-6 (IL-6)[97,98], interleukin-8 (IL-8)[99], tumor necrosis factor α (TNF-α)[100], and transforming growth factor ß (TGF-ß)[101]. In addition, the expression of the intercellular adhesion molecule 1 (ICAM-1), which permits leukocytes to adhere to endothelium, to leave the bloodstream, and infiltrate the skin, proved to be increased in the microvasculature and epidermis of psoriatic skin[102]. Current interest of psoriasis research focusses on cytokines and adhesion molecules as an integrated regulatory circuit[103].

Metabolic abnormalities:

The levels of cyclic AMP and cyclic GMP are changed. cAMP is decreased and cGMP is increased in psoriatic epidermis[104,105], and various investigations support the possibility of a defective cAMP system[106,107]. Lithium is known to inhibit the formation of cAMP, and exacerbates psoriasis[108]. Certain polyamine levels are raised in psoriasis[109]. Putrescine and spermidine levels are increased, as well as their rate-limiting synthetic enzyme, ornithine-decarboxylase[109]. Various data suggest a disturbed protease-anti-protease equilibrium in psoriasis[110-112].

THERAPY

Psoriasis is a chronic relapsing benign skin disease and administration of a drug may continue for a long time. Therefore therapeutics are needed with a small risk of side effects. Stable psoriasis should first be treated with extensive topical therapy. Phototherapy or photochemotherapy is indicated in cases of more widespread psoriasis. If the psoriasis is extensive, more intensive topical therapy should be considered in a day-care unit or hospital. In cases of severe psoriasis, systemic treatment with for example retinoids or methotrexate is indicated.

Coal tar

Coal tar has been used in topical therapy for more than a century. It is assumed to have an antimitotic effect[113]. Tar can be incorporated in various vehicles such as creams, ointments, lotions, gels and shampoos. It is a safe and effective treatment[114,115]. Disadvantages are the colour and odour of coal tar, the induction of folliculitis and enhanced sunburn.

Dithranol (anthralin)

A century ago the value of chrysarobin, a tree bark extract, in psoriasis was discovered accidentally. Dithranol is a synthetic analogue of chrysarobin and is working maximally when formulated in a stiffened base[3]. The exact working mechanism is unknown. Its effect is however tightly linked with its side effects: staining of the skin and textiles by oxidation products, and irritation of the skin.

Dithranol inhibits key enzymes of cell metabolism, interacts with mitochondriae, and has an antiproliferative effect in psoriasis[116]. Formulations with increasing strength should be used to avoid excessive irritation of the skin. Several regimes have been used. For out-patient treatment, short-contact therapy using dithranol in an ointment base is useful. This therapy is based on the hypothesis that dithranol is absorbed quicker by lesional psoriatic skin than by unaffected skin[116]. Dithranol therapy requires intensified control and instruction. It has no systemic effects and is a very effective treatment.

Corticosteroids

Corticosteroids possess anti-proliferative, anti-inflammatory and immunosuppressive properties[117,118]. They are very effective in the treatment of psoriasis and widely used. After binding to a cytoplasmatic receptor, the corticosteroid is transported to the nucleus, and lipocortins are induced, a group of biomolecules with phospholipase A2 inhibiting activity[119]. Disadvantages of corticosteroid therapy (especially when applicating the stronger formulations used in the treatment of psoriasis) are the induction of dermal atrophy, perioral dermatitis, suppression of the pituitary-adrenal axis, and the rapid relapse of treated lesions[117].

Photo(chemo)therapy

UVB as well as UVA in combination with psoralens are effective in the treatment of psoriasis. UV-light induces free radicals and subsequently inflammation. The number of Langerhans cells decreases during treatment and the shape of these cells changes. The duration of the remission after photo(chemo)therapy is relatively long compared to corticosteroid treatment. Prolonged and intensive use of UV-light increases the risk of skin malignancies, especially of squamous cell carcinomas[3,120].

Methotrexate

Methotrexate (MTX) inhibits DNA-synthesis by competing as a substrate for dihydrofolate reductase and acts on the rapidly dividing epidermal cells of the psoriatic lesion. MTX is also capable of inhibiting PMN-chemotaxis and preventing their metabolic burst[121,122]. Long-term MTX therapy is very effective in the treatment

of psoriasis, however the risk of liver damage and cirrhosis limits the indication for MTX therapy[4].

Retinoids

Retinoids are derivatives of vitamin A, and are bound by a cytoplasmatic receptor. Subsequently they are transported to the nucleus, bind to a nuclear receptor[123] and act via modulation of transscription and translation of DNA. The process of keratinization is altered by retinoids. The best results are achieved in pustular psoriasis or in guttate psoriasis of recent onset. These drugs are teratogenic, which has an important impact for women of childbearing age. Side effects are dose-related and include cheilitis, hair loss, generalized pruritus, dryness of the skin, hypertriglyceridemia, and hyperostosis. Relapses occur usually after treatment is discontinued[4,124].

Cyclosporin

Cyclosporin is an immunosuppressive drug that reversibly suppresses the immunomodulating functions of cells of the immune system. It is effective in the treatment of psoriasis, although side effects may limit its use, e.g. hypertension and impairment of renal function. It is especially useful in patients with extensive psoriasis. Lesions re-occur after discontinuation of therapy[125,126].

Vitamin D_3

Vitamin D_3 and analogues decrease proliferation and increase differentiation of keratinocytes in culture[127]. In addition, it posesses immunomodulating properties in vivo[128]. This hormone also posesses a cytoplasmatic receptor, is afterwards bound to a nuclear receptor, and acts via interference with transcription and translation[129]. It is effective in the treatment of psoriasis, but can show serious side effects. The results of treatment with analogues having less systemic actions such as MC903 are promising[130,131].

Although all these treatment modalities are very useful in the treatment of psoriasis, it is important to search for new or modified classical treatments which are more effective and/or show less side effects.

AIM OF THE THESIS

Despite all the investigations on the pathogenesis of psoriasis, the specific role of various cell types in epidermal and dermal compartment is not known. In this thesis, epidermal growth in combination with inflammation in epidermal and dermal compartment were studied using immunohistochemical techniques. In order to achieve further information on the pathogenetic processes involved in psoriasis the following aims were formulated:

1. To investigate the phases of the psoriatic process during the evolution of a lesion, using immunohistochemical techniques for assessment of epidermal growth and inflammation.
2. Elucidation of the order of events concerning epidermal growth and inflammation during normalization of a psoriatic lesion.
3. Evaluation of the clinical efficacy of new and modified classical therapies for psoriasis.
4. To establish to what extent the markers for epidermal growth and inflammation are of diagnostic relevance with respect to psoriasis.

METHODOLOGY AND TECHNIQUES

Approach:

1. In order to study the phases in the induction of psoriasis, two in vivo models were used. Firstly, the transition zone between lesional and clinically uninvolved skin from a spreading psoriatic plaque was investigated (chapter 2.1). Secondly, application of leukotriene B_4 on normal skin was carried out. This results in features as seen in the psoriatic lesion[132-134]. The different phases of inflammation and epidermal growth can be studied carefully using this model. The changes induced by application of leukotriene B_4 and the relevance of this model as an in vivo model for psoriasis is discussed in chapter 2.2.

2. Before and during treatment courses with various anti-psoriatic therapies, biopsies were taken at different time intervals and processed for immunohistochemistry. This approach permits the monitoring of changes in inflammation and epidermal growth during regression of psoriatic lesions (Chapter 3). In addition, the effect of one of these therapies (dithranol) on normal skin has been investigated using the same immunohistochemical markers. The relevance of each marker for monitoring therapies and the correlation between histological features and clinical scores were studied. The value of a new marker for monitoring anti-psoriatic therapies, keratin 17, is evaluated in chapter 3.7.

3. To investigate the clinical effect of various new or modified classical anti-psoriatic therapies, patients from the out-patient department were treated and monitored using the Psoriasis Area and Severity Index (PASI-score). This score is calculated from the percentage of the body surface involved:

   A: 0 = no involvement, 1 = < 10% involvement, 2 = 10-29%, 3 = 30-49%, 4 = 50-69%, 5 = 70-89%, 6 = 90-100%,

   and from the amount of erythema (E), desquamation (D), and infiltration (I): 0 = no involvement, 1 = slight involvement, 2 = moderate involvement, 3 = severe involvement, 4 = severest possible involvement.

   PASI = upper extremities: 0.2 x A(E+D+I) +
   trunk: 0.3 x A(E+D+I) +
   lower extremities: 0.4 x A(E+D+I).

   The capacity to reduce PASI-scores of new and modified classical therapies is evaluated in chapter 3.

4. Epidermal growth and inflammation were assessed in three other erythematosquamous skin disorders. To establish to what extent the changes as seen in psoriasis are specific, the following three skin diseases were investigated:

   1. Inflammatory Linear Verrucous Epidermal Nevus (ILVEN) versus linear psoriasis (Chapter 4.1). ILVEN is a congenital nevus and has been suggested to be a separate disease entity. The delineation from linear

psoriasis has been discussed in the literature. So far the question as to the nosological independency of ILVEN remains unsettled[135].

2. Pityriasis Rubra Pilaris (PRP). The relationship between psoriasis and PRP has been discussed by many authors. Firstly, it can be rather difficult to differentiate both conditions on clinical grounds, secondly, the transition of the one in the other has been suggested[136].
3. Discoid Lupus Erythematosus (DLE). Initial lesions of DLE show sharply demarcated erythema, hyperkeratosis, and elevation. If not adequately treated, a marked atrophy results. In the etiology of DLE auto-immunity plays an important role[137].

These three skin diseases show clinically overlap, especially in the initial phase. DLE however, ends in a contrasting state.

Immunohistochemistry:

Monoclonal antibodies:

To assess epidermal **proliferation** the monoclonal antibody Ki-67 was chosen. This antibody is directed against a nuclear antigen present in cycling cells. This antigen is presented in the late G1-phase, S-phase, G2-phase and M-phase of the cell cycle. It is an appropriate marker for changes in proliferation[138].

To assess epidermal **differentiation** antibodies against cytokeratins were used. These are proteins which are components of the cytoskeleton[139]. The pattern of keratins expressed by keratinocytes changes as they mature[140,141]. In this thesis we have selected antibodies against keratin 10 (RKSE60), a keratin that is present in supra-basal differentiated keratinocytes[49], and against keratin 13 and 16 (Ks8.12). Keratin 13 is absent in normal adult human skin[141], whereas keratin 16 is expressed by hyperproliferative keratinocytes in vitro as well as in vivo[48,142]. Keratin 16 was reported to correlate well with clinical scores for psoriasis[143]. Two other markers for epidermal differentiation are involucrin and filaggrin. Involucrin is a cytoplasmatic protein precursor of the cornified envelope[144], filaggrin is involved in the formation of keratohyalin granules[145]. In normal skin, involucrin is found in the granular layer and in the upper stratum spinosum. Filaggrin is present in the stratum corneum and in the granular layer. These two proteins were stained using a polyclonal antibody

against involucrin and a monoclonal antibody against filaggrin. In one study, a monoclonal antibody against keratin 17 was used (E3)[146]. Keratin 17 is not present in normal human epidermis, but is restricted to the outer root sheath of the hair follicle and to myoepithelial cells of the sebaceous gland[147]. In Chapter 3.7 it's distribution in psoriatic lesions is investigated. An indirect peroxidase technique was used for staining with this antibody.

Inflammation: A panel of antibodies was chosen to visualize cells involved in inflammation. T-lymphocytes were stained with a pan-T cell marker, directed against CD2 (Dako T11)[148]. B-lymphocytes were stained using an antibody against CD22 (Dako Pan-B)[94]. A CD14-antibody was chosen to stain monocytes and macrophages (WT14)[149]. For assessment of PMN, a polyclonal antibody against elastase was used[150]. Elastase is not totally specific for PMN. Monocytes are reported to contain 5% of the elastase activity of monocytes[151]. Finally, Langerhans cells were stained using the antibody OKT6, directed against CD1a[152].

Dermal extracellular matrix proteins: Three important components of the extracellular matrix are heparansulphate, fibronectin, and the recently described protein tenascin[78-80]. Staining was performed using the antibodies JM-72, anti-fibronectin, and T2H5 respectively.

Staining techniques:

The antibodies used in this thesis are indicated in Table I.

Biopsies were embedded in Tissue Tek OCT compound (Miles Scientific, Naperville, USA), snap frozen in liquid nitrogen and stored at -80 °C until use.

Skin sections of 7 μm were cut. The slides were fixed for 10 minutes in acetone/-ether 60/40 vol% for Ki-67, or in acetone for staining with the other antibodies.

Staining with Ks8.12, RKSE60, Ki-67, anti-elastase, T2H5, JM-72, anti-fibronectin, anti-involucrin, anti-filaggrin and E3 were performed using a peroxidase-technique. In brief, slides were incubated with the antibodies for 30 minutes (Ki-67, Ks8.12, RKSE60, anti-elastase, anti-involucrin, anti-filaggrin, E3) or for 60 minutes (T2H5, JM-72, anti-fibronectin) at room temperature in the dark. After 3 washes with phosphate buffered saline (PBS) slides were incubated with rabbit-anti-mouse antibody (RAMPO, Dakopatts, Copenhagen, Denmark), or with swine-anti-rabbit-immuno-

Table 1.

ANTIBODY (MANUFACTURER)	SPECIFICITY	DILUTION	TECHNIQUE
Ki-67 (Dakopatts, Copenhagen, Denmark)	nuclear antigen in late G1, S, G2 and M phase	1:40 - 1:100	indirect peroxidase
Ks8.12 (Sigma, St Louis, USA)	keratin 13 and 16	1:25 - 1:40	indirect peroxidase
RKSE60 (F Ramaekers, Maastricht, NL)	keratin 10	1:10	indirect peroxidase
Anti-elastase (Serotec, Oxford, England)	elastase in polymorphonuclear leukocytes	1:100	direct peroxidase
T11 (Dakopatts, Copenhagen, Denmark)	T-lymphocytes (CD2)	1.100	PAP*
Pan-B (Dakopatts, Copenhagen, Denmark)	B-lymphocytes (CD22)	1·100	PAP
WT14 (W Tax, Nijmegen, NL)	monocytes/macrophages (CD14)	1:100	PAP
OKT6 (Ortho Diagn Systems, Raritan, USA)	Langerhans cells (CD1a)	1:100	PAP
T2H5 (A Verstraeten, Amsterdam, NL)	tenascin	1:2000	indirect peroxidase
JM-72 (J vd Born, Nijmegen, NL)	heparansulphate	1:500	indirect peroxidase
Anti-fibronectin (Sigma, St Louis, USA)	fibronectin	1:500	indirect peroxidase
Anti-involucrin (Biomed Techn Inc, Stoughton, USA)	involucrin	1:10	indirect peroxidase
Anti-filaggrin (Biomed Techn Inc, Stoughton, USA)	filaggrin	1:500	indirect peroxidase
E3 (S Troyanovski, Moscow, USSR)	keratin 17	1:10	indirect peroxidase

* PAP = peroxidase-anti-peroxidase

globulin (Dakopatts, Copenhagen, Denmark, for staining with involucrin), conjugated with peroxidase 1:50 in phosphate buffer containing 5% human AB-serum for 30 minutes. This incubation step was skipped in the elastase-staining as the anti-elastase antibody is directly conjugated with peroxidase. After 3 more washes with PBS and pre-incubation with sodium acetate buffer, pH 4.9, slides were stained in sodium acetate buffer containing 200 mg/l 3 amino-9-ethyl-carbazole solution and 0.01% H_2O_2 prepared freshly (AEC-solution) for 10-15 minutes at room temperature in the dark.

Staining with T11, Pan-B, WT14 and OKT6 were carried out using a peroxidase-anti-peroxidase technique (PAP-technique). Slides were incubated with the monoclonal antibodies (dilution 1:100) for 60 minutes. After 3 washes in PBS, incubation followed with rabbit-anti-mouse immunoglobulins (RAM-Ig, Dakopatts, Copenhagen, Denmark) 1:25 in PBS for 20 minutes. After 3 more washing steps incubation with peroxidase-anti-peroxidase complexes (PAP, Dakopatts, Copenhagen, Denmark) 1:100 in PBS for 20 minutes was performed. Incubation with RAM-Ig and PAP was repeated and slides were stained with AEC-solution after 2 washes with PBS and pre-incubation with sodium acetate buffer pH 4.9.

All slides were finally washed with demineralized water and counterstained with Mayer's haematoxylin (Sigma, St.Louis, USA). Slides were mounted in glycerine gelatin and studied by light microscopy.

Histological examinations

The number of Ki-67 stained nuclei was counted per mm length of section.

Staining with Ks8.12, RKSE60, anti-involucrin, anti-filaggrin and E3 was assessed in the basal and suprabasal epidermal compartment using a semi-quantitative scale (Chapter 2, 3 and 4).

Inflammatory cells (PMN, T- and B-lymphocytes, CD14-positive cells and Langerhans cells) were assessed in dermis and epidermis using a semi-quantitative scale (Chapter 2, 3 and 4).

Distribution of extracellular matrix proteins was described compared to classical histological features (Chapter 2.1).

Statistical analysis

For statistical analysis were used: the Mann-Whitney test , the Wilcoxon ranking test for paired data, and the Kruskal-Wallis test for group means.

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CHAPTER 2

SEQUENTIAL STUDIES ON IN VIVO MODELS FOR PSORIASIS

This chapter is based on the following publications:

1. De Jong EMGJ, Schalkwijk J, van de Kerkhof PCM. Epidermal proliferation and differentiation, composition of the inflammatory infiltrate and the extracellular matrix in the margin of the spreading psoriatic lesion. European Journal of Dermatology 1991; 1: 221-227.
2. De Jong EMGJ, van Erp PEJ, van Vlijmen IMJJ, van de Kerkhof PCM. The interrelation between inflammation and epidermal proliferation in normal skin following epicutaneous application of leukotriene B_4; An immunohistochemical study. Clin Exp Dermatol 1992; 17: 413-420.

2.1 EPIDERMAL PROLIFERATION AND DIFFERENTIATION, COMPOSITION OF THE INFLAMMATORY INFILTRATE AND THE EXTRACELLULAR MATRIX IN THE MARGIN OF THE SPREADING PSORIATIC LESION.

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SUMMARY

Changes seen in uninvolved skin adjacent to spreading psoriatic lesions represent early events in the evolution of a lesion.
In the margin of spreading psoriatic plaques, epidermal proliferation as well as keratinization (keratin 16, keratin 10), the inflammatory infiltrate, and extracellular matrix proteins (tenascin, heparansulphate, fibronectin) were analyzed using immunohistochemistry.
Dermal changes (expression of tenascin) and the inflammatory infiltrate (T-lymphocytes, Langerhans cells) appeared peripherally to the epidermal changes (acanthosis, keratin 16 expression, hyperproliferation).
The present study indicates that the stroma is an important target for studies on initial events in psoriasis.

Early changes taking place in the evolution of a psoriatic lesion, are difficult to catch. Therefore, various investigative approaches have been evaluated to study the incipient events of psoriasis. Tape-stripping[1,2], injection[3] or application of leukotriene-B_4[4,5], and taking biopsies from early lesions[6-9] have been used in the past. In addition, studying changes in the uninvolved skin adjacent to a spreading psoriatic lesion, can reveal new information[10-12].

Whether the epidermal or the dermal compartment show the earliest changes, remains controversial. In previous studies it has been shown that dermal infiltrate cells[12] and dermal vascular changes (increased blood flow[11], increased alkaline phosphatase[10]) can be found distant from the clinically involved skin. On the other hand, epidermal alterations can also be found outside the boundaries of the macroscopic psoriatic lesion[12].

In this study, three different units have been investigated: the epidermal compartment, the inflammatory infiltrate, and dermal extracellular matrix proteins (ECM).

In the epidermis, parameters for proliferation and keratinization were studied. Epidermal proliferation was characterized using the monoclonal antibody Ki-67, which stains a nuclear antigen present in cycling cells. Keratinization was investigated by studying the expression of keratin 16 and keratin 10 with the antibodies Ks8.12 and RKSE60 respectively.

The various components of the inflammatory infiltrate were studied using antibodies against T-lymphocytes (T11, CD2), against polymorphonuclear leukocytes (anti-elastase), against monocytes/macrophages (WT14, CD14), and against Langerhans cells (OKT6, CD1a).

The expression and localization of extracellular matrix proteins were also investigated. Tenascin, which is a recently described ECM, is present in the subepidermal dermis of normal skin and is discontinuously distributed[13-17]. In psoriasis and in other hyperproliferative skin disorders, an increased expression of tenascin is present[18]. In addition, the distribution of heparansulphate and fibronectin were investigated in the margin of active psoriatic lesions.

Patients and biopsies

Six patients (three male, three female; mean age 48 (27-76) years) suffering from chronic plaque psoriasis participated in the study. They had used no topical treatment for at least two weeks, and no systemic treatment for at least one year. In all patients the disease was in an unstable phase. From each patient one lesion was selected that, according to the patients history, had been extending during the last week. A punch biopsy (ϕ 6mm) was taken after local anaesthesia, across the margin of the spreading lesion. The biopsy was embedded in Tissue Tek OCT Compound (Miles Scientific, Naperville, USA), snap frozen in liquid nitrogen and stored at -80°C until use. From each biopsy consecutive sections of 7 μm were cut and fixed in acetone/ether 60/40 % for staining with the antibody Ki-67, or in acetone (0 °C) for 10 minutes for staining with the other antibodies.

Antibodies

Epidermal proliferation For assessment of epidermal proliferation the antibody Ki-67 was used (Dakopatts, Copenhagen, Denmark) which stains a nuclear antigen present in cycling cells.

Epidermal keratinization For assessment of epidermal keratinization, the antibody Ks8.12 (Sigma, St Louis, USA) was used, directed against keratin 13/16. Keratin 16 is expressed by hyperproliferative keratinocytes[19]. Keratin 13 is not present in adult human skin[20]. In addition, the antibody RKSE60 (gift of F. Ramaekers) was used, directed to keratin 10, which is expressed by differentiated keratinocytes.

Inflammation For assessment of inflammation in epidermis and dermis, antibodies against T-lymphocytes (T11, anti-CD2, Dakopatts, Copenhagen, Denmark), against polymorphonuclear leukocytes (anti-elastase, Serotec, Oxford, UK), against Langerhans cells (OKT6, anti-CD1a, Ortho Diagnostic Systems, Raritan, USA) and against monocytes/macrophages (WT14, anti-CD14, Dept of Medicine, Division Nephrology, University Hospital Nijmegen, The Netherlands[21]) were used.

Extracellular matrix proteins A monoclonal antibody against tenascin (T2H5) was obtained from AA Verstraeten, The Netherlands Cancer Institute, Amsterdam, The Netherlands[18], and used in combination with a monoclonal antibody (JM-72) to

heparansulfate core protein (J vd Born, Department of Nephrology, University Hospital Nijmegen), and a monclonal antibody to human plasma fibronectin (Sigma, St Louis, USA (clone F7387, ascites fluid)).

Staining procedures

For staining with the antibodies Ki-67 (1:100 in phosphate buffer), Ks8.12 (1:40), RKSE60 (1:10), anti-tenascin (1:2000), anti-heparansulfate (1:500) and anti-fibronectin (1:500), an indirect peroxidase technique was used. For staining PMN (anti-elastase, 1:100), a direct peroxidase technique was carried out. Slides were incubated with the primary antibodies for 30 minutes (for staining with Ki-67, Ks8.12, RKSE60 or anti-elastase); or for 60 minutes (antibodies to tenascin, heparansulphate, fibronectin) at room temperature in the dark. After washing in phosphate buffer the slides were incubated with a solution of rabbit-anti-mouse-immunoglobulin conjugated with peroxidase 1:50 in phosphate buffer containing 5% human AB-serum for 30 minutes, except for the anti-elastase antibody. After 3 washing steps in phosphate buffer and preincubation in sodium acetate buffer pH 4.9, slides were stained in a solution of 3-amino-9-ethyl-carbazole (AEC) in sodium acetate buffer containing 0.01% H_2O_2 for 10 minutes. All slides were counterstained with Mayer's haematoxylin (Sigma, St Louis, USA) and mounted in glycerin gelatin. Staining with T11, OKT6 and WT14 was carried out using a peroxidase-anti-peroxidase technique. Slides were incubated for 60 minutes with the monoclonal antibodies in a dilution of 1:100. Three washing steps in phosphate buffer were followed by incubation with rabbit-anti-mouse-immunoglobulin (Dakopatts, Copenhagen, Denmark) 1:25 in phosphate buffer for 20 minutes. This was followed by a washing step and incubation with peroxidase-anti-peroxidase complexes (Dakopatts, Copenhagen, Denmark) 1:100 in phosphate buffer for 20 minutes. The last two incubation steps were repeated. The slides were stained in the same AEC-solution in sodium acetate buffer and H_2O_2 and counterstained with Mayer's haematoxylin. Finally they were mounted in glycerin gelatin.

Histological examination

The immunohistochemical stainings were compared with the localization of parakeratosis, the reappearence of the granular layer, with the amount and localization of the inflammatory infiltrate, and with the development of initial acanthosis and full acanthosis.

RESULTS

Figure 1 shows the margin of a psoriatic lesion. The point where the parakeratotic horny layer changed into an orthokeratotic stratum corneum was an abrupt transition point in all biopsies. In addition, the granular layer reappeared immediately adjacent to the parakeratosis transition point. Full acanthosis developed more centrally than a parakeratotic stratum corneum.

In the suprabasal compartment of the epidermis, staining with the antibody Ks8.12 (keratin 13/16) was seen in the periphery of the lesion, well before the transition point of parakeratosis and full acanthosis in all six biopsies. In the psoriatic lesion, staining with RKSE60 (keratin 10) was diminished and discontinuous. The transition to the staining pattern of normal skin was seen abruptly at the site where the granular layer reappeared in all biopsies.

Initial acanthosis was the first histological change in the epidermis. In the basal cell compartment of the epidermis, the number of Ki-67 stained nuclei was high in the psoriatic lesion, and low in the uninvolved skin. In 4 out of 6 patients, Ks8.12 staining in the suprabasal compartment extended into the uninvolved skin beyond the point where a high number of Ki-67 stained nuclei was found. In 2 out of 6, the reverse was seen, high numbers of Ki-67 stained cells were found peripherally from suprabasal Ks8.12 staining.

The dermal inflammatory infiltrate was maximally present at the parakeratosis transition point in five out of six biopsies, but the infiltrate extended beyond the lesion into the clinically uninvolved skin in all patients (Fig 2). The infiltrate was composed mainly of T-lymphocytes. Above the dermal infiltrates, considerable epidermotropism was seen, mainly in the involved areas. In the uninvolved areas, rarely epidermotropism was found. Langerhans cells constituted only a small part of the

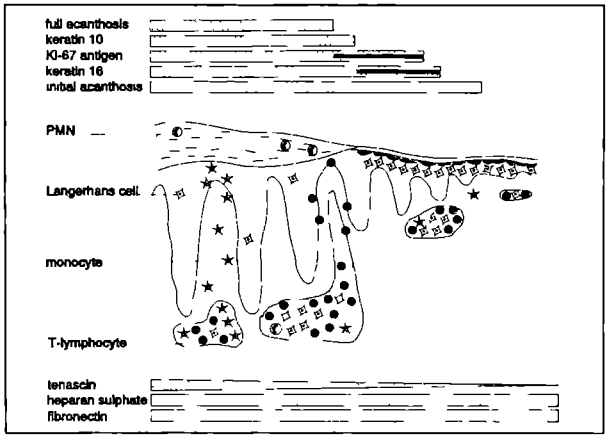


Figure 1. Margin of the spreading psoriatic lesion. Shaded bars represent the localization of the various parameters. Black bars (Ki-67 antigen, keratin 16) represent variable expression.

infiltrate in the center of the psoriatic lesion. In the infiltrates at the margin and in uninvolved skin, their contribution was more substantial (Fig 3). Epidermal Langerhans cell numbers were low in the lesion compared to uninvolved epidermis. The transition from low to high numbers was located outside the border of the lesion as defined by full acanthosis and parakeratosis in one patient, and at the parakeratosis transition point in 2 patients. In 3 patients, a gradual transition was seen. CD14-cells were extensive in the dermis of the psoriatic lesion. Also the lesional epidermis contained large numbers of CD14-cells. Towards uninvolved skin, a gradual decrease was seen in the dermis. In the epidermis of uninvolved skin, only sporadically CD14-cells were seen. PMN were seen sparsely in the stratum corneum in the psoriatic lesion. The inflammatory infiltrate contained only sporadically PMN.

The extracellular matrix protein heparansulphate was restricted to basal membranes both in the lesional and in the non-lesional skin; no differences in expression were seen. Fibronectin was present diffusely throughout the dermis; however no differences between lesional and non-lesional skin were found. Tenascin was found in a continuous pattern not only in the papillary dermis and reticular dermis of the psoriatic lesion, but also far beyond the parakeratosis transition point in the uninvolved skin. In three out of six biopsies, dermal presence of tenascin decreased gradually, and became discontinuous in two out of these three, at the most distant edge of the sections (Fig 4). In the other three patients, tenascin expression in the

dermis remained high in the distant uninvolved dermis. This is in contrast to the pattern of expression of tenascin in normal skin[18], where a discontinuous expression is seen just under the basal membrane.

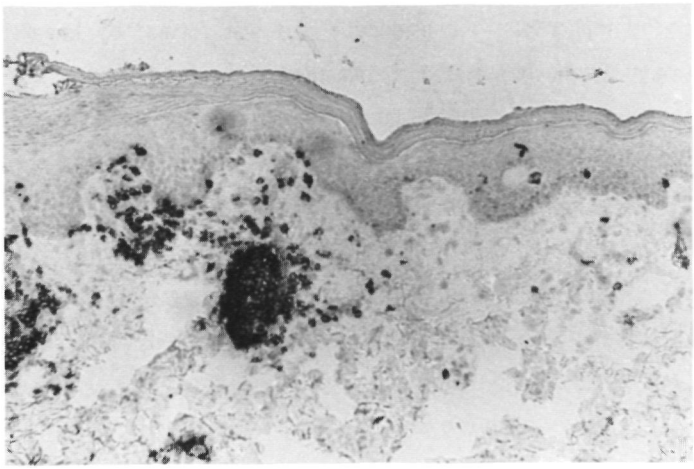


Figure 2. T11 staining (T-lymphocytes) in the margin of the psoriatic lesion (x 200)

DISCUSSION

Changes seen in uninvolved skin adjacent to the spreading psoriatic lesion represent early events in the evolution of such a lesion. In this investigation, epidermal proliferation, keratinization in combination with the inflammatory infiltrate in the margin of psoriatic lesions was studied. In addition, the extracellular matrix proteins tenascin, heparansulphate and fibronectin were investigated.

In the epidermal compartment, keratin 16 expression was seen peripherally from the parakeratosis transition point and full acanthosis, however not extending as far as the increased infiltrate in the dermis or as the increased tenascin expression. In the epidermis, discontinuous keratin 10 expression was seen confined to the full acanthotic epidermis. This is in agreement with previous studies[12]. Nuclear Ki-67 staining, indicating epidermal proliferation, showed a more variable pattern than in an earlier study[12]. In all patients, initial acanthosis was seen before the number of Ki-67 stained nuclei was high, and in 4 out of 6 patients, Ks8.12 staining was seen more peripherally than high numbers of Ki-67 stained nuclei. Possibly a lag time between entering S-phase and the expression of the antigen recognized by the

antibody Ki-67, as postulated[22], can explain this discrepancy. Alternatively, a delay in differentiation of keratinocytes might explain the early onset of initial acanthosis, especially since keratin 16 is seen early in the evolution of a lesion.

The composition of the dermal inflammatory infiltrate in the uninvolved skin consisted of T-lymphocytes, a considerable part was formed by Langerhans cells, and CD14 cells. In the lesion, the composition of the infiltrate was different from that in the uninvolved skin. It consisted of only few Langerhans cells, many T-lymphocytes and numerous CD14 cells. CD14 cells were also diffusely distributed in the papillary and reticular dermis and along skin adnexes. The maximal presence of the infiltrate, in the margin of the lesion, contained considerably more Langerhans cells than the infiltrate in the central part of the lesion. In this respect it is of interest that in a previous study, CD1a positive Langerhans cells were found in high amounts in the infiltrate of initial psoriatic lesions (50-60%), and consisted only 10% of the infiltrate cells in chronic psoriasis[23]. Langerhans cell numbers in the epidermis were low in the lesion. This is in agreement with earlier studies[23]. An active migration of Langerhans cells in psoriasis has been postulated. CD14 positive cells (monocytes, macrophages) show a dense pattern in the dermis and epidermis of the central part of the lesion. In the uninvolved epidermis, they are not present. A previous study proposed a role of macrophages in the inition of a lesion[24], as they appeared before T-lymphocytes and PMN were seen. In the present study, monocytes/macrophages were seen maximally in the fully developed lesion, and no epidermal CD14 cells were seen outside the involved section. The low number of PMN in the infiltrates of psoriatic lesions and uninvolved skin is striking. Most PMN seen were trapped in the stratum corneum of the psoriatic lesion. This is in disagreement with earlier studies, where PMN were the first cells invading the epidermis in the initiation of a lesion[9,25].

The extracellular matrix proteins in the dermis were differentially distributed. Heparansulphate was evenly distributed over the total length of the biopsy along the basement membrane, showing the pattern of normal skin[26]. Fibronectin showed no difference in distribution comparing involved and uninvolved dermis. Its expression in the uninvolved skin was high compared to that of normal skin[27]. Tenascin expression however, was strong in papillary and reticular dermis of the lesion, and gradually diminished towards the periphery in three out of the six biopsies. Tenascin

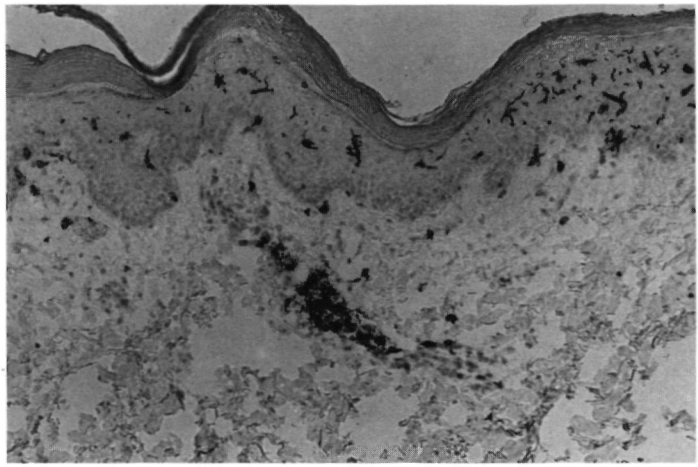


Figure 3. OKT6 staining (Langerhans cells) in the margin of the psoriatic lesion (x 200)

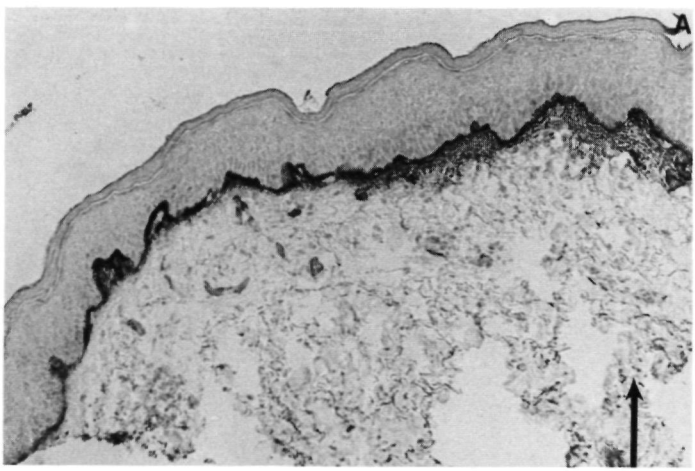
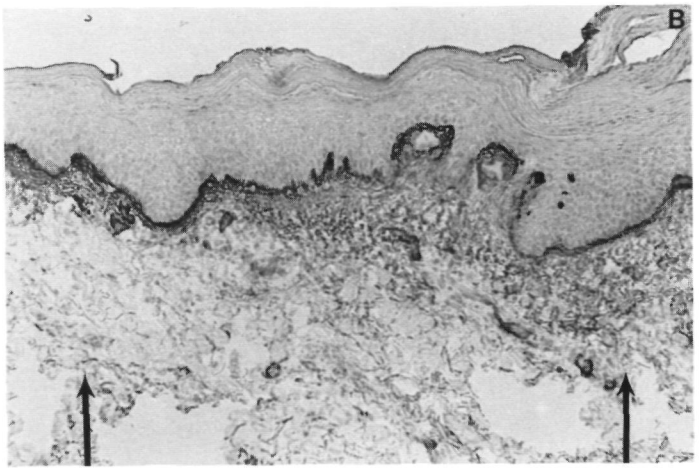
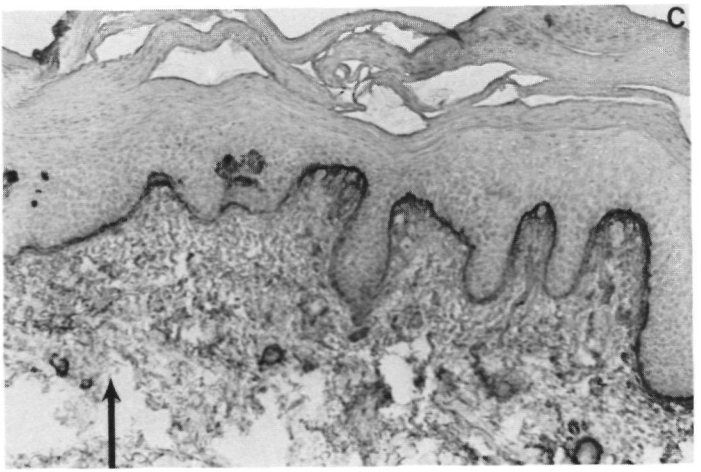


Figure 4. T2H5 staining (tenascin) in the psoriatic lesion and in clinically uninvolved skin (x 200). Reference points are marked with an arrow. A: Clinically uninvolved skin at the left



B: transition zone



C: psoriatic lesion at the right.

has shown to be increased in hyperproliferative skin diseases[18] and is probably involved in wound healing[16]. In general, tenascin seems to correlate with remodelling of the epidermis. The observation that tenascin is high in the uninvolved skin adjacent to the psoriatic lesion suggests that it is involved in the early phase of the evolution of a lesion. Whether the first signal for induction of tenascin is derived from the inflammatory cells or from the epidermis is unknown.

This study demonstrated in the periphery of the lesions, an increase in the extracellular matrix protein tenascin and an increase in the dermal inflammatory infiltrate. In previous studies also dermal changes have been detected in the clinically uninvolved skin surrounding a psoriatic lesion, such as an increase in vascular endothelium measured by alkaline phosphatase[10], and an increased dermal blood flow using Laser Doppler Flowmetry[11]. The maximum of the inflammatory infiltrate was present at the margin of the lesion, just beneath the parakeratosis transition point. The early expression of tenascin and the peripheral presence of the inflammatory infiltrate containing many Langerhans cells in the dermis, in combination with the apparent undisturbed epidermis, provide further evidence that the dermal compartment is early involved in the pathogenesis of psoriasis.

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2.2 THE INTERRELATION BETWEEN INFLAMMATION AND EPIDERMAL PROLIFERATION IN NORMAL SKIN FOLLOWING EPICUTANEOUS APPLICATION OF LEUKOTRIENE B_4; AN IMMUNOHISTOCHEMICAL STUDY.

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SUMMARY

Topical application of leukotriene B_4 (LTB_4) on normal skin has been used as an in vivo model to investigate cutaneous inflammation and epidermal proliferation, which are important phenomena in the pathogenesis of psoriasis. The aim of the present investigation is to further elucidate the interrelation between inflammation and epidermal proliferation, using specific monoclonal antibodies as markers for different cell types involved and to investigate to what extent this model reflects changes as observed in the psoriatic lesion.

Aliquots of LTB_4 were applied on the upper arms of 8 healthy volunteers. After LTB_4-application, biopsies were taken at consecutive time intervals. On frozen sections, epidermal proliferation was assessed by Ks8.12- (keratin 16) and Ki-67-binding (cycling cells), inflammation was characterized using anti-elastase (PMN), T11 (T-lymphocytes), pan-B (B-lymphocytes), WT 14 (CD14-positive cells) and OKT 6 (Langerhans cells).

New observations were that the density of CD14-positive cells was increased already after 8 hours and decreased slightly at 72 hours. A striking rearrangement of Langerhans cells was seen in close vicinity to intraepidermal accumulation of PMN. Remarkably an increased density of these cells in the dermis at 72 hours was seen and a decrease in the epidermis. In line with previous studies, the accumulation of PMN reached a maximum 24 hours after LTB_4-challenge. The identity of the mononuclear infiltrate cells which have been reported 48 to 72 hours after LTB_4 proved to be T-lymphocytes. No B-lymphocytes were observed. Ki-67-positive nuclei were maximally increased 72 hours after LTB_4-application, which implies that recruitment of cycling cells is of relevance for the LTB_4-induced proliferation in vivo. The hyperproliferation-related keratin 16 was expressed variably in the suprabasal compartment. The present study demonstrates that CD14 cells and Langerhans cells participate in acute LTB_4 induced inflammation.

The relevance of leukotriene B4 induced changes in normal skin for studies on the pathogenesis of psoriasis is discussed. In particular events in the initial psoriatic lesion are approached by this model.

INTRODUCTION

Leukotrienes are mediators that are generated in the inflammatory response in various tissues, including skin[1-4]. They are produced from arachidonic acid present in the membrane of cells including keratinocytes[5-7] via a lipoxygenase pathway. Of particular significance in this respect is the 5-lipoxygenase product leukotriene B_4 (LTB_4), which has been shown to have a potent chemoattractant activity for both polymorphonuclear leukocytes (PMN) and monocytes[8]. In addition, LTB_4 stimulates epidermal growth in vitro[9].

Topical application of LTB_4 on normal skin produces an acute inflammation, dominated by PMN[10], followed by a perivascular mononuclear cell infiltrate after 48 hours[11] and epidermal proliferation[12], reaching a maximum after 72-96 hours. A dose-response relationship has been shown between these phenomena and the concentration of LTB_4 applied to the skin. Therefore this approach has been used as an in vivo model to investigate the influence of therapies such as corticosteroids,

dithranol, photochemotherapy, methotrexate end retinoids on transcutaneous migration of PMN[13].

Inreased amounts of LTB_4 have been found in lesional skin of atopic dermatitis, allergic contact dermatitis, urticaria and psoriasis[3,14-16]. In psoriasis, a skin disorder characterized by epidermal proliferation and inflammation, increased production of 12-HETE, 15-HETE, platelet activating factor, interleukin-8, and LTB_4 has been demonstrated in lesional skin[2-4,16,17]. In view of the relatively high biologic activity of LTB_4 compared to other leukotrienes, this compound seems of particular relevance for inflammation control.

The aim of the present investigation is to further elucidate the interrelation between inflammation and epidermal proliferation after epicutaneous application of LTB_4, using monoclonal antibodies as markers for different cell types involved. In particular, the following questions were adressed:

(i) what is the identity of infiltrate cells of the LTB_4-induced inflammation and what is their relation with epidermal proliferation and keratinization.

ii) to what extent are these changes compatible with the abnormalities observed in the psoriatic lesion.

MATERIALS AND METHODS

Subjects

A group of 8 healthy subjects participated in the study. They had no history or signs of skin disease. The group consisted of 2 females and 6 males. Their age was between 21 and 28 years. Prior to the experiment, approval of the Ethical Committee was obtained.

LTB_4-application and biopsy procedures

Aliquots of 100 ng of LTB_4 (Paesel GmbH, Frankfurt, FRG) diluted in 10 μl of ethanol were applied on the outer surface of the upperarms of the volunteers via a glass cylinder (5.5 mm diameter) and the ethanol was evaporated under a stream of nitrogen[18]. The test sites were covered with impermeable dressings (Silver patch,

van der Bend BV, Brielle, The Netherlands) held at place with leukosilk tape (Beiersdorf, Hamburg, FRG).

Before LTB_4-application and at time intervals of 8, 24, 48 and 72 hours biopsies were taken using a razor blade in conjunction with a metal guard. Chloraethyl spray was used as a local anaesthetic (Aethylchlorid, Adroka, Switzerland).

The biopsies were snap frozen in liquid nitrogen, embedded in Tissue Tek OCT compound (Miles Scientific, Naperville, USA) and stored at -80 °C.

Staining procedures

To assess epidermal proliferation staining with the following monoclonal antibodies was performed

i) Ks8.12 (Sigma, St Louis, USA) to assess keratin 16, which is expressed in hyperproliferative epidermis[19,20]

ii) Ki-67 (Dakopatts, Copenhagen, Danmark) to detect a nuclear antigen present in actively cycling cells.

Inflammation was characterized with the antibodies

iii) Anti-elastase (Serotec, Oxford, England) a marker for polymorphonuclear leukocytes[21]

iv) T11 (Dakopatts, Copenhagen, Denmark) to assess T-lymphocytes (CD2)

v) Pan-B (Dakopatts, Copenhagen, Denmark) to assess B-lymphocytes (CD22)

vi) WT14 (Dept. Medicine, Div.Nephrol. University Hospital Nijmegen)[22] to demonstrate CD14-positive cells which represent activated monocytes and macrophages

vii) OKT6 (Ortho Diagnostic Systems, Raritan, USA) to assess Langerhans cells (CD1a)

Skin sections of 7 μm were cut. The slides were fixed for 10 minutes in acetone/a-ether 60/40 vol% for Ki-67, or in acetone for staining with the other antibodies.

Staining with Ks8.12, Ki-67, and anti-elastase were performed using a peroxidase-technique. In brief, slides were incubated with the monoclonal antibodies for 30 minutes, and after 3 washes with phosphate buffered saline (PBS) incubated with rabbit-anti-mouse antibody (Dakopatts, Copenhagen, Denmark) conjugated with peroxidase for 30 minutes except for the anti-elastase antibody, which is directly conjugated with peroxidase. After 3 more washes with PBS and pre-incubation with

sodium acetate buffer, pH 4.9, slides were finally stained in sodium acetate buffer containing 200 mg/l 3-amino-9-ethyl-carbazole solution and 0.01% H_2O_2 prepared freshly (AEC-solution) for 15 minutes at room temperature in the dark.

Staining with T11, Pan-B, WT14 and OKT6 were done using a peroxidase-anti-peroxidase technique (PAP-technique). For this technique slides were incubated with the monoclonal antibodies for 60 minutes. After 2 washes with PBS an incubation step followed with rabbit-anti-mouse immunoglobulins (RAM-Ig, Dakopatts, Copenhagen, Danmark) and after 2 more washes an incubation with peroxidase-anti-peroxidase complexes (PAP, Dakopatts, Copenhagen, Danmark) was performed. The incubation with RAM-Ig and PAP were repeated and the slides stained with AEC-solution after 2 washes with PBS and pre-incubation with sodium acetate buffer pH 4.9.

All slides were finally washed with demineralized water and counterstained with Mayer's haematoxylin (Sigma, St.Louis, USA). Slides were mounted in glycerine gelatin and studied by light microscopy.

Histological examinations

The density of PMN, T- and B-lymphocytes, CD14-positive cells and Langerhans cells were assessed blind by two investigators in dermis and epidermis using a 5-point scale: 0 = no positive cells observed, 1 = only sporadic staining of cells, 2 = minimal presence, 3 = moderate presence, 4 = pronounced presence. The dermal infiltrate was subdivided in perivascular and diffuse localization.

The number of actively cycling cells was assessed by counting Ki-67-positive nuclei per mm length of section.

Ks8.12-binding of the suprabasal and basal compartments was assessed using a 5-point scale: 0 = no staining, 1 = sporadic staining, 2 = minimal staining, 3 = moderate staining, 4 = pronounced staining.

Statistical analysis

The Mann-Whitney test was used for statistical analysis.

RESULTS

Clinical response

Apart from a small blister in one of the volunteers at 72 hours after LTB_4-application, no signs of inflammation were seen.

Inflammation

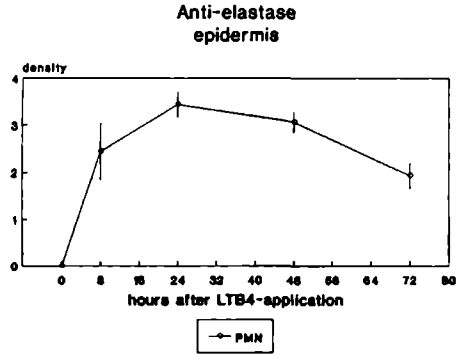
Density and localization of inflammatory cells following LTB_4-application, are summarized in Figure 1a-1d.

PMN (Fig.1a) A reproducable accumulation of PMN in dermis and epidermis was found, with a maximum at 24 hours after application of LTB_4. In the epidermis the accumulation was already present and distributed over basal and suprabasal compartment at 8 hours. At 24 hours, maximum accumulation, the microabscesses were predominantly localized in the subcorneal zone (Fig.3). After 48 hours and 72 hours the microabscesses became progressively flattened and faded away in all patients. The dermal PMN-accumulation was in phase with the epidermal infiltration, however, at 8 hours relatively more cells were seen in the dermal compartment. At 72 hours, the PMN-extravasation had considerably diminished but was still present.

CD14-positive cells (Fig. 1b) In the epidermis, CD14-positive cells were only sporadically present at all time intervals. In contrast, dermal CD14-positive cells already were increased diffusely ($p = 0.05$) at 8 hours after LTB_4-application (Fig. 4a-b). Their numbers remained increased until 48 hours, and diminished at 72 hours.

Langerhans cells (Fig. 1c) In the epidermis the CD1a-positive Langerhans cells were present at all time intervals. The density of these cells in the epidermal compartment showed a statistically significant decrease at 72 hours ($p < 0.01$). The Langerhans cells showed in all subjects consistently the following migration pattern (Fig 5a,b,c,): Before and 8 hours after LTB_4-application the Langerhans cells were seen midepidermally. After 24 hours they lined up at the margins of the microabscesses, high in the epidermis. At 48 hours they were distributed diffusely in the epidermis, and after 72 hours mainly in the midepidermal compartment and basal layer. No clustering of Langerhans cells was observed at any time interval. In the dermis at 8 hours Langerhans cells were seen in increased amounts compared to unchallenged skin ($p < 0.03$). Remarkably, at 72 hours a substantial increase was

A


Anti-elastase
epidermis
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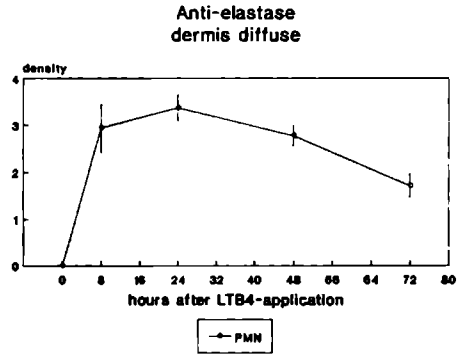
PMN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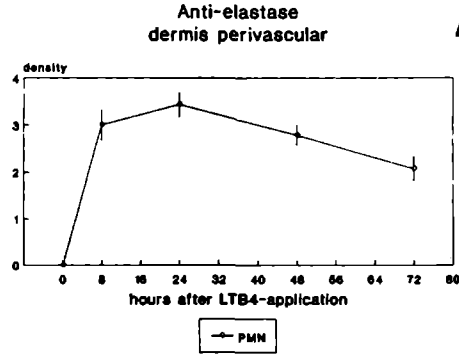
Anti-elastase
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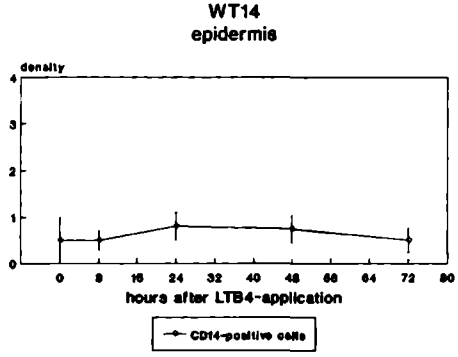
PMN

B


WT14
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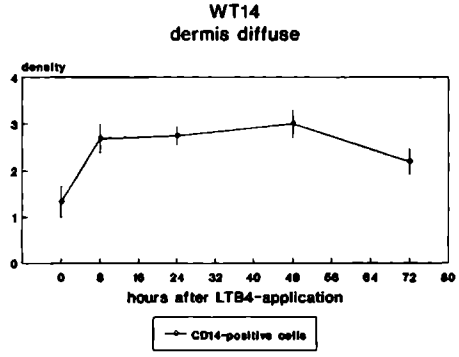
CD14-positive cells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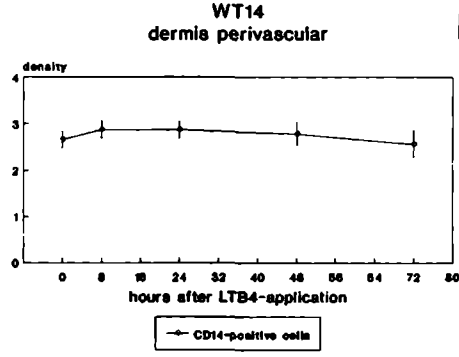
WT14
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CD14-positive cells

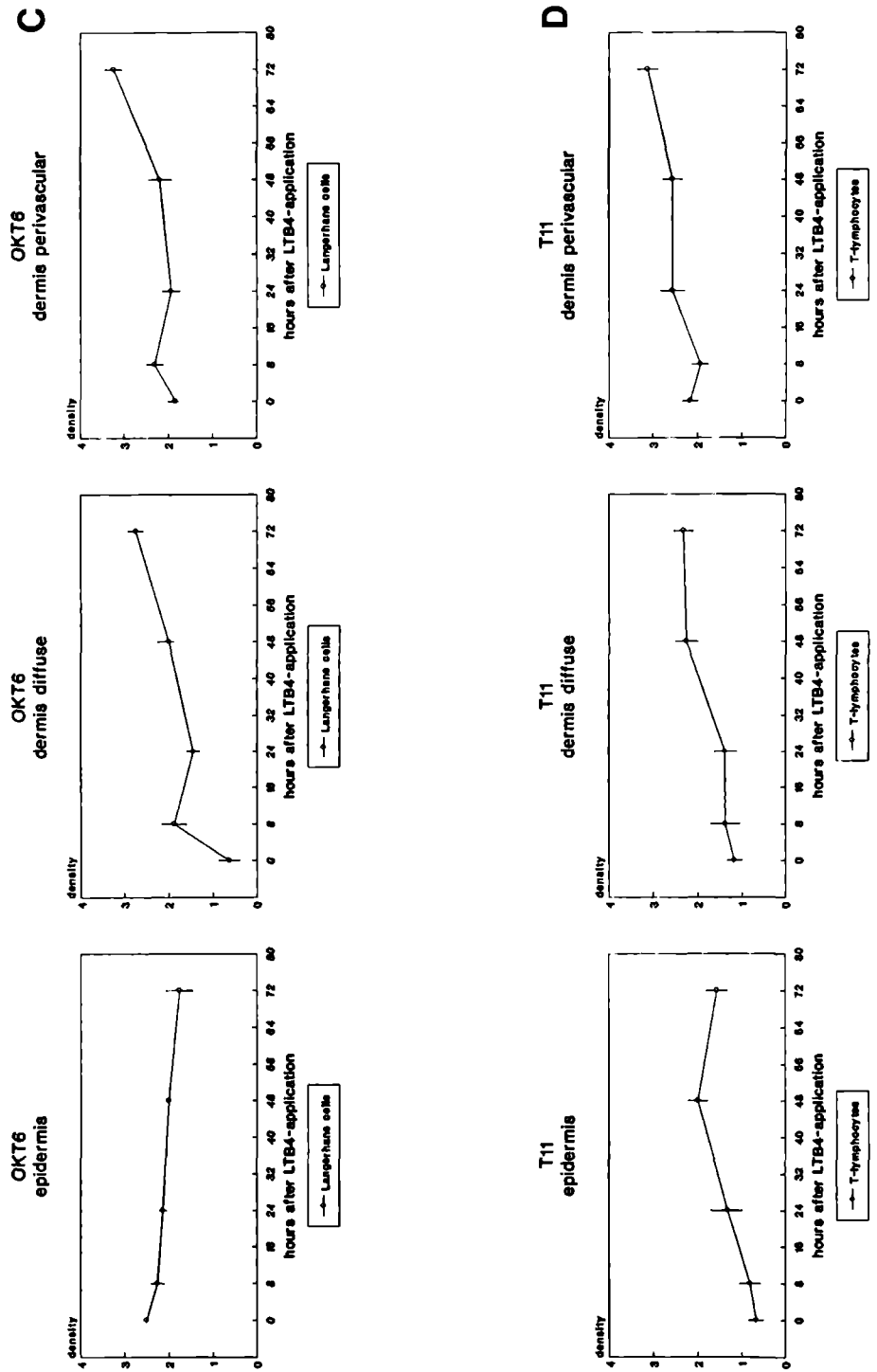

Figure 1: Schematic representation of the density of infiltrate cells at time intervals following LTB_4-application (mean ± SEM); (a) PMN, (b) CD14-positive cells, (c) Langerhans cells, (d) T-lymphocytes.

observed in the perivascular zone and diffusely in the dermis ($p < 0.02$, $p < 0.01$, respectively).

T-lymphocytes (Fig. 1d) In the epidermis only sporadic T-lymphocytes were seen at 8 hours after LTB_4-application. Their number increased gradually and at 48 hours a maximum was seen. At 72 hours T-lymphocytes were still present. Dermal perivascular and diffuse T-lymphocytes, however, showed an increase in number (Fig. 6 a-b) and a maximum was reached at the end of the observation period ($p < 0.05$, $p < 0.02$, resp.).

B-lymphocytes No B-lymphocytes were seen in the epidermis and dermis of normal skin after LTB_4-application.

Proliferation

Numbers of Ki-67-positive nuclei and Ks8.12-binding are summarized in figure 2.

Ks8.12 (Fig. 2a) The pattern of Ks8.12-binding after LTB_4-application was variable. A slight increase was seen of basal and suprabasal staining, however, the increase was not statistically significant. The interindividual variability was considerable.

Ki-67 (Fig. 2b and Fig. 7a + b) The number of Ki-67-positive nuclei in the epidermal basal layer did not increase significantly during the first 24 hours, increased slightly after 48 hours ($p < 0.05$), and markedly after 72 hours ($p < 0.02$).

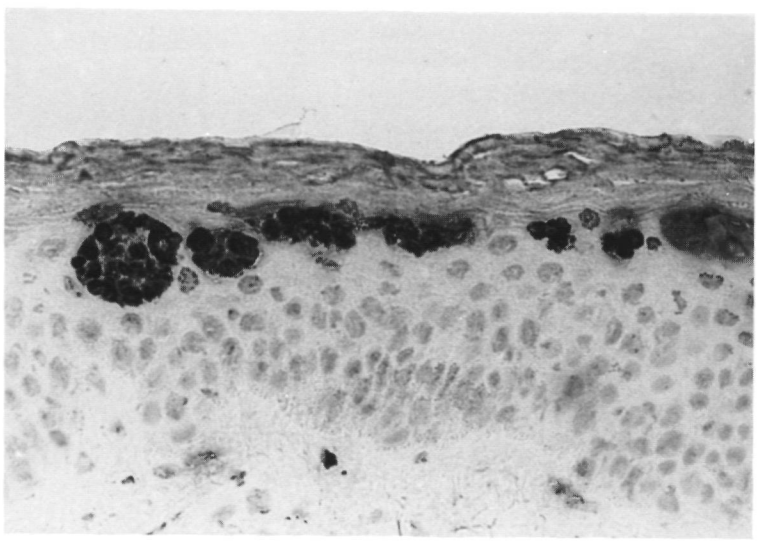

Figure 3: Staining with anti-elastase, showing maximal accumulation of PMN 24 hours after LTB_4- application (x 800).

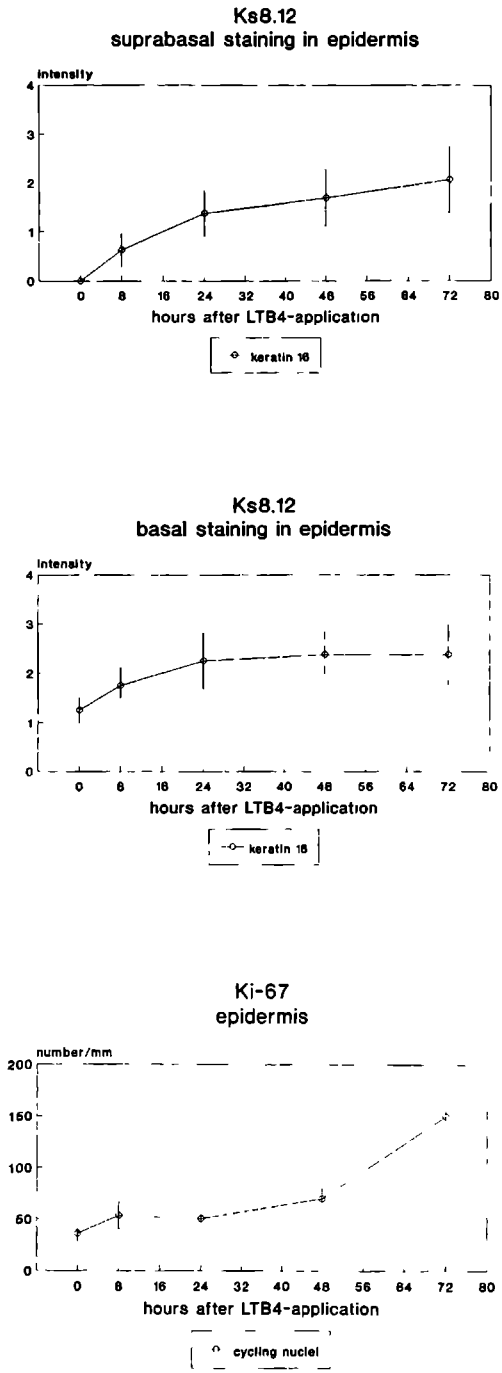

Figure 2: Markers for epidermal proliferation at time intervals following LTB_4-application (mean ± SEM); (a) Ks8.12-binding, (b) Ki-67-expression.

The dynamics of inflammation and epidermal growth following epicutaneous application of LTB_4 have been studied extensively with respect to PMN-accumulation and DNA-synthesis using standard histological, enzymological and flow-cytometrical methods[10,21,23]. The infiltrate has been studied on H and E sections[11]. In the present investigation different components of inflammation and epidermal growth are studied together, using the immunohistochemical approach which permits the assessment of the topography of the changes.

The PMN-accumulation following LTB_4-application has been studied using standard histological methods (H and E sections)[10,18] and by enzymological assessment of the enzyme elastase[21,23]. Elastase is a marker for PMN. Monocytes contain some leukocyte elastase but in substantially lower amounts (5%) compared to PMN[24,25]. The present study confirms the dynamics of PMN-accumulation observed in these studies with a maximum after 16-24 hours.

Remarkably, in contrast to previous studies[11], monocytes/macrophages (CD14-positive cells) appeared simultaneously with PMN, within the first 8 hours. In this respect it is of importance that both PMN and monocytes demonstrate a chemotactic response to LTB_4 in vitro[8].

To the best of our knowledge, a chemotactic response of Langerhans cells to LTB_4 has never been reported. Unexpectedly, an increased density of CD1a positive Langerhans cells was observed in the dermis 8 hours after LTB_4-application. An alternative explanation might be the expression of CD1a, by dermal macrophages in some phases of the inflammatory process[26]. However, at 8 hours, CD1a-positive cells were restricted to the subepidermal zone, whereas CD14-positive cells were distributed more uniformly over the dermis.

The Langerhans cell has a well-established position as an antigen-presenting cell and as an initiator of the immune response and is involved in aspecific inflammation[27,28]. At 24-48 hours after LTB_4-challenge, Langerhans cells marginated the PMN-accumulation. The functional aspect of this rearrangement of Langerhans cells could well be a response to altered structures in the epidermis, that are subsequently presented as antigen to the T-lymphocytes. Alternatively, Langerhans cells might phagocytose products released from the PMN or damaged structures of the epider-

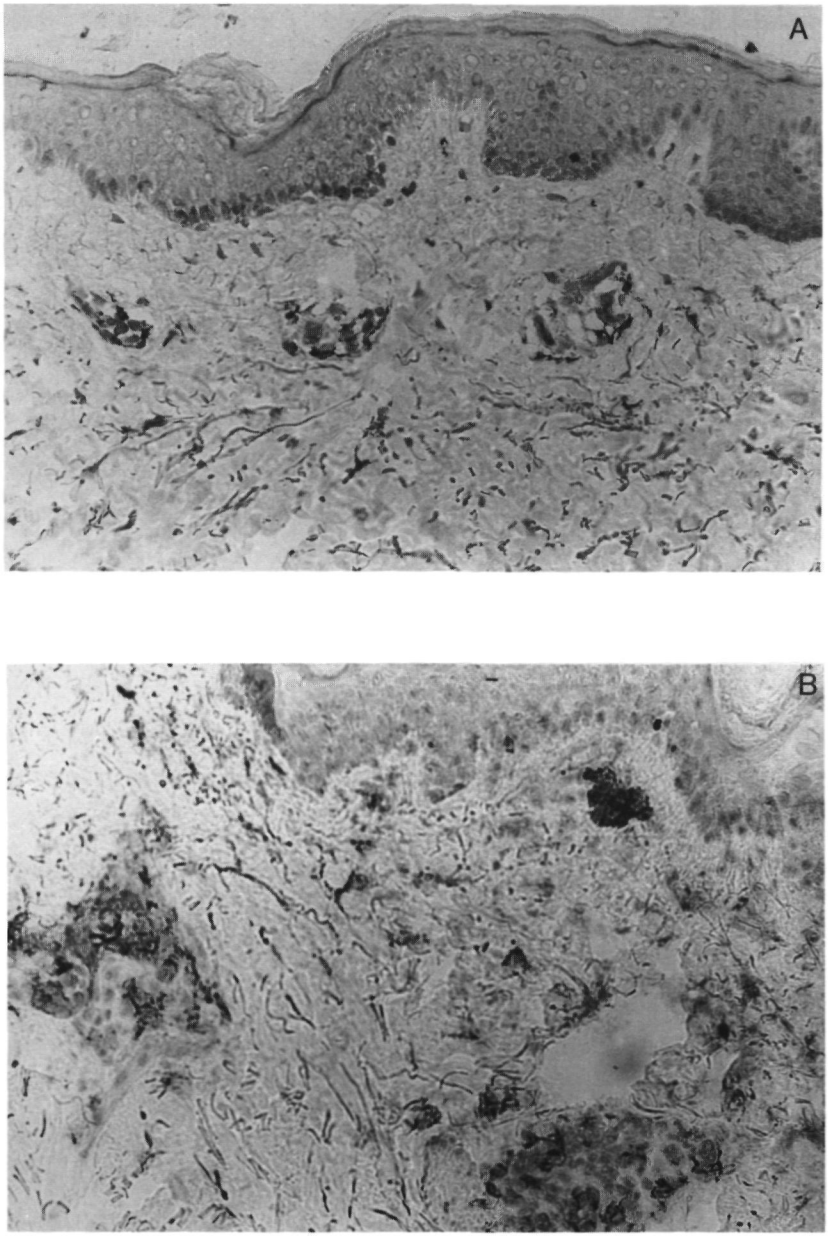

Figure 4: Staining with WT14, showing CD14-positive cells before (a) and 8 hours (b) after application of LTB_4 (x 400).

mis. Indeed, it has been shown that Langerhans cells phagocytose exogenous proteins[29], including peroxidase. The increase of Langerhans cells in the dermis and a decrease in the epidermal compartment at 72 hours after LTB_4-application could be an indication that Langerhans cells migrate from the epidermis into the dermis. Alternatively, the possibility can not be ruled out that the dermal increase of Langerhans cells is due to new influx, possibly as a reaction to epidermal loss of Langerhans cells. The observations in the present study confirm the function of Langerhans cells in non-specific inflammation involving the epidermis. Their possible migration pattern from the epidermal microabscesses to the dermis might be an indication that the Langerhans cells are part of an immunological reaction in this model.

A direct interference of LTB_4 with T-lymphocyte migration and proliferation never has been demonstrated. In the present study, an increased number of T-lymphocytes is observed as late as 48-72 hrs after LTB_4-challenge (Fig 1d). It is attractive to speculate that increased density of T-lymphocytes is the result of activation of Langerhans cells. In vitro, Langerhans cell activation results in T-cell proliferation[30]. Release of interleukine-1 has been reported to occur following Langerhans cell activation[31]. This cytokine induces the production of interleukin-2 by T-lymphocytes; interleukin-2 promotes T-cell proliferation[32]. In addition, interleukin-1 is a chemotactic substance for T-lymphocytes[33]. Therefore, Langerhans cell activation is likely to result in T-cell accumulation in vivo.

In line with a previous study on DNA-synthesis[12] the present investigation showed an increased number of cycling cells. Epidermal hyperproliferation might be the result of LTB_4 itself[9]. Alternatively, the invasion of the epidermis by PMN might desintegrate the epidermis and hence induce a recovery-response comparible to the situation following standardized injury such as sellotape stripping. The increased number of Ki-67-positive nuclei implies an increased recruitment of cycling cells from the resting G_0-population. Hyperproliferative keratinocytes in culture and in vivo express keratin 16[19,20]. Ks8.12 is a monoclonal antibody which detects keratin 16 and 13[34], however, keratin 13 is not expressed in adult human skin[35]. In contrast, in 3 out of 8 subjects no suprabasal staining was found at 72 hours after LTB_4-application, although the marker Ki-67 showed a pronounced nuclear binding. Therefore, we conclude that the suprabasal expression of keratin 16 is not always associated with epidermal hyperproliferation in vivo.

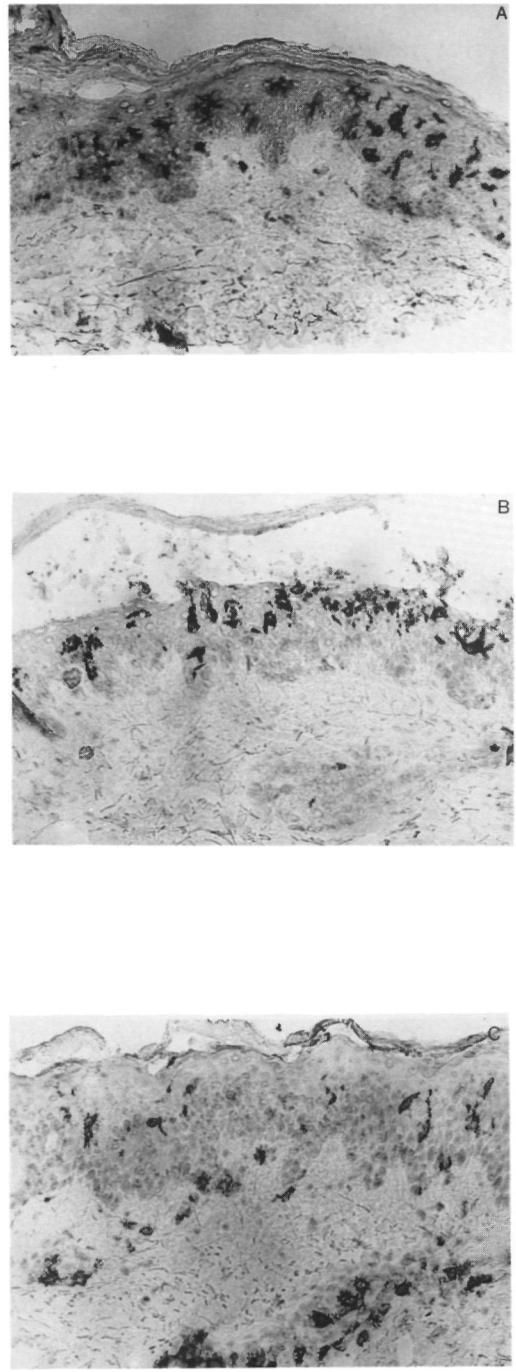

Figure 5: Langerhans cells before application of LTB_4 (a); after 24 hours in the vicinity of micro-abscesses (b); and after 72 hours decreased density in epidermis, increased density in dermal compartment (c) (x 400).

Inflammation with intraepidermal invasion of PMN and T-lymphocytes and participation of monocytes are well established features of the psoriatic lesion. The observation that the PMN are seen relatively frequent in the acute phase and relatively seldom in the chronic phase of the psoriatic lesion[36,37] is well reflected in the dynamics of this model. In the psoriatic lesion[38] and in normal skin after LTB_4-application no B-lymphocytes were observed. In the early phase of the psoriatic lesion a pronounced accumulation of Langerhans cells in the dermal compartment and a decrease in the epidermis were observed by several investigators[39,40]. A similar redistribution was observed following LTB_4-application on normal skin. In the chronic psoriatic lesion Langerhans cell density was only slightly increased in the dermis compared to normal skin, and the epidermal Langerhans cell counts were markedly decreased[40]. An increase in the number of cycling cells is observed in the psoriatic plaque[41] and following LTB_4-application. On the other hand differences exist between the psoriatic lesion and the normal skin following LTB_4-challenge. Firstly, the general architecture of the skin is strikingly different. Papillomatosis with tortuous capillaries are consistent findings in the psoriatic lesion but are not present in LTB_4 treated skin. The appearance of LTB_4-induced micropustules is well demarcated in contrast to the spongiform pustules of Kogoj in psoriasis. The suprabasal expression of keratin 16 is a consistent phenomenon in the psoriatic lesion. However, in the suprabasal compartment of LTB_4-treated skin, keratin 16, visualized by Ks8.12-binding, is not present in 3 out of 8 subjects after 72 hours.

Epicutaneous application of LTB_4 induces several changes as observed in the initial psoriatic lesion. Therefore, it can be used as a model for the acute stages. The in vivo response comprizes the invasion of CD14 positive cells and a rearrangement of Langerhans cells. In addition, the model is of importance as an inflammation model to assess the potency of anti-inflammatory drugs, including antipsoriatic therapies.

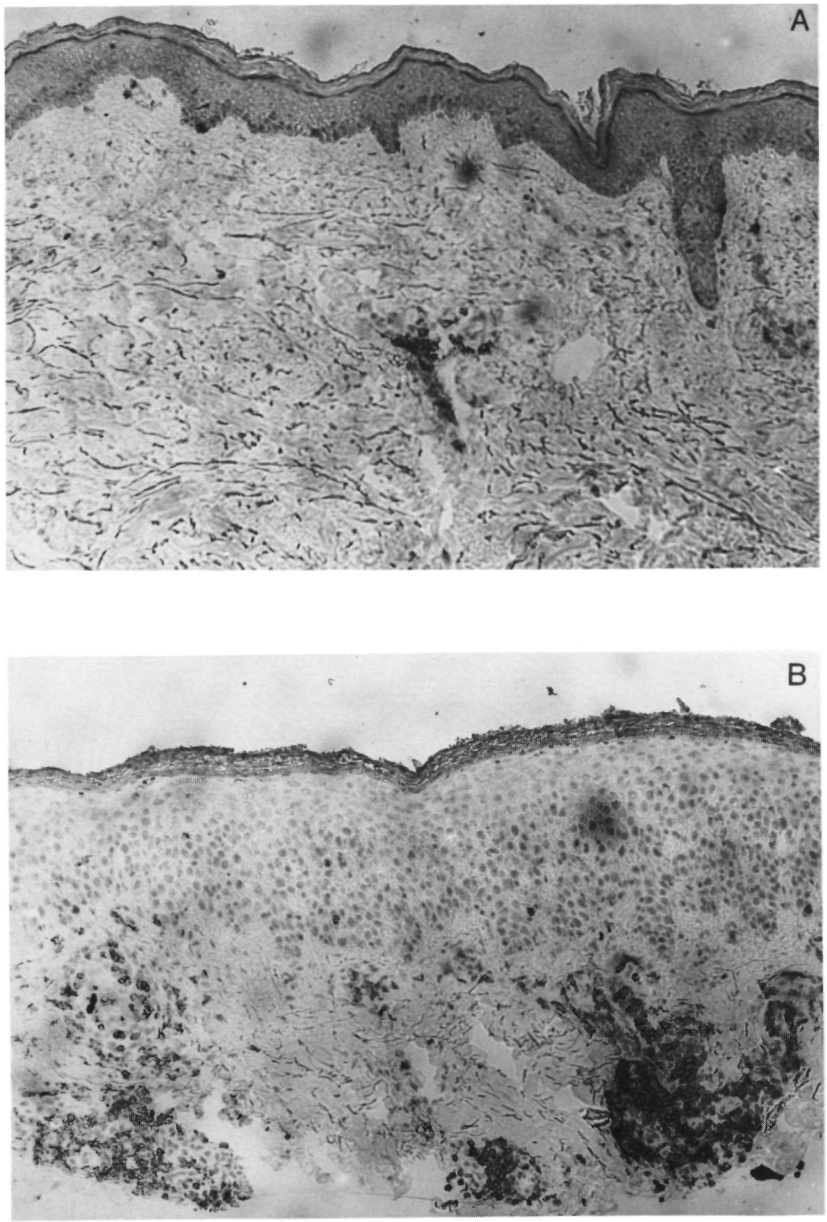

Figure 6: T11 staining, showing T-lymphocytes at 0 hours (a) and at 72 hours after LTB_4-application (b) (x 200).

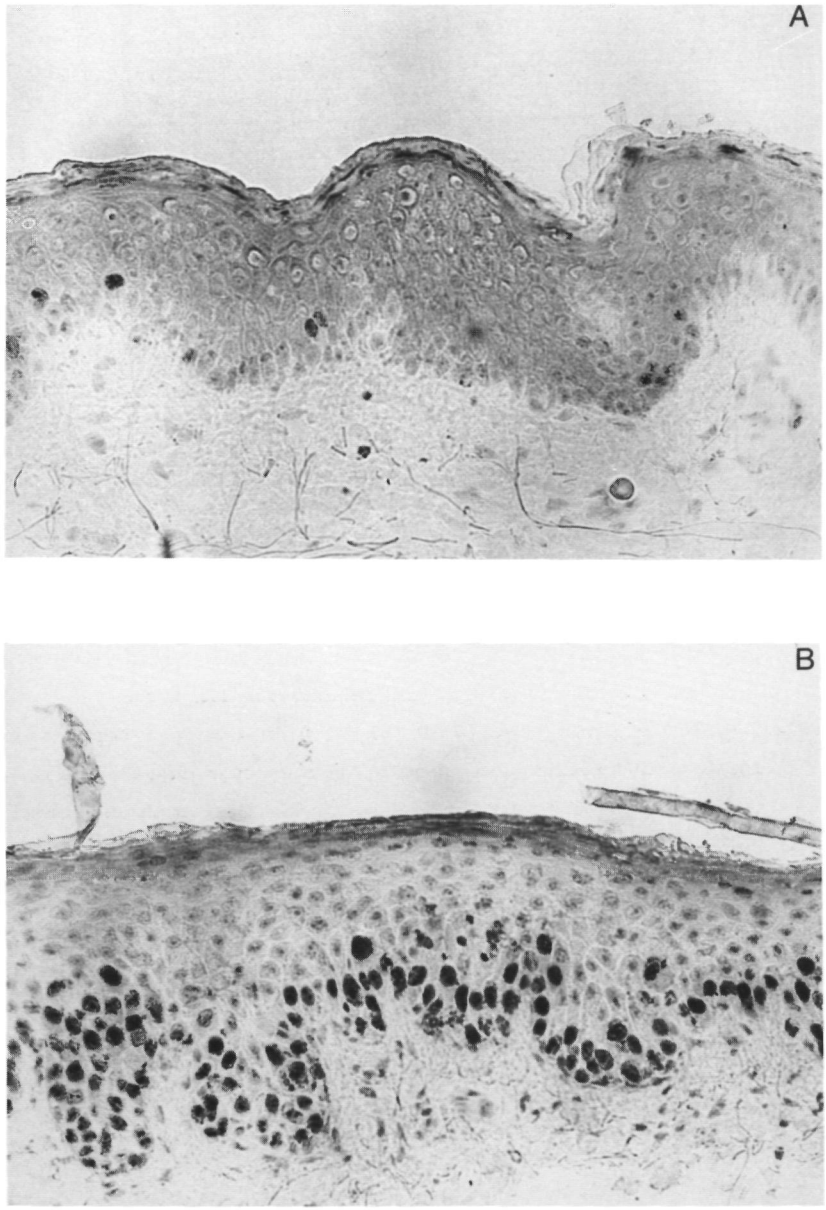

Figure 7: Ki-67 expression before (a) and 72 hours after the application of LTB_4 (b) (x 400).

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CHAPTER 3

CLINICAL AND HISTOLOGICAL CHANGES DURING ANTI-PSORIATIC TREATMENT

This chapter is based on the following publications:

1. De Jong EMGJ, van de Kerkhof PCM. Simultaneous assessment of inflammation and epidermal proliferation in psoriatic plaques during long-term treatment with the vitamin D_3 analogue MC903: modulations and interrelations. Br J Dermatol 1991; 124: 221-229.
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3.1 SIMULTANEOUS ASSESSMENT OF INFLAMMATION AND EPIDERMAL PROLIFERATION IN PSORIATIC PLAQUES DURING LONG-TERM TREATMENT WITH THE VITAMIN D_3 ANALOGUE MC903: MODULATIONS AND INTERRELATIONS.

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SUMMARY

The influence of topical application of MC903, an analogue of 1α,25-dihydroxyvitamin D_3, on psoriatic plaques, was investigated during a long-term treatment study. Parameters for epidermal growth and for inflammation were assessed on frozen sections using immunohistochemical methods. The aim was to elucidate the modulations of these parameters in time, and interrelations between the different cell types involved during MC903 treatment.

Biopsies were taken before treatment and after 1, 2, 4 and 12 weeks of treatment. Monoclonal antibodies against the hyperproliferation-associated keratin 16, against cycling cells, and against T-lymphocytes, B-lymphocytes, Langerhans cells and CD14-positive cells were used in combination with a polyclonal antibody against polymorphonuclear leukocyte (PMN) - elastase.

The earliest change was a statistically significant decrease of PMN after one week of treatment followed by a decline of cycling cells after two weeks. These changes preceded the decrease of T-lymphocytes which occurred after 4 weeks. Keratin 16-content tended to diminish after 4 weeks of treatment. The number of CD14-positive

cells decreased slightly during the observation period, whereas the number of Langerhans cells tended to increase. No B-lymphocytes were found.
These results suggest that MC903 influences not primarily T-lymphocytes; PMN and epidermal growth seem to be more directly affected.

INTRODUCTION

The observation in a patient with psoriasis and senile osteoporosis, treated with 1α,25-dihydroxycholecalciferol, who showed clearing of her psoriasis, by Morimoto *et al.* in 1985[1], has led to a new group of therapeutics for psoriasis. Several trials have been carried out with different derivatives of vitamin D_3[2,3,4]. In order to minimize side effects on blood-calcium and -phosphate, an analogue of 1α,25-dihydroxyvitamin D_3 was developed which is 100 times less active on calcium and phosphate metabolism compared to 1α,25-dihydroxyvitamin D_3[5]. In clinical trials this analogue, MC903, has shown to improve psoriasis[6,7,8] (figure 1).
In psoriasis both inflammation and epidermal proliferation are the well known

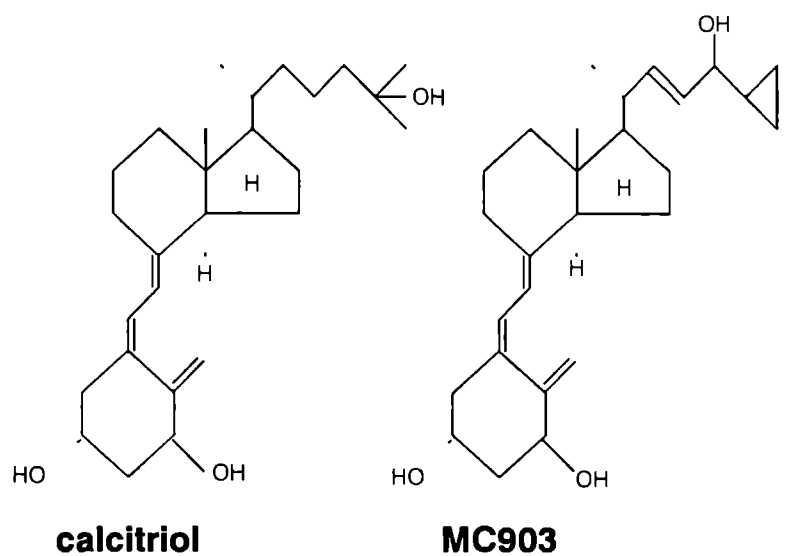

Figure 1. Chemical structure of the vitamin D_3 analogue MC903 and 1α,25-dihydroxycholecalciferol (calcitriol).

pathological features. Epidermal proliferation of the lesion can be defined as an increased recruitment of cycling cells[9], and the expression of hyperproliferative keratins (keratin 16)[10]. Cutaneous inflammation in psoriasis is a complex interplay of polymorphonuclear leukocytes (PMN), T-lymphocytes, Langerhans cells and monocytes. The order in which inflammation and proliferation appear during the pathogenesis and disappear during treatment is not yet elucidated.

Although the clinical efficacy of MC903 is well established, its anti-psoriatic working mechanism is not yet totally unravelled. In vitro studies showed the anti-proliferative and differentiation-inducing effect of 1α,25-dihydroxyvitamin D_3 on a HL60-cell line[11] and on cultured keratinocytes[12,13]. In addition, various investigations also showed an immunomodulating effect, such as interference with cytokine production and T-cell proliferation, of 1α,25-dihydroxyvitamin D3[14,15,16,17,18] as well as MC903[19,20]. However, the in vivo relevance of these in vitro observations remains to be explored.

The aim of the present investigation is to visualize changes in inflammation and epidermal proliferation using immunohistochemical methods during long-term treatment of psoriasis with the vitamin D_3 analogue MC903. From consecutive biopsies frozen sections were stained with monoclonal antibodies against T- and B-lymphocytes, CD14-positive cells and Langerhans cells, and with a polyclonal antibody against PMN-elastase, in combination with monoclonal antibodies against keratin 13 and 16, and against cycling cells. The order of events in epidermal and dermal compartment, and the interactions between different cell types involved, were studied.

MATERIALS AND METHODS

Patients

Eight patients suffering from psoriasis were treated with MC903 ointment (50 μg/g) twice daily (Leo Pharmaceutical Products, Copenhagen, Denmark). These patients had shown to respond well to MC903-treatment in a previous double-blind in-patient comparison study using MC903 and betamethasone[21]. The patients were included in a multi-centre trial covering 12 months of treatment. Their age was between 27 and

85 years, and they had received no local anti-psoriatic treatment within 4 weeks or systemic treatment within 2 months before the start of the study. The patients had normal blood calcium, phosphate, albumin, bilirubin, alkaline phosphatase, aspartate amino transferase, creatinine and urate. White and red blood cell counts, haemoglobin, platelets and differential counts were within normal ranges. These parameters were measured every 4 weeks. Clinical improvement was described using the PASI-score.

Biopsies

Punch biopsies (3 mm diameter) were taken from psoriatic lesions of 6 patients before treatment and after 1 week, 2 weeks, 4 weeks and 12 weeks of treatment with MC903. Biopsies were embedded in Tissue Tek OCT compound (Miles Scientific, Naperville, USA), snap frozen in liquid nitrogen and stored at -80°C. Sections of 7 μm were cut and fixed for 10 minutes in acetone-ether 60/40 vol% for staining with the antibody Ki-67 and with acetone for the remaining antibodies.

Monoclonal antibodies

To assess epidermal proliferation monoclonal antibodies were used against a nuclear antigen present in cycling cells (Ki-67, Dakopatts, Copenhagen, Denmark)[22], and against keratin 13 and 16 (Ks8.12, Sigma, St Louis, USA). Keratin 16 is present in hyperproliferative epidermis[23], meanwhile keratin 13 has not been found in adult human skin[24]. To characterise the inflammatory infiltrate staining was performed with antibodies against leukocyte-elastase which visualises PMN (Serotec, Oxford, England); against T-lymphocytes (CD2) (T11, Dakopatts, Copenhagen, Denmark); against B-lymphocytes (CD22) (pan-B, Dakopatts, Copenhagen, Denmark); against CD14-positive cells which represent monocytes and macrophages (WT14, Dept. Medicine, Div. Nephrol. University Hospital Nijmegen)[25]; and against Langerhans cells (OKT6, Ortho Diagnostic Systems, Raritan, USA)[26].

Staining procedures

An indirect peroxidase technique was used for staining with Ks8.12 and Ki-67. In brief, slides were incubated for 30 minutes with the monoclonal antibodies and after a washing step with phosphate buffered saline (PBS) incubated with rabbit-anti-

mouse-immunoglobulin conjugated with peroxidase (RAMPO) for 30 minutes. A solution of 3-amino-9-ethylcarbazole (AEC) in sodium acetate buffer pH 4.9 containing 0.01% H_2O_2 was added for 15 minutes after preincubation with sodium acetate buffer pH 4.9. Staining with anti-elastase was done using a direct immunoperoxidase technique. After an incubation step of 30 minutes and 3 washes in PBS, AEC-solution was added after preincubation with sodium acetate buffer pH 4.9. Staining with T11, pan-B, WT14 and OKT6 was performed using a peroxidase-anti-peroxidase technique (PAP-technique). Slides were incubated for 60 minutes with the monoclonal antibodies. After a wash-step with PBS an incubation cyclus followed of 20 minutes with rabbit-anti-mouse-immunoglobulin (RAM-Ig, Dakopatts, Copenhagen, Denmark), 2 washes with PBS, and 20 minutes with peroxidase-anti-peroxidase-complexes (PAP, Dakopatts, Copenhagen, Denmark). This cyclus was repeated one more time, slides were preincubated with sodium acetate buffer pH 4.9 and finally stained in AEC-solution. All slides were counterstained with Mayer's haematoxylin (Sigma, St Louis, USA) and mounted in glycerin gelatin.

Histological examinations

Epidermal proliferation was measured by counting the number of Ki-67 positive nuclei per mm length of section, and by assessing Ks8.12-staining in suprabasal and basal layers of the epidermis using a 5-point scale: 0 = no staining, 1 = sporadic staining, 2 = minimal staining, 3 = moderate staining, 4 = pronounced staining.
Dermal inflammation (PMN, T-lymphocytes, B-lymphocytes, Langerhans cells, CD14-positive cells) was semiquantitatively enumerated by expressing the number of positively stained cells as a percentage of the total number of infiltrate cells similar to Synkowski and Provost[27]: 0 = no positive cells, 1 = sporadic, 2 = 1-25%, 3 = 26-50%, 4 = 51-75%, 5 = 76-99%, 6 = 100%. Inflammatory cells in the epidermis were assessed using the 5-point scale as used for Ks8.12-staining.

Statistical analysis

For statistical evaluation the Mann-Whitney test was used.

Clinical response

In all patients MC903 resulted in an improvement of the psoriatic lesions. Total clearance was not achieved in any of them after 12 weeks of treatment. The amelioration was most pronounced in the first 4 weeks of treatment ($p < 0.05$). Continuation beyond this period resulted in a modest improvement only. No side effects were observed during treatment. No flare ups were seen in this period. Clinical results from this multi-center study covering 12 months will be available in the near future.

Epidermal growth

The effect of MC903 on parameters of epidermal growth are summarized in figures 2, 4 and 5. The number of actively cycling epidermal cells (Ki-67-positive nuclei) showed a statistically significant decrease after 2 weeks of treatment ($p < 0.03$). After this initial improvement the number of Ki-67-positive nuclei only decreased slightly. Values as found in uninvolved psoriatic skin by other investigators[28] were not reached in the 12 weeks observation period. Keratin 16, seen as Ks8.12-staining was high in the suprabasal layers of the epidermis. The intensity of this staining remained pronounced and diminished only in the period between 4 and 12 weeks of treatment. However, this decrease was not statistically significant. Basal staining was almost unchanged throughout the whole observation time, and varied only from no staining to sporadic staining in all biopsies. The distribution and intensity of keratin 16 did not switch to the pattern characteristic for clinically uninvolved psoriatic skin which consists of sporadic staining of the basal layer and no staining of the suprabasal compartment[10].

Inflammation

Density of inflammatory cells are shown in figures 3a,b,c,d and 6. In untreated psoriatic plaques, PMN as shown by the antibody anti-elastase, were present as micro-abscesses in the stratum corneum, and dispersed in the epidermis. Already after 1 week of treatment with MC903, only sporadic and small micro-abscesses were seen. After 4 weeks the number of PMN had decreased strikingly and no

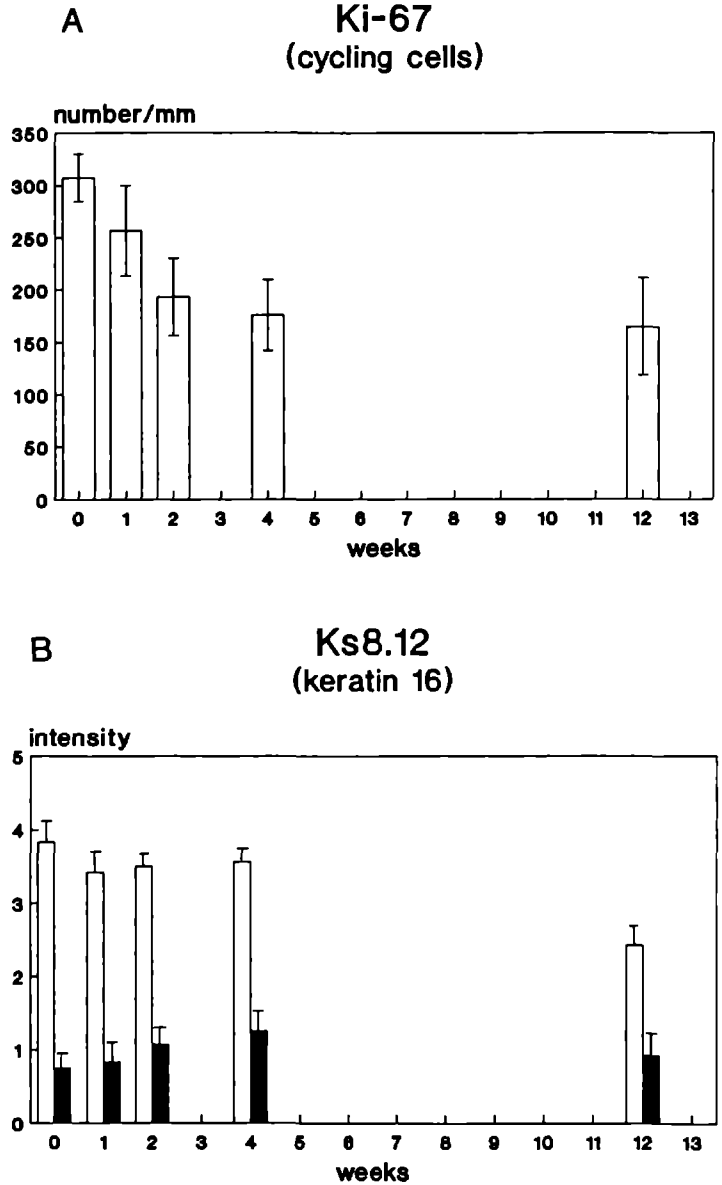

Figure 2. Markers for epidermal growth before and during treatment of psoriatic plaques with MC903, expressed as means ± sem. a) Ki-67 (cycling epidermal cells), b) Ks8.12 (epidermal cells expressing keratin 16), □ = suprabasal staining, ■ = basal staining.

microabscesses were found anymore ($p < 0.01$). Dermal PMN also diminished in this treatment period. Remarkably, after 12 weeks an increase of epidermal as well as in dermal PMN was measured (Fig 3a). T-lymphocytes (T11-positive cells) were

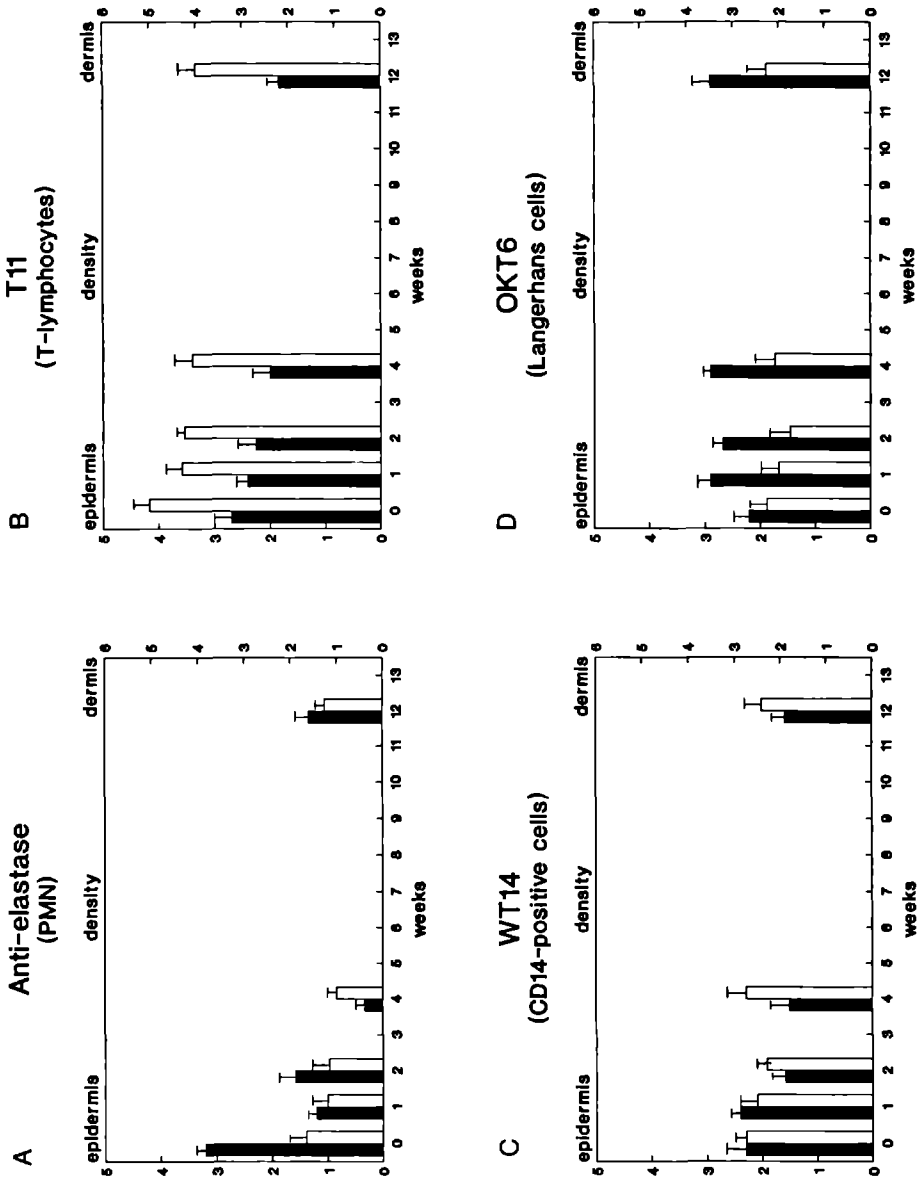

Figure 3. Markers for inflammation before and during treatment of psoriatic plaques with MC903, expressed as means ± sem. ■ = epidermal cells, □ = dermal cells. a) anti-elastase (PMN), b) T11 (T-lymphocytes), c) WT14 (CD14-positive cells), d) OKT6 (Langerhans cells).

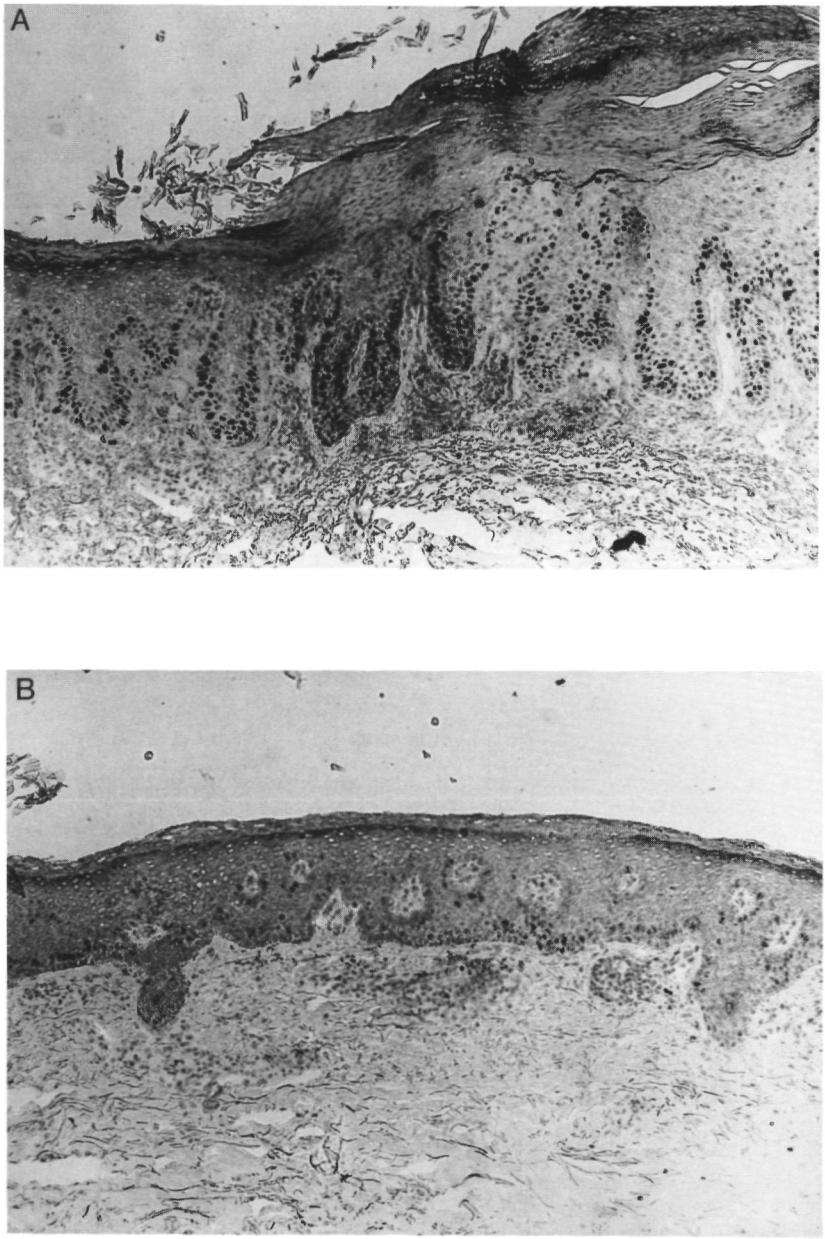

Figure 4. Ki-67 staining (155x), a) before treatment, b) after 12 weeks of treatment with MC903.

numerous in the dermis and epidermis of untreated psoriatic lesions. Of the dermal infiltrate, 76-90% of the cells were T-lymphocytes. Also a considerable epidermotropism was seen. In the period of treatment with MC903, T-lymphocytes diminished gradually in both dermal and epidermal compartment, resulting in a borderline significant decrease after 4 weeks ($p = 0.05$). B-lymphocytes (pan-B-positive cells) were not found in psoriatic skin. CD14-positive cells, assessed by the monoclonal antibody WT14, were seen diffusely in as well dermis as epidermis. Their numbers decreased slightly however not statistically significant. Their distribution pattern did not show a consistent change. Epidermal Langerhans cells, visualised by OKT6, tended to increase within the first week ($p > 0.05$) of treatment and remained at the same level during the observation period of 12 weeks. The pattern of distribution of these cells correlated with the general histological appearance: pretreatment biopsies, showing marked acanthosis, papillomatosis and parakeratosis, demonstrated focal aggregates of Langerhans cells intermingled with Langerhans cell-free zones whereas biopsies of treated skin with a more modest degree of involvement, showed a more diffusely distributed network of Langerhans cells. Dermal Langerhans cells decreased slightly during treatment with MC903.

Relation between clinical and histological scores

If the clinical response and scores for different histological parameters are compared, it can be seen that the decrease of the number of epidermal PMN and the number of Ki-67 positive nuclei, preceded the decrease in PASI-score. T-lymphocytes diminished simultaneously with the clinical scores. Also Ks8.12 binding tended to diminish at the same time as the PASI-score.

The patient group was divided into a group showing marked improvement (PASI after 12 weeks < 3, $n=4$) and a group showing slight to moderate improvement (PASI after 12 weeks > 3, $n=2$). If these two groups are compared, it can be seen that the decrease in Ki-67 positive nuclei is twice as high in the group showing marked improvement compared to the decrease in the group showing slight to moderate improvement (decrease in stained nuclei/mm length of section 157 and 70 respectively). The reduction of epidermal PMN in the group showing marked improvement was also twice as high as the reduction in the other group (reduction of 2.25 and 1 respectively). The number of T-lymphocytes reduced substantially in the

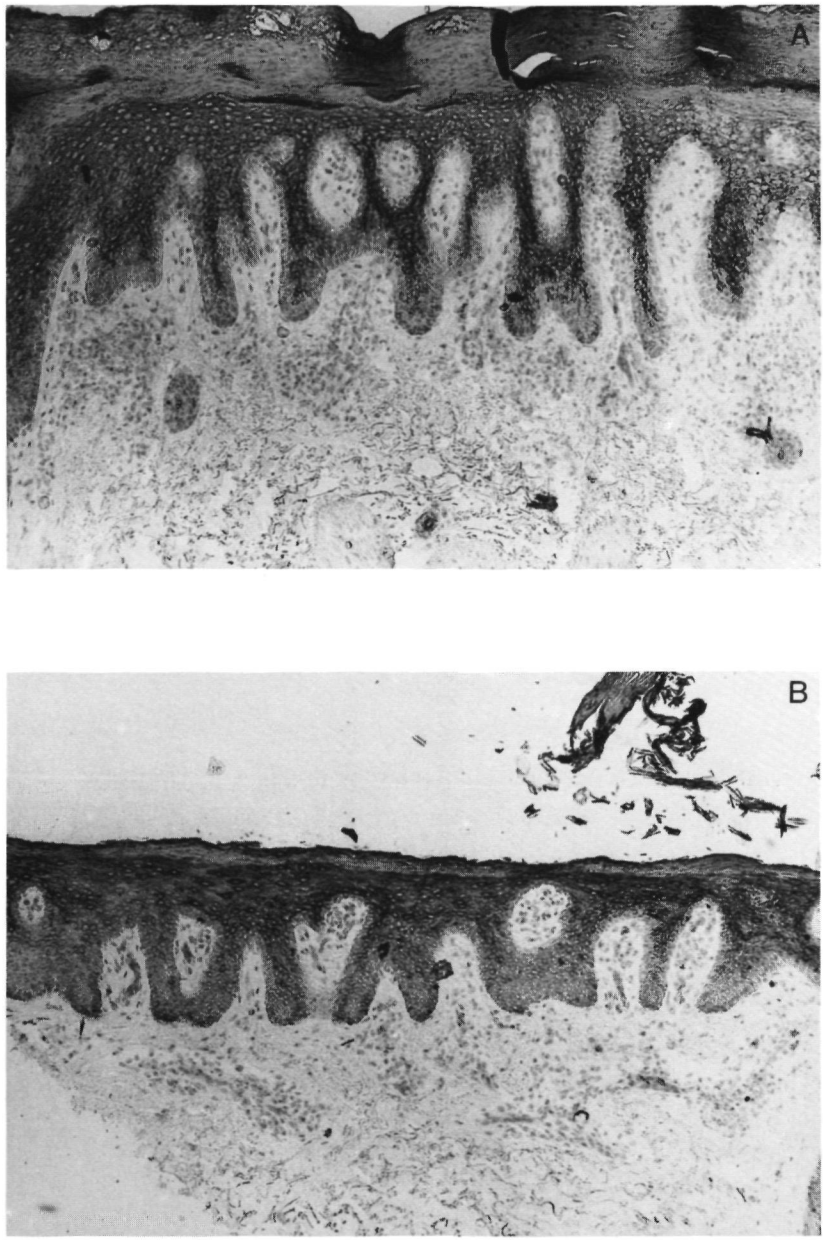

Figure 5. Ks8.12 staining (155x), a) before treatment, b) after 12 weeks of treatment with MC903.

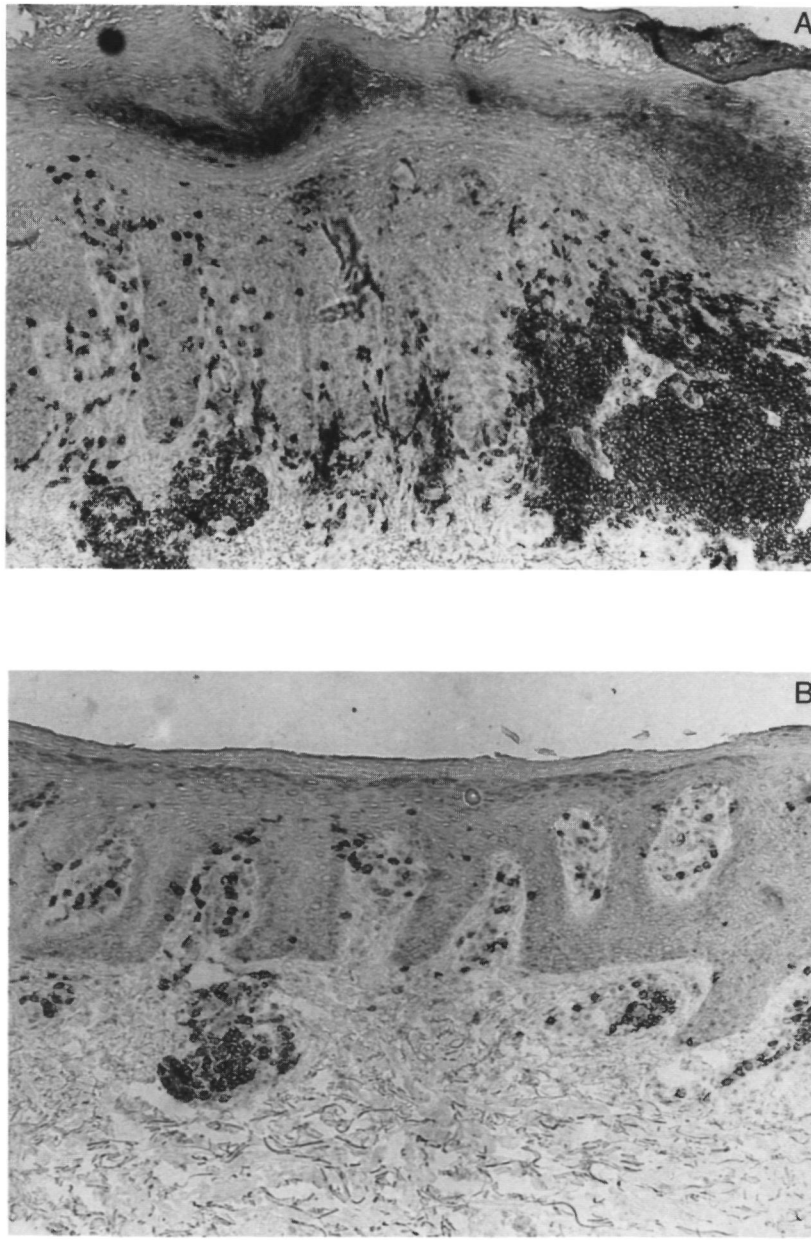

Figure 6. T11 staining (200x), a) before treatment, b) after 4 weeks of treatment with MC903.

first group, whereas the decrease in the second group was marginal (reduction of 1.4 and 0.1 respectively). Ks8.12 binding diminished in both groups to the same extent (2.4 and 2.8 respectively).

DISCUSSION

The dynamics of the changes observed in psoriatic lesions during treatment with MC903, are described in figure 2 and 3. In the order of events the earliest change is a pronounced decrease of PMN, which is already statistically significant after one week of treatment ($p < 0.05$). Simultaneously a tendency can be seen of the number of Langerhans cells to increase, however this change is not statistically significant. After two weeks a significant decrease in Ki-67-positive nuclei occurs ($p < 0.05$) of almost 40%. Following this event, PASI-scores diminished after 4 weeks of treatment and a reduction of T-lymphocytes was observed, reaching borderline significancy ($p = 0.05$). Ks8.12-staining diminishes only in the period between 4 and 12 weeks of treatment. Although all parameters tended to improve during further treatment, the number of PMN showed a slight increase in this period ($p > 0.05$). The group of patients reaching the lowest PASI-scores after 12 weeks of treatment showed the most pronounced reduction of the number of Ki-67 positive nuclei, epidermal PMN and T-lymphocytes.

The skin plays an important role in vitamin D metabolism. It is not only the site where vitamin D is produced[29], but keratinocytes also have receptors for 1α,25-dihydroxyvitamin D_3 and function as a target organ[30]. Growing evidence indicates that 1α,25-dihydroxyvitamin D_3 can suppress proliferation and induce differentiation of different cell types including keratinocytes[11,12,13]. It is not clear through which working mechanism 1α,25-dihydroxyvitamin D_3 influences proliferation and differentiation of skin. 1α,25-dihydroxyvitamin D_3 possesses a cytosolic receptor[31] that is transported to the nucleus after binding, and is thought to work through DNA-transcription[32]. In addition, several authors reported a rapid effect of 1α,25-dihydroxyvitamin D_3 on intracellular calcium transport[33] and on cGMP in human skin fibroblasts[34]. These observations suggest that more immediate mechanisms, not mediated via the genom, might be of relevance for the mode of action of 1α,25-

dihydroxyvitamin D_3. MC903 is very similar to the natural metabolite 1α,25-dihydroxyvitamin D_3. They possess the same receptor and they induce cell differentiation and inhibit cell proliferation at the same concentrations in vitro. In addition, MC903 is at least 100 times less potent in its effect on calcium metabolism in vivo[5].

In the present investigation a rapid decrease of the number of proliferating cells, measured as Ki-67-positive nuclei, was found after two weeks, resulting in a total decrease of 48% after 12 weeks. This result shows that the in vitro effect on proliferation of MC903 is also found in the in vivo situation. These findings are in accordance with values found by de Mare et al who observed a decrease in DNA-content after 6 weeks of treatment with MC903 using flowcytometrical methods. This effect was comparable with the effect of betamethason[21]. Ks8.12-binding, in psoriasis revealing keratin 16 expression, only showed a tendency to diminish after 4-12 weeks of treatment. In a previous investigation, using flowcytometrical quantification, Ks8.12-binding proved to be reduced significantly after 6 weeks of treatment with MC903[21]. The discrepancy between the immunohistochemical and flowcytometrical assessment can be explained by the precise quantification which is provided by the flowcytometrical approach. Although the number of cycling cells is reduced in an early stage of MC903-treatment, the reduction of the intraepidermal accumulation of PMN proved to anticipate the antiproliferative effect in vivo.

Receptors for 1α,25-dihydroxyvitamin D_3 are present in cell types involved in immunological reactions, such as activated T-lymphocytes, B-lymphocytes, monocytes and thymocytes[15]. Cytokine production such as IL-1, IL-2, IL-6 and EGF-ß, is modulated by 1α,25-dihydroxyvitamin D_3 and / or MC903[14,16-20]. It has been suggested that IL-1 might induce epidermal proliferation[35]. The present study demonstrates the effect of MC903 on the immune system in vivo. At first, the accumulation of PMN diminishes and after the most significant reduction of epidermal proliferation (two weeks) T-lymphocytes appear less frequently (4 weeks). CD14-positive cells tend to diminish slightly and gradually during the 12-week treatment with MC903, however without reaching a statistically significant reduction. The reappearance of PMN and the slight reduction of the remaining parameters during 4-12 weeks of treatment is intriguing. Further studies are needed to elucidate this phenomenon. The molecular basis of the interference of MC903 with inflammation in vivo remains speculative. However, based on in vitro observations, it is

feasible that MC903 might work via modulation of IL-1 and IL-2 production or by interference with the response of keratinocytes to interleukin-1.

Well-known antipsoriatic therapies such as UV-irradiation and corticosteroids[36], decrease epidermal Langerhans cells during treatment. In this light it is intriguing that Langerhans cells did not diminish but increased during therapy with MC903. In several biopsies, in which the histological appearance normalised to a large extent, the distribution of Langerhans cells returned to the normal pattern of a diffuse midepidermal network. Therefore, a reduction of the number of Langerhans cells does not seem a conditio sine qua non necessary for the improvement of psoriatic plaques.

The present study suggests that the effect of the vitamin D_3 analogue MC903 is not primarily via interference with the number of T-lymphocytes. However, MC903 might alter the response of keratinocytes to interleukin-1, resulting in a down regulation of epidermal proliferation. The PMN and epidermal growth seem to be targets with substantial relevance for MC903 in psoriasis.

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3.2 TOPICAL APPLICATION OF 13-CIS-RETINOIC ACID IN THE TREATMENT OF CHRONIC PLAQUE PSORIASIS.

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SUMMARY

Topical retinoids have been suggested to be of potential value in the treatment of psoriasis. The aim of the present study was to find out whether topical application of 13-cis retinoic acid (13-cis-RA) has an antipsoriatic effect. Nine patients participated in the investigation. In each patient, two comparable psoriatic lesions (5 x 5 cm or more) were selected for treatment with either 13-cis-RA in a 0.1 % cream base or with the vehicle only (placebo), using a double-blind approach. The investigation was a left-right within-subject comparison. Before and after four weeks the lesions

were recorded for clinical scores. Punch biopsies were taken in 8 patients before and after treatment and examined using immunohistochemical methods to assess epidermal proliferation and keratinization, and to assess inflammation. 13-cis-RA treatment resulted in a mild decrease of scaling and induration. Erythema however increased. No statistically significant difference in biological effects was reached between 13-cis-RA and placebo treated lesions and no changes in expression of the immunohistochemical markers were seen.

INTRODUCTION

Systemic application of retinoids is a well-established treatment for severe acne, disorders of keratinization and psoriasis[1-4]. Side effects however restrict the use of this treatment to the most severe expressions of these disorders. Therefore, the search for topical administration of retinoids seemed worthwhile.

Several clinical studies in plaque-type psoriasis have shown that treatment with topically applied all-trans retinoic acid (All-trans RA) results in a modest therapeutic effect which can be observed after 2 weeks of treatment[5-12]. Nevertheless, the clinical usefulness of topically applied all-trans RA in psoriasis is limited by the appearance of skin irritation ("retinoid dermatitis") in most patients.

Topical 13-cis-retinoic acid (13-cis-RA) proved to be moderately effective in the treatment of acne[13]. Recently, Steijlen et al. reported a beneficial effect of 13-cis-RA 0.1% cream in Darier's disease[14]. Topical application of a cream containing 13-cis-retinoic acid 0.1% has been shown in a previous study to inhibit inflammation induced by leukotriene B_4 (LTB_4). Following treatment of normal skin with this cream, the intraepidermal accumulation of polymorphonuclear leukocytes (PMN) after epicutaneous application of LTB_4 proved to be remarkably inhibited[15]. In addition, topically applied 13-cis-RA as a 0.1% cream has been shown to be well tolerated in human volunteers[16]. Since LTB_4 induced migration of PMN has been suggested to be of importance as an antipsoriatic target[17], and the characteristics concerning the local tolerability of 13-cis-RA signify a potential advantage over all-trans RA, a study was conducted aimed at evaluating the efficacy and safety of topical 13-cis RA in plaque-type psoriasis and estimating to which extent this

treatment modulates immunohistochemical parameters for epidermal growth and inflammation.

MATERIALS AND METHODS

Patients

Nine patients, aged 33 to 67 years with chronic plaque psoriasis, participated in the study. The group consisted of 5 males and 4 females. In 5 patients, the involvement of the skin was below 10% of the body surface, and in 4 patients the involvement ranged between 10 and 30% . Before starting the trial, no systemic treatment had been administered for at least 4 weeks, and the patients had refrained from topical treatment for at least 2 weeks. Prior to the experiment, approval of the medical ethical committee was obtained.

Application of creams

In each patient, two comparable psoriatic plaques (5 x 5 cm or more) were selected for the investigation. Plaques were selected on areas which are usually not exposed to sunlight. As the study was double-blind, the identity of the cream was not known to the patient and investigators throughout the observation period. During 4 weeks; a 0.1% 13-cis-RA cream (F. Hoffmann-La Roche AG, Basel, Switzerland) or the vehicle only (placebo) were applied twice daily thinly and evenly on the selected areas without occlusion.

Clinical monitoring

Before treatment and at the end of the investigation, a photographic documentation was performed. During the treatment, the severity of the lesions was recorded using a 5-point scale for erythema, scaling and induration, as follows: 0 = no involvement, 1 = slight involvement, 2 = moderate involvement, 3 = severe involvement, 4 = severest possible involvement.

Cellbiological assessment

Before and at the end of the treatment punch biopsies (3 mm diameter) were taken from the psoriatic lesions treated with 13-cis-RA cream and placebo. The biopsies were embedded in tissue Tek OCT Compound (Miles Scientific, Naperville, USA), snap frozen in liquid nitrogen and stored at -80°C. Sections of 7 μm were cut and fixed for 10 minutes in acetone/ether 60/40% for staining with the antibody Ki-67 and with acetone for staining with the remaining antibodies.

For immunohistochemical studies a panel of monoclonal antibodies was used.

Inflammation was characterized with the antibodies:

(i) Anti-elastase (Serotec, Oxford, England), a marker for polymorphonuclear leukocytes (PMN)

(ii) T11 (Dakopatts, Copenhagen, Denmark) against T-lymphocytes (CD2)

(iii) WT14 (Dept. Medicine, Div. Nephrol., University Hospital Nijmegen) against CD14 positive cells which represent monocytes and macrophages[18]

(iv) OKT6 (Ortho Diagnostic Systems, Raritan, USA) to assess Langerhans cells (CD1a)

Epidermal proliferation was characterized with the antibody Ki-67 (Dakopatts, Copenhagen, Denmark) which binds with a nuclear antigen present in cycling cells.

Keratinization was characterized with the antibodies:

(i) Ks8.12 (Sigma, St. Louis, USA) to assess keratin 16, which is expressed in hyperproliferative epidermis.

(ii) RKSE60 (Gift from Dr. Fr. Ramaekers, Dept. of Pathology, Nijmegen) against keratin 10, a keratin present in differentiated epidermis.

For staining with RKSE60, Ks8.12 and Ki-67, an indirect peroxidase technique was used. In brief, the slides were incubated with the monoclonal antibodies for 30 minutes, washed three times with phosphate buffered saline (PBS) and incubated with rabbit-anti-mouse antibody (RAMPO, Dakopatts, Copenhagen, Denmark) conjugated with peroxidase for 30 minutes. After another washing step with PBS and preincubation with sodium acetate buffer pH 4.9, slides were stained in sodium acetate buffer, which contained 200 mg/l 3-amino-9-ethyl-carbazole-solution and 0.01% H_2O_2 (AEC-solution) for 15 minutes. A direct immunoperoxidase technique was used for staining with anti-elastase. The slides were incubated for 30 minutes with the antibody; and after washing with PBS and a preincubation with sodium

acetate buffer, pH 4.9, AEC-solution was added. Staining with T11, WT14 and OKT6 was performed using a peroxidase-anti-peroxidase technique (PAP-technique). After an incubation of 60 minutes with the antibody and a washstep with PBS, the slides were incubated for 20 minutes with rabbit-anti-mouse-immunoglobulin (RAM-Ig, Dakopatts, Copenhagen, Denmark), washed two times with PBS, incubated for 20 minutes with peroxidase-anti-peroxidase-complexes (PAP, Dakopatts, Copenhagen, Denmark) and again washed with PBS. After repeating the incubation with RAM-Ig and PAP, the slides were preincubated with sodium acetate buffer, pH 4.9, and finally stained in AEC-solution. At last, all slides were counterstained with Mayer's haematoxylin (Sigma, St. Louis, USA) and mounted in glycerin gelatin.

Histological examination

The number of Ki-67 positive nuclei was counted per mm length of section. To assess Ks8.12 and RKSE60-staining in suprabasal and basal layers of the epidermis, we used a 5-point scale: 0 = no staining, 1 = sporadic staining, 2 = minimal staining, 3 = moderate staining, 4=pronounced staining. The same scale was used for assessment of anti-elastase, T11, WT14 and OKT6-staining in the epidermis. In the dermis we assessed staining with anti-elastase, T11, WT14 and OKT6 by semiquantitative enumeration of positively stained cells as a percentage of the total number of infiltrate cells[19]: 0 = no positive cells; 1 = sporadic; 2= 1-25%; 3= 26-50%; 4= 51-75%; 5= 76-99%; 6= 100%.

Statistical analysis

Statistical analysis was carried out using the Wilcoxon Ranking Test for paired data.

RESULTS

Before treatment the clinical severity scores for erythema, scaling and induration were similar in the lesions to be treated with 13-cis-RA and the placebo cream. Topical treatment with 13-cis-RA and its placebo cream was well tolerated. In one patient a tenderness was experienced on both sides. The overall improvement was inconspicious.

Figure 1 summerizes clinical scores. Scaling and induration decreased statistically significant following treatment with 13-cis-RA ($P<0,05$). In contrast, scaling and induration in patients treated with placebo cream decreased without reaching statistical significancy. Erythema, however showed a tendency to increase ($P>0,05$) after treatment both with 13-cis-RA and placebo cream. The differences were not statistically significant. No erythema was seen on the perilesional skin.

The histological examination of the pretreatment and posttreatment biopsies showed a characteritic psoriatic appearance. Papillomatosis, acanthosis and parakeratosis of the epidermis were distinct features in both pretreatment and posttreatment biopsies. Ki-67 staining showed average numbers of 200 actively cycling cells per mm length of epidermis. In additon, an intense suprabasal Ks 8.12 staining (keratin 16) demonstrated the hyperproliferative state of the epidermis. Staining with RKSE60 (keratin 10) also showed suprabasal staining and distinct locations without keratin 10. These loci were observed in epidermis overlying dermal papillae. This distribution was also found in a previous study[20]. The untreated and treated psoriatic lesions showed a dermal infiltrate consisting mainly of T-lymphocytes. Polymorphonuclear leukocytes in epidermis and dermis were also found. Langerhans cells were seen focally in the epidermis and in the dermal infiltrate. CD14-positive cells were seen predominantly in the dermis. These cells were not restricted to the perivascular infiltrate, but were diffusely distributed in the dermis.

No statistically significant changes in the parameters for epidermal proliferation (Ki-67), keratinization (Ks8.12, RKSE60) or for inflammation (anti-elastase, T11, WT14, OKT6) could be found in 13-cis-RA versus placebo treated lesions and in pretreatment versus posttreatment (13-cis-RA and vehicle only) lesions.

DISCUSSION

The present study demonstrates a mild decrease of scaling and induration following topical application of 13-cis-RA. On the other hand, erythema tended to increase following this treatment. Immunohistochemical markers were not changed after treatment with 13-cis-RA.

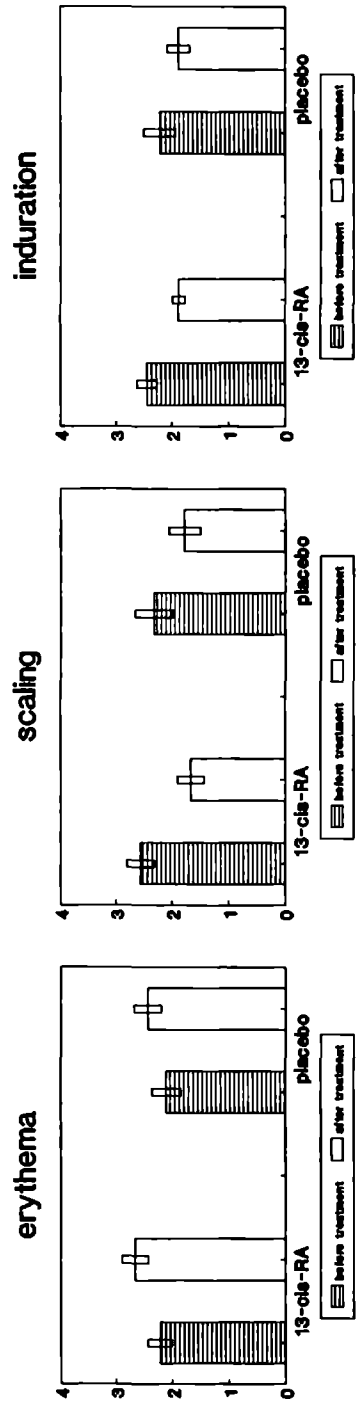

Figure 1. Clinical scores (means ± sem) before and after treatment with 13-cis-RA or placebo.

The slight improvement of scaling and induration is compatible with the previous observation of a beneficial effect of 13-cis-RA in Dariers disease[14]. Topical 13-cis-RA also causes slight improvement in acne[13]. In addition, topical application of this retinoid on normal skin reduced the LTB_4-induced PMN accumulation[15]. However, in the present study the density and composition of the infiltrate did not change during therapy with the same preparation.

Concentrations of 13-cis-RA between 0.05 % and 0.1 % in a cream base proved to have a therapeutic benefit in acne, and in Darier's disease 13-cis-RA in the same concentration and same vehicle as used in the present study proved to be effective[14]. In addition, a clear effect on LTB_4-induced accumulation of PMN was shown with the same preparation[15]. In contrast, 0.1 % 13-cis-RA in a cream base showed no convincing amelioration of clinical and histological parameters in psoriatic lesions (present study). An increase of the concentration of 13-cis-RA or an ointment base might add to the therapeutic effect. In conclusion, the clinical value of topical 13-cis-RA (0.1% cream base) in the treatment of psoriasis is limited. This parallels the observations in acne where systemic therapy is far more efficacious than topical treatment.

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3.3 SHORT-CONTACT DITHRANOL TREATMENT OF PSORIASIS, USING A NOVEL EMULSIFYING OINTMENT (HERMAL-AW®): CHANGES IN CLINICAL SCORES AND IN PARAMETERS FOR INFLAMMATION, PROLIFERATION AND KERATINIZATION.

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SUMMARY

In this communication the clinical and immunohistochemical effects of short contact applications of dithranol (0.5-5%) in the novel emulsifying ointment Hermal-AW are reported. Twenty patients were treated and biopsies were taken before treatment (n=16), after two (n=5) and eight weeks (n=16) for immunohistochemical assessment of proliferation, keratinization and inflammation. Among the patients 75% showed a satisfactory clinical response. Of the parameters investigated, Ks8.12 binding (keratin 13 and 16) showed the most pronounced correlation with the clinical scores. Although T-lymphocytes and CD14-cells persisted up to 8 weeks, epidermal polymorphonuclear leukocytes diminished in an earlier phase. The epidermal changes preceded the dermal changes.

INTRODUCTION

Dithranol is a well-established treatment for chronic plaque psoriasis[1,2]. In-patient treatment with dithranol monotherapy or combined regimes of dithranol with ultraviolet radiation and tar ("Ingram scheme") have a remarkably high clearing efficiency and are safe[3]. However, irritation of the skin and staining limit the classical application largely to in-patient departments or day care centres.

During the last 15 years, short contact therapy with dithranol has become an important alternative for the classical longterm applications[4-13]. The limited application periods (10-30 minutes) using relatively high doses of dithranol have revolutionized out-patient dithranol therapy. Staining of clothes, textiles and bathroom equipment however, has seriously decreased the first enthusiasm of short contact therapy.

The development of new formulations which permit an efficient removal of dithranol from the skin has opened new perspectives for short contact dithranol treatment. In the present study the clinical efficiency and staining properties of dithranol (0.5-5%) in the emulsifying ointment Hermal-AW (Hermal, Hamburg, FRG) is evaluated using clinical severity scores.

The effect of dithranol on classical histological parameters has been studied in the past. Proliferation has been investigated measuring epidermal thickness using H & E staining, the number of mitoses or 3[H]-thymidine incorporation[14-16]. The inflammatory infiltrate has been studied mainly on H & E sections[17].

In the present study, the effect of dithranol-treatment in a new ointment base on specific markers for proliferation, keratinization and inflammation was studied using immunohistochemistry. Especially the correlations between clinical scores and the different markers was of our interest.

MATERIALS AND METHODS

Patients

A group of 20 patients, 11 females and 9 males, aged between 19-73 years, participated in the investigation. The percentage of their body surface involved with

psoriasis varied between few palm-sized plaques to 49%. The Psoriasis Area and Severity Index (PASI) before treatment varied between 5 and 27, averaging 13.2. All patients had been untreated for at least two weeks. Patients had refrained from systemic treatments (retinoids, methotrexate) for at least six months. Patients with exsudative eczematized or pustular manifestations were excluded from the study. Permission from the Ethics Committee and written informed consent from all patients were obtained.

Treatment protocol

Dithranol Hermal AW applications were carried out on an out-patient basis using a short contact scheme. The ointments were applied daily by the patients at home. The duration of the application was 20 minutes per day. Dithranol 0,5%, 1%, 2%, 3%, 4% and 5% were manufactured by the pharmacist in Hermal-AW (Hermal, Hamburg, FRG). The concentrations were increased after 3 days of application if no burning or stinging sensation was reported. If the concentration of 5% was reached and no further improvement was observed the application periods were extended two hours. The ointments were removed with water and a detergent. Changes in the duration of the application period were done at three day intervals. No increase of the concentration or lengthening of the application period was done in case the patient experienced a burning sensation and/or in case of the appearance of a pronounced erythema around the plaques.

During the phase that the concentrations of dithranol were increased, the patient visits were three times a week. If an optimal concentration was reached the patient visited the department once a week. The duration of treatment was eight weeks.

During the treatment the clinical response was monitored at every visit. Clinical monitoring was done using the PASI[18]. To express the relative improvement of the individual patient, the following equation was used:

$$\frac{\text{PASI before} - \text{PASI after}}{\text{PASI before}} \times 100\% = \text{relative improvement.}$$

The appearance of irritation and staining was monitored on a 3-point scale (-/+/++). Following discontinuation of treatment the patient was seen at regular intervals. A relapse was defined as the reappearance of new lesions.

Biopsy procedures and immunohistochemical staining

In all 20 patients, biopsies were taken from the lesions before and after treatment. An additional biopsy was taken from a subgroup of 5 patients after two weeks of treatment. The biopsies were snap frozen in liquid nitrogen and stored at -80°C until use. Slides of 7 μm were cut and fixed in acetone or in acetone/ether 60/40% for 10 minutes. Staining to assess epidermal proliferation, keratinization and epidermal and dermal inflammation, was done using the following antibodies:

Proliferation

Ki-67 (Dakopatts, Copenhagen, Denmark): binds to a nuclear antigen present in cycling cells

Keratinization

Ks8.12 (Sigma, St Louis, USA): binds to keratin 13 and 16. Keratin 16 is expressed by hyperproliferative keratinocytes[19]. Keratin 13 is not present in adult human skin[20].

RKSE60 (a gift of F. Ramaekers): binds to keratin 10. Keratin 10 is expressed by differentiated keratinocytes.

Inflammation

Anti-elastase (Serotec, Oxford, England): staining leukocyte elastase, present in polymorphonuclear leukocytes (PMN)

T11 (Dakopatts, Copenhagen, Denmark): stains CD2, present on T-lymphocytes

OKT6 (Ortho Diagnostic Systems, Raritan, USA): stains CD1a, present on Langerhans cells

WT14 (Dept. Medicine, Div. Nephrol. University Hospital Nijmegen): directed against CD14, present on monocytes and macrophages[21].

A direct peroxidase technique was used to visualize PMN. An indirect peroxidase technique was used for staining with the antibodies Ki-67, Ks8.12 and RKSE60. Slides were incubated for 30 minutes with the antibodies in a dilution of 1:100, 1:40, 1:25 and 1:10 respectively. After washing in phosphate buffer the slides were incubated with a solution of rabbit-anti-mouse-immunoglobulin conjugated with

peroxidase 1:50 in phosphate buffer containing 5% human AB-serum for 30 minutes, except for the anti-elastase antibody. After 3 washing steps in phosphate buffer and preincubation in sodium acetate buffer pH 4.9, slides were stained in a solution of 3-amino-9-ethyl-carbazole (AEC) in sodium acetate buffer containing 0.01% H_2O_2 for 10 minutes. All slides were counterstained with Mayer's haematoxylin (Sigma, St Louis, USA) and mounted in glycerin gelatin.

Staining with T11, OKT6 and WT14 was carried out using a peroxidase-anti-peroxidase technique. Slides were incubated for 60 minutes with the monoclonal antibodies in a dilution of 1:100. After washing in phosphate buffer the slides were incubated with rabbit-anti-mouse-immunoglobulin (RAM-Ig, Dakopatts, Copenhagen, Denmark) 1:25 in phosphate buffer for 20 minutes. This was followed by a washing step and incubation with peroxidase-anti-peroxidase complexes (PAP-complexes, Dakopatts, Copenhagen, Denmark) 1:100 in phosphate buffer for 20 minutes. Once more the slides were incubated with RAM-Ig, followed by PAP-complexes. The slides were stained in AEC-solution in sodium acetate buffer and H_2O_2 and counterstained with Mayer's haematoxylin. Finally they were mounted in glycerin gelatin.

Histological changes in the epidermal and dermal compartment were assessed using a semi-quantitative scale:

Epidermis: 0= no staining, 1= sporadic staining, 2= minimal staining, 3= moderate staining, 4= moderate-pronounced staining, 5= pronounced staining, 6= whole epidermis stained.

Dermis: 0= no stained cells, 1= sporadic, 2= 1-25% of the infiltrate cells stained, 3= 26-50%, 4= 51-75%, 5= 76-99%, 7= 100%.

Ki-67-positive nuclei were counted per mm length of epidermis.

Statistical analysis

The Wilcoxon ranking test for matched pairs was used for the statistical analysis.

RESULTS

Clinical response

The clinical response to the treatment is summarized in figure 1 and table I. In two patients treatment was discontinued due to non-compliance of the patients. In two

other patients dithranol had induced a worsening of the lesions due to an intercurrent instability of psoriasis.

In 16 patients the treatment had resulted in some improvement (figure 1). PASI scores reduced from 13.3 +/- 1.5 (SEM) before treatment, to 3.1 +/- 0.8 after 8 weeks of treatment. An average reduction of PASI-scores of 10.1 was obtained. A satisfactory response was observed in twelve patients (Table I). The degree of improvement was not correlated with the extent of the lesions.

The remission period in patients showing a satisfactory response, varied from two weeks to four months, averaging 6.3 weeks.

The final concentrations reached in the patients were 3% (one patient), 4% (three patients) and 5% (twelve patients). In three patients the treatment was complicated by an irritation of the surrounding skin. A pronounced and dysfigurating staining was observed in three patients. In 11 patients a moderate staining was observed which was well-accepted by the patients. In two patients no staining was seen at all. The removal of the ointments with water and a detergent was relatively easy and complete. Minimal staining of clothing or furniture was recorded by any of the patients.

Histological findings

The immunohistochemical observations before treatment (n=16), after 2 weeks of treatment (n=5) and 8 weeks after treatment (n=16) are summarized in Figure 2.

After two weeks of treatment with dithranol, already a statistically significant decrease in Ki-67 positive nuclei could be seen ($p < 0.05$, figure 3), in com-

Table I. Clinical results expressed as the number of patients showing different degrees of relative improvement.

	<50%	50-90%	>90%	Drop out
Present study	4	10	2	4

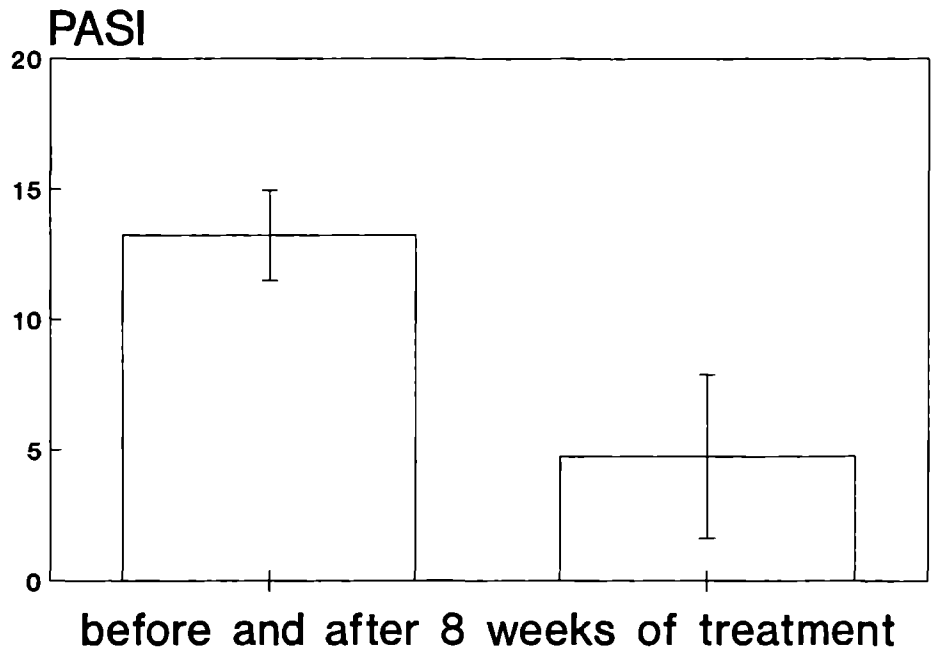

Figure 1: Clinical scores before and after treatment with dithranol in emulsifying ointment (means +/- SEM).

bination with a decrease in Ks8.12 binding ($p < 0.05$, figure 4). In addition, epidermal PMN diminished ($p < 0.05$). However the other parameters showed no significant changes in the early phase of dithranol treatment.

After 8 weeks of treatment with dithranol, a pronounced effect on proliferation could be seen. Ki-67 positive nuclei decreased in a statistically significant manner ($p < 0.04$). Keratinization assessed by the antibodies Ks8.12 (keratin 16) and RKSE60 (keratin 10) also showed a statistically significant change at the end of the treatment period ($p < 0.04$ and $p < 0.03$ respectively). Different cell types involved in inflammation in epidermal and dermal compartment were evaluated. The number of PMN decreased as well in epidermis as in dermis ($p < 0.02$, $p < 0.03$ respectively). T-lymphocytes showed the same pattern, a diminution in both compartments ($p < 0.02$ in epidermis, $p < 0.03$ in dermis, figure 5). CD14 cells showed no significant change in number, distribution or shape in the dermal compartment, but decreased statistically significant in the epidermis after 8 weeks. The number of Langerhans cells showed no significant change. However another remarkable

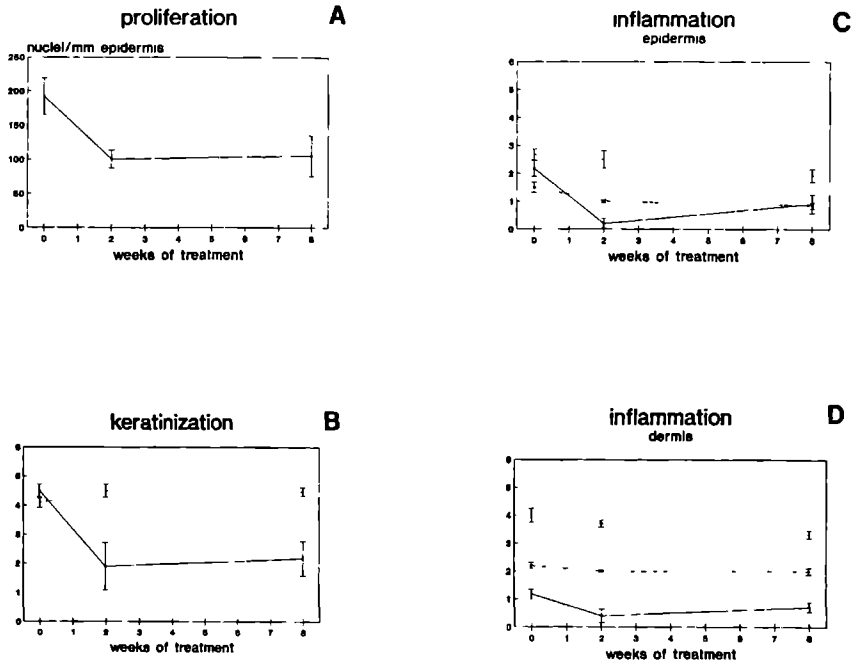

Figure 2: Scores for proliferation, keratinization and inflammation during dithranol treatment (means +/- SEM). A : Number of nuclei per mm length of epidermis stained with Ki-67; B: Ks8.12-binding (———) and RKSE60-binding (.....), representing keratin 16 and keratin 10 respectively; C: Expression of anti-elastase (———), T11 (.....) and WT14 (—), representing PMN, T-lymphocytes and CD14-positive cells in the epidermis; D: Expression of anti-elastase (———), T11 (.....) and WT14 (----), representing PMN, T-lymphocytes and CD14-positive cells in the dermis.

phenomenon was observed. After dithranol therapy, the staining pattern using OKT6 showed intercellular distribution of the antibody (figure 6).

Linear regression analysis was carried out to assess correlation between clinical severity scores and the immunohistochemical parameters showing a statistically significant change during treatment. Ks8.12 proved to be correlated with clinical improvement in all patients, The remaining parameters (Ki-67 staining, keratin 10 staining, PMN and T-lymphocytes) were less indicative for clinical resolution.

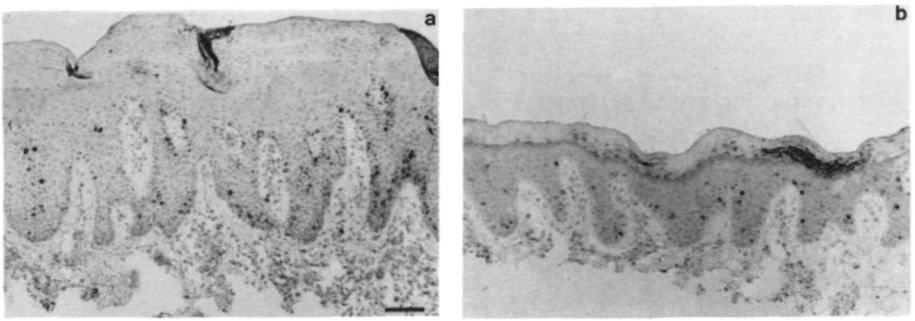

Figure 3: Staining with the antibody Ki-67, marker for proliferation; (a) psoriasis before treatment, (b) same patient after two weeks of treatment with dithranol. Bar = 4 μm.

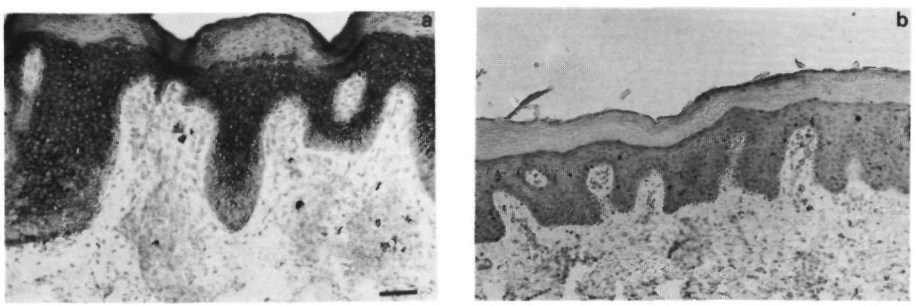

Figure 4: Staining with the antibody Ks8.12, showing keratin 16; (a) psoriasis before treatment, (b) same patient after two weeks of treatment with dithranol. Bar = 5 μm.

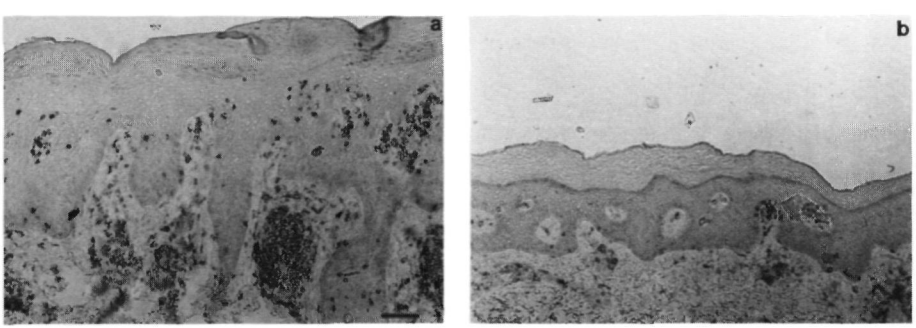

Figure 5: Staining with the antibody T11, staining T-lymphocytes; (a) psoriasis before treatment, (b) same patient after eight weeks of treatment with dithranol. Bar = 5 μm.

Short contact therapy with dithranol in paste, paraffin and cream bases have been reported to be successful in the treatment of chronic plaque psoriasis[4-13,22]. If short contact therapy is carried out at an in-patient department, its effectivity has been reported to be equal or even better compared to classical dithranol therapy[5,7,10]. If carried out at the out-patient department with supervision and concentration adjustments three times a week, a satisfactory response (complete or almost complete clearing) was seen in 15 out of 20 patients, using Psoralon (Hermal) preparations[12]. However, less frequent out-patients supervisions yielded a far lower therapeutic effectivity reaching 10% clearing[9,23].

In the present study dithranol was manufactured in Hermal-AW by the local pharmacist. This monotherapy proved to have a satisfactory response in 12 out of 20 patients. An average reduction of PASI scores of 10.1 was obtained. The clinical efficacy of the present treatment is comparable with the results obtained using Psoralon (12). The remission period of 6.3 weeks following dithranol Hermal-AW is in line with the average remission period of 6 weeks following the classical Ingram Scheme, using the same criterion for an initial relapse[24].

Following short contact treatment removal of dithranol from the skin is essential in order to prevent staining of textiles, clothing etc. Using stiffened paraffin base, the paste and also the various cream bases, postapplication staining is an important drawback. Using Psoralon MT and dithranol Hermal-AW, postapplication staining of textiles proved to be minimal. In this respect the possibility of manufacturing dithranol in Hermal-AW by the local pharmacist opens new possibilities for individualising modern dithranol treatment at an out-patient basis.

Proliferation assessed by Ki-67 binding, diminished statistically significant after two weeks of treatment. This confirms earlier studies in which a 50% decrease in ^{3}[H] thymidine incorporation was seen after 2 weeks (16). Also in the present study the number of Ki-67 positive nuclei decreased with about 50% after two weeks of treatment. In previous studies the number of mitoses reduced already after 4 days[15]. Some investigators found a decrease in DNA-content after dithranol therapy[25,26] whereas others found no change[12,27,28]. A possible explanation for this phenomenon is an arrest of cells in the S phase[12].

Keratinization during dithranol therapy showed a clear change. In a previous study using flowcytometry, a decrease in Ks8.12 binding was demonstrated after 6 weeks of treatment[12]. In the present study, Ks8.12 binding diminished already statistically significant after two weeks of treatment. Ks8.12 binding was very low after two weeks of treatment selectively in patients who showed a considerable further improvement between 2 and 8 weeks. This observation suggests that reduction of Ks8.12 binding might predict further clinical improvement. RKSE60 binding (keratin 10) increased during therapy. RKSE60 binding correlated well with restoration of the general histology. A remarkable feature was that in lesions showing complete clinical clearance after dithranol therapy, still some histological abnormalities were seen: acanthosis, papillomatosis and a noticeable hyperorthokeratosis.

Inflammation persisted relatively long during dithranol treatment. However epidermal PMN diminished already in the early phase. After 8 weeks also dermal PMN and T-lymphocytes in both skin compartments reduced. CD14-positive cells diminished in the epidermal compartment. The epidermal changes therefore, precede the dermal changes during dithranol treatment. In a previous study Braun Falco et al reported on "the persisting cellular inflammatory reaction" during dithranol treatment[17]. In the present study a dissociation between the reduction of PMN versus T-lymphocytes and CD14-cells could be shown. In vitro studies suggested an effect of dithranol on PMN[29,30] and lymphocytes[30]. The present in vivo study suggests that the interference with PMN migration is relevant in the early phase of dithranol treatment. In the present study the number of Langerhans cells did not change. Remarka-

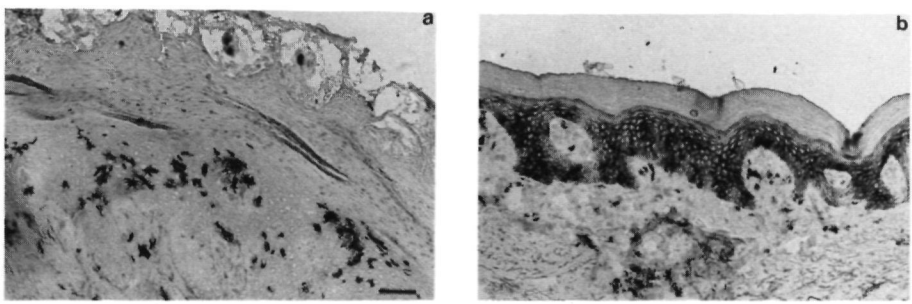

Figure 6: Staining with the antibody OKT6, staining Langerhans cells; (a) psoriasis before treatment, (b) same patient after eight weeks of treatment with dithranol. Bar = 4 μm.

bly, after dithranol therapy, staining of Langerhans cells in the epidermis using OKT6 showed an intercellular distribution. This has been described by other investigators in various skin diseases such as cutaneous B cell lymphoma, a variety of inflammatory skin disorders and in cutaneous T-cell lymphoma[31,32]. To the best of our knowledge, this phenomenon has not been described in untreated nor treated psoriatic lesions. The presence of intercellular OKT6 binding seemed to be associated with large numbers of Langerhans cells. However in our study the number of Langerhans cells did not increase statistically significant. Possibly damage or irritation of Langerhans cells by dithranol might cause leakage of the T6 antigen. Induction of CD1a on keratinocytes might be an alternative explanation.

It can be concluded that out-patient treatment with dithranol in the emulsifying ointment Hermal-AW is an efficient treatment modality. The order of changes of parameters for inflammation, proliferation and keratinization indicate that epidermal changes precede the dermal changes. In particular, a decrease of nuclear Ki-67 staining, Ks8.12 binding and a decrease in PMN-accumulation constitute relatively early changes.

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3.4 TOPICAL APPLICATION OF DITHRANOL ON NORMAL SKIN INDUCES EPIDERMAL HYPERPROLIFERATION AND INCREASED Ks8.12-BINDING.

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SUMMARY

Although dithranol has been used for 75 years in the treatment of psoriasis, its working mechanism is still not resolved. In order to further define the mode of action of dithranol the interference with normal skin was studied. The effect of dithranol on epidermal proliferation, keratinization and inflammation was examined using immunohistochemistry.

Punch biopsies from six volunteers who applied dithranol 0.5% in petrolatum, were taken before application, after 48 and 96 hours. Biopsies were processed for assessing epidermal proliferation by Ki-67-binding (cycling cells), for keratinization by Ks8.12-binding (keratin 13 and 16, keratin 16 is expressed by hyperproliferative keratinocytes) and RKSE60-binding (keratin 10). For assessing inflammation the antibodies anti-elastase (PMN), T11 (T-lymphocytes, CD2), OKT6 (Langerhans cells, CD1a) and WT14 (monocytes/macrophages, CD14) were used.

Ki-67-staining started to increase between 48 and 96 hours whereas Ks8.12-binding had increased already between 0 and 48 hours. RKSE60 staining showed a decline between 48 and 96 hours. Inflammation in the dermis showed an increase after 48 hours, and continued to increase. In the inflammatory infiltrate, the accumulation of

PMN was limited compared to the pronounced infiltration of T-lymphocytes. Langerhans cell shape and epidermal position altered in four volunteers.
Application of dithranol on normal skin produces analogies and discrepancies compared to application of dithranol on psoriatic lesions. Direct interference with epidermal growth and differentiation seems less likely as the anti-psoriatic principle.

INTRODUCTION

Dithranol is a classical and time-honoured treatment for chronic plaque psoriasis. The anti-psoriatic principle, so far, is not exactly understood.
Previous studies have shown interference of dithranol with DNA-synthesis, effects on mitochondrial function, and on cytosolic enzymes[1-4]. The ultimate result of dithranol therapy in psoriatic lesions is a down-regulation of epidermal proliferation and a decrease of inflammatory cells in the dermis[5-9]. In contrast, dithranol induces erythema in the surrounding skin of a psoriatic lesion[10].
In normal skin, however, the effects of dithranol are largely unknown. In order to achieve better insight in the mode of action, the events occurring in normal skin after application of dithranol were studied. Monoclonal antibodies were used on frozen sections to assess epidermal proliferation and keratinization (keratin 10 and 16). Keratin 10 is expressed by differentiating keratinocytes and can be seen in the suprabasal layers of normal epidermis[11]. Ks8.12 binds to keratin 13 and 16. Keratin 13 is absent in adult human skin[12]. Keratin 16 is expressed by hyperproliferative keratinocytes in vitro as well as in vivo[13]. In addition the inflammatory infiltrate was studied (polymorphonuclear leukocytes (PMN), T-lymphocytes, Langerhans cells, monocytes and macrophages).

Volunteers

Six healthy volunteers with no signs or history of skin disease participated in the study (3 females, 3 males, age varying between 22 and 32 years, mean age 26.7 years). They applied dithranol 0.5% in petrolatum thinly and evenly on an area of 5 x 5 cm once a day for five consecutive days. Punch biopsies (4mm) were taken before application of dithranol, and at 48 and 96 hours application of dithranol. In addition, clinical signs were recorded such as discolouration, erythema, or itching.

Staining procedures

Biopsies were embedded in Tissue Tek OCT Compound (Miles Scientific, Naperville, USA) and snap frozen in liquid nitrogen. Sections of 7 μm were cut and fixed in acetone (0 °C) for 10 minutes, or in acetone/ether 60/40% (for Ki-67 staining). The antibodies used are indicated in Table I. A direct peroxidase (anti-elastase), indirect peroxidase (Ki-67, Ks8.12, RKSE60) or PAP technique was used (T11, OKT6, WT14). For staining with the antibodies anti-elastase, Ki-67, Ks8.12 and RKSE60, slides were incubated for 30 minutes with the primary antibodies. After washing in phosphate buffer the slides were incubated with a solution of rabbit-anti-mouse-immunoglobulin conjugated with peroxidase 1:50 in phosphate buffer containing 5% human AB-serum for 30 minutes, except for the anti-elastase antibody. After 3 washing steps in phosphate buffer and preincubation in sodium acetate buffer pH 4.9, slides were stained in a solution of 3-amino-9-ethyl-carbazole (AEC) in sodium acetate buffer containing 0.01% H_2O_2 for 10 minutes. All slides were counterstained with Mayer's haematoxylin (Sigma, St Louis, USA) and mounted in glycerin gelatin.

Staining with T11, OKT6 and WT14 was carried out using a peroxidase-anti-peroxidase technique. Slides were incubated for 60 minutes with the monoclonal antibodies. After washing in phosphate buffer the slides were incubated with rabbit-anti-mouse-immunoglobulin (RAM-Ig, Dakopatts, Copenhagen, Denmark) 1:25 in phosphate buffer for 20 minutes. This was followed by a washing step and incubation with peroxidase-anti-peroxidase complexes (PAP-complexes, Dakopatts, Copenhagen, Denmark) 1:100 in phosphate buffer for 20 minutes. Once more the

slides were incubated with RAM-Ig, followed by PAP-complexes. The slides were stained in AEC-solution in sodium acetate buffer and H_2O_2 and counterstained with Mayer's haematoxylin. Finally they were mounted in glycerin gelatin.

Histological scores

Histological changes in the epidermal and dermal compartment were assessed using a semi-quantitative scale:

Epidermis: 0= no staining, 1= sporadic staining, 2= minimal staining, 3= moderate staining, 4= moderate-pronounced staining, 5= pronounced staining, 6= whole epidermis stained.

Dermis: 0= no stained cells, 1= sporadic, 2= 1-25% of the infiltrate cells stained, 3= 26-50%, 4= 51-75%, 5= 76-99%, 7= 100%.

The number of Ki-67-stained nuclei was counted per mm length of section.

Statistical analysis

The Wilcoxon ranking test for matched pairs was used for the statistical analysis.

Table I

Antibody	Specificity	Dilution	Manufacturer
Ki-67	nuclear antigen in late G1-, S-, G2- and M-phase	1:40	Dakopatts, Copenhagen, Denmark
Ks8 12	keratin 13 and 16	1:25	Sigma, St Louis, USA
RKSE60	keratin 10	1:10	F Ramaekers, Dept of Pathol, Nijmegen
Anti-elastase	neutrophil elastase	1:100	Dakopatts, Copenhagen, Denmark
T11	T-lymphocytes (CD2)	1:100	Dakopatts, Copenhagen, Denmark
OKT6	Langerhans cells (CD1a)	1:100	Dakopatts, Copenhagen, Denmark
WT14	monocytes/macrophages (CD14)	1:100	W Tax, Dept Nephrol, Nijmegen

RESULTS

Volunteers

All six volunteers showed after 48 hours a slight brown discolouration of the skin and some erythema. After 96 hours, the discolouration had increased and the erythema was less pronounced than at 48 hours. One volunteer showed vesicles at the application site, one other showed slight peeling of the stratum corneum.

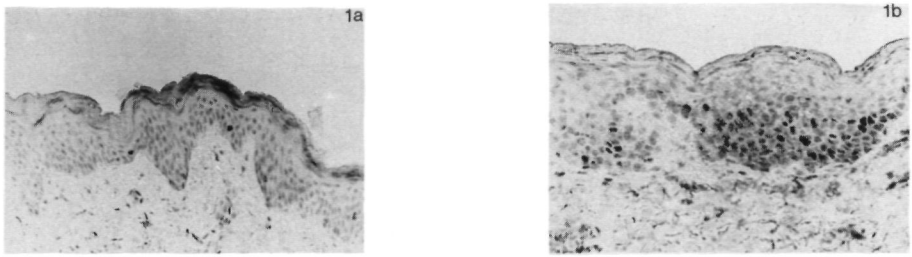


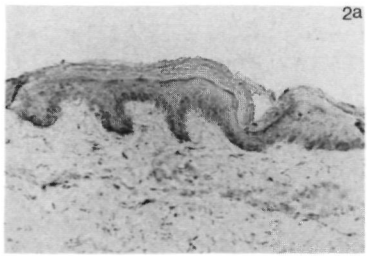
Figure 1: Staining with the antibody Ki-67, marker for proliferation. A: normal skin before application of dithranol, B: normal skin after 96 hours application of dithranol. Bar = 20 μm.

Histological findings

Epidermal proliferation Before application of dithranol, the number of Ki-67-positive nuclei numbered 16 +/- 2 (mean +/- SEM) per mm. After 48 hours, this number had not increased. After 96 hours, a statistically significant ($p<0.03$) increase in stained nuclei was seen up to 47 +/- 11 (mean +/- SEM) per mm. This is shown in figure 1.

Epidermal keratinization Before application, staining with the antibody Ks8.12 (keratin 13 and 16) showed only sporadical staining in the basal layer. No suprabasal staining was seen at all. After 48 hours, suprabasal staining had increased up to 1.2 +/- 0.3 (mean +/- SEM), $p<0.05$. After 96 hours, Ks8.12 staining in the suprabasal compartment had increased further (1.8 +/- 0.5, mean +/- SEM, $p<0.05$), whereas staining of the basal cell layer decreased ($p<0.05$) (figure 2). Staining with the antibody RKSE6O (keratin 10) showed no significant change between 0 and 48 hours, but diminished between 48 and 96 hours application of dithranol ($p<0.05$).

Inflammation in epidermis and dermis Intraepidermal accumulation of PMN (anti-elastase-staining) did not occur during the observation period. In the dermis a significant increase of PMN was seen after 48 and 96 hours compared to the pretreatment biopsy ($p< 0.04$ and $p< 0.03$, respectively). In contrast to PMN, T-lymphocytes (T11) showed a substantial increase in the epidermal as well as dermal compartment after 48 and 96 hours. In the epidermis, these cells increased from 0.5 +/- 0.2, via 2.1 +/- 0.3 after 48 hours, to 2.7 +/- 0.6 (means +/- SEM, $p< 0.05$)



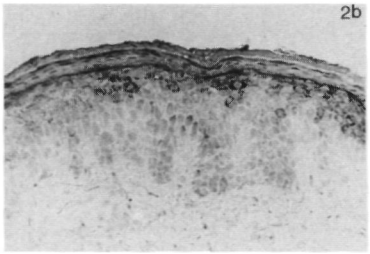
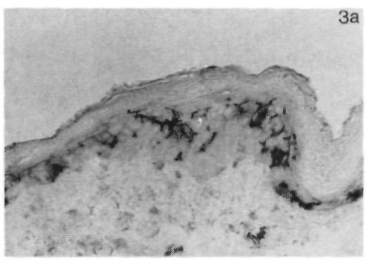


Figure 2: Staining with the antibody Ks8.12, showing keratin 16. A: normal skin before application of dithranol, B: normal skin after 96 hours application of dithranol.



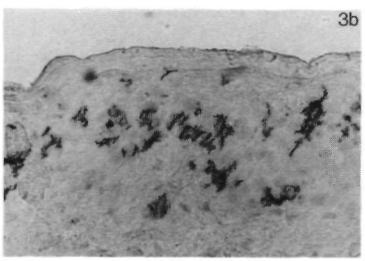


Figure 3: Staining with the antibody OKT6, showing Langerhans cells. A: normal skin before apllication of dithranol, B: normal skin after 48h application of dithranol. OKT6 positive Langerhans cells can be seen in the basal layer of the epidermis.

after 96 hours. In the dermal compartment, an increase from 1.4 +/- 0.3, via 2.8 +/- 0.3, up to 4.8 +/- 0.2 after 96 hours was demonstrated (means +/- SEM, $p<0.05$ and $p<0.03$, respectively). Cells staining with the antibody WT14 (monocytes, macrophages) also increased in epidermis as well as in dermis after 48 ($p<0.03$) and 96 hours ($p<0.03$). Langerhans cells (OKT6) showed no statistically significant change in number in the epidermis. Their shape, however, altered compared to the shape of these cells in the untreated skin, e.g. their dendrites became smaller (figure 3). In four volunteers a rearrangement of Langerhans cells was seen. In two of these Langerhans cells were seen mainly in the basal layer of the epidermis after 48 hours (figure 3), in another volunteer this picture was found after 96 hours. In the fourth volunteer, Langerhans cells were seen in the vicinity of

small vesicles in the epidermis. In the dermal compartment, Langerhans cells showed a statistically significant increase after 96 hours ($p < 0.03$).

The inflammatory infiltrate showed an increase mainly in the dermis. T-lymphocytes contributed most to the increase of cells in the dermis. WT14-stained cells also showed a substantial increase. The contribution of PMN to the infiltrate remained limited.

DISCUSSION

The various events taking place after application of dithranol, can be summarized as follows: Ki-67-staining started to increase between 48 and 96 hours whereas Ks8.12-binding had increased already between 0 and 48 hours. RKSE60 staining showed a decline between 48 and 96 hours. Inflammation in the dermis showed an increase after 48 hours, and continued to increase up to 96 hours. In the inflammatory infiltrate, the accumulation of PMN was limited compared to the pronounced infiltration of T-lymphocytes.

In the treatment of psoriasis, the application of dithranol is an established therapy with few side effects. In previous studies, a decrease in ^{3}[H]-thymidine incorporation, a decrease in DNA-content and cells in SG2M-phase, and a decrease in cells staining with the antibody Ki-67 has been observed during treatment of psoriatic lesions with dithranol[5,9,15,16]. Also keratinocytes in culture, showed a decrease in growth after incubation with dithranol[17]. In the present study, normal skin in vivo showed a hyperproliferative response to dithranol applications, as demonstrated by an increase of Ki-67 stained nuclei. In addition, an increase of Ks8.12 staining in the suprabasal compartment was observed already after 48 hours. In this respect, it is of interest that Ks8.12 staining occurs already well before hyperproliferation is observed, indicated by Ki-67 staining. The explanation for the discrepancy as to the dynamics of dithranol-induced proliferation and the expression of keratin 16 -so far believed to be a proliferation associated event- is not clear. Some investigators have proposed an association of keratin 16 expression with a more general disturbance of the epidermal condition and may not necesseraly be hyperproliferation associated [15]. Such a condition could be induced by dithranol.

Due to the so far unknown induction time for the epitope recognized by the Ki-67 antibody, we cannot exclude the possibility that the transition from resting cell into cycling cell might occur earlier than at 96 hours. In hyperproliferative epidermis, such as psoriasis or normal skin after sellotape stripping[18,19], the content of keratin 10 is diminished. In the present investigation, also a decline in keratin 10 is shown that coincides with the increase in Ki-67 staining between 48 and 96 hours.

Langerhans cells were seen mainly in the basal cell layers of the epidermis after application of dithranol in three volunteers. In particular, Langerhans cells are very sensitive to damage of the mitochondria by dithranol[20]. Possibly the Langerhans cells in the upper layers of the epidermis are destructed or have lost their CD1a antigen.

In the inflammatory infiltrate, the limited accumulation of PMN compared to T-lymphocytes is striking. Histologically, a toxic or irritative reaction shows in general a greater content of PMN[21]. In this respect, it is of interest that dithranol has shown to inhibit chemotaxis of PMN in vitro[22], and the leukotriene B_4 induced accumulation of PMN in normal skin in vivo[23].

Comparing the responses of normal and lesional psoriatic skin, we are faced with analogies as well as discrepancies. PMN decreased after two weeks of dithranol treatment of the psoriatic lesion and T-lymphocytes persisted beyond this period [15]. In normal skin treated with dithranol few PMN accumulated but T-lymphocytes proved to be pronounced. Whereas epidermal proliferation and keratin 16 expression decrease, and keratin 10 expression increases during treatment of the lesion[15,16], the normal skin treated with dithranol shows the reverse. Therefore, a direct interference with epidermal growth and differentiation seems less likely as the antipsoriatic principle.

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3.5 CLINICAL AND BIOCHEMICAL EFFECTS OF AN ORAL LEUKOTRIENE BIOSYNTHESIS INHIBITOR (MK886) IN PSORIASIS.

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SUMMARY

Antipsoriatic agents have been shown to decrease skin levels of arachidonic acid and its metabolites including 12-monohydroxy-eicosatetranoic acid (12-HETE), and leukotriene B_4 (LTB_4). In addition, specific systemic and topical lipoxygenase inhibitors have been reported to be effective in the treatment of psoriasis.
The objective of this study was to investigate the effect of a potent oral leukotriene biosynthesis inhibitor (MK886) in patients with chronic plaque psoriasis. Clinical response together with the changes of LTB_4 levels in lesional skin biopsy specimens, and urinary leukotriene E_4 (LTE_4) excretion was evaluated. In addition, markers of inflammation, proliferation and keratinization were studied immunohistochemically.

No change in clinical scores, or lesional LTB_4 levels were observed with a 10 1/3 day course of MK886. A statistically significant reduction in urinary LTE_4 excretion was observed (mean LTE_4 ng/hr were 5.14 and 1.51 pretreatment and day 11 with MK886 respectively; and 7.55 and 6.57 pretreatment and on day 11 with placebo treatment respectively). Epidermal accumulation of polymorphonuclear leukocytes (PMN) tended to diminish in the MK886 treatment group. These results indicate that despite a significant reduction (>70%) in systemic leukotriene levels, and epidermal PMN accumulation, no correlative changes in clinical scores or LTB_4 levels in skin lesions were found with a short course of MK886.

INTRODUCTION

In the search for the pathogenesis of psoriasis, many cell types and substances have been investigated. One remarkable biochemical deviation in the psoriatic lesion is the increased amount of leukotrienes[1,2]. These leukotrienes have been studied in normal skin, and in involved and uninvolved psoriatic skin using different methods[3].

Leukotriene B_4 (LTB_4), seems to be of primary importance as it is increased in psoriatic skin compared to normal skin. LTB_4 is a potent chemoattractant for polymorphonuclear leukocytes (PMN) in vitro and in vivo[4]. In addition, application of LTB_4 on normal skin can produce microabscesses of PMN[5,6], an inflammatory infiltrate[7,8], and epidermal proliferation[9]. These features can also be found in the psoriatic lesion. Topical application of LTB_4 to uninvolved and involved psoriatic skin induces a reaction different from normal skin. In psoriasis, a habituation to continuously high amounts of LTB_4 seems to take place[7]. Various anti-psoriatic therapies can inhibit the LTB_4-induced PMN-accumulation in normal skin[10]. Therefore, their mechanism of action may in part be due to inhibition of LTB_4 induced inflammation.

In the past, different therapeutics have been investigated that interfered with LTB_4-metabolism. LTB_4 is produced from arachidonic acid via the 5-lipoxygenase pathway (figure 1). Therapeutics that inhibit 5-lipoxygenase (5-LO) have been used in the past[11,12], but had serious side effects. Recently, a successful trial with a topical 5-lipoxygenase inhibitor has been published[13].

The effect of a potent oral 5-lipoxygenase inhibitor MK886 (3-[1-(p-chlorobenzyl)-3-t-butylthio-5-isopropylindol-2-yl]-2,2-dimethylpropanoic acid), Merck Frosst (Montreal, Canada), was studied in patients with chronic plaque psoriasis. MK886 is an indirect leukotriene biosynthesis inhibitor whose mechanism of action is through the binding of a membrane-bound 5-LO-activating protein (FLAP) thereby inhibiting the translocation and activation of 5-LO[14,15]. MK886 is a potent inhibitor of leukotriene biosynthesis in vitro in human PMN (IC50 = 2.5 nM) and in human whole blood (EC50 = 1.1 μM). At this concentration no effect is seen on cyclooxygenase or 12-lipoxygenase[16]. Studies in normal volunteers have demonstrated its ability to inhibit calcium ionophore stimulated LTB_4 synthesis ex vivo in whole blood[17].

The objectives of this study were to investigate the effects of MK886 on: (1) the clinical appearance of psoriatic lesions; (2) LTB_4 levels in lesional skin biopsy specimens; (3) inhibition of systemic leukotriene biosynthesis as reflected in the primary leukotriene metabolite in the urine (LTE_4)[18,19]; and on skin immunohistochemical markers of inflammation, proliferation and keratinization.

MATERIALS AND METHODS

Study design

This was a double-blind, placebo-controlled, parallel group, randomized study in which sixteen patients with chronic plaque psoriasis were treated with MK886 or placebo for ten and one third days. Eight patients received MK886 orally at a dose of 150 mg three times a day, and eight patients received matching placebo for a total of 31 doses per patient. Approval from the Medical Ethical Committee at the University Hospital Nijmegen was obtained.

Clinical and laboratory safety measurements

Prior to starting therapy, a clinical examination including EKG, and laboratory measurements were carried out. The laboratory measurements consisted of haematology (total and differential white blood cell counts, haemoglobin, haematocrit, platelet count) and blood chemistry (ASAT, ALAT, bilirubin, gammaGT, alkaline phosphatase, urea, creatinine, total protein and fasting blood glucose). In addition,

urinalysis was done (pH, protein, glucose, sediment). During the treatment period, the same haematology and blood chemistry measurements were carried out at day 1, 3, 5, 8 and 11. After the treatment period, clinical examination and laboratory measurements were repeated.

Patients

A group of 16 patients with chronic plaque psoriasis participated in this study. Informed consent was obtained from all patients. Their age varied from 25 years to 66 years (mean 47.9 years). The group consisted of 11 males and 5 females who were postmenopausal or surgically sterilized. They had used no topical treatment for at least two weeks, or systemic treatment for at least two months.

Clinical assessment and biopsy procedures

Two symmetrical psoriatic lesions were selected for each patient. Before and after the treatment period a photograph was taken and a clinical assessment was made using a four point scale for scaling, erythema and plaque elevation (see Table I). The mean scores for these three parameters were used for analysis. Clinical assessment was performed again on days 5 and 11 of therapy. Just before the first dose of the test drug, two biopsies were taken from one of the selected psoriatic lesions. Two hours after the last dose (dose 31), two biopsies were taken from the second lesion. One pre- and posttreatment biopsy was used for measurement of LTB_4. The remaining two biopsies were used for immunohistochemical processing. Biopsies for LTB_4 measurements were taken using a keratotome after local anaesthesia with chlooraethyl spray, and after gently removing the scales with a scalpel, because scales contain large amounts of LTB_4. Biopsies for immunohistochemistry were taken using a razor blade in conjunction with a metal guard after local anaesthesia with chlooraethyl spray. Biopsies were snap frozen in liquid nitrogen and stored at -80°C until use.

Leukotriene Analysis

Synthetic leukotrienes, leukotriene metabolites, hydroxy eicosatetranoic acid isomers, and rabbit antiserum against LTB_4, LTC_4 and LTE_4 were obtained from Dr. Robert Young, Merck Frosst Canada.

LTB_4 in skin biopsies: LTB_4 in skin biopsies were extracted as described[20], with minor modifications. Briefly, samples were ground under liquid nitrogen in a FREEZER/MILL^R vial (SPEX Industries, Edison, USA) modified with a resealable aspiration hole to allow direct extraction with 2 x 1 ml 75% methanol: 25% 0.1 M Tris-HCL, 1mM EDTA, pH 3. Diluted extracts were partially purified on preconditioned, disposable 500 mg C-8 columns (Amersham International, Amersham, UK). HPLC separation of leukotrienes and quantitative measurements of LTB_4 by RIA were carried out as described previously[17,21].

Urinary LTE_4: Urine was collected over a four hour period before treatment with MK886 from zero to four hours after the last dose of MK886 on day 11. The total volume was measured and a 40 ml aliquot was stabilized and prepared for analysis as described previously[20].Sample clean-up on C-8 columns and HPLC separation were carried out as described for skin LTB_4. Quantitative measurements of LTE_4 by RIA employed the procedure decsribed for LTB_4 except for the substitution with antiserum against LTE_4, [^{3}H]-LTE_4 and a lower charcoal concentration (0.1% w/v final concentration) used for removal of unbound counts[22].

Table I: Psoriasis lesion scores.

	Scaling	Erythema	Plaque elevation
1	none	none	flat
2	minimal	very mild	barely perceptible elevation
3	moderate	moderate	elevation
4	severe	strong	high elevation

Immunohistochemical staining

Biopsies were cut into sections of 7μm and fixed in acetone for 10 minutes. Staining with the following antibodies was carried out:

Ki-67 (Dakopatts, Copenhagen, Denmark): a proliferation marker, staining a nuclear antigen present in late G1, S, and G2M-phase

Ks8.12 (Sigma, St Louis, USA): directed against keratin 13 and 16

RKSE60 (gift of Dr F Ramaekers, Dept of Pathology, University Hospital Nijmegen): directed against keratin 10

Anti-elastase (Serotec, Oxford, England): staining elastase-positive cells (polymorphonuclear leukocytes, PMN)

T11 (Dakopatts, Copenhagen, Denmark): a pan-T-lymphocyte marker

WT14 (Dept Medicine, Div Nephrol, University Hospital Nijmegen): staining CD14 positive cells (monocytes and macrophages)[23]

OKT6 (Ortho Diagnostic Systems, Raritan, USA): staining Langerhans cells.

In brief, staining with anti-elastase was carried out using a direct peroxidase-technique, staining with Ki-67, Ks8.12 and RKSE60 was done using an indirect peroxidase technique, and staining with T11, WT14 and OKT6 was done using a PAP-technique. The staining procedures are described previously[24]. Ki-67 positive nuclei were counted per mm length of section. Presence in the epidermis and dermis of the remaining stained structures were assessed using a 6-point scale:

Epidermis: 0 = no presence, 1 = sporadically present, 2 = minimal presence, 3 = moderate presence, 4 = moderate-pronounced presence, 5 = pronounced presence, 6 = whole epidermis stained.

Dermis: 0 = no presence, 1 = sporadically present, 2 =1-25% of infiltrate cells stained, 3 = 26-50% of infiltrate cells stained, 4 = 51-75% of infiltrate cells stained, 5 = 76-99% of infiltrate cells stained, 6= 100% of infiltrate cells stained.

Statistical analysis

Statistical analysis was done using the Wilcoxon ranking test for paired data, and the Kruskal-Wallis test for group means.

RESULTS

Safety

No serious clinical adverse experiences were reported, MK886 was overall well tolerated. A heavy feeling in the stomach was reported more frequently in the MK886 group (4 patients) than the placebo group (0 patients). One patient had to be

withdrawn from the trial because of an elevation of alkaline phosphatase (123 U/l before treatment, 153 U/l after 5 days of treatment, normal values 0-120 U/l). After breaking the code, this patient turned out to be in the placebo group. In the other patients, all safety parameters remained within normal ranges.

Clinical effect

No significant change in any of the parameters of the lesion score were observed in the MK886 or placebo treated group after ten and one third days of treatment. Mean scores before treatment were 2.99 +/- 0.13 (sem) for the MK886-group, and 3.33 +/- 0.15 for the placebo group. On day 11 these scores were 2.73 +/- 0.1 (p=0.097) and 2.93 +/- 0.20 (p=0.470) respectively.

Measurements of LTB_4 in skin biopsies and urinary LTE_4

Baseline levels of LTB_4 in skin biopsies, and urinary LTE_4 excretion in the MK886-treated group and the placebo treated group were comparable.

Measurements of LTB_4 in the psoriatic lesions were in line with the clinical effect of MK886. No significant reduction in the amount of LTB_4 was seen after MK886-treatment (p=0.5) (see Table II).

In contrast, the amount of the metabolite LTE_4 diminished in a statistically significant manner in the MK886 treated group. These results are shown in Table III. Mean values for urinary LTE_4 in ng per hour were 4.67 +/- 0.67 (sem) and 5.0 +/- 2.24 for the MK886 and the placebo group respectively at day 1; and 0.96 +/- 0.36 (p=0.02) and 3.73 +/- 1.99 (p=0.72) respectively at day 11. The amount of ng LTE_4 per mmol creatinine was 8.5 +/- 0.38 before treatment with MK886, and 8.51 +/- 3.64 in the placebo group. After treatment, the mean concentrations were 1.72 +/- 0.44 (p=0.02) and 7.345 +/- 1.9 (p=0.72) respectively.

Histological examinations

Results of staining with the different monoclonal antibodies can be seen in Table IV. No change in Ki-67-positive nuclei could be found. Keratin 10 and keratin 16 expression did not differ before and after treatment. In addition, the amount of T-lymphocytes, Langerhans cells and CD14-positive cells remained unchanged. The number of epidermal polymorphonuclear leukocytes (PMN) reduced in the MK886-

Table II: LTB_4 in psoriatic biopsies (pg LTB_4/mg wet tissue), mean +/- sem

	before treatment	after treatment	p
MK886	11.6 +/- 1.85	12.6 +/- 3.70	0.50
Placebo	24.8 +/- 10.60	13.6 +/- 3.03	0.14

Table III: LTE_4 excretion in urine (ng LTE_4/hour), mean +/- sem

	before treatment	after treatment	p
MK886	8.50 +/- 0.38	1.72 +/- 0.44	0.02
Placebo	8.51 +/- 3.64	7.33 +/- 1.99	0.72

treated biopsies reaching borderline significancy (p=0.05), whereas epidermal PMN in the placebo treated biopsies remained unchanged.

DISCUSSION

Eicosanoids are mediators of inflammation and are generated from arachidonic acid, which is present in the plasma membrane[25]. Three different enzymes produce 3 groups of eicosanoids: via 12-lipoxygenase 12-HETE is produced, via cyclo-oxygenase prostaglandins and thromboxane are produced, and via 5-lipoxygenase, leukotriene A_4, B_4, C_4, D_4 and E_4 are generated (figure 1). Based on former investigations, leukotriene B_4 seems to be of importance in the pathogenesis and maintenance of psoriasis[1,3,26]. LTB_4 has been measured in biologically active amounts in chronic plaque skin lesions (33.1 +/- 9.7 ng/g wet tissue in scale and 5.3 +/- 2.0 ng/g wet tissue in lesional skin without scale)[27]. This LTB_4 appears to be derived from both epidermis and PMN[1,25,26]. Arachidonic acid and its metabolites have been

Table IV: Immunohistochemical staining, results of proliferation (positive nuclei per mm length of section), keratinization and inflammation (score 0-6), mean +/- sem.

	Antibody	Before treatment MK886	Before treatment Placebo	After treatment MK886	After treatment Placebo
PROLIFERATION	Ki-67 (nuclear antigen)	56 +/- 7	67 +/- 10	62 +/- 7	59 +/- 11
KERATINIZATION	Ks8.12 (keratin 16)	3.7 +/- 0.4	4.7 +/- 0.3	4.0 +/- 0.3	4.2 +/- 0.5
	RKSE60 (keratin 10)	4.0 +/- 0	3.4 +/- 0	4.0 +/- 0	3.6 +/- 0.6
INFLAMMATION	EPIDERMAL				
	Anti-elastase (PMN)	2.3 +/- 0.5	2.5 +/- 0.5	1.4 +/- 0.3	2.4 +/- 0.6
	T11 (T-lymphocytes)	2.3 +/- 0.1	2.3 +/- 0.3	2.3 +/- 0.2	2.6 +/- 0.2
	WT14 (CD14 + cells)	1.4 +/- 0.2	1.7 +/- 0.2	1.6 +/- 0.2	1.8 +/- 0.1
	OKT6 (Langerhans cells)	2.8 +/- 0.3	2.4 +/- 0.2	2.8 +/- 0.3	2.2 +/- 0.3
	DERMAL				
	Anti-elastase (PMN)	1.1 +/- 0.3	1.3 +/- 0.3	1.0 +/- 0.2	1.1 +/- 0.2
	T11 (T-lymphocytes)	3.0 +/- 0.2	3.3 +/- 0.2	3.3 +/- 0.3	3.1 +/- 0.2
	WT14 (CD14 + cells)	1.9 +/- 0.1	2.0 +/- 0	2.0 +/- 0	2.0 +/- 0
	OKT6 (Langerhans cells)	2.0 +/- 0	2.0 +/- 0	2.0 +/- 0	1.9 +/- 0.1

quantitated in skin chamber fluid from lesional and non-lesional psoriatic skin[28,29]. In this model 12R-HETE and LTB_4 are the most abundant products found. LTB_4 is one of the most potent chemoattractants for PMN, 12R-HETE is less potent but is still a highly active neutrophil chemoattractant. Benoxaprofen which is a 5, 12, and 15-LO inhibitor, and a cyclooxygenase inhibitor (the mechanism is unclear) produced amelioration of psoriasis, however side effects limited continued study of this compound[12].

This study investigated the effect of a potent oral 5-lipoxygenase inhibitor on clinical scores and histological parameters of proliferation, keratinization and inflammation, LTB_4 levels in psoriatic lesions and urinary LTE_4 excretion. Baseline levels of LTB_4 in skin biopsies and urinary LTE_4-excretion were comparable in the MK886 and placebo-treated group. LTB_4 in the psoriatic lesions before treatment with MK886 were lower than values reported by Duell et al.[20] who did not remove scales, a source of skin LTB_4[27], but higher than the values reported by Fogh et al.[27]. In conclusion, no significant changes in clinical scores were seen. In accordance with these findings, no change in the amount of LTB_4 in psoriatic lesions was measured. However a statistically significant decrease in urinary LTE_4 was seen. All parameters for proliferation, keratinization and inflammation remained unchanged after therapy, and no statistically significant difference between MK886 and placebo treated patients could be seen. Only the number of epidermal PMN decreased and reached borderline significance.

Benoxaprofen appeared to be clinically effective[11,12] and inhibits the migration and motility of PMN isolated from peripheral blood in vitro[30]. Its effects on histological parameters was not investigated. As benoxaprofen is not a specific 5-LO inhibitor, other mechanisms might be involved in its antipsoriatic potential. It is of interest that in this study a decrease in epidermal PMN was found, whereas the amounts of LTB_4 in skin biopsies did not decrease. It is possible that MK886 reached concentrations in skin that were not sufficient to produce a statistically significant decrease of LTB_4, while an effect on PMN migration could have occured via an alternative mechanism. In this respect it might be of relevance that MK886 reaches peak drug levels at 1-2h post dose, and that the elimination half- time of MK886 is relatively short[17]. The concentration of MK886 in peripheral blood was sufficient to inhibit systemic leukotriene synthesis as reflected in the $>$ 70% decrease in urinary LTE_4

excretion. This decrease in the systemic level of leukotrienes may have had an effect on PMN in peripheral blood, thereby altering their migration into the skin.

In the study of Black et al[13] a topical 5-lipoxygenase inhibitor decreased the amount of LTB_4 in skin chamber fluid in treated psoriatic lesions. A clear clinical effect was also shown. In their study, the decrease in the amount of LTB_4 diminished before a clinical effect was seen. The clinical improvement was statistically significant after 14 days, compared to the vehicle treated lesions. Although topical application of a 5-lipoxygenase inhibitor can induce substantial clinical improvement[13], the present study demonstrates that short term systemic administration of a selective 5-LO inhibitor, which produced a >70% inhibition of systemic leukotriene biosynthesis does not result in an anti-psoriatic effect. As MK886 is not a direct inhibitor of 5-LO, but prevents its activation by binding to FLAP this might be an explanation for the differences in clinical effect between this study and the study of Black et al[13]. Another explanation might be the relatively short treatment period of 10 1/3 days. Otherwise, it is possible that the LTB_4-like material found in the skin is not derived from the 5-LO-pathway, but may be derived either through lipid peroxidation or through a cytochrome P450-catalyzed pathway. Further investigation in this area is warranted.

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3.6 EFFECT OF TOPICAL TREATMENT WITH BUDESONIDE (PREFERID[R]) ON CLINICAL SCORES, AND ON PARAMETERS FOR PROLIFERATION, KERATINIZATION AND INFLAMMATION IN PSORIASIS.

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SUMMARY

Corticosteroids are important in the treatment of inflammatory dermatoses, such as psoriasis. They have anti-inflammatory, anti-proliferative and immunosuppressive actions. In order to elucidate which of these working principles are of relevance for corticosteroid treatment in psoriasis, six patients were treated for three weeks with Budesonide 0.025% ointment (Preferid[R]).
Clinical effects were recorded using the PASI-score (Psoriasis Area and Severity Index). Biopsies from psoriatic lesions were taken before and during treatment and processed for immunohistochemistry. Antibodies were used to assess epidermal proliferation (Ki-67 binding) and keratinization (keratin 16 (Ks8.12) and keratin 10

(RKSE60)). In addition, inflammatory cells were stained; T-lymphocytes (T11), monocytes/macrophages (WT14), polymorphonuclear leukocytes (PMN, anti-elastase) and Langerhans cells (OKT6).

PASI-scores reduced significantly after one week and three weeks of treatment. Epidermal proliferation decreased after one week already, whereas the inflammatory infiltrate reduced in a later stage of treatment (after 3 weeks).

Keratinocyte hyperproliferation is an important target of corticosteroid treatment in psoriasis. The relative long persistance of the infiltrate cells may explain the relapse seen after corticosteroid therapy.

INTRODUCTION

Corticosteroids have been of much benefit in the treatment of inflammatory dermatoses. They posess anti-proliferative, anti-inflammatory and immunosuppressive properties[1,2]. Their working mechanism is mediated via a cytoplasmatic receptor and transportation to the nucleus, which results in transcription of DNA to mRNA and translation of mRNA to proteins. In addition a non-nuclear working mechanism has been postulated[3-5].

Topical application of corticosteroids in the treatment of chronic plaque psoriasis has proven to be an effective treatment[6]. While used for chronic dermatoses, local and systemic side effects (dermal atrophy, suppression of adrenal function) form an important disadvantage. Therefore, the search for potent formulations with few local and/or systemic side effects still goes on.

One of these new generation corticosteroids is Budesonide (Preferid^{R}) 0.025% ointment[7-14], which has shown to be at least as effective as betamethasone-17-valerate 0.01% ointment[9], and to have little effect on plasma cortisol levels and urinary cortisol excretion[14]. In the present investigation, the effect on clinical parameters of topical treatment with Budesonide 0.025% ointment has been investigated in six patients with chronic plaque psoriasis at the out-patient department.

The effects of corticosteroid treatment on histological and biochemical parameters in epidermis and dermis have been investigated previously[1,15-18]. In psoriasis, epidermal hyperproliferation, disturbed keratinization and inflammation are important and well-

known aberrations. More recently, it became clear that various cytokines play an important role in homeostasis and in pathology of skin. In psoriasis, interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-8 (IL-8), tumour necrosis factor-α (TNFα), and transforming growth factor-ß (TGF-ß) seem to play an important role in the pathogenesis and regression of a lesion[19-23]. How these cytokines are involved in the antipsoriatic action of corticosteroids remains to be solved.

In this study, the effect of treatment with Budesonide 0.025% ointment on histological parameters for proliferation and keratinization were studied, using antibodies against an epitope on the nucleus of proliferating keratinocytes (Ki-67), against keratin 13/16 (Ks8.12) and keratin 10 (RKSE60). The inflammatory cells were analyzed using monoclonal antibodies against T-lymphocytes (T11), monocytes/macrophages (WT14), polymorphonuclear leukocytes (PMN) (anti-elastase) and Langerhans cells (OKT6).

MATERIALS AND METHODS

Patients

Six patients with chronic plaque psoriasis participated in the study, one male and five females. Their age varied from 24 to 67 with an average of 47.5 years. They had used no systemic treatment for at least two months, and no topical treatment for at least two weeks. Patients applied Budesonide ointment 0.025% (Preferid^{R}, Gist Brocades, Delft, The Netherlands) twice daily thinly and evenly to the psoriatic lesions during three weeks. Clinical scores were recorded before treatment, after one week and after three weeks of treatment for the lesions on the upper extremities, trunk and lower extremities. Erythema (E), infiltration (I) and scaling (S) of the lesions were assessed using a 5-point scale: 0= no cutaneous involvement, 1= slight, 2= moderate, 3= severe, 4= severest possible involvement. The area (A) of the body surface that was involved was recorded using a 7-point scale: 0= no involvement, 1= <10%, 2= 10-29%, 3= 30-49%, 4= 50-69%, 6= 70-89%, 6= 90-100% of the body surface involved.

The PASI-score was calculated as follows:

upper extremities: 0.2 x A(E+I+S) +

trunk: 0.3 x A(E+I+S) +

lower extremities: 0.4 x A(E+I+S)

Punch biopsies (ϕ 4 mm) were taken from a lesion before treatment, after one week and after three weeks of treatment.

Immunohistochemical stainings

The biopsies were immediately embedded in Tissue Tek OCT Compound (Miles Scientific USA) and snap frozen in liquid nitrogen. Slides were cut of 7 μm, fixed in acetone or in acetone/ether 60/40% (Ki-67 staining) for 10 minutes and stored at -80 °C until use. Staining to assess epidermal proliferation, keratinization and epidermal and dermal inflammation, was carried out using the following antibodies:

-Ki-67 (Dakopatts, Copenhagen, Denmark): binds to a nuclear antigen present in cycling cells

-Ks8.12 (Sigma, St Louis, USA): binds to keratin 13 and 16. Keratin 16 is expressed by hyperproliferative keratinocytes[19]. Keratin 13 is not present in adult human skin[20].

-RKSE60 (a gift of F. Ramaekers): binds to keratin 10. Keratin 10 is expressed by differentiated keratinocytes.

-Anti-elastase (Serotec, Oxford, England): staining leukocyte elastase, present in polymorphonuclear leukocytes (PMN)

-T11 (Dakopatts, Copenhagen, Denmark): stains CD2, present on T-lymphocytes

-OKT6 (Ortho Diagnostic Systems, Raritan, USA): stains CD1a, present on Langerhans cells

-WT14 (Dept. Medicine, Div. Nephrol. University Hospital Nijmegen): directed against CD14, present on monocytes and macrophages[21].

A direct peroxidase technique was used to visualize PMN. An indirect peroxidase technique was used for staining with the antibodies Ki-67, Ks8.12 and RKSE60. Slides were incubated for 30 minutes with the antibodies in a dilution of 1:100, 1:40, 1:25 and 1:10 respectively. After washing in phosphate buffer the slides were incubated with a solution of rabbit-anti-mouse-immunoglobulin conjugated with

peroxidase 1:50 in phosphate buffer containing 5% human AB-serum for 30 minutes, except for the anti-elastase antibody. After 3 washing steps in phosphate buffer and preincubation in sodium acetate buffer pH 4.9, slides were stained in a solution of 3-amino-9-ethyl-carbazole (AEC) in sodium acetate buffer containing 0.01% H_2O_2 for 10 minutes. All slides were counterstained with Mayer's haematoxylin (Sigma, St Louis, USA) and mounted in glycerin gelatin.

Staining with T11, OKT6 and WT14 was carried out using a peroxidase-anti-peroxidase technique. Slides were incubated for 60 minutes with the monoclonal antibodies in a dilution of 1:100. After washing in phosphate buffer the slides were incubated with rabbit-anti-mouse-immunoglobulin (RAM-Ig, Dakopatts, Copenhagen, Denmark) 1:25 in phosphate buffer for 20 minutes. This was followed by a washing step and incubation with peroxidase-anti-peroxidase complexes (PAP-complexes, Dakopatts, Copenhagen, Denmark) 1:100 in phosphate buffer for 20 minutes. Once more the slides were incubated with RAM-Ig, followed by PAP-complexes. The slides were stained in AEC-solution in sodium acetate buffer and H_2O_2 and counterstained with Mayer's haematoxylin. Finally they were mounted in glycerin gelatin.

Scores

Histological changes in the epidermal and dermal compartment were assessed using a semi-quantitative scale:

Epidermis: 0= no staining, 1= sporadic staining, 2= minimal staining, 3= moderate staining, 4= moderate-pronounced staining, 5= pronounced staining, 6= whole epidermis stained.

Dermis: 0= no stained cells, 1= sporadic, 2= 1-25% of the infiltrate cells stained, 3= 26-50%, 4= 51-75%, 5= 76-99%, 6= 100%.

Ki-67-positive nuclei were counted per mm length of epidermis.

Statistical analysis

The Wilcoxon ranking test for matched pairs was used for the statistical analysis.

Clinical response

The clinical response to Preferid treatment is summarized in figure 1. The treatment was well-tolerated. No local adverse effects were seen. After one week of treatment, the PASI-score had diminished in a statistically significant manner ($p<0.03$) from 5.36 +/- 0.98 (mean +/- sem) to 3.63 +/- 0.67. After three weeks of treatment, the average PASI-score measured 2.12 +/- 0.58, $p<0.03$ compared to before treatment.

Immunohistochemical observations

Scores before treatment, after 1 week of treatment and 3 weeks after treatment in the epidermis were as follows. The number of Ki-67 stained nuclei diminished from 153 +/- 24 to 44 +/- 10 after one week of treatment ($p<0.03$). After three weeks, their numbers had diminished further to 30 +/- 11 per mm length of epidermis ($p<0.03$,

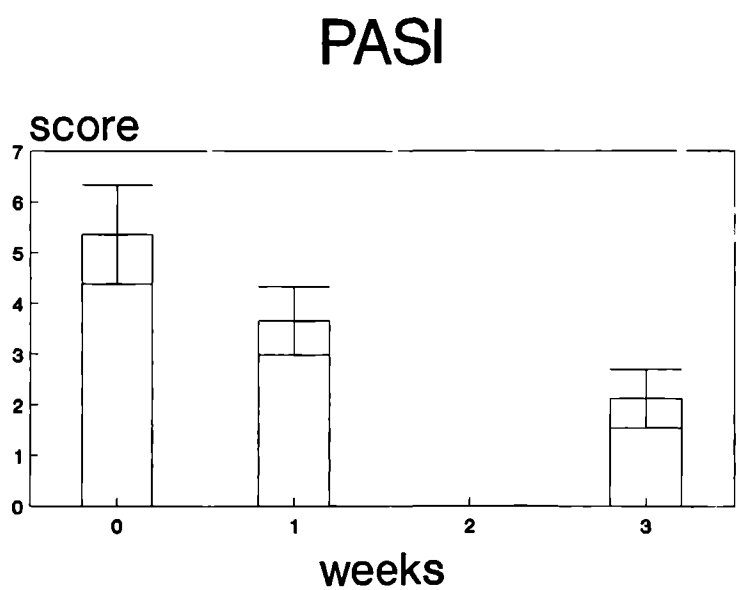

Figure 1. PASI-scores (+/- SEM) before treatment, after one week and three weeks of treatment (n=6). * = first statistically significant reduction.

Table I. Markers for epidermal proliferation (means +/- SEM).

Antibody	t = 0	t = 1 week	t = 3 weeks
Ki-67	153 +/- 24	44 +/- 10	30 +/- 11
Ks8.12	4.5 +/- 0.6	2.5 +/- 0.8	2.8 +/- 1.8

Table I). Staining with Ks8.12 (keratin 16) diminished significantly after one week ($p < 0.03$) and continued to decline between one and three weeks of treatment (Table I). In contrast, keratin 10 showed no significant changes during treatment with Budesonide. Epidermal PMN, T-lymphocytes, and Langerhans cells did not show any statistically significant change. Otherwise, epidermal WT14-stained cells diminished in a statistically significant manner ($p < 0.03$) after three weeks.

In the dermal compartment, the presence of the infiltrate declined between 0 and 3 weeks ($p < 0.05$). The number of PMN diminished already after one week significantly ($p < 0.05$), in contrast to the percentage of T-lymphocytes, WT14 stained cells and Langerhans cells, which remained unchanged during the observation period.

DISCUSSION

Corticosteroids are very effective in the treatment of psoriasis, and possess anti-proliferative, anti-inflammatory and immunosuppressive actions. In this study, the clinical effectiveness of Budesonide 0.025% ointment was confirmed[9-13]. After one week of treatment, already a statistically significant reduction in PASI-score was accomplished, which extended during two weeks of further treatment.

It is not clear, which of the three working principles of corticosteroids are of relevance for the antipsoriatic potential. The order in which markers for proliferation, keratinization and inflammation change during treatment with corticosteroids, might provide further information on this issue. In the present investigation, biopsies

from psoriatic lesions were taken before treatment, after one week and after three weeks of treatment with Budesonide 0.025% ointment.

The early decline of proliferating keratinocytes (Ki-78 positive nuclei) and of keratin 16 expression after one week, indicates that keratinocyte proliferation is an important target of corticosteroid treatment in psoriasis. Expression of keratin 10 did not change during this observation period. In addition, epidermal inflammatory cells (T-lymphocytes, PMN) and Langerhans cell numbers remained unchanged. In contrast, the number of WT14 staining cells (monocytes/macrophages) diminished in the epidermis after three weeks of treatment. Epidermal accumulation of PMN is suggested to be important in the anti-psoriatic working mechanism of corticosteroids[24]. The authors reached this conclusion based on an in vivo model for PMN migration. In the present investigation, although epidermal PMN remained constant during the first week of treatment, a dramatic decrease of markers for proliferation was observed. Therefore, it is likely that epidermal PMN migration is not an early target for corticosteroid treatment in psoriasis.

The dermal inflammatory infiltrate diminished between one and three weeks of treatment. The relative persistance of dermal changes despite clinical success resulting from Budesonide treatment might provide an explanation for the relatively fast relapse following cessation of treatment with corticosteroids[25-27].

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3.7 KERATIN 17: A USEFUL MARKER IN ANTI-PSORIATIC THERAPIES.

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SUMMARY

Keratin 17 is not present in normal epidermis but occurs in the hair follicle, in myoepithelial cells, in psoriatic scalp, and in basal cell carcinomas.
The aim of the present study was to investigate to what extent this keratin subtype was present in chronic plaque psoriasis, and to evaluate whether it's staining pattern changed during antipsoriatic treatment, using the antibody E3 on frozen sections.
In 6 patients suffering from psoriasis, biopsies were taken before and after treatment with anthralin or with the vitamin D_3 analogue MC903. Clinical evaluations were carried out using the PASI-score.

In all pretreatment biopsies pronounced staining was found high in the suprabasal compartment, while after treatment no staining was found in 4 out of 6 patients. One treated patient showed sporadically cells expressing keratin 17, and one patient a moderate staining of the suprabasal compartment after treatment. This latter patient showed the smallest reduction in PASI-score.
This study suggests that keratin 17 is a useful marker to monitor success of anti-psoriatic therapies.

INTRODUCTION

The investigation of changes that take place during development or therapeutical regression of psoriatic lesions can give us a more clear insight into processes which are of importance in the pathogenesis and treatment of this disease.
In previous studies, different parameters have been used to investigate increased epidermal cell proliferation and disturbed differentiation in psoriasis. The histological appearance[1-3], percentage of cells in SG_2M-phase[4], incorporation of ^{3}[H]-thymidine[5], or BrdUrd[6], Ki-67-staining[6,7], and up- or down-regulation of distinct keratins[8] have been used as parameters.
The aim of the present study was to further investigate the effect of anti-psoriatic therapies on keratin expression in human epidermis. In psoriasis, keratins are expressed that can not -or only in small amounts- be found in normal human skin. Also down-regulation of keratins which are related to differentiation takes place[9].
Assessing the amount of keratins no. 2, 10, 16 and 18 in psoriatic skin proved to be a useful approach in monitoring therapies. Especially keratin 16 correlates well with progression or regression of the lesions[8,10].
Keratin no. 17 is a constituent which is not present in normal human epidermis. In skin it is restricted to the lower part of the outer root sheath of the hair follicle, and to myoepithelial cells of the sebaceous gland. It is also expressed by basal cell carcinomas and might be associated with hyperproliferation[11,12]. Recently the expression of keratin 17 was shown in lesional psoriatic epidermis of the scalp[13].

In this study the presence and localization of keratin 17 in psoriatic lesions was further investigated and the effect of anti-psoriatic therapies on keratin 17 expression in such lesions was studied using monoclonal antibody E3 on frozen sections.

MATERIALS AND METHODS

Six patients suffering from psoriasis participated in this study. They had not received topical treatment for at least two weeks, and no systemic treatment for at least two months. Three patients were treated with anthralin in a cream-base and three patients were treated with the new vitamin D_3-analogue MC903. Clinical response was measured using the PASI-score. Biopsies from psoriatic lesions were taken before therapy and after 8 weeks of anthralin-treatment or after 12 weeks of MC903-treatment. The biopsies were snap-frozen in liquid nitrogen, and stored at -80°C. Frozen sections of 7 μm were cut and fixed in acetone (4°C, 10 minutes). Staining with the mouse monoclonal antibody E3 to keratin 17[11] was performed using a dilution of 1:10 in phosphate buffered saline (PBS). Incubation for 30 minutes at room temperature in the dark was followed by 3 washing steps with PBS. Thereafter the sections were incubated for 30 minutes with a peroxidase-conjugated rabbit-anti-mouse antibody (Dakopatts, Copenhagen, Denmark) diluted 1:50 in PBS, containing 5% human AB-serum. After 3 more washing steps in PBS and preincubation with sodium acetate buffer (pH 4.9) E3 binding was visualized in sodium acetate buffer containing 200 mg/l 3-amino-ethyl-carbazole (AEC) and 80 μl/l H_2O_2. Finally slides were counterstained with Mayers haematoxylin and mounted in Kaisers glycerin gelatin.

RESULTS

Results of the staining reactions with the antibody E3 in untreated psoriatic skin and in anthralin or MC903 treated psoriasis are shown in Table I. Staining with this keratin 17 antibody, showed a consistent pattern in all biopsies of untreated psoriatic skin. Keratin 17 was present in the upper layers of the suprabasal compartment, and

Table I. PASI-scores and keratin 17 expression before and after treatment.

Patient	Treatment	PASI before	PASI after	Keratin 17 before	Keratin 17 after
1	anthralin	20	6	pronounced expression	no expression
2	anthralin	5	0.5	pronounced expression	no expression
3	anthralin	27	8	moderate-pronounced expression	no expression
4	MC903	7.2	1.8	pronounced expression	sporadically present
5	MC903	5 4	3	pronounced expression	moderate expression
6	MC903	5 8	1.8	pronounced expression	no expression

was evenly distributed over the total length of the sections (figure 1A). Post-treatment biopsies showed a clear reduction in keratin 17 (figure 1B). In four out of six patients no keratin 17 expression could be found at all after treatment (three patients treated with anthralin, one patient treated with MC903). In one patient only sporadic keratin 17 positive cells were present after therapy, while the remaining patient still showed considerable staining for keratin 17 in the post-treatment biopsy. This patient also showed the smallest reduction in PASI-score. The presence of keratin 17 was most pronounced in areas that showed distinct hyperparakeratosis. No keratin 17 expression was seen in the basal layers of the epidermis.

All patients showed a marked improvement of their psoriatic lesions during the treatment period. PASI-scores are also shown in Table I. The average reduction in PASI-score was 8.2, varying from 2.4 to 19.

DISCUSSION

Keratin expression in different epithelia has been investigated extensively during the last decade. Various types of epithelia show different patterns of keratin expression. In addition, many tumors and cultured cell lines partly retain the keratin composition and distribution of their tissue of origin[14].

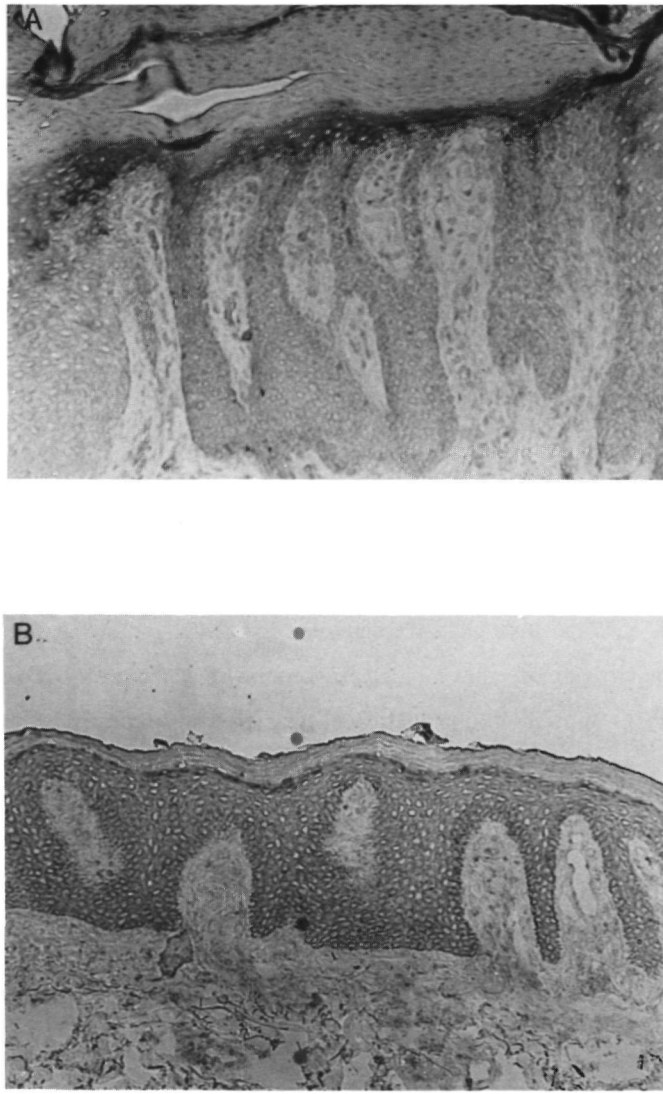

Figure 1: A: E3 staining reaction showing keratin 17 in an untreated lesion of patient 1 (x250), B: E3 staining reaction after 8 weeks of treatment with anthralin in the same patient (x250). Bar = 40 μm.

In this study the presence and localization of keratin 17 in psoriatic skin was investigated, using the monoclonal antibody E3. In addition, changes in keratin 17 content in response to antipsoriatic therapy were examined.

In all biopsies of untreated psoriatic lesions, E3-staining was observed in the most superficial layers of the living epidermis, just underneath the stratum corneum. Keratin 17 is not present in normal interfollicular epidermis, but can be found in the outer root sheath of the hair follicle and in myoepithelial cells of the sebaceous gland. Also in other epithelia exclusive staining of the basal cell compartments was reported[11]. Keratin 17 is also expressed in basal cell carcinomas which has led to the hypothesis that basal cell carcinomas have a follicular origin[12]. In earlier studies also a correlation of keratin 17 expression and hyperproliferation has been suggested[12]. The results of the present investigation suggest an association with abnormal terminal differentiation of keratinocytes in psoriasis, since keratin 17 is seen exclusively in the upper layers of the stratum spinosum.

During treatment with anthralin and treatment with MC903, also the expression of keratin 16 is reduced[10,15], a well established marker for hyperproliferation of keratinocytes[16]. Based on immunohistochemical studies using the antibody Ks8.12, keratin 16 was found to be present not only in the most superficial layers of the psoriatic lesion, but in the entire suprabasal compartment. Following treatment Ks8.12 staining was largely diminished[7,10,15]. Treatment with anthralin or MC903 proved to result in an even more marked decrease of keratin 17 expression, which correlated well with the clinical response.

The present study suggests that keratin 17 is a useful and sensitive marker to monitor improvement of psoriatic lesions.

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CHAPTER 4

EPIDERMAL GROWTH AND INFLAMMATION IN PSORIASIS-RELATED SKIN DISORDERS

This chapter is based on the following publications:

1. De Jong EMGJ, Rulo HFC, van de Kerkhof PCM. Inflammatory Linear Verrucous Epidermal Nevus (ILVEN) versus linear psoriasis. A clinical, histological and immunohistochemical study. Acta Derm Venereol (Stockh) 1991; 71: 343-346.
2. Van de Kerkhof PCM, de Jong EMGJ. Topical treatment with the vitamin D_3 analogue MC903 improves pityriasis rubra pilaris: a clinical and immunohistochemical observation. Br J Dermatol 1991; 125: 293-294.
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4.1 INFLAMMATORY LINEAR VERRUCOUS EPIDERMAL NEVUS (ILVEN) VERSUS LINEAR PSORIASIS; A CLINICAL, HISTOLOGICAL AND IMMUNOHISTOCHEMICAL STUDY.

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SUMMARY

Inflammatory Linear Verrucous Epidermal Nevus (ILVEN) has been suggested to be a separate disease entity. However, the delineation from linear psoriasis has been discussed in the literature of the last decades.

The aim of the present study was to investigate in addition to the clinical and histological criteria, the immunohistochemical aspects of inflammation, epidermal proliferation and keratinization. From a clinical and histological point of view ILVEN and psoriasis, according to the established criteria, proved to overlap. The immunohistochemical study suggest that the following procedures have an additional diagnostic impact: assessment of elastase positive cells, assessment of keratin 16 and of keratin 10.

INTRODUCTION

Since the first description of inflammatory linear verrucous epidermal nevus (ILVEN) by Unna[1], various investigators endeavoured to formulate both clinical and histopathological criteria to delineate ILVEN from psoriasis[2,3].

The clinical appearance of ILVEN varies from lichenoid to psoriasiform, from scaling to markedly verrucous[2,4,5]. The nosology becomes even more complex by the observation that linear lesions might coexist with typical psoriatic lesions[6].

At the histological level the alternation of orthokeratosis and parakeratosis has been suggested to be typical for ILVEN, although such pattern can be seen in psoriasis as well. On the other hand, micropustules of Munro, a characteristic feature of the psoriatic lesion, can be seen in ILVEN.

In order to delineate ILVEN from linear psoriasis we investigated a group of 6 patients suffering from linear erythematous lesions with scaling and or a verrucous appearance.

In addition to clinical and histological description the aim of the present study was to assess inflammation, proliferation and keratinization using immunohistochemical methods.

MATERIALS AND METHODS

CASE REPORTS

Clinical features of the patients are summarized in table I. Table II specifies the histological observations.

Case 1

The first patient was a 77-year-old female who had had extensive symmetrical erythematosquamous papular lesions all over her body ever since she was 5 years old. On her right arm, right upper leg and right half of the thorax the lesions were linearly arranged. The patient suffered from severe pruritus. In some biopsies the histopathological findings were compatible with ILVEN and in other biopsies with

psoriasis. Treatment with PUVA and dithranol did reduce psoriasis, but failed to influence ILVEN. Multiple excisions of linear lesions were carried out.

Case 2

The second patient was a 28-year-old female with erythematous hyperkeratotic papular eruptions in the right groin and on the vulva. These lesions, which had been present since birth, caused severe itching and were both clinically and histopathologically classified as a linear verrucous nevus. Topical treatment with all-trans-retinoid acid, solutio carbonis detergens, corticosteroids, or treatment with argon laser or cryotherapy, did not bring any positive effect.

Case 3

The third patient was a 28-year-old female with slightly scaling linear erythematosquamous papules on the right half of the trunk extending into the medial right leg (Figure 1). Pruritus was absent. Clinically the lesions, which were present during one year, were compatible with lichen striatus. Histopathologically the lesions could not be designated with certainty as ILVEN or lichen striatus. Without any therapy, a partial regression of the lesions was seen.

Case 4

The fourth patient was a 37-year-old male with linear erythematosquamous lesions, which were diagnosed as ILVEN. The lesions appeared at the age of 24 and were localised on the left arm and axilla. Severe pruritus was experienced. Cryotherapy and several topical therapies did not improve his condition. No spontaneous regression was seen. Multiple excisions reduced the extent of the lesions.

Case 5

The fifth patient was a 26-year-old female who had a one years history of ILVEN-like lesions on the left arm and left half of the thorax following the lines of Blaschko. These lesions caused minimal pruritus. Histologically the diagnosis lichen striatus was made. Treatment with all-trans-retinoic acid caused much irritation, but smoothened the surface of the lesions. A partial regression was seen.

Case 6

The sixth patient was a 46-year-old male who had slightly itching linear erythematosquamous lesions on his left leg and trunk since the age of 14 (Figure 2). He also had signs of classical psoriasis. Histologically both diagnoses could be confirmed. Dithranol reduced the psoriatic lesions. The linear verrucous lesions did not respond.

IMMUNOHISTOCHEMICAL INVESTIGATIONS

In each patient a punch biopsy (3mm) was taken from the linear lesions and snap frozen in liquid nitrogen. Staining was performed using the following antibodies in order to characterize inflammation and proliferation. A direct or indirect peroxidase technique, or a peroxidase-anti-peroxidase technique was used on acetone-fixed slides of 7 μm.

Inflammation

Anti-elastase (Serotec, Oxford, England) to assess elastase, a marker enzyme for polymorphonuclear leukocytes (PMN)

T11 (Dakopatts, Copenhagen, Denmark) to assess T-lymphocytes (CD2)

WT14 (Dept. Medicine, Div. Nephrol., University Hospital Nijmegen) to assess CD14-positive cells (monocytes and macrophages)[7],

OKT6 (Ortho Diagnostic Systems, Raritan, USA) to assess Langerhans cells (CD1a)

Stained cells in the epidermal compartment were assessed using a scale ranging from 0 to 4 points: 0 = no expression, 1 = sporadic expression, 2 = minimal expression, 3 = moderate expression, 4 = pronounced expression.

Stained cells in the dermis were expressed as a percentage of the total number of infiltrate cells: 0 = no cells stained, 1 = sporadically cells stained, 2 = 1-25% of the infiltrate cells stained, 3 = 26-50% of the infiltrate cells stained, 4 = 51-75% of the infiltrate cells stained, 5 = 76-99% of the infiltrate cells stained, 6 = 100% of the infiltrate cells stained.

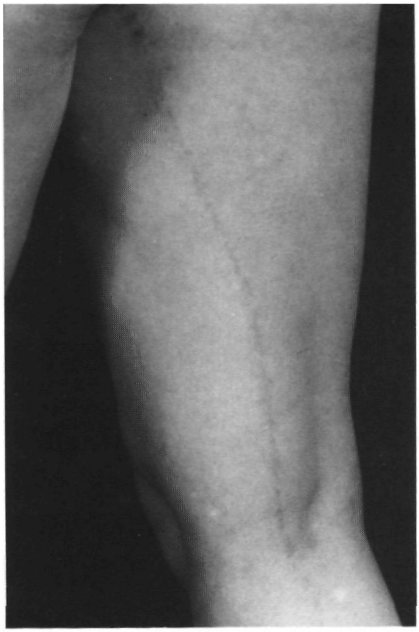

Figure 1. Linear lesion on the right leg of patient 3.

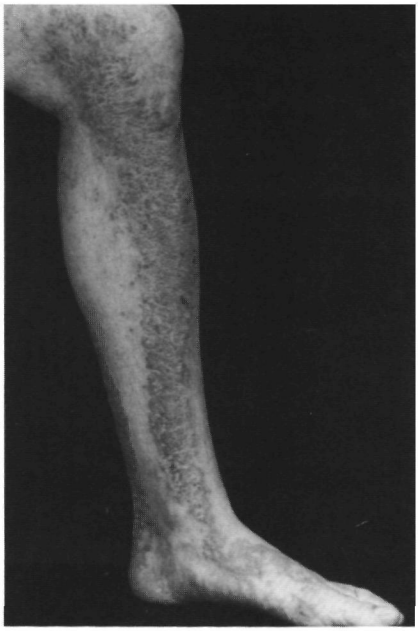

Figure 2. Linear lesion on the left leg of patient 6.

Proliferation:

Ki-67 (Dakopatts, Copenhagen, Denmark) was used to visualise nuclei of cycling cells

The number of Ki-67 positive nuclei was counted per mm length of epidermis.

Keratinization:

Ks8.12 (Sigma, St. Louis, USA), an antibody directed against keratin 13 and 16. Keratin 16 is present in hyperproliferative epidermis[8], whereas keratin 13 is absent in human adult skin[9].

RKSE60 (a generous gift of Dr. F.C.S. Ramaekers), directed against keratin 10, present in differentiated epidermal cells.

Staining with Ks8.12 and RKSE60 were scored using the same scale as used for inflammation-parameters in the epidermal compartment. Staining of suprabasal (differentiated) and basal (undifferentiated) compartment were assessed separately.

Table I. Clinical Characteristics

Pat.	Age of onset (years)	Duration (years)	Refractoriness to treatment	Spontaneous regression	F/M	Localization	Pruritus	Psoriatic lesions
1	5	72	yes (trunk)	no	F	right arm + leg	yes	yes
2	0-1	28	yes (upper leg)	no	F	vulva + right groin	yes	no
3	27	1	not treated	partial	F	right abdomen	no	no
4	24	13	yes	no	M	left arm + axilla	yes	no
5	25	1	?	partial	F	left arm + trunk	slight	no
6	14	32	partly (trunk)	no	M	left leg + trunk	yes	yes

Table II. Histological characteristics

Patient	Papillomatosis	Acanthosis	Epidermal PMN	Hyperkeratosis ortho/para	Inflammatory infiltrate	Remarks	Histological diagnosis
1a	+	+	+	+/+	pronounced		ILVEN
1b	+	+	+	+/+	moderate		psoriasis
2	+/-	+	-	+/+	moderate		ILVEN
3	+/-	+	-	+/+	band-like, pronounced	saw tooth	ILVEN/lichen striatus
4	+ +,-	+ +,-	-	+/+	mild, exocytosis	follicular plugging	ILVEN
5	-	+,-	-	+/-	band-like, pronounced	saw tooth	lichen striatus
6a (Verrucous lesion)	+	+ +	-	+/+	moderate		linear porokeratosis
6b (Psoriatiform lesion)	+	+	+	+/+	mild		ILVEN/-psoriasis

RESULTS

The results of the immunohistochemical investigations are presented in table III.
Staining with the monoclonal antibody Ks8.12, assessing keratin 16, showed foci of suprabasal staining coinciding with areas of parakeratosis in 3 out of the 4 patients suffering from ILVEN (patients 1,4,6). In the lesions diagnosed as lichen striatus (patients 3 and 5), a diffuse suprabasal staining was found in patient 5, the focal pattern was seen in the other patient.
The number of Ki-67 positive nuclei was increased slightly to markedly in all biopsies as well in ILVEN as in lichen striatus. Both conditions could not be differentiated using this marker. In contrast to the focal distribution pattern of the hyperproliferation related keratin 16 in the suprabasal compartment, the germinative zone showed an equal distribution of Ki-67 positive nuclei.
Keratin 10 was equally distributed in the suprabasal compartment, only in one biopsy of a psoriatiform lesion (6b) distinct areas, overlying dermal papillae showing less intense staining were seen.
Infiltrate analysis revealed a consistent composition in all biopsies. However, in patient 6 who had a superimposed psoriasis, the occurrence of PMN in the more psoriasiform lesion was increased. The identity of the mononuclear infiltrate cells proved to be predominantly T-lymphocytes. CD14-positive cells and Langerhans cells were found in the dermal infiltrate as well as in the epidermal compartment.

DISCUSSION

Clinical criteria for the diagnosis Inflammatory Linear Verrucous Epidermal Nevus (ILVEN) have been described by Altman and Mehregan in 1971[2]. In a study of 25 patients they defined as diagnostic criteria: (i) early age of onset, (ii) a predominance in females (f : m = 4 : 1) (iii) frequent involvement of the left lower extremity, (iv) substantial pruritus, (v) persistency of the lesions and (vi) refractoriness to treatment.
From these criteria, criteria (ii) and (iii) have reported to be associated with ILVEN in larger surveys. In the individual patient, however, both criteria have a limited diagnostic value.

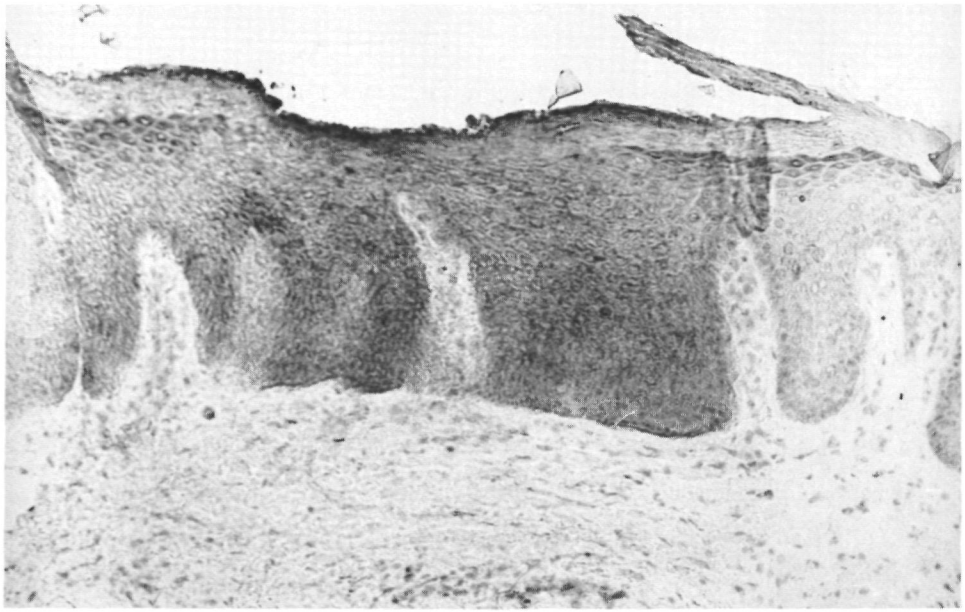

Figure 3. Immunohistochemical staining with the antibody Ks8.12 (keratin 16) showing a focal distribution pattern (x200).

If we analyze the clinical findings of the present series (table I) using the 4 discriminative criteria (i, iv, v, vi), 2 out of 6 patients (patients 1 and 2) appeared to be "classical cases" of ILVEN. Two out of 6 patients (patients 3 and 5) fulfilled to none of the 4 criteria, the clinical characteristics in these patients were compatible with the diagnosis lichen striatus[10]. The two remaining patients (patients 4 and 6) did show some of the criteria. Firstly a relatively late age of onset was mentioned by patients 4 and 6. However, relatively minor manifestations of ILVEN may have been overlooked by the patient. In patient 6 superimposed psoriasis is complicating the picture. Therefore, the partial regression mentioned, can be explained as a reduction of the psoriatic component.

Histological criteria for the diagnosis ILVEN have been defined by several authors[2,3]. Altman and Mehregan found in biopsies an inflammatory and psoriasiform appearance. Acanthosis, papillomatosis, spongiosis and exocytosis leading to spotty areas of parakeratosis were often seen. Sometimes small Munro-abscesses were found. Areas of parakeratosis with loss of the granular layer, and of orthokeratosis

Table IIIa. Immunohistochemical observations

	Proliferation	Keratinization			
Antibody	Ki-67 number/mm, mean +/- SEM	Ks8.12 suprabasal	basal	RKSE60 suprabasal	basal
Patients					
1	counting not possible	0-4	0-3	3.5	0
2	102.8 +/- 8.8	0-2.5	1	3.25	0
3	174.3 +/- 11.3	0-3	0-1	2	0
4	48.7 +/- 5.4	3	0	3	0
5	70.6 +/- 13.1	3	0	2	0
6a	224.5 +/- 26.7	2	2	2.5	0
6b	490.0 +/- 102.0	3.5	1	2	0

Table IIIb. Immunohistochemical observations. E = epidermal, D = dermal.

	Inflammation							
Antibody	Anti-elastase		T11		WT14		OKT6	
	E	D	E	D	E	D	E	D
Patients								
1	1	1	3	5	1	2	3	2
2	1	1	2.5	4	1	2	3	2
3	0	1	3	5	1	3	3.5	2
4	0	1	3	5	0	2.5	3	2
5	0	1	2.5	5	1	3	3.5	2
6a	0	0	3	3	0	2	2.5	2
6b	2	1.5	3	3	1	3	3	2

with a prominent granular layer with a sharp demarcation were seen. A mild perivascular infiltrate consisting mainly of T-lymphocytes was present. Dupré and Christol further developed these criteria. They stated that specific and non-specific lesions of ILVEN exist. Essential features of specific lesions were: alternation of hypergranulotic, depressed areas with overlying othohyperkeratosis, and of areas of agranulosis with overlying parakeratosis. Papillomatosis, acanthosis and a lymfohistocytic infiltrate were non-specific features.

In psoriasis the criteria designated as "non-specific for ILVEN" by Dupré and Christol are usually expressed. The "specific criteria for ILVEN" can be observed occasionally in psoriasis. The micro-abscesses of Munro are supposed to be a "specific criterion for psoriasis"[11]. However this feature occasionally has been reported in ILVEN. Therefore delineation between ILVEN and psoriasis is difficult on histological grounds.

In the present series, the 2 patients with classical ILVEN on clinical grounds (patients 1 and 2) showed the "specific histological ILVEN hallmarks". In the patients with incomplete clinical presentation of ILVEN (patients 4 and 6) again this specific picture was observed. In the 2 patients with a clinical presentation of lichen striatus (patients 3 and 5), a saw tooth dermo-epidermal delineation was observed. In 2 patients with ILVEN superimposed by psoriasis, intra-epidermal PMN accumulation was seen.

The present immunohistochemical study demonstrates that abnormal keratinization and epidermal hyperproliferation are dissociated in ILVEN. No direct topographical relation was observed between the localization of the infiltrate and the epidermal changes. However, studies on incipient lesions might reveal such a relationship. The remarkable focal distribution pattern of Ks8.12 (keratin 16 expression, Figure 3) suggests that further studies on the keratinization process in ILVEN are worthwhile.

From an immunohistochemical point of view ILVEN and psoriasis show certain differences. In ILVEN we observed a relatively low occurrence of elastase positive cells (PMN), a focal staining pattern of Ks8.12 (anti-keratin 16) and a homogeneous distribution of RKSE60 (anti-keratin 10). In psoriasis the occurrence of elastase positive cells is more substantial, Ks8.12 staining is homogeneous and RKSE60 staining shows foci of decreased staining[12]. It is attractive to speculate that the relatively low occurrence of PMN in ILVEN is the cellbiological explanation for

refractoriness to anti-psoriatic treatment. In conclusion, this investigation suggests that visualizing PMN, keratin 16 and keratin 10 adds valuable information to distinguish ILVEN from psoriasis.

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4.2 TOPICAL TREATMENT WITH THE VITAMIN D_3 ANALOGUE MC903 IMPROVES PITYRIASIS RUBRA PILARIS; A CLINICAL AND IMMUNOHISTOCHEMICAL OBSERVATION.

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SUMMARY

A patient is described who presented with a clinical picture compatible with psoriasis, later shifting to pityriasis rubra pilaris. He was treated with the vitamin D_3 analogue MC903, and biopsies were taken before, after 1, 2, 4 and 12 weeks of treatment. Monoclonal antibodies to assess epidermal proliferation, differentiation and inflammation were applicated. A substantial clinical improvement was seen. Immunohistochemically epidermal proliferation, keratin 16 content and T-lymphocytes decreased. This case report indicates that the vitamin D_3 analogue MC903 might have more applications than chronic plaque psoriasis alone.

INTRODUCTION

Topical treatment with the vitamin D_3 analogue MC903 has been shown to be effective in psoriasis[1,2]. Several mechanisms of action may be of relevance: interference with the T cell response[3], inhibition of epidermal proliferation[4] and or interference with keratinization[5]. Pityriasis rubra pilaris could well be responsive to

MC903 as abnormal keratinization, increased epidermal proliferation and infiltration of T lymphocytes characterize the histological picture in this disorder.

The relationship between psoriasis and pityriasis rubra pilaris has been discussed by many authors. Firstly it can be rather difficult to differentiate both conditions on clinical grounds[6,7,8], secondly the transition of the one in the other has been suggested[6,9].

Recently we had the opportunity to investigate the effect of longterm topical treatment with MC903 in a patient who was initially diagnosed as psoriasis but developed a classical manifestation of pityriasis rubra pilaris during the course of the treatment.

CASE REPORT: CLINICAL OBSERVATIONS

A 81-year-old man presented with an erythematosquamous skin disorder of many years duration which had been diagnosed as psoriasis. Previous treatments with topical corticosteroïds, photochemotherapy and MC903 ointment had resulted in substantial but temporary improvement. In april 1989 the disorder covered 10-29% of the skinsurface of the arms and legs. The lesions consisted of sharply demarcated erythematosquamous plaques. The nails had a normal appearance. As the clinical appearance at that time was compatible with psoriasis, the patient was included in a trial on the longterm efficiency of MC903 in this disease. The patient was treated for 10 months and at monthly intervals the Psoriasis Areas Severity Index (PASI) was recorded. Figure 1 shows PASI scores before and during the treatment. At these visits blood investigations (haematology and biochemistry including calcium and phosphate) were carried out and no abnormalities were observed. After one month a substantial improvement was seen, however the clinical picture adopted more and more a non-psoriatic appearance. Follicular hyperkeratotic papules with a perifollicular erytheme, coalescing to erythematous plaques dominated the picture.

As the clinical diagnosis was not compatible anymore with psoriasis, a biopsy was taken for histological assessment (H and E staining). The epidermis showed some hyperkeratosis with perifollicular parakeratosis. A perivascular mononuclear infiltrate was seen in particular around the hair follicles. No elongation of rete

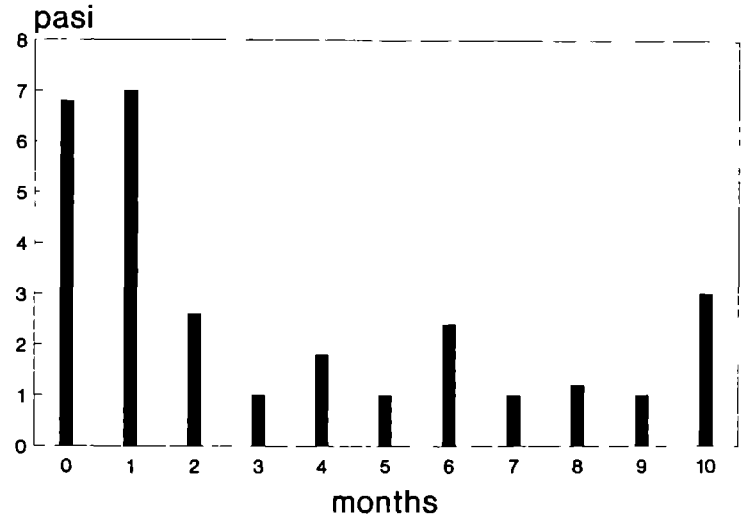

Figure 1. PASI scores before and at monthly intervals during treatment with MC903.

ridges, no papillomatosis and no accumulation of polymorphonuclear leukocytes were observed.

IMMUNOHISTOCHEMICAL OBSERVATIONS

Before and after 1, 2, 4 and 12 weeks of treatment, punch biopsies (3 mm diameter) were taken. Monoclonal antibodies were used against a nuclear antigen present in cycling cells (Ki-67, Dakopatts Copenhagen, Denmark)[10,11], and against keratin 13 and 16 (Ks8.12, Sigma, St Louis, USA), to assess epidermal proliferation. Keratin 16 is present in hyperproliferative epidermis[12], meanwhile keratin 13 has not been found in adult human skin[13]. To characterise the inflammatory infiltrate staining was performed with antibodies against leukocyte-elastase which visualizes PMN (Serotec, Oxford, England); against T-lymphocytes (CD2) (T11, Dakopatts, Copenhagen, Denmark); against B-lymphocytes (CD22) (Pan-B, Dakopatts, Copenhagen, Denmark); against CD14-positive cells which represent monocytes and macrophages (WT14, Dept. Medicine, Div. Nephrol. University Hospital Nijmegen)[14]; and against Langerhans cells (OKT6, Ortho Diagnostic Systems, Raritan, USA)[15].

Stainings were carried out using an indirect peroxidase-, a direct immunoperoxidase- or a peroxidase-anti-peroxidase technique.

Epidermal proliferation was measured by counting the number of Ki-67 positive nuclei per mm length of section, and by assessing Ks8.12-staining in suprabasal and basal layers of the epidermis using a 5-point scale: 0= no staining, 1= sporadic staining, 2= minimal staining, 3= moderate staining, 4= pronounced staining. Dermal inflammation (PMN, T-lymphocytes, B-lymphocytes, Langerhans cells, CD14-positive cells) was semiquantitatively enumerated by expressing the number of positively stained cells as a percentage of the total number of infiltrate cells similar to Synkowski and Provost[16]: 0= no positive cells, 1= sporadic, 2= 1-25%, 3= 26-50%, 4= 51-75%, 5= 76-99%, 6= 100%. Inflammatory cells in the epidermis were assessed using the 5-point scale as used for Ks8.12-staining.

RESULTS

Table I summarizes the immunohistochemical observations. Before treatment and after 1 week treatment, nuclear binding of Ki-67 (indicator for proliferation) is

Table I. Binding of monoclonal antibodies, characterizing inflammation, epidermal proliferation and keratinization, before and at regular time intervals during MC903 treatment. E= Epidermal binding; D= Dermal binding. Ki-67 positive nuclei per mm length of epidermis.

Weeks	Ki-67	Ks8.12	Anti-elas-tase	T11	WT14	OKT6
0	53.3	2-4	E:0, D:0	E:1.5, D:3	E:0, D:2	E:3.5,D:2.5
1	148.4	4	E:0, D:1	E:1.5, D:3	E:0, D:0	E:3.5,D:2.5
2	159.4	1-2	E.0, D:1	E:1, D:2.5	E:0, D:0	E:3, D:2.5
4	114 6	2	E:0, D:1	E:1, D:1.5	E:0, D:0	E:3.5,D:1.5
12	90.3	2	E·0, D:0	E:1, D:2.5	E:0, D:0	E:3.5, D:2
0-12	↑	↓	=	↓	=/↓	=/↓

substantially lower than values as observed in untreated psoriatic plaques (305 ± 32)[17]. After 2-4 weeks treatment nuclear binding to Ki-67 increased but remained below the psoriatic range. Remarkably, no anti-elastase binding (PMN) was observed before nor during MC903 treatment. The complete absence of elastase in all biopsies is not compatible with the diagnosis psoriasis. Ks8.12 binding (keratin 16 expression) is reduced after 2 weeks treatment and the binding of T11 (T cells) and WT14 (monocytes and macrophages) showed a reduction within the first 2 weeks.

DISCUSSION

A patient presented with a clinical picture compatible with the diagnosis psoriasis, well responding to antipsoriatic therapies and therefore was included in a trial on the longterm efficacy of the vitamin D_3 analogue MC903. During the trial some clinical characteristics of pityriasis rubra pilaris became more and more prominent. The ultimate diagnosis pityriasis rubra pilaris was confirmed histologically.

In biopsies taken before the initiation of MC903 treatment, absence of elongated rete ridges and papillomatosis, the relatively low nuclear binding of Ki-67 (proliferation) and especially the absence of anti-elastase binding (PMN), are not compatible with psoriasis and indicative for pityriasis rubra pilaris.

The differentiation of pityriasis rubra pilaris from psoriasis can be difficult[6-9]. In addition to the difficulty to separate two disease entities from each other the possibility of an 'overlap' between the entities has been suggested:"Some patients seem to start with pityriasis rubra pilaris and end up with psoriasis"[6]. The present case suggests the reversed transition. Niemi et al. reported a 27% incidence of HLA-B13 in a series of patients with pityriasis rubra pilaris[9]. The authors suggested that this association was of interest in the light of the interrelation between psoriasis and pityriasis rubra pilaris, however, the series of Niemi et al. was to small to reach statistical significancy[9]. Although an overlap between psoriasis and pityriasis rubra pilaris has been suggested, a distinct separation of the two entities is possible on clinical, histological and cell kinetic criteria[20]. The folliculair hyperkeratotic papules and the general histological appearance without any PMN, virtually excludes the diagnosis psoriasis.

In psoriasis intraepidermal accumulation of PMN is a characteristic feature[21]. In chronic plaque psoriasis however polymorphonuclear leukocytes may be sparse[22]. Using elastase as a marker enzyme for PMN, the sensitivity of PMN detection is markedly increased as PMN remnants will also be detected. In previous series of untreated psoriatic plaques binding to anti-elastase was a consistent finding[17]. The cell proliferation (labeling index) of lesional skin in pityriasis rubra pilaris is raised, however the values were below the level of the psoriatic lesion[9,20,23-26]. In this study we used nuclear Ki-67 binding as a parameter for epidermal proliferation. So far, we are not aware of a report on keratin 16 expression in pityriasis rubra pilaris. This cytokeratin is expressed in hyperproliferative skin in general[27]. MC903 treatment has been shown to be effective in psoriasis[1-2]. In an immunohistochemical study of skin biopsies from psoriatic plaques taken at intervals during MC903 treatment we observed a decrease of PMN accumulation after 1 week, a reduction of nuclear Ki-67 binding after 2 weeks, after 4 weeks a reduction of T-lymphocytes and between between 4 and 12 weeks some reduction of Ks8.12 binding. In pityriasis rubra pilaris (present case), the reduction of Ks8.12 proved to be an early event which was accompanied by a reduction of T lymphocytes. The nuclear binding of Ki-67 remained above the normal range (24.7 ± 2.2)[11].

Baised on the present case report it is feasible that the vitamin D_3 analogue MC903 might have more applications than psoriasis. Further studies on the efficacy in disorders of keratinization seem worthwhile.

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4.3 IMMUNOHISTOCHEMICAL DETECTION OF PROLIFERATION AND DIFFERENTIATION IN DISCOID LUPUS ERYTHEMATOSUS.

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SUMMARY

Discoid lupus erythematosus (DLE) lesions show hyperkeratosis and atrophy, which may reflect abnormal epidermal proliferation and/or differentiation. In this investigation, markers for epidermal proliferation and differentiation were studied in cutaneous lesions of DLE. Frozen sections of biopsies from 20 patients were examined immunohistochemically regarding Ki-67-staining and keratin 16 expression (parameters for proliferation), and the expression of keratin 10, involucrin and filaggrin (parameters for differentiation). Using these markers, epidermal proliferation was found to be increased in DLE. Keratin 10 expression, a marker for early differentiation, showed the pattern of normal skin. Involucrin and filaggrin, markers for terminal differentiation, were expressed already in the lower part of the stratum spinosum, whereas in normal skin these markers were restricted to the stratum granulosum and the upper layers of the stratum spinosum, and the stratum granulosum and corneum, respectively. We conclude that in cutaneous lesions of DLE, hyperproliferation is combined with normal early differentiation and premature terminal differentiation of keratinocytes.

INTRODUCTION

Cutaneous lesions of discoid lupus erythematosus (DLE) show a clinical picture of well-defined erythematous patches, with adherent scales and follicular plugging. If not treated, a marked atrophy results[1].

In two previous investigations, an increased incorporation of ^{3}[H]-thymidine was shown in lesional skin of DLE[2,3]. This seems in contrast to the marked atrophy that results from these lesions.

Very little is known about the epidermal differentiation in DLE. Apparently, in this disease, hyperproliferation does not result in acanthosis or in a parakeratotic horny layer. In contrast, atrophy and orthokeratosis are established histological features of DLE.

In this investigation therefore, the proliferation and differentiation-processes were examined using monoclonal antibodies on frozen sections. Ki-67, an antibody to a nuclear antigen, present in cycling cells has been used as a marker for proliferation[4], in combination with an antibody directed against keratin 13 and 16. Keratin 16 is expressed by hyperproliferative keratinocytes as well in vitro as in vivo[5]. As markers for differentiation, antibodies were used against keratin 10, which is expressed in the suprabasal compartment of normal skin[6]; to involucrin, a cytoplasmatic protein precursor of the cornified envelope[7]; and to filaggrin, a protein involved in the formation of keratohyalin granules[8]. In normal skin, involucrin is expressed in the upper stratum spinosum and granulosum[9]. Filaggrin is present in stratum corneum and the granular layer[10]. In addition, the distribution of Langerhans cells, T-lymphocytes and monocytes/macrophages was studied to correlate epidermal changes with the degree of inflammation.

MATERIALS AND METHODS

Patients and biopsies

Twenty patients with a clinical picture of discoid lupus erythematosus (DLE) were studied. Two punch biopsies were taken from lesional skin. One was fixed in formalin and processed for Haematoxylin and Eosin stained (H&E) sections, one

was embedded in Tissue Tek OCT Compound (Miles Scientific, Naperville, USA) and snap frozen in liquid nitrogen for immunofluorescence and for additional staining on cryostat sections of 7 μm with monoclonal antibodies.

Immunohistochemical techniques

Epidermal proliferation was studied with the antibodies: Ki-67 (Dakopatts, Copenhagen, Denmark), that stains a nuclear antigen expressed by cycling cells; and with Ks8.12 (Sigma, St Louis, USA) that binds to keratin 13 and 16.

Epidermal differentiation was studied using the following antibodies: RKSE60 (a gift of Dr F Ramaekers, Maastricht, The Netherlands) binding to keratin 10; anti-involucrin (Biomedical Technologies Inc., Stoughton, USA), directed against involucrin, a cytoplasmatic protein precursor of the cornified envelope; and anti-filaggrin (Biomedical Technologies Inc., Stoughton, USA), directed against filaggrin, a protein involved in the formation of keratohyalin granules.

Slides were fixed in acetone/ether (60/40%) for staining with Ki-67 or in acetone for staining with Ks8.12, RKSE60, anti-involucrin and anti-filaggrin. Slides were rehydrated in phosphate buffer (pH 7.0), and incubated with the antibodies at room temperature in the dark in a dilution of 1:40, 1:25, 1:10, 1:10 and 1:500 in phosphate buffer, respectively. After 3 washing steps in phosphate buffer, slides were incubated for 30 minutes with swine-anti-rabbit-immunoglobulin conjugated with peroxidase (for involucrin-staining, a polyclonal antibody) or with rabbit-anti-mouse-immunoglobulin conjugated with peroxidase, diluted 1:50 in phosphate buffer containing 5% human AB-serum. Three more washing steps in phosphate buffer and preincubation in sodium acetate buffer (pH 4.9) were followed by final staining in a solution of 3-amino-ethyl-carbazole (AEC) in sodium acetate buffer containing 0.01% H_2O_2 for 10 minutes. The slides were counterstained with Mayer's haematoxylin (Sigma, St Louis, USA) and mounted in glycerin gelatin.

Inflammation was investigated in epidermis and dermis using the following antibodies: T11 (Dakopatts, Copenhagen, Denmark) for T-lymphocytes (CD2); OKT6 (Ortho Diagnostic Systems, Raritan, USA) for Langerhans cells (CD1a); and WT14 (Dr WJ Tax, Dept. Medicine, Div. Nephrol. University Hospital Nijmegen) for CD14-positive cells (monocytes and macrophages)[11].

Slides were dried at room temperature for 30 minutes and fixed in acetone for 10 minutes. After rehydration in phosphate buffer (pH 7.0) slides were incubated for 60 minutes with the antibodies in a dilution of 1:100. A peroxidase-anti-peroxidase technique was used consisting of an incubation step with rabbit-anti-mouse-immunoglobulin (RAM-Ig, Dakopatts, Copenhagen, Denmark) 1:25 in phosphate buffer for 20 minutes, washing in phosphate buffer and incubation for 20 minutes with peroxidase-anti-peroxidase complexes (PAP, Dakopatts, Copenhagen, Denmark) 1:100. These last two incubation steps were repeated to amplify the signal. Finally slides were stained in AEC-solution, counterstained with Mayer's haematoxylin and mounted in glycerin gelatin.

Scores

On H&E sections, scoring for different histological features was done using a four-point scale: 0= not present, 1= slight, 2= moderate, 3= heavy. The features reviewed were the degree of atrophy, acanthosis, hyperkeratosis, follicular plugging, hydropic degeneration, patchy infiltrate, perifollicular infiltrate and epidermotropy.
Immunohistochemical stainings were assessed using a 7-point scale. For the dermal compartment: 0= no staining, 1= sporadically cells present, 2= 1-25% of the infiltrate cells stained, 3= 26-50%, 4= 51-75%, 5= 76-99%, 6= 100%. In the epidermal compartment: 0= no staining, 1= sporadically stained, 2= minimal staining, 3= moderate staining, 4= moderate-pronounced staining, 5= pronounced staining, 6= whole epidermis stained.
Ki-67-positive nuclei were counted and expressed per mm length of section.

RESULTS

Patients

The group consisted of 14 women and 6 men. Their age varied between 24 and 70 years with an average of 41 years. The time that the patients had been affected varied between two weeks and 30 years, with an average duration of 6 years. Sixteen patients used no therapy at the time of the investigation, one patient applied hydrocortisone-17-butyrate (Locoid^{R}) topically, one patient used prednisone 7.5 mg

daily, one patient used etretinate (Tigason^{R}) 35 mg daily, and one patient used hydrochloroquinesulfate (Plaquenil^{R}) 600 mg daily. Biopsies were taken from lesional skin from various locations (9 x face, 5 x arm, 3 x back, 1 x leg, 1 x scalp, 1 x neck).

H&E sections and immunofluorescence

Immunofluorescence studies showed a pattern compatible with DLE in all patients. H&E sections of the 20 patients showing positive immunofluorescence revealed a classic picture of DLE in 18, in two specimens the features of subacute cutaneous lupus erythematosus were seen.

Immunohistochemistry (Table I)

Epidermal proliferation Ki-67-positive nuclei were uniformly distributed in the basal layer of the epidermis. The mean number of stained nuclei was 56.1 +/- 8.2 per mm length of epidermis (mean +/- sem). This is illustrated in Figure 1. A substantial variation between different biopsies existed, however. The highest number counted came to 151 nuclei/mm, the lowest to 9 nuclei/mm. Substantial Ks8.12-binding (keratin 13 and 16), was demonstrated in the suprabasal compartment, mean score +/- sem was 3.25 +/- 0.4. In the basal layers only sporadically Ks8.12-binding was seen (Fig 2).

Epidermal differentiation RKSE60 (keratin 10) was expressed in the suprabasal compartment and showed a homogeneous pronounced binding (Fig 3) in all biopsies. The mean score for RKSE60-binding was 4.5 +/- 0.2 (mean +/- sem). Binding of anti-involucrin was seen not only in the stratum granulosum and upper stratum spinosum, but also in lower cell layers (Fig 4). In some biopsies, binding was seen immediately above the cells of the basal layer. In 7 out of the 20 patients, the increase in stained cell layers coincided with a decrease in staining of the higher cells of the stratum granulosum. The average score for anti-involucrin-binding was 4.3 +/- 0.3 (mean +/- sem). Anti-filaggrin was also expressed in cell layers below the granular layer (Fig 5). However, staining was never seen in the cell-layers just above the basal keratinocytes. The extension of its binding pattern was less pronounced than the extension of binding of anti-involucrin. Scores for expression of anti-filaggrin were 2.9 +/- 0.1 (mean +/- sem).

Inflammation In all biopsies the infiltrate consisted mainly of T-lymphocytes (about 75%) as shown by T11-staining (score 4.3 +/- 0.12, mean +/- sem). T-lymphocytes were present in the epidermis as well in 19 out of 20 biopsies (score 1.6 +/- 0.2, mean +/- sem). WT14-expression, showing CD14-positive cells (monocytes and macrophages) accounted for less than 25% of the infiltrate (score 2.1 +/- 0.04, mean +/- sem). These cells could sporadically be found in the epidermal compartment as well (score 0.7 +/- 0.1, mean +/- sem). OKT6-staining Langerhans cells showed sporadically expression in the dermal compartment (score 0.8 +/- 0.1, mean +/- sem), and were consistently present in the epidermis (2.0 +/- 0.2, mean +/- sem).

Correlations

The number of Ki-67-positive nuclei indeed was increased compared with normal skin, but tended to diminish when the degree of atrophy was greater, as seen in

Table I: Immunohistochemical scores for proliferation, differentiation and inflammation in DLE (means +/- SEM). Ki-67 staining is expressed as number of stained nuclei per mm length of epidermis, remaining antibodies are expressed using a semi-quantitative 7-point scale.

Antibody	Specificity	Score
Ki-67	cycling cells	56.1 +/- 8.2
Ks8.12	keratin 16	3.3 +/- 0.4
RKSE60	keratin 10	4.5 +/- 0.2
Anti-involucrin	involucrin	4.3 +/- 0.3
Anti-filaggrin	filaggrin	2.9 +/- 0.1
T11	T-lymphocytes	epidermis 1.6 +/- 0.2
		dermis 4.3 +/- 0.1
WT14	monocytes/macrophages	epidermis 0.7 +/- 0.1
		dermis 2.1 +/- 0.04
OKT6	Langerhans cells	epidermis 2.0 +/- 0.2
		dermis 0.8 +/- 0.1

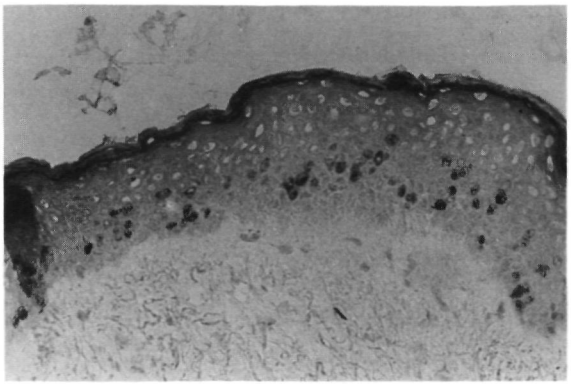

Figure 1. Staining with the antibody Ki-67 in lesional skin of DLE (x500).

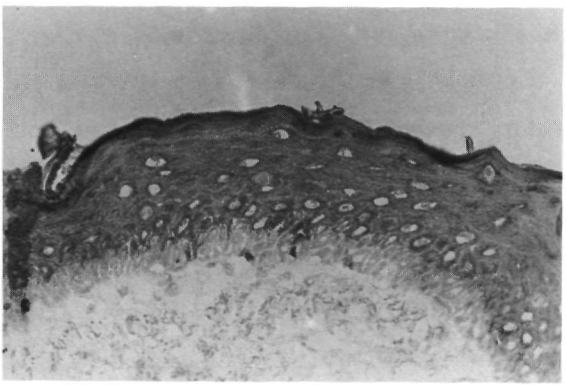

Figure 2. Ks8.12-staining in the suprabasal compartment (keratin 16) of a DLE-lesion (x625).

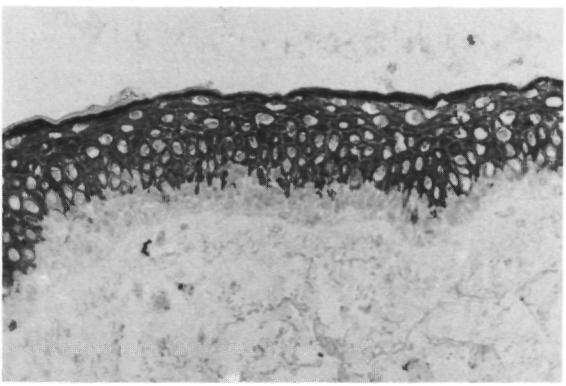

Figure 3. Homogeneous expression of keratin 10 in DLE (x500).

H&E-sections. In addition, the number of Ki-67-stained nuclei correlated well with suprabasal Ks8.12-binding (keratin 13 and 16), and with involucrin- and filaggrin-expression. Filaggrin expression correlated well with the degree of hyperkeratosis. The remaining histological hallmarks of DLE did not correlate with markers for proliferation and differentiation. Differentiation markers did not correlate with either the degree of atrophy or the amount of inflammation. The four patients who were receiving treatment showed values representative of the total group. Clinical data, such as the age of the patient or the years a patient had been affected, were not correlated with histologic aberrations.

DISCUSSION

For this study 20 patients with DLE were selected. The immunohistochemical results were compared with results in normal skin as reported in literature. Preliminary experiments by our group in normal skin revealed the same staining pattern as described in the literature.

Markers for inflammation conformed in all patients to the established features of the infiltrate in DLE[11]. The infiltrate consisted mainly of T-lymphocytes and of monocytes and macrophages, whereas only a few Langerhans cells were present. In this study, the observation of a hyperproliferative state of the epidermis in discoid lupus erythematosus lesions measured by ^{3}[H]-thymidine[1,2], has been confirmed and extended using the antibodies Ki-67 and Ks8.12 as markers. The number of Ki-67-stained nuclei was increased compared to normal skin of healthy individuals (14.7 +/- 2.2), but lower compared to the psoriatic lesion (196.9 +/- 22.4)[13]. A homogeneous distribution of the Ki-67-positive nuclei through the basal layer of the skin was seen. However, the number of Ki-67-stained nuclei tended to diminish as the degree of atrophy was higher. The occurence of some Ki-67 positive nuclei just above the basal layer suggests an extension of the germinative pool. A similar situation has been observed in psoriatic lesions[14]. The expression of Ks8.12 in the suprabasal compartment of the epidermis of DLE provides further support to the observation that DLE is hyperproliferative. Ks8.12-binding (keratin 13 and 16) is seen in psoriatic lesions and in normal skin after tape-stripping and decreases during

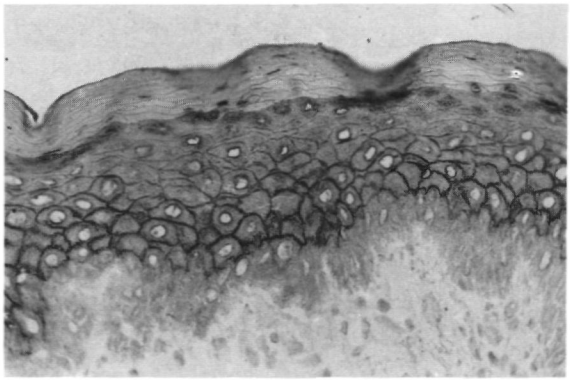

Figure 4. Staining with anti-involucrin in the lower layers of the stratum spinosum of DLE (x625).

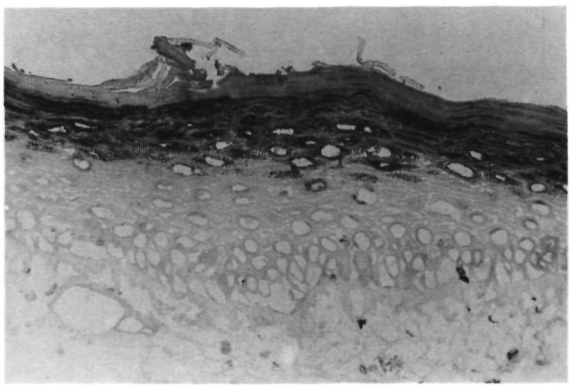

Figure 5. Anti-filaggrin staining in the upper stratum spinosum of DLE (x500).

anti-psoriatic therapy[15,16]. Keratin 16 is expressed by hyperproliferative keratinocytes in vitro as well as in vivo[5]. The hyperproliferative state of the epidermis in DLE might be a repair mechanism in an attempt to restore the damage of the basal cell layer. Possibly this reaction ultimately extinguishes and results in atrophy.

In a previous investigation a normal differentiation pattern and a shortened transit time of keratinocytes has been postulated in DLE[2]. In the present study it is shown that epidermal differentiation is disturbed compared to normal skin. RKSE60-binding (keratin 10) is pronounced, but involucrin and filaggrin are expressed in lower cell layers of the epidermis compared to normal skin[9,10]. Keratin 10 is a marker for early differentiating keratinocytes, whereas involucrin and filaggrin are markers for the

later phase of differentiation. Keratin 10 is diminished in psoriasis and after sellotape-stripping of normal skin[17,18]. Differentiation in these two situations is altered, which is also reflected by a parakeratotic horny layer. In DLE, orthokeratosis occurs and we observed a binding pattern of RKSE60 as seen in normal skin[6]. The early differentiation phase therefore, seems to proceed normally. However, although orthokeratotic cornification takes place, markers for the late phase of differentiation -involucrin and filaggrin- are expressed in lower cell layers compared to normal skin. This staining pattern of involucrin is also found in hyperproliferative skin disorders such as benign neoplasms, malignant tumours and psoriasis[9]. The increase in involucrin-expression is thus in agreement with the high number of Ki-67-stained nuclei and with the increased Ks8.12-expression in DLE. In a previous study on various skin disorders, increased filaggrin expression correlated with hyperkeratosis and hypergranulosis[10]. In DLE, the distribution of filaggrin is extended. High magnification examination revealed that filaggrin is present in the stratum granulosum and in cell layers beneath it. In the present study, filaggrin expression correlated well with hyperkeratosis, but no thickening of the stratum granulosum was seen.

Markers for terminal differentiation are expressed early in DLE. From in vitro studies, some regulatory mechanisms have been postulated which influence differentiation of keratinocytes. Loss of contact with the basal membrane and extracelluar matrix (ECM) constituents such as laminin, collagens and proteoglycans is needed to differentiate[19]. When human keratinocytes are cultured in suspension, withdrawal from the cell-cycle and the synthesis of involucrin is induced[19,20]. Possibly a decrease in contact of the basal cells with the basal membrane and ECM, caused by infiltrating cells or by upward pressure from dividing basal keratinocytes, induces the early expression of late differentiation markers by suprabasal keratinocytes.

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CHAPTER 5

DISCUSSION

5.1 ON THE IMMUNOHISTOCHEMICAL APPROACH

In psoriasis, the histological abberations are considerable compared to normal skin. In this thesis immunohistochemical methods have been used to investigate epidermal growth and inflammation during evolution and regression of psoriatic lesions. A panel of monoclonal and polyclonal antibodies directed against cellular constituents involved in epidermal proliferation and differentiation, and against inflammatory cells and dermal extracellular matrix proteins has been used on frozen sections. This approach has advantages and disadvantages.

ADVANTAGES:

One of the most important advantages is that topographical information is preserved. This permits to investigate interrelations between different cell types, such as between Langerhans cells and PMN (Chapter 2.1). In addition, when untreated psoriatic skin is compared to treated, or to normal skin, the divergence is sufficient for reliable assessment using a semi-quantitative method. This approach permits to carry out multiparameter analysis in time and/or place.

LIMITATIONS:

A histological picture is not easily quantitated. Especially when multiple parameters are assessed, or when cells are abundantly present, counting of cells raises serious difficulties and is fairly impossible. Quantitative approaches such as flowcytometry or enzyme measurement of marker enzymes are more sensitive. This is illustrated in Chapter 3.1, where during treatment with MC903, no significant reduction in Ks8.12-binding was seen, whereas a clear decline was demonstrated by flowcytometrical assessment using the same antibody[1]. However limitations of flowcytometry and measurements of marker enzymes such as elastase[2] are loss of topography. Using the immunohistochemical markers chosen in this thesis, less information is obtained about functional aspects or about the timing of regulation/dysregulation of the phenomena assessed, which may take place at the molecular level. In addition, possible masking of epitopes and cross reactivity limit the interpretations.

SYNTHESIS:

In understanding and investigating diseases, histology remains the point of departure. In this respect, quantitative and qualitative methods are complementary. Measurements at the RNA level might be useful techniques in future investigations. Multiparameter immunohistochemical analysis, as used in this thesis, might provide an indication of the dynamics of events important in induction and regression of psoriatic lesions.

5.2 ON THE PASI-SCORE

The Psoriasis Area and Severity Index is introduced by Fredriksson in 1978[3], and is a score to describe the clinical condition of a patient, by integrating the degree of erythema, desquamation, infiltration and body surface involved. A standardized clinical description, internationally accepted, is useful in comparing results of various anti-psoriatic therapies, and in comparing different investigations. Nevertheless the disadvantage of such a score is that it is subjective and thus varies with the investigator. A considerable variation can exist in the clinical picture of two patients with the same PASI-score. For example, a PASI-score of 8.4 can be either a patient with extensive (50-69%) psoriasis, with only slight erythema, desquamation and infiltration, or a patient with only a few lesions (<10%) that show the severest possible erythema, desquamation and infiltration. It might therefore be useful to compare not exclusively the PASI-scores, but in addition the constituing components.

5.3 SEQUENTIAL STUDIES ON IN VIVO MODELS FOR PSORIASIS

INTRODUCTION

In this thesis two investigations were carried out to study the phases of the psoriatic process during evolution of a lesion (Chapter 2). The approaches used were firstly

studying the transition zone between involved and uninvolved skin of spreading psoriatic plaques, and secondly simulation of a psoriatic lesion by application of leukotriene B_4 on normal skin. In the following paragraphs firstly the abberations known to be present in distant uninvolved skin from psoriatic patients are reviewed, followed by changes seen in the fully developed psoriatic lesion. A part of this is described in Chapter 3. Subsequently, the two in vivo models used in this thesis are discussed.

DISTANT UNINVOLVED SKIN

From a clinical point of view, uninvolved skin from psoriatic patients, seems essentially normal. However, the Koebner phenomenon indicates that uninvolved psoriatic skin is intrinsically abnormal. Morphologic examination of uninvolved skin using H&E staining, reveals no abnormalities in epidermal or dermal compartment. However, cellkinetic studies on epidermal growth demonstrated a slight but significant increase in percentage of keratinocytes in SG_2M-phase in biopsies taken from uninvolved skin[4]. In addition the number of mitoses[5] and the incorporation of tritiated thymidine[6] is reported to be increased compared to normal skin. Biochemically several abnormalities compared to normal skin have been postulated. Increased amounts of phospholipase A_2[7,8], increased release of arachidonic acid metabolites[9], increased ornithine decarboxylase activity[10] and polyamine synthesis[10] were demonstrated, whereas the activity of protein kinase C proved to be reduced[11]. Recently, fibroblasts from uninvolved psoriatic skin proved to be abnormal in culture[12]. Barton et al. suggested that the endothelium in clinically uninvolved skin of psoriatic patients was abnormal with respect to vascular mass and vessel dilatation[13].

THE PSORIATIC LESION

In the fully developed psoriatic lesion, morphological changes in epidermis as well as in dermis are prominent. Acanthosis, papillomatosis, hyperparakeratosis, and an inflammatory infiltrate are the most striking features (Chapter 1). From cellkinetic studies it is clear that a large increase in the number of epidermal cells and in the

rate of production of these cells exists in psoriatic lesions. This is probably due to an increase in the absolute size of the germinative compartment and to recruitment of G_0 cells. Nowadays the cell cycle time in psoriatic epidermis is believed to be relatively normal[14]. Biochemical abnormalities in the lesional skin include increased amounts of arachidonic acid metabolites[9], increased activity of phospholipase A_2[7,8], phospholipase C[15], and ornithine decarboxylase with an increased production of polyamines[10]. In addition, decreased activity of protein kinase C[11] and adenylate cyclase[16] have been reported. The level of calmodulin, the calcium binding protein, is increased[17]. Calmodulin-calcium enhances the activities of phospholipase A_2 and adenylate cyclase. In the psoriatic lesion many inflammatory changes are found[18,19]. Recently, it has been shown that TNF-α, IFN-τ and IL-8 induce the expression of TGF-α[20]. It has been hypothesized by prof. P. Mier that inflammation and epidermal proliferation can be triggered by activation of the TGF-α loop[21].

In the present thesis we could confirm that inflammation and proliferation is profoundly disturbed in lesional psoriatic skin (Chapter 2 and 3). The expression of keratin 16 and 17 is increased in the epidermis. At the same time the expression of keratin 10 is decreased. Epidermal proliferation, indicated by the number of nuclei staining with the antibody Ki-67 is increased. Numbers of T-lymphocytes and PMN are raised in as well epidermis as dermis. Langerhans cells and monocytes/macrophages are present in the dermal infiltrate and in the epidermis of psoriatic lesions. In the epidermal compartment Langerhans cells show focal aggregation.

THE TRANSITION ZONE BETWEEN UNINVOLVED AND LESIONAL SKIN

In the transition zone of spreading psoriatic plaques, simultaneous assessment of epidermal growth, inflammation and the extracellular matrix components was carried out in consecutive sections using immunohistochemistry (Chapter 2.1). In this study, dermal changes preceeded epidermal changes. An inflammatory infiltrate composed mainly of T-cells was found in the more peripheral sections of the transition zone in combination with increased expression of the extracellular matrix protein tenascin. This was observed more distantly too, that means before any epidermal alteration was seen. The first epidermal change was a slight acanthosis, followed by keratin 16 expression and increased binding of the antibody Ki-67. A striking observation is

that nor the dermal infiltrate nor the epidermal compartment contained PMN in the distant uninvolved skin adjacent to psoriatic lesions. This is in contrast to studies on pre-pinpoint lesions, pointing out the early involvement of PMN in the initiation of a lesion[22,23].

In a previous study on markers for epidermal proliferation and keratinization in the margin of the psoriatic lesion, the first epidermal change was also initial acanthosis, followed by increased staining with the antibody Ks8.12[1]. Other investigators studying dermal changes in spreading psoriatic lesions, showed that increased alkaline phosphatase (marker enzyme for vascular endothelium) can be found distant from the psoriatic lesion[24]. An increased blood flow measured by laser-doppler flowmetry is detectable at sites where no change in the number of T-lymphocytes or Langerhans cells can be seen[25].

Alltogether, the dermal compartment is likely to be the tissue, showing the earliest changes in the evolution of a psoriatic lesion.

SIMULATION OF THE PSORIATIC LESION

One of the most striking features of a psoriatic lesion is the accumulation of PMN in the epidermis, as microabscesses of Munro or Kogoj[26]. A potent chemoattractant for PMN is leukotriene B_4 (LTB_4)[27], a product derived from arachidonic acid via the enzyme 5-lipoxygenase[28], and found in high amounts in lesional psoriatic skin compared to uninvolved or normal skin[9]. Epicutaneous application of leukotriene B_4 on normal skin produces an acute inflammation dominated by PMN[29], followed by hyperproliferation of the epidermis[30], features as seen in the psoriatic lesion. Anti-psoriatic therapies proved to inhibit LTB_4-induced accumulation of PMN[31]. In Chapter 2.2 the interrelations between cells involved in epidermal growth and inflammation are investigated. A migration of Langerhans cells to intra-epidermal microabscesses containing PMN was shown. The composition of the inflammatory infiltrate changed from predominantly PMN and monocytes after 8 and 24 hours, to T-lymphocytes and Langerhans cells after 48 and 72 hours. Changes as seen in initial psoriatic lesions were approached using this model. As a model for chronic plaque psoriasis it is less suitable.

SYNTHESIS

The uninvolved skin from psoriatic patients shows no morphological but some cellkinetic and biochemical deviations. Uninvolved skin seems to be in an adaptive state as epicutaneous application of leukotriene B_4 induces less accumulation of PMN in uninvolved psoriatic skin compared to normal skin[32]. Uninvolved skin can be triggered by a still unknown stimulus (microtrauma) to evolve to subclinically involved skin. This is characterized by dermal alterations such as increased presence of T-lymphocytes, increased expression of the extracellular matrix protein tenascin, and increased blood flow. Subclinical epidermal changes are augmented acanthosis and Ks8.12-binding. In the initial lesion, early events are again Ks8.12-binding in the suprabasal compartment of the epidermis, and increased binding of Ki-67 indicating hyperproliferation in the epidermis. The maximal inflammatory infiltrate is located at the edge ot the psoriatic lesion, consisting of T-lymphocytes, Langerhans cells and PMN. In the chronic lesion, the classical psoriatic features are present: considerable hyperparakeratosis, acanthosis and papillomatosis, PMN trapped in the stratum corneum, and a heavy inflammatory infiltrate consisting of T-lymphocytes and monocytes.

HYPOTHESIS

The concerted finding of T-lymphocytes and epidermal acanthosis, accompanied by an abnormal composition of the extracellular matrix, early in the transition from uninvolved to involved skin suggests that interactions between lymphocytes and keratinocytes, and between lymphocytes, keratinocytes and extracellular matrix are of pathogenetic significance in the transition from uninvolved to involved skin. Further studies on signal transduction and modulation of interactions (cytokines, mediators of inflammation, intercellular adhesion, biochemical parameters) might unravel this process. So far it remains unclear whether the epidermal compartment or the dermal compartment is master in inducing psoriatic features.

5.4 CLINICAL AND HISTOLOGICAL CHANGES DURING ANTI-PSORIATIC TREATMENT

5.4a CLINICAL CHANGES

In the therapeutical battle against psoriasis many alternative treatment modalities are available. Unfortunately no absolute cure exists. As psoriasis is a chronic, life-long disease, treatment should meet high standards for safety and tolerability. Therefore, the search for better new or modified classical therapies goes on. In this thesis, various topical and systemic experimental treatments have been investigated.

Clinically effective treatments

MC903

The observation of Morimoto et al in 1985[33] of a psoriastic patient ameliorating after treatment with oral $1\alpha,25(OH)_2$vitamin D_3, in combination with the knowledge about the proliferation-inhibiting and differentiation-inducing effect of vitamin D_3 on cultured keratinocytes[34], led to a wave of trials with oral and topical vitamin D_3. Systemic side effects limited its use, however. Therefore the vitamin D_3 analogue MC903 has been developed in 1987. MC903 binds to the same cytoplasmatic receptor, is as effective on proliferation and differentiation of cultured keratinocytes, but is 100x less active on calcium and phosphate metabolism in vivo as $1\alpha,25(OH)_2$vitamin D_3[35]. Topical treatment with MC903 proved to be a succesful treatment for chronic plaque psoriasis[36,37]. In a study on 345 patients MC903 was at least as efficacious as betamethasone valerate[38] assessed by the severity of symptoms and percentage body surface involved (PASI-score). In Chapter 3.1 the results of topical treatment of nine patients with MC903 ointment 0.05% during 12 weeks is reported. PASI-scores diminished from 7.7 +/- 1.2 (mean +/- SEM) before treatment, to 2.3 +/- 0.5 after 12 weeks of treatment, an average reduction of 5.4. The improvement was most pronounced in the first 8 weeks of treatment. No total clearing was achieved in any of them. This trial was part of a multicenter trial, from which clinical results are not yet available. Systemic side effects have not been found. One patient had to be withdrawn from treatment with MC903 because of a repeated

burning sensation after applying MC903 on to the skin. The patient compliance was very good, as they were satisfied with the clinical results and as the ointment was easy applicable. MC903 appears to be an effective, well-tolerable, and safe addition to the therapeutic arsenal in the treatment of chronic plaque psoriasis. It is registered as a new anti-psoriatic treatment modality in the Netherlands in september 1992.

Dithranol

Ever since the first use of dithranol for psoriasis, people have endeavoured to find formulations which would be easier to apply. Short-contact therapy has made dithranol treatment available for the out patient department[39]. Nevertheless staining of textile is a serious practical problem. Therefore, developing dithranol-formulations that exert a good clinical effect on the psoriatic lesions, but that are easy to wash off, remains important. In this thesis (Chapter 3.3) the clinical effect of a new formulation (dithranol in a new emulsifying ointment in the concentrations 0.5%-5%) has been investigated, which permits efficient removal of dithranol from the skin. This therapy proved to have a satisfactory response in 12 out of 20 patients. Only two patients dropped out because of non-compliance. In the remaining patients this dithranol treatment was well tolerated. An average reduction of the PASI-score of 10.1 was obtained. The remission period averaged 6.3 weeks. These results are comparable with previous studies on the efficacy of dithranol[40,41].

Budesonide

Topical application of corticosteroids in the treatment of psoriasis has proven to be an effective treatment[42]. As psoriasis is a chronic dermatose, one has to bear in mind the accumulative impact of local and systemic side effects. Budesonide is a new corticosteroid with little effect on plasma cortisol levels and urinary cortisol excretion[43]. The clinical efficacy of Budesonide ointment 0.025% in a three weeks treatment period has been described in Chapter 3.6. An average reduction in PASI-scores of 3.24 was recorded (from 5.3 +/- 1 to 3.6 +/- 0.7, means +/- SEM). No side effects were seen. Patient compliance was very good, as corticosteroids are easy to apply, do not smell or stain. Prolonged use however, is still to be avoided in view of the local side effects.

Clinically ineffective treatments

13-cis-retinoic acid

Systemic treatment of psoriasis with retinoids has nowadays a well-established position[44]. Because of the serious side effects that can occur, their use has been limited to patients with extensive psoriasis. Topical administration of retinoids might exert fewer side effects. In Chapter 3.2 an investigation on the efficacy of topical 13-cis-retinoic acid 0.1% in a cream base has been described. During a four weeks period of treatment, no improvement could be demonstrated. Nevertheless, 13-cis-retinoic acid showed to improve Darier's disease[45]. Also a beneficial effect of topically applied all-trans-retinoic acid in the treatment of chronic plaque psoriasis has been shown[46,47]. Possibly, increasing the concentration of 13-cis-retinoic acid, or changing the cream base into an ointment base might improve its clinical effect. So far, the value of topical 13-cis-retinoic acid 0.1% in a cream base in the treatment of chronic plaque psoriasis remains unsubstantial.

MK886

In psoriatic skin, increased amounts of products from the arachidonic acid cascade have been found, inclusing leukotriene B_4[48]. Inhibitors of the key enzyme in the production of leukotriene B_4, 5-lipoxygenase, have proven to ameliorate psoriasis[49]. Side effects forced a withdrawal of this inhibitor. In Chapter 3.5 the effect of a new oral leukotriene biosynthesis inhibitor (MK886)[50] on chronic plaque psoriasis has been investigated. Despite a significant reduction in systemic leukotriene levels of more than 70%, no changes in the clinical parameters were detectable. In addition, levels of leukotriene B_4 in lesional skin did not change. Possibly, MK886 reached concentrations in skin that were not sufficient to inhibit leukotriene biosynthesis, due to either a suboptimal concentration of MK886, or to a too short treatment period.

Synthesis

In this thesis, five experimental therapies for treating chronic plaque psoriasis have been examined. The objective was to acquire new, or improved classical effective therapeutics with few side effects.

Treatment with the vitamin D_3 analogue MC903 is as effective as a class 3 corticosteroid, and has until now exerted no systemic side effects. Local side effects (burning sensation) are seen in about 3% of the patients. MC903 is therefore an important novel addendum in the treatment of psoriasis.

Short-contact treatment using dithranol in a new cream base proved to be as effective as treatment with the specialité Psoralon (Hermal, Hamburg, FRG) and to induce a remission period comparable with that after classical Ingram therapy. An important advantage is that this new formulation is easy to apply in the out-patient department, can well be removed and can be manufactured by the local pharmacologist. Therefore this formulation opens new perspectives for the out-patient treatment with dithranol.

Tretment with the new corticosteroid Budesonide proved to be very effective. It is positioned as a class 3-4 steroid. In view of local side effects Budesonide is comparable to other steroids from that class.

Topical treatment with 13-cis-retinoic acid (0.1% in cream) and systemic treatment using a leukotriene biosynthesis inhibitor (MK886) were not clinically effective, and the value of these formulations so far is limited in the treatment of chronic plaque psoriasis.

5.4b HISTOLOGICAL CHANGES

In order to obtain information on working mechanisms of anti-psoriatic treatments, the order of events taking place during therapeutical regression of psoriatic lesions was studied (Chapter 3). During the treatment course, biopsies were taken and processed for immunohistochemistry. Changes in time in topography and quantity of cells involved in epidermal growth and inflammation were analyzed.

The application of dithranol on to psoriatic lesions induces regression of those lesions. It seems contradictory that this effect is reached using concentrations which induce some inflammation. To further analyze the inflammatory action of dithranol, we have investigated the effect of this application on normal skin in Chapter 3.4.

In addition, the impact of a new histological marker in psoriasis, keratin 17, has been described in Chapter 3.7.

The various changes in numbers of cells involved in epidermal growth and inflammation during treatment with the investigated anti-psoriatic therapies are summarized in Table I.

MC903

$1\alpha,25(OH)_2$vitamin D_3 and its analogue MC903 both bind to the same cytoplasmatic receptor, are transported to the nucleus resulting in transcription and translation of proteins involved in calcium homeostasis and growth control[35,51,52]. In addition a direct and rapid effect on intracellular calcium and cGMP has been reported[53,54]. Many cells possess receptors for vitamin D_3, including keratinocytes[55]. $1\alpha,25(OH)_2$-vitamin D_3 as well as MC903 affect proliferation and differentiation of cultured cells[34], and both show an immunomodulating effect[56,57]. Whether the anti-psoriatic effect of MC903 is exerted primary via regulation of proliferation and differentiation of keratinocytes, or via regulation of the number of inflammatory cells, is investigated in Chapter 3.1. As the number of epidermal PMN, and the number of nuclei stained with the antibody Ki-67, diminished early in the treatment period, while the number of T-lymphocytes, Langerhans cells and CD14-positive cells remained unchanged, it is feasible that MC903 interferes firstly with PMN and epidermal growth. In a later stage of treatment, the number of T-lymphocytes decreased. However, the immunoregulatory function of these cells might be altered before a decline in number is detected.

Dithranol

The working principle of dithranol in the treatment of psoriasis, is despite many investigations still not exactly known. Inhibition of epidermal proliferation and inflammation is the ultimate result[58,59]. But in contrast, dithranol also induces erythema and inflammation[60]. Which cell or cellular component forms the main target for dithranol is not known. When epidermal growth and inflammation are investigated simultaneously (Chapter 3.3), it becomes apparent that the first changes, seen after two weeks of treatment, are visible in the epidermal compartment. Epidermal proliferation (Ki-67 staining, Ks8.12-staining) and the number of epider-

mal PMN show then an already statistically significant decrease. Inflammation persisted relatively long during treatment with dithranol, especially T-lymphocytes, CD14-positive cells, Langerhans cells, and dermal PMN.

When dithranol is applied to normal skin (Chapter 3.4) some analogies and some contrasting findings are seen compared to psoriasis. As in psoriasis, an inflammatory infiltrate is induced. The infiltrate, however consists mainly of T-lymphocytes and of relatively few PMN. In contrast to psoriasis, the expression of keratin 13/16 (Ks8.12-binding) and hyperproliferation (Ki-67-binding) is increased. Strikingly, in accordance with the treatment of psoriatic plaques with dithranol, in normal skin epidermal changes are induced before dermal changes occur.

Budesonide

Corticosteroids are anti-proliferative, anti-inflammatory, and immunosuppressive[42,61]. They possess a cytoplasmatic receptor and are transported to the nucleus after binding, but also non-nuclear working mechanisms are described[62]. In Chapter 3.6 it is shown that topical application of Budesonide 0.025% ointment induces a significant decrease in epidermal proliferation (Ki-67-binding, Ks8.12-binding) and in dermal accumulation of PMN already after one week. The remaining inflammatory cells remained relatively high. Epidermal proliferation therefore, seems an important target in the anti-psoriatic working principle of Budesonide.

13-cis-retinoic acid

Vitamin A and derivatives bind to a cytoplasmatic receptor and act at the level of gene transcription[63]. Orally administered etretinate and acitretin have previously shown a clear antipsoriatic effect in about 60% of the patients[44,64]. Topical treatment with 13-cis-retinoic acid 0.1% in a cream base showed no clinical and histological improvement of lesions and resulted in a status quo of epidermal growth and inflammation (Chapter 3.2). The untreated and treated psoriatic lesions showed a characteristic psoriatic appearance without any difference between pre- and posttreatment biopsies.

MK886

MK886 is an indirect leukotriene biosynthesis inhibitor which acts through binding of a membrane-bound 5-lipoxygenase-activating protein (FLAP), and thereby inhibits the translocation and activation of 5-lipoxygenase[50,65]. Before and after treatment with MK886, no significant changes in epidermal proliferation, differentiation or in inflammation parameters could be seen in biopsy specimens from psoriatic lesions (Chapter 3.5). Solely a decrease in the number of PMN in the epidermis, reaching borderline significancy ($p=0.05$) was detectable.

Synthesis

A quite uniform pattern of immunohistochemically detected changes was seen during treatment with the various anti-psoriatic therapies.

In the early phases of treatment (from 0 to 2 weeks), mainly changes in epidermal cells occured. The features involved in this early change were the number of Ki-67 stained nuclei, of polymorphonuclear leukocytes (PMN), and the expression of keratin 16. The most consistent finding was the strikingly rapid decrease of the number of Ki-67-positive nuclei in the epidermis in all effective treatments. The amount of keratin 16 in the suprabasal epidermal compartment showed a decrease during treatment with dithranol and budesonide, but not during treatment with MC903. PMN in the epidermis decreased after MC903- and dithranol-treatment, but not after topical application of budesonide. This caused in contrast a decline in dermal PMN. Markedly, the number of PMN in the epidermis during the clinically ineffective systemic treatment with MK886 decreased, reaching borderline statistical significance.

In the later phases of treatment, the number of T-lymphocytes in dermis as well as epidermis decreased, in combination with a decline in dermal PMN.

Epidermal CD14-positive cells (monocytes/macrophages) showed a statistically significant decrease after two weeks of treatment with budesonide, and after eight weeks of treatment with dithranol. Dermal CD14-positive cells showed no major changes. OKT6-positive Langerhans cells tended to increase in the epidermis, and to decrease in the dermal compartment during all effective treatments, but never reached statistical significance.

Hypothesis

It is apparent that epidermal immunohistochemical changes dominate the picture in the early phases of anti-psoriatic treatment. In the early phase of treatment, epidermal growth and PMN are important targets. T-lymphocytes persist relatively long during therapy, and are still prominent even when a clear clinical effect is reached. These observations raise the question whether T-lymphocytes are important in relapses of psoriasis after therapy. Possibly a major reduction in the number of T-lymphocytes is needed for a prolonged remission period.

Keratin 17

In Chapter 3.7 we have described the presence, localization, and changes of keratin 17 in psoriatic lesions before and after therapy. Discovering new aberrations in psoriasis, and studying these aberrations during regression of lesions, might provide more knowledge about, and insight in, psoriasis.

Keratin 17 is not present in normal human epidermis. It can only be found in the outer root sheath of the hair follicle and in myoepithelial cells of the sebaceous gland[66,67]. In untreated psoriatic lesions, keratin 17 was present in the upper layers of the epidermis. After MC903 or dithranol therapy, a clear reduction was found.

Decline in keratin 16 content and Ks8.12 binding is used as a marker for monitoring therapies[37,68]. Expression of keratin 16 is known to be associated with hyperproliferation of keratinocytes[69,70]. From some investigations however, evidence arises that Ks8.12 binding might be sign of a more general disturbance of the epidermal condition[40]. The functional meaning of keratin 17 is not known. Because of its location, the expression of this keratin might be associated with abnormal differentiation, more than with abnormal proliferation. Further studies on the functional significance of keratin 17 expression are indicated. Assessing keratin 17 content in psoriatic lesions before and after therapy might be a useful approach in monitoring anti-psoriatic therapies at the level of epidermal differentiation.

Table I. Timing of the first statistically significant changes of histological markers during various anti-psoriatic treatments. x = investigated time points, ep = epidermal, de = dermal.

Treatment	Time weeks												
	0	1	2	3	4	5	6	7	8	9	10	11	12
MC903	x	x PMN (ep)	x Ki-67		x T11 (ep/de) PMN (ep/de)								x
Dithranol	x		x Ki-67 Ks8.12 PMN(ep)						x RKSE60 T11 (e/d) PMN (de) WT14 (ep)				
Budesonide	x	x Ki-67 Ks8.12 PMN (de)		x WT14 (ep)									
13-cis-RA	x				x								
MK886	x	x PMN (ep)											

5.5 EPIDERMAL GROWTH AND INFLAMMATION IN PSORIASIS-RELATED SKIN DISEASES

The clinical picture of psoriasis is well-defined and often easy to recognize[71]. Nevertheless, sometimes the picture may be difficult to delineate from other erythematosquamous skin disorders. Histologically, using H&E staining, the exact diagnosis can mostly be achieved. In skin disorders showing clinically overlap with psoriasis, our question was whether parameters for epidermal growth and inflammation were similar or distinctive. In Chapter 4 we have investigated three of these skin disorders.

Inflammatory Linear Verrucous Epidermal Nevus (ILVEN) versus linear psoriasis

The delineation of ILVEN from psoriasis has been discussed by many authors[72,73,74]. In Chapter 4.1 we have investigated six patients with linear erythematosquamous lesions. From a clinical point of view, ILVEN and linear psoriasis proved to overlap. Immunohistochemically, a distinction between ILVEN and psoriasis could be made. In ILVEN, we observed focal suprabasal staining with Ks8.12 (keratin 16), homogenous staining using the antibody RKSE60 (keratin 10), and a relatively low occurrence of PMN. In psoriasis, keratin 16 is distributed homogeneously in the suprabasal compartment, keratin 10 is absent in distinct areas, and PMN are a more consistent finding. All other immunohistochemical parameters revealed no differences between ILVEN and psoriasis. In previous investigations, it has been shown that various antipsoriatic therapies inhibit the intraepidermal accumulation of PMN[31]. It is attractive to hypothesise that the absence of PMN in ILVEN might form an explanation for its refractoriness to treatment.

Pityriasis Rubra Pilaris

Extensive psoriasis and pityriasis rubra pilaris (PRP) can be difficult to differentiate clinically, especially since transition from the one in to the other has been described[75]. In chapter 4.2 of this thesis we describe a patient who was first diagnosed as

having psoriasis, but the clinical picture developed into pityriasis rubra pilaris. Immunohistochemically, a low number of Ki-67 stained nuclei and absence of PMN were discriminative.

Dicoid Lupus Erythematosus

Clinically, discoid lupus erythematosus (DLE) is characterized by sharply demarcated erythematosquamous lesions. In contrast to psoriasis, scales are adherent and follicular plugging is present. Ultimately DLE results in atrophy[76], whereas psoriasis does not. In Chapter 4.3, biopsies from twenty patients with a clinical picture of DLE have been investigated immunohistochemically. Epidermal proliferation measured by Ki-67 staining turned out to be increased compared to normal skin, but decreased compared to psoriasis[77]. In addition, the amount of keratin 16 in the suprabasal compartment was high. Lesions of DLE are in a hyperproliferative state, but this tends to diminish with the degree of atrophy. Early epidermal differentiation, measured as expression of keratin 10 is unchanged compared to normal skin. A homogenous distribution in the suprabasal compartment is present, whereas in psoriasis keratin 10 is absent in foci[1]. In DLE, parameters for terminal differentiation (filaggrin and involucrin) are expressed early compared to normal skin[78,79]. In psoriasis, filaggin expression is decreased[79], whereas involucrin expression is increased[78]. In conclusion, in DLE hyperproliferation (however not as extreme as in psoriasis) is associated with normal early differentiation and premature terminal differentiation of keratinocytes. In psoriasis, pronounced hyperproliferation is associated with abnormal early differentiation[80] in combination with abnormal terminal differentiation[78,79].

5.6 GENERAL CONCLUSIONS

1. During the induction of a psoriatic lesion, immunohistochemically detectable dermal changes precede epidermal changes. Increased expression of tenascin and increased numbers of T-lymphocytes are the earliest detectable aberrations in the margin zone of spreading psoriatic plaques. Secondly, a slight epidermal acanthosis is present. Therefore, mechanisms involved in T-lymphocyte-keratinocyte interaction, and the interrelations between T-lymphocytes, keratinocytes and extracellular matrix should form an important part of future studies.

   Epicutaneous application of LTB_4 is rather a model for acute inflammation than a selctive model for psoriasis, especially since in the margin zone of spreading psoriatic plaques, PMN were virtually absent using elastase as a marker enzyme.

2. During treatment of psoriatic lesions, epidermal changes precede dermal changes. Epidermal growth is early affected during treatment, in combination with the number of epidermal PMN. The early reduction of PMN accumulation during treatment is intruiging. T-lymphocytes persist relatively long. This indicates that a more substantial reduction of the number of T-lymphocytes could well induce prolonged remission periods.

3. The immunohistochemical investigation of markers described in this thesis provide a "fingerprint" of epidermal growth and inflammation in various skin disorders. The contribution of these markers to the diagnosis of psoriasis and psoriasis-related skin diseases is relatively limited, as a reasonable overlap exists. Extra information is given by assessment of PMN, Ks8.12 binding and RKSE60 binding in ILVEN (Chapter 4.1), and of PMN in pityriasis rubra pilaris (Chapter 4.2). In DLE, a close look at the epidermal differentiation process shows aberrations compared to normal skin and variations compared to psoriasis. It is intruiging that in both acanthotic psoriatic lesions, and in atrophic DLE-lesions, increased Ki-67 binding and altered differentiation is

seen. Studying the regulation of these processes uncovers more of the cellbiological and molecular causes involved.

4. The observation that corticosteroids, vitamin A and -derivatives, and vitamin D and -derivatives are effective in the treatment of psoriasis, suggest that the steroid receptor supergene family induces an important collective working mechanism, which can implicate that this family is relevant for the understanding of the pathogenesis of psoriasis.

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SUMMARY

In this thesis, parameters for epidermal growth and inflammation have been investigated in the induction and regression of psoriasis. In addition, clinical efficacy of some new and modified classical treatments were studied. The described parameters were also examined in three psoriasis-related skin diseases.

In Chapter 1, psoriasis is introduced by describing its epidemiological, aetiological and genetical features. Clinical and histological aspects of this skin disease are pictured as well. In the pathogenesis of psoriasis, still many controversies exists as to which cells or events are crucial. The role of various cell types is described. The established and some new treatments are presented. The applied methods used for clinical and immunohistochemical assessment in this thesis are introduced.

In Chapter 2, the induction of psoriatic lesions has been investigated using two different in vivo models: the margin zone of spreading plaques, and epicutaneous application of leukotriene B_4 (LTB_4) on normal skin. In the margin zone, increased expression of the extracellular matrix protein tenascin and increased numbers of T-lymphocytes in the dermis preceded initial acanthosis of the epidermis. Epicutaneous application of LTB_4 on normal skin induces an acute inflammatory reaction, which partly corresponds with changes as seen in initial psoriatic lesions. The low numbers of polymorphonuclear leukocytes (PMN) in the margin zone using elastase as a marker enzyme raises doubts as to the specificity of the LTB_4 model.

In Chapter 3, the effects of new anti-psoriatic therapies on clinical parameters and on markers for epidermal growth and inflammation are described. Treatment with the vitamin D_3 analogue MC903 proved to be an effective and safe acquisition. Short-contact treatment with dithranol in a new cream base, proved to be as effective as other dithranol treatment schemes. Advantages of this formulation are that it can be manufactured by the local pharmacologist, and that it is easy to remove from skin. Budesonide, a class 3-4 corticosteroid proved to be very effective in the treatment of psoriasis. Topical application of 13-cis-retinoic acid 0.1% in a cream base and oral administration of a leukotriene biosynthesis inhibitor (MK886) showed

no clinical effect. Corticosteroids, vitamin A and -derivatives, and vitamin D and -derivatives are effective therapies. Therefore the steroid receptor supergene family can provide important information for understanding more of the mechanisms involved in regression of psoriatic lesions and maybe in it's pathogenesis.
Immunohistochemical investigations concerning epidermal growth and inflammation during anti-psoriatic treatment revealed that the epidermal compartment shows changes before the dermal compartment is altered. Especially epidermal growth and the number of PMN are early affected. T-lymphocytes persist relatively long. Langerhans cells and monocytes/macrophages show slight but not statistically significant changes in number. These observations might indicate that more substantial reduction in the number of T-lymphocytes induces prolonged remission periods.

In Chapter 4 three skin disorders are investigated that show clinically overlap with psoriasis: inflammatory linear verrucous epidermal nevus (ILVEN) versus linear psoriasis, pityriasis rubra pilaris (PRP), and discoid lupus erythematosus (DLE). The immunohistochemical stainings concerning epidermal growth and inflammation, compared to those in psoriasis, revealed corresponding and deviating features. Extra information is given by assessment of PMN, Ks8.12- and RKSE60-binding in ILVEN, by measuring PMN in PRP, and by studying proliferation and differentiation markers in DLE.

In Chapter 5 the immunohistochemical approach and the PASI-score used for assessment of clinical parameters are discussed. A synthesis of the interrelation between parameters for epidermal growth and inflammation during induction and regression of the psoriatic lesion is made. During induction, dermal changes preponderate, whereas during regression epidermal changes do. Epidermal growth and PMN play an important role in the initial therapeutic effect. As after treatment still dermal aberrations exist, especially concerning T-lymphocytes, a more substantial decline of these cells is suggested to be important for prolonged remission. Studying regulation of epidermal growth and inflammation in psoriasis-related skin disorders provides additional information on the mechanisms involved.

in dit proefschrift zijn parameters voor epidermale groei en ontsteking bestudeerd tijdens ontstaan en tijdens regressie van psoriasis. Eveneens is het klinisch effect van enkele nieuwe en gemodificeerde klassieke therapieen onderzocht. Bovengenoemde parameters zijn tevens bij drie aan psoriasis gerelateerde ziektebeelden bekeken.

In Hoofdstuk 1 wordt het ziektebeeld psoriasis geintroduceerd en worden de epidemiologische, aetiologische en genetische kenmerken beschreven. Klinische en histologische aspecten van deze huidziekte worden behandeld. Over de pathogenese van psoriasis bestaan nog steeds verschillen van inzicht wat betreft de vraag welke cellen of gebeurtenissen cruciaal zijn. De rol van verschillende celtypes worden besproken. De klassieke, gevestigde en enkele nieuwe behandelingsmethoden worden gepresenteerd. Bovendien worden de klinische en immunohistochemische methodes, toegepast in dit proefschrift, geintroduceerd.

In Hoofdstuk 2 is het ontstaan van psoriasislaesies onderzocht met behulp van twee in vivo modellen: de randzone van groeiende plaques en epicutane applicatie van leukotriene B_4 (LTB_4) op normale huid. In de randzone werd toegenomen expressie van het extracellulaire matrix eiwit tenascin, en een toegenomen aantal T-lymfocyten in de dermis gezien. Deze gebeurtenissen gingen vooraf aan beginnende acanthose van de epidermis. Epicutane applicatie van LTB_4 op normale huid induceert een acute ontstekingsreactie, die gedeeltelijk overeenkomt met de veranderingen die te zien zijn bij vroege psoriasislaesies. De lage aantallen polymorfkernige leukocyten (PMN) die in de randzone gevonden worden wanneer elastase als marker enzym wordt gebruikt, doen twijfelen aan de specificiteit van het LTB_4-model voor psoriasis.

In Hoofdstuk 3 wordt het effect van nieuwe anti-psoriatische behandelingen op klinische parameters en op parameters voor epidermale groei en ontsteking beschreven. Behandeling met het vitamine D_3 analoog MC903 heeft bewezen een effectieve en veilige aanwinst te zijn. Korte duur-behandeling met dithranol in een nieuwe crème basis bleek even effectief als andere dithranol-behandelinsschema's. Voordeel

van dit nieuwe preparaat is dat het kan worden gemaakt door de lokale apotheek, en dat het gemakkelijk van de huid kan worden verwijderd. Budesonide, een klasse 3 à 4 corticosteroid bleek zeer effectief bij de behandeling van psoriasis. Lokale applicatie van 13-cis-retinoinezuur 0.1% in een crèmebasis en orale toediening van een leukotriene-biosynthese-remmer (MK886) lieten geen klinisch effect zien. Corticosteroiden, vitamine A en -afgeleiden en vitamine D en -afgeleiden zijn effectieve behandelingen voor psoriasis. De steroid receptor supergene family lijkt belangrijke extra informatie te kunnen brengen wat betreft therapeutische regressie en mogelijk ook de pathogenese van psoriasis.
Immunohistochemisch onderzoek van epidermale groei en ontsteking tijdens behandeling van psoriasis bracht aan het licht dat het epidermale compartiment veranderingen liet zien voordat veranderingen te zien waren in de dermis. Met name epidermale groei en het aantal PMN veranderden vroeg in de tijd. T-lymfocyten bleven relatief lange tijd aanwezig. Langerhans cellen en monocyten/macrofagen veranderden in aantal, echter dit was niet statistisch significant. Uit deze observaties zou kunnen blijken dat een meer substantiele reductie van het aantal T-lymfocyten mogelijk een langere remissie-periode geeft.

In Hoofdstuk 4 zijn drie huidziektes onderzocht die klinisch overeenkomsten laten zien met psoriasis: inflammatoire lineaire verruceuze epidermale naevus (ILVEN) versus lineaire psoriasis, pityriasis rubra pilaris (PRP), en discoide lupus erythematodes (DLE). De immunohistochemische kleuringen wat betreft epidermale groei en inflammatie werden vergeleken met de resultaten hiervan bij psoriasis, en lieten zowel overeenkomsten als verschillen zien. Extra informatie geeft bepaling van PMN en kleuring met behulp van de antilichamen Ks8.12 en RKSE60 bij ILVEN, het aantal PMN bij PRP, en het bestuderen van proliferatie- en differentiatie-parameters bij DLE.

In hoofdstuk 5 worden de immunohistochemische kleuringen en de PASI-score (Psoriasis Area and Severity Index) bediscussieerd. Een synthese van de interrelatie tussen parameters voor epidermale groei en ontsteking tijdens ontstaan en verbetering van de psoriasis laesie wordt gepresenteerd. Tijdens inductie staan dermale veranderingen op de voorgrond, terwijl dit bij regressie de epidermale veranderingen

zijn. Epidermale groei en het aantal PMN spelen een belangrijke rol bij het vroege therapeutische effect van een behandeling. Aangezien na behandeling nogal eens dermale afwijkingen blijven bestaan, met name wat betreft T-lymfocyten, lijkt verdere vermindering van deze cellen belangrijk voor langere remissie-periodes. Het onderzoeken van regulering van epidermale groei en ontsteking bij aan psoriasis gerelateerde huidziektes, zorgt voor uitbreiding van onze kennis over de mechanismes die hierbij betrokken zijn.

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CURRICULUM VITAE

Elke de Jong werd geboren op 16 maart 1963 te Gilze en Rijen. Zij behaalde in 1981 het eindexamen Gymnasium-ß aan het van Maerlantlyceum te Eindhoven. Aansluitend startte zij met de studie geneeskunde aan de Katholieke Universiteit Nijmegen, waar zij in februari 1989 het artsexamen behaalde. Vanaf mei 1989 was zij werkzaam als wetenschappelijk onderzoekster op de afdeling Dermatologie van het Academisch Ziekenhuis Nijmegen, waar zij onder leiding van Prof. Dr. P.C.M. van de Kerkhof werkte aan dit proefschrift. Sinds 1 juni 1991 is zij in opleiding tot Dermatologe te Nijmegen.

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