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Expression of Interleukin-6-like Molecules and Tumour Necrosis Factor after Topical Treatment of Psoriasis with a New Vitamin D Analogue (MC 903)

ANNEMETTE OXHOLM,[1,3] PETER OXHOLM,[2,3] BENT STABERG[4]
and KLAUS BENDTZEN[2]

[1]Department of Dermatology, Gentofte Hospital, [2]Laboratory of Medical Immunology TTA, Rigshospitalet, University Hospital, the [3]Bartholin Institute, Kommunehospitalet, Copenhagen, and [4]Bispebjerg Hospital, Copenhagen, Denmark

Skin biopsies from 5 patients with chronic plaque psoriasis were examined to test the effect of topical treatment with a new synthetic vitamin D_3 analogue, MC 903, on epidermal interleukin 6 (IL-6) and tumour necrosis factor α (TNFα). Skin biopsies, taken before therapy and after one and 2 weeks of therapy were examined immunohistologically. IL-6 was visualized using a partially purified polyclonal antiserum to crude supernatants of activated human blood monocytes before and after absorption with recombinant human IL-6. TNFα was demonstrated using a specific polyclonal antiserum to human recombinant TNFα. Both the intensity and extension of staining for IL-6, but not for TNFα, were increased in lesions compared with unaffected skin. During therapy with MC 903 a decline in the staining intensity for IL-6, but not for TNFα, was observed in both lesional and unaffected skin. Placebo-treated lesions, however, remained unchanged.

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A. Oxholm, Department of Dermatology, Gentofte Hospital, DK-2900 Hellerup, Denmark.

Recent investigations have indicated that keratinocytes secrete several different immuno-inflammatory mediators (cytokines), including interleukin 6 (IL-6) and tumour necrosis factor α (TNFα) (1–3). These and other cytokines are known to participate in a number of immunoinflammatory processes (1–6). By the use of an immunohistological technique we recently showed that 'IL-1-like molecules' and TNF could be demonstrated in the epidermis of healthy individuals (7). Further experiments revealed that IL-1-like molecules were most likely identical with IL-6 (8).

Recent studies suggest an immunoregulatory role of the vitamin D metabolite $1,25(OH)_2D_3$ (9), and in vitro studies have shown that this hormone and a synthetic analogue MC 903 influence the production and function of cytokines (10).

The serum concentration of $1,25(OH)_2D_3$ in patients with disseminated psoriasis is reduced suggesting an abnormal vitamin D metabolism in these patients (11). Moreover, local and systemic treatments with vitamin D metabolites are beneficial in the treatment of psoriasis (12, 13).

We have therefore studied the influence of topical treatment with MC 903 on IL-6 and TNFα expression in the skin of psoriasis patients.

MATERIALS AND METHODS

Patients and design

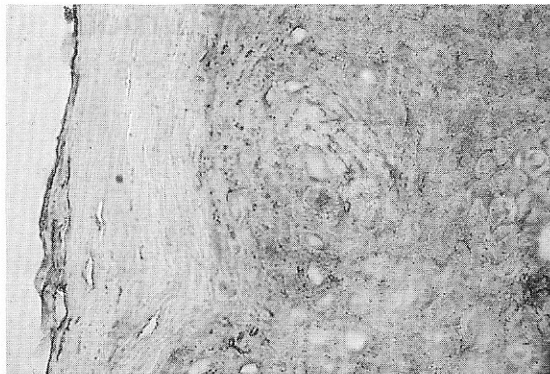
Five informed outpatients (mean age 46 years, range 40–54) with symmetrical, plaque-type psoriasis vulgaris were selected for the study. None of the patients had received antipsoriatic treatment for at least 3 weeks prior to the study. All had normal serum levels of calcium, creatinine, aspertate aminotransferase and alkaline phosphatase.

MC 903 cream (water/oil emulsion) (vitamin D analogue, Leo Pharmaceutical Company, Copenhagen, Denmark) (14, 15) containing 1 200 µg MC 903 per g cream and the control cream base were evenly applied with a fingertip (about 0.125 g) twice daily on 100 cm² large symmetrical psoriasis plaques. MC 903 was applied on an equal sized unaffected skin area. Treatment with MC 903 and cream base was continued for 2 weeks. As controls, 3 healthy individuals applied equal amounts of cream base on healthy skin for 2 weeks. The treatment was 'blind' to the patient but 'open' to the investigators. All patients received concomitant topical treatment with moisturing cream on the remaining part of the body. However, no other antipsoriatic treatment was permitted.

Blood samples for standard laboratory examinations, including serum calcium and serum phosphate, were repeated after treatment.

Clinical assessment

Clinical assessment of infiltration, erythema, and scaling of the selected psoriatic plaques was performed by one of the authors at the onset of therapy and after one and 2 weeks of therapy. Each clinical parameter was given a score (0: not present, 1: slight, 2: moderate, 3: severe). The clinical score



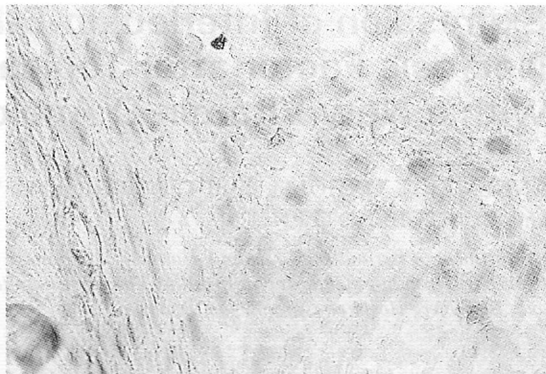


Fig. 1. Epidermal IL-6-like molecules demonstrated by a biotin–avidin–peroxidase technique using an anti-MK-antiserum. (*a*) Uninvolved skin before treatment (original, ×800). (*b*) Lesional skin before treatment. In both cases prior absorption of the antiserum with rIL-6 abolished staining.

was calculated as the sum of these parameters, the highest possible score would be 9.

Skin biopsies

Punch biopsies (4 mm) of psoriasis plaques (treated with MC903 or placebo) and of unaffected skin (treated with MC903) were obtained, using infiltration anaesthesia with 2% lidocaine in a ring around the biopsy site. The biopsies were taken before treatment, and after one and 2 weeks of therapy. From the controls, biopsies were taken before and after 2 weeks of treatment with placebo. The tissues were quick-frozen and stored at -70°C, before staining with hematoxylin–eosin and biotin–avidin.

Production of antisera to human monokines (anti-MK) and to human rTNFα

The specific antiserum to human rTNF*α* was produced and donated by Dr C. A. Dinarello (Tufts New Engl. Med. Center, Boston, Mass., USA) (6, 7). This antiserum does not react with IL-1*α*, IL-1*β*, TNF*β*, interleukin 2 (IL-2), or interferon-*γ* (Bendtzen K, unpublished data).

The rabbit-antiserum against crude supernatants of *Staph. albus*-activated human blood monocytes was produced by Dr C. A. Dinarello (16). This antiserum was purified by sequential absorption with antigens from leukocyte supernatants obtained after 30 min of incubation with serum and staphylococci, with leukocyte supernatants obtained after 18 h of incubation in 2.5 μg of cycloheximide per ml, which inhibits the synthesis of various monokines, and with fresh human AB serum. This absorbed antiserum (anti-MK) reacts with both major forms of IL-1 (IL-1*α* and IL-1*β*) and, in addition, with IL-6 but not with IL-2, interferon-*γ*, TNF*α*, or TNF*β* (8, Bendtzen, unpublished).

Biotin–avidin technique for demonstration of tissue-bound IL-6 and TNFα

4–6 μm sections of the skin were cut in a cryostat. The samples were air-dried for 5 min, fixed in acetone for 1 min, and then incubated for 5 min with 3% hydrogen peroxide in distilled water, followed by repeated washing (5×1 min) in distilled water and, finally, in Tris buffer (0.05 M Tris-HCl, pH 7.6) for 5 min. The sections were then incubated in Tris-buffer in a humidity chamber for 20 min with 20% normal swine serum (Dakopatts, Copenhagen, Denmark). Excess serum was removed, and the sections were incubated for 30 min (20°C) with diluted anti-MK, anti IL-1*α* or *β* (two-fold dilutions 1:40–1:640), or anti-rTNF*α* antiserum (two-fold dilutions 1:20–1:320) (see below). The sections were then washed in Tris buffer (3×5 min) followed by incubation for 30 min (20°C) with biotinylated swine antirabbit immunoglobulin (Dakopatts) diluted 1:300 in Tris buffer. After washing in Tris buffer (3×5 min), the sections were incubated with ABComplex (Dakopatts) for 30 min (20°C) and washed as mentioned above. The sections were then incubated with 0.038% 3-amino-9-ethylcarbazol (Sigma, St. Louis, Mo, USA) and 0.014% hydrogen peroxide for 5 min, washed in water for 10 min, counterstained with hematoxylin for 2 ½

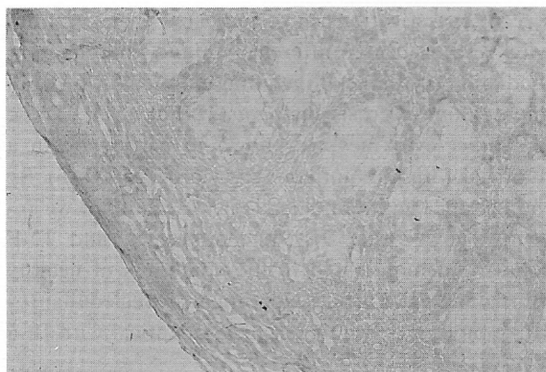
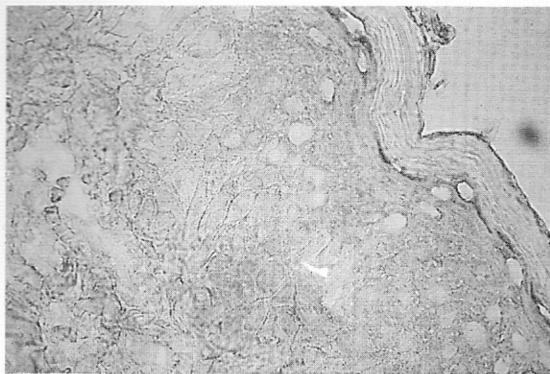


Fig. 2. Negative control. Involved skin. The primary layer antiserum was replaced by normal rabbit serum (original, ×800).



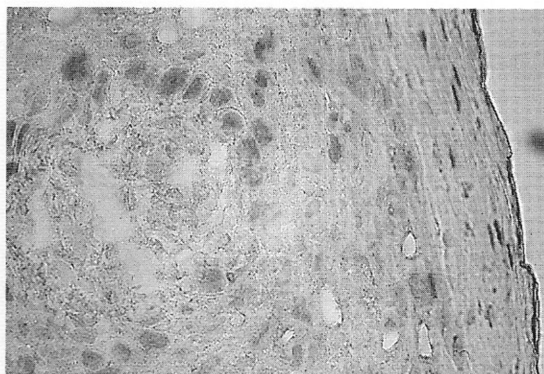


Fig. 3. Epidermal IL-6-like molecules demonstrated by a biotin–avidin–peroxidase technique using an anti-MK-antiserum. Skin samples taken after one week of treatment with MC 903. (*a*) Uninvolved skin, (*b*) lesional skin.

min and, finally, washed in water for 10 min. The samples were mounted with Gurr aquamount (BDH Chemical, Poole, England).

Absorption of anti-MK with human rIL-6/anti-rTNFα with rTNFα

Prior to experiments, 1 μl anti-MK antiserum (1 : 10 dilution) was incubated with 100 U (=20 ng) of human rIL-6 at 20°C for 1 h followed by incubation at 4°C for 1 h. The rIL-6 preparation was kindly provided by Dr T. Hirano (Osaka University, Osaka, Japan) (*4*).

Preincubation of anti-rTNFα-antiserum with human rTNFα (kindly provided by Dr G. R. Adolf, Boehringer Inst., Vienna, Austria) abolished the staining caused by this antiserum.

Immunohistological controls

The specific staining was also lost if either the primary layer (the anti-MK or anti-rTNFα antiserum) or the secondary layer antibody was omitted, or if the primary antibody was replaced by Tris-buffer or normal rabbit serum (Dakopatts).

RESULTS

Anti-MK and anti rTNFα antiserum staining patterns in untreated and placebo-treated skin

In unaffected and untreated skin a granular staining of the intercellular space of the epidermis was found in all specimens. Staining was localized either to membranes of single cells or to groups of cells. Staining was most pronounced in the upper layers and only occasionally included the basal layers of the epidermis (Fig. 1 *a*).

If the anti-MK-antiserum was first absorbed with rIL-6, the staining was abolished in specimens from both normal skin and psoriatic skin lesions.

In biopsies from lesional skin stained with anti-MK-antiserum both the staining intensity and its distribution were increased compared with unaffected skin (Fig. 1 *b*). The staining now included the basal layers, although irregularly distributed, and further dilution of the primary layer antibody was still capable of staining the skin. This, however, was not the case in biopsies stained for TNFα. The staining patterns for both cytokines were unaffected of treatment for one and 2 weeks with placebo cream in both lesional skin and skin from controls.

Staining of both normal and lesional psoriatic skin with anti-rIL-1α or anti-rIL-1β was negative.

Anti-MK and anti-rTNFα antiserum staining patterns in MC 903-treated skin

In biopsies from both *lesional* and *unaffected* skin the staining pattern for IL-6-like molecules was altered after one and 2 weeks of treatment with MC 903 cream. A marked decrease was observed in staining intensity of the upper viable layers of the epidermis (stratum granulosum and spinosum). Indeed, in two of the biopsies from unaffected skin there was no detectable staining in the upper epidermal layers in the dilutions used. Weak staining was preserved in the basal layer in all biopsies from lesions (Figs. 3 *a*, *b* and 4). There was no decline in the stratum corneum (SC) staining in skin lesions after one week of treatment. However, after 2 weeks a decline was observed in parallel with the histologic normalization of SC. Like in untreated skin anti-MK-antiserum staining was abolished after absorption with IL-6.

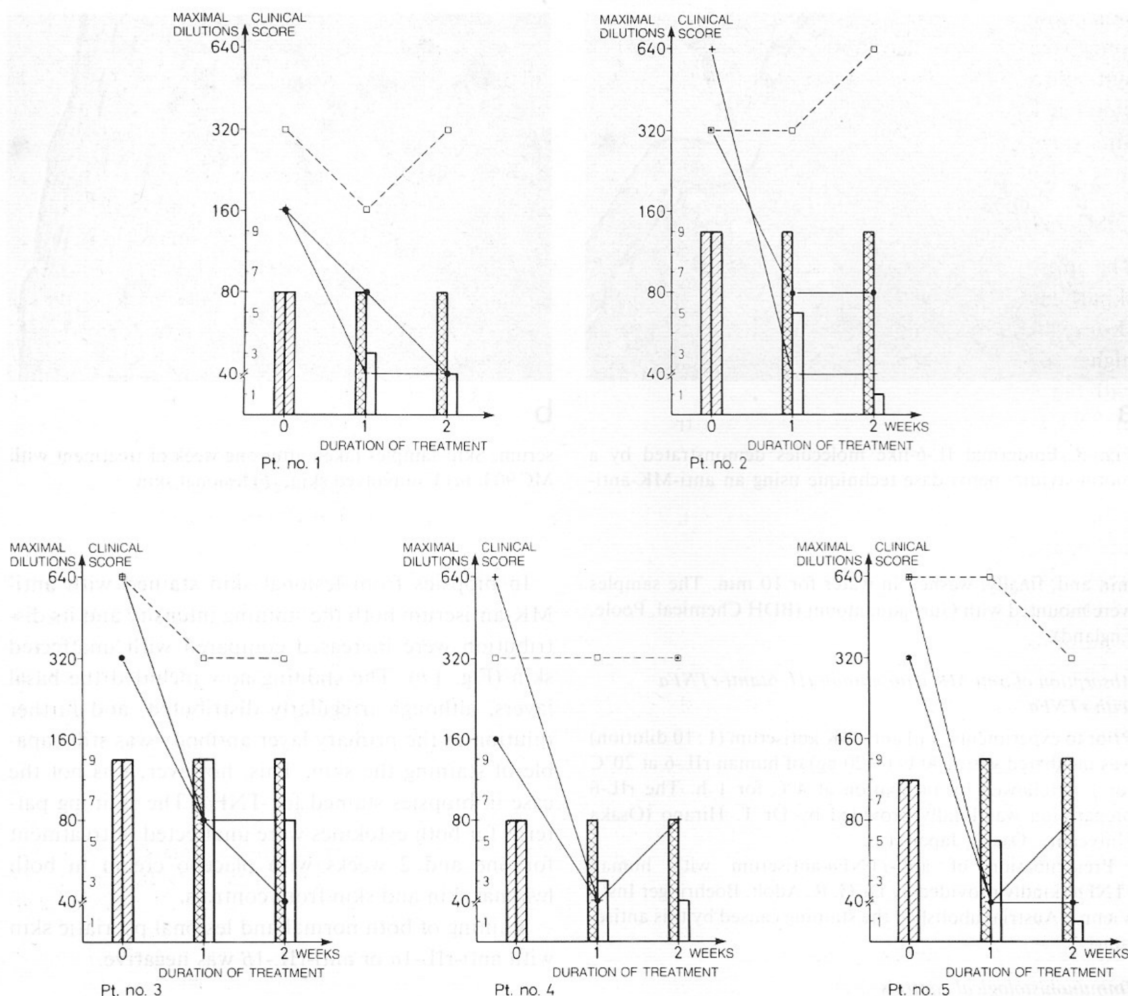


Fig. 4. Maximum dilutions of primary layer anti-MK-antiserum giving epidermal membrane staining using the biotin–avidin technique. Skin samples were taken before therapy, after one week and after 2 weeks of treatment with MC 903/placebo. ●, Uninvolved skin treated with MC 903; +, lesional skin treated with MC 903; □, lesional skin treated with placebo cream. Columns indicate clinical scores. Hatched bars: Placebo-treated lesions; □, MC 903-treated lesions.

Staining with anti-rTNFα antiserum was unaffected by treatment with MC 903.

Histology of hematoxylin/eosin-stained skin specimens

Biopsies from unaffected skin showed no histopathological abnormalities of the epidermis. In some biopsies a discrete perivascular infiltrate of mononuclear cells was present in the dermis, but no changes of the vessels or papillae were observed. The histology of unaffected skin was unchanged during treatment with both placebo and MC 903 cream.

Biopsies from plaques showed typical psoriatic changes. After one week of treatment with MC 903 only minor signs of normalization occurred. After 2 weeks, however, the response to therapy was evident with a marked decrease in thickness of stratum corneum and of the viable epidermal cell layers.

Clinical response

The overall clinical responses in the 5 patients are shown in Fig. 4. Treatment with MC 903 cream induced a gradual decrease in the clinical score. However, there was only an incomplete clearing of the le-

sions after the 2 weeks of treatment. There was no improvement of the placebo-treated plaques. Local or systemic adverse reactions were not observed, and the blood analyses were within normal limits before and after treatment.

DISCUSSION

The present paper shows that in lesional psoriatic skin the in vivo bound epidermal cytokine, probably IL-6, was more widely distributed and present at a higher concentration, especially in the SC, compared with the uninvolved skin. In contrast, the distribution of TNFα in lesional skin did not differ from that visualized in unaffected skin. During treatment with a vitamin D_3 analogue, MC 903, the amount and distribution of epidermal IL-6 declined in both lesional and unaffected psoriatic skin compared with placebo-treated skin. In contrast, the amount and distribution of TNFα did not change under the same experimental conditions.

The immunohistological technique used in the present study has been discussed previously (7, 8). The present findings strongly suggest that the major cytokine visualized in the skin, by anti-MK-antibodies is similar to, or identical with IL-6. Thus, the binding of anti-MK antibodies was completely inhibited by absorption with purified human rIL-6 and not with rIL-1α or β.

IL-6 induces proliferation of many cell types, including $CD4^+$ T-lymphocytes, where the mediator may contribute to the induction of IL-2 receptors (4, 5) and, possibly, explain the recruitment of $CD4^+$ cells in active psoriatic lesions. Recently the immunoregulatory role of $1,25(OH)_2D_3$ and MC 903 has been elaborated in vitro (9, 10). Both hormones selectively inhibit the T-cell stimulating function of IL-1 and thus IL-2 production. In contrast, the biological activity of TNFα is unaffected (10). We also detected a differentiated effect of MC 903, namely on the staining of IL-6 and TNFα in epidermis. The decrease in concentration and distribution of IL-6 during treatment with MC 903 cream was observed in the most mature layers of the epidermis, while staining was preserved in the basal layer.

$1,25(OH)_2D_3$ affects the function of cells via specific cytoplasmatic and nuclear receptors in many tissues including the skin (9, 12). The fact that the expression of IL-6 decreased both in lesional and in unaffected skin during treatment with MC 903 indicates that the drug acts directly on keratinocytes and not necessarily via an effect on lymphocytes infiltrating the psoriatic lesions.

The molecular mechanisms underlying the decreased expression of IL-6 during treatment with MC 903 are unknown. A direct effect of MC 903 on the production of IL-6 and/or an effect on the production or function of IL-1, a potent inducer of IL-6 (4, 5), are both possible. The calcium metabolic activity of vitamin D_3 analogues may also be involved, but it is noteworthy that the calcium metabolic effect of MC 903 is approximately 100 times lower than that of $1,25(OH)_2D_3$, at least in rats (15).

Recent studies have shown that $1,25(OH)_2D_3$ causes a time- and dose-dependant decrease in cell proliferation and a stimulation of terminal differentiation in cultured human keratinocytes isolated from normal as well as from psoriatic skin (12, 17). On the basis of the present findings it seems relevant to ask whether IL-6 is involved as an endogenous regulator of keratinocyte growth. If so, IL-6 may be of importance in the pathophysiology of psoriasis. However, it should be borne in mind that immunohistological techniques do not allow exact conclusions concerning the functional activity of the demonstrated cytokines.

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