British Journal of Dermatology 2003; **149:** 853–857.

CONCISE COMMUNICATION

Pimecrolimus does not affect Langerhans cells in murine epidermis

J.G.MEINGASSNER, E.KOWALSKY, H.SCHWENDINGER , A.ELBE-BÜRGER* AND A.STÜTZ

*Novartis Forschungsinstitut, Brunnerstrasse 59, A-1235 Vienna, Austria, and *DIAID, Department of Dermatology, University of Vienna Medical School, Vienna, Austria*

Accepted for publication 29 April 2003

Summary

Background Langerhans cells (LCs) function as specialized antigen-presenting cells in the epidermis, and therefore play a critical role in cutaneous immunological reactions. Topical treatment with corticosteroids is associated with a decrease in epidermal LC number and antigen-presenting capacity in laboratory animals and humans.

Objectives To examine whether pimecrolimus, a nonsteroidal inflammatory cytokine inhibitor recently introduced for the topical treatment of atopic dermatitis, differs from corticosteroids in effects on LCs.

Methods Groups of BALB/c mice were treated twice daily on one to five consecutive days on the inner surface of the right ear with 10 μL of ethanolic solutions of the test compounds at their clinically used concentrations (1% pimecrolimus, 0·1% betamethasone-17-valerate, 1% hydrocortisone and 0·05% clobetasol propionate) or with the vehicle (controls) alone. At selected time points after the treatment epidermal sheets were prepared and examined histomorphometrically for LCs immunolabelled with antibodies to major histocompatibility complex (MHC) class II and DEC 205, and adenosine diphosphatase staining.

Results No changes in number or morphology of LCs were observed in epidermal sheets of mice treated for 5 days with pimecrolimus. In contrast, an almost complete depletion of LCs was observed in skin samples treated with hydrocortisone, betamethasone or clobetasol. Even a single-day treatment schedule with hydrocortisone, betamethasone or clobetasol caused a significant reduction in MHC class II+ LCs, by 31%, 62% and 87%, respectively.

Conclusions It is therefore unlikely that topically applied pimecrolimus affects epidermal LCs, in contrast to corticosteroids.

Key words: corticosteroids, epidermis, Langerhans cells, mouse, pimecrolimus

Topical application of drugs aims at local therapeutic intervention while avoiding potential unwanted effects associated with systemic administration of medicinal products. Nevertheless, local treatment regimens resulting in local or systemic toxicity after percutaneous absorption have been reported. Side-effects of glucocorticoids, which are widely used topically in the treatment of inflammatory skin conditions, are well-known examples. Striae, epidermal and dermal atrophy, acne, perioral dermatitis, rosacea, purpura or telangiectasia are gross clinical signs frequently observed after use of glucocorticoids.[1] Microscopic changes are a decrease in numbers of epidermal Langerhans cells (LCs).[2–6] LCs function as specialized antigen-presenting cells in the epidermis, and therefore play a critical role in cutaneous immunological reactions.[7,8] Additionally, LCs may function as anti-inflammatory agents because a decrease in number and function has been associated with an enhanced inflammatory reaction of the skin.[9]

Correspondence: Josef G. Meingassner.
E-mail: josef.meingassner@pharma.novartis.com

As the new nonsteroidal cream pimecrolimus (SDZ ASM 981, Elidel®) has recently been introduced for the topical treatment of atopic dermatitis,[10–12] we were interested to know if it would have a different effect on LCs than the older treatments, topical corticosteroids. We therefore investigated the effect of topical administration of pimecrolimus, an inflammatory cytokine inhibitor specifically developed for the treatment of inflammatory skin diseases, with that of corticosteroids of various potencies on LC density in skin of treated mice. All compounds were tested in the same experimental setting at their clinically used concentrations.

Materials and methods

Laboratory animals

Ten- to 12-week-old female BALB/cAnNCrl mice were used. The animals were supplied by Charles River (Sulzfeld, Germany) and maintained conventionally under standard animal house conditions with standard laboratory chow (SNIFF® R/M-H diet for mice and rats; Soest, Germany) and drinking water given *ad libitum*. In each study, groups of four mice were used after an acclimatization period of 8–10 days. The animals were used according to an approved protocol under licence no. MA 4008/99 (Magistratsabteilung Nr. 58, Amt der Wiener Landesregierung).

Test compounds, formulation and application

Pimecrolimus was synthesized at Novartis Pharma AG (Basle, Switzerland). Hydrocortisone, betamethasone-17-valerate and clobetasol propionate were obtained from Sigma-Aldrich (Vienna, Austria). The test compounds were dissolved daily in ethanol at their clinically used concentrations (1% pimecrolimus, 1% hydrocortisone, 0·1% betamethasone-17-valerate and 0·05% clobetasol propionate), and 10 μL of the solutions or vehicle (controls) were applied to the inner surface of the right auricle, twice a day on five consecutive days. In follow-up studies mice were treated only for 1, 2, 3 or 4 days in order to evaluate the minimal treatment period associated with a significant reduction of LC numbers.

Immunocytological/histochemical labelling of Langerhans cells in epidermal sheets

Antibodies against murine major histocompatibility complex (MHC) class II and DEC 205 (a multilectin receptor for adsorptive endocytosis, expressed in murine dendritic cells[13]) as well as adenosine diphosphatase (ADPase) staining were used to label LCs in epidermal sheets. Epidermal sheets from all test sites were prepared with an ammonium thiocyanate separation technique[14] 3 days after the last application in all treatment groups and for recovery studies 11, 27 or 45 days after the last application of the 5-day treatment schedule. LCs were labelled with a single (anti-MHC class II) or a double (anti-MHC class II and anti-DEC 205) immunostaining procedure.

Single immunolabelling. The acetone-fixed sheets were incubated for 1 h at 37 °C with fluorescein isothiocyanate (FITC)-labelled antimouse MHC class II monoclonal antibody (mAb) ($I\text{-}A^d$; clone AMS-32.1; PharMingen, San Diego, CA, U.S.A.; 1 : 500), washed, and then mounted dermal side up in Dako Fluorescent Mounting Medium (Dako, Glostrup, Denmark) on glass slides for fluorescence microscopy.

Double immunolabelling. The acetone-fixed sheets were incubated with a biotinylated antimouse MHC class II mAb (as above) for 16 h at 4 °C, then reacted with Texas red/streptavidin (Amersham, Freiburg, Germany; 1 : 600) for 1 h at 37 °C. After washes the sheets were counterstained with a rat antimouse DEC 205 mAb (clone NLDC-145; Serotec, Oxford, U.K.; undiluted hybridoma culture supernatant) for 16 h at 4 °C, followed by a FITC-conjugated $F(ab)_2$ goat antirat IgG antibody (Biosource, Camarillo, CA, U.S.A.; 1 : 750) for 1 h at 37 °C and processed as above. Immunocytochemical controls included irrelevant isotype-matched mAbs; these consistently yielded negative results.

Adenosine diphosphatase staining. ADPase staining of paraformaldehyde-fixed sheets was performed according to the technique of Chaker *et al.*[15] with the substrate at 0·33 mmol L^{-1} concentration.

Counting. Immunocytologically/histochemically labelled cells were counted in 10–30 randomly chosen microscopic fields [× 400 and × 650 (ADPase), respectively] in each epidermal sheet with the help of rectangular grids and expressed as cells mm^{-2} epidermal sheet. The evaluation was performed in a coded fashion.

Statistical analysis. For statistical analyses the square roots of the data were compared using ANOVA, and the Tukey *post hoc* test was applied for pairwise comparisons using the software program Systat V10.

Results

In the first series of studies, in which a 5-day treatment schedule was applied, depletion of MHC class II+ cells was almost total in epidermal sheets examined 3 days after the last application of 1% hydrocortisone, 0·1% betamethasone-17-valerate or 0·05% clobetasol propionate. In contrast, neither significant numerical nor morphological changes in LCs were observed in test sites exposed to topical 1% pimecrolimus (Fig. 1). Because the deleterious effect of the corticosteroids was similar, independently of their different potencies, we evaluated the recovery period and the minimal treatment schedules resulting in LC depletion. A tendency towards recovery after the 5-day treatment schedule was seen earliest in hydrocortisone-treated mice, on day 32 (45% of controls, Fig. 2). On day 50, the density of MHC class II+ cells in clobetasol-treated samples still differed significantly (by 74%) from controls, whereas recovery in hydrocortisone- or betamethasone-treated animals had almost been achieved (Fig. 2). A significant reduction in epidermal MHC class II+ LCs by corticosteroids was also seen in mice treated twice daily for less than 5 days. Hydrocortisone, for example, applied twice daily on two consecutive days, resulted in an LC reduction of 81% (data not shown). Even after a single-day treatment, MHC class II+ cell counts (or ADPase+ cell counts) were reduced by 31 (38)%, 62 (74)% and 87 (82)% compared with vehicle-treated skin in hydrocortisone-, betamethasone- and clobetasol-treated skin, respectively (Figs 3 and 4).

We therefore used a 1-day treatment schedule in follow-up studies. To test whether the reduction in LCs was also reflected by another marker, we included DEC 205 in our studies. Dual immunofluorescence staining revealed that MHC class II+ and DEC 205+ cells were significantly reduced in the epidermis treated with

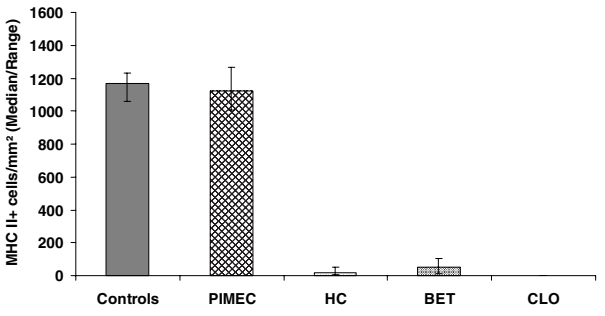

Figure 1. Major histocompatibility complex (MHC) class II+ cells (median values and ranges, $n = 4$) in auricular epidermis treated topically with ethanol (vehicle, controls), pimecrolimus (1%, PIMEC), hydrocortisone (1%, HC), betamethasone-17-valerate (0·1%, BET) or clobetasol propionate (0·05%, CLO) twice daily on days 1–5. Evaluation was performed on day 8.

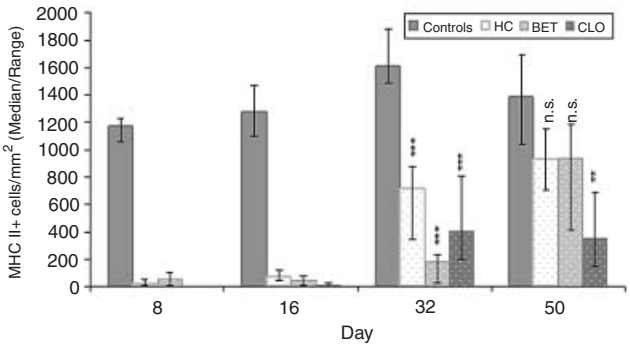

Figure 2. Major histocompatibility complex (MHC) class II+ cells (median values and ranges, $n = 4$) in auricular epidermis treated topically with ethanol (vehicle, controls), hydrocortisone (1%, HC), betamethasone-17-valerate (0·1%, BET) or clobetasol propionate (0·05%, CLO) twice daily on days 1–5. Evaluation was performed as indicated. n.s., not significantly different from controls; $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$ vs. controls.

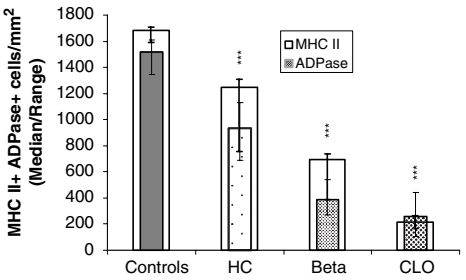

Figure 3. Major histocompatibility complex (MHC) class II+ or adenosine diphosphatase (ADPase)+ cells (median values and ranges, $n = 4$) in auricular epidermis treated topically with ethanol (vehicle, controls), hydrocortisone (1%, HC) betamethasone-17-valerate (0·1%, BET) or clobetasol propionate (0·05%, CLO) twice on day 1. Evaluation was performed on day 4. $^{***}P < 0{\cdot}001$.

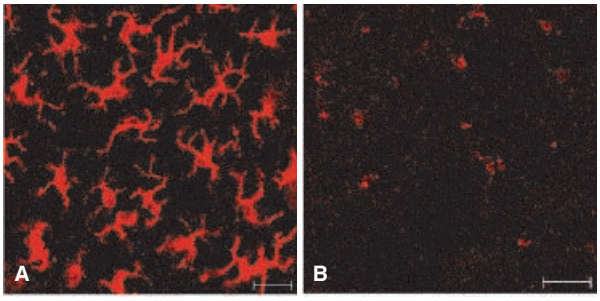

Figure 4. Major histocompatibility complex class II+ cells (Texas red/streptavidin staining) in epidermal sheets of an untreated control (A) and a mouse treated with 0·05% clobetasol (B) twice on day 1 and evaluated on day 4. Scale bar = 20 μm.

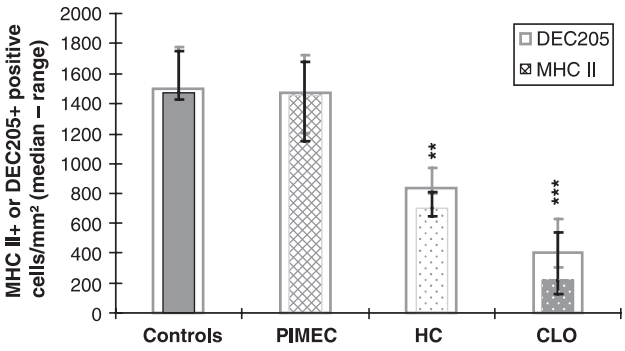

Figure 5. Major histocompatibility complex (MHC) class II+ and DEC 205+ cells (median values and ranges, $n = 6$ or 7) in auricular epidermis treated topically with ethanol (vehicle, controls), pimecrolimus (1%, PIMEC), hydrocortisone (1%, HC) or clobetasol propionate (0·05%, CLO) twice on day 1. Evaluation was performed on day 4. $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$ vs. controls.

hydrocortisone (53 and 45%) or clobetasol (85 and 73%) compared with pimecrolimus- or vehicle-treated samples (Fig. 5). Pimecrolimus-treated samples did not differ significantly from controls using these LC labels.

Interestingly, DEC 205 expression was less impaired by the corticosteroid treatment than MHC class II expression. MHC class II+ and DEC 205+ cell counts differed significantly ($P < 0{\cdot}01$) in hydrocortisone- and clobetasol-treated sites (Fig. 5). MHC class II expression was frequently absent or only faint in DEC 205+ cells, whereas all MHC class II+ cells expressed DEC 205 (Fig. 6).

Discussion

The present study shows that the application of 1% pimecrolimus twice daily for five consecutive days had no effect on epidermal LC counts in murine epidermis even though the >100-fold concentration inhibiting allergic contact dermatitis in mice was used.[10] In contrast, hydrocortisone, betamethasone and clobetasol propionate, also applied at their clinically used concentrations, almost totally eliminated LCs, identified by MHC class II, DEC 205 and ADPase labelling. Reduction of MHC class II+ expression and antigen-presenting capacity in murine or human epidermal LCs by topically applied corticosteroids has been reported previously.[2–6] The observation that even a 1-day treatment schedule with the low-potency hydrocortisone resulted in a significant reduction in MHC class II+ or DEC 205+ cells is a new and unexpected finding. The data from the single-day treatment study as well as the results from the recovery study confirmed previous observations that the impact of corticosteroids on LCs is potency dependent.[6] Because two different cell surface markers and an additional biochemical label have been applied to identify LCs, we provide strong experimental evidence that the reduction in cell numbers is due to a treatment-related depletion of the cells in the epidermis and not just to a downregulation of certain surface markers. It remains to be investigated whether this depletion is due to LC necrosis or apoptosis *in situ* or whether LCs emigrate from the epidermis following application of corticosteroids. Our preliminary data indicate that LC depletion most probably results from apoptosis rather than from emigration. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labelling staining of epidermal sheets revealed fragmented DNA in most LCs.[16] Accumulation of MHC class II+ cells in a 'string of pearls' fashion, which would be indicative of LC emigration, was not observed microscopically in dermal sheets (W.Hötzenecker and A.Elbe-Bürger, unpublished observation).

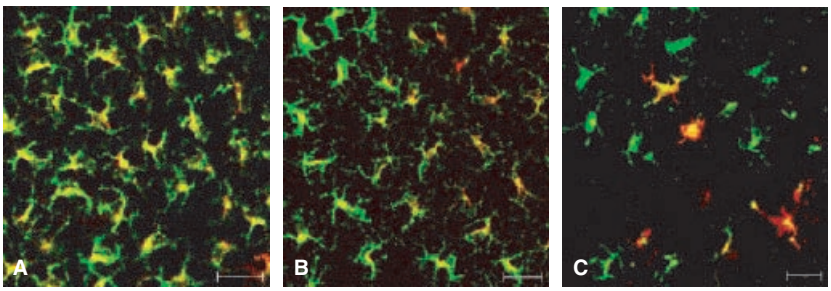

Figure 6. Langerhans cells (LCs) immunolabelled with antimajor histocompatibility complex (MHC) class II antibody (red fluorescence) and anti-DEC 205 (green fluorescence) in auricular epidermis treated with vehicle (controls, A), pimecrolimus (1%, B) or clobetasol propionate (0·05%, C) twice on day 1 and evaluated 3 days after the last application. (A) and (B) show similar features; (C) shows MHC class II+ LCs absent except a few round or dendritic shaped cells arranged focally. Intact DEC 205 expression is seen on many LCs lacking MHC class II immunolabelling. Scale bar = 20 μm.

Furthermore, the observation that the expression of DEC 205 is less impaired by corticosteroids than MHC class II expression is under more detailed investigation. The cell surface protein DEC 205 has been reported to be involved in the capture and endocytosis of extracellular glycoprotein antigens and in their delivery to MHC class II-containing compartments of antigen processing and presentation.[17,18] The different effects of corticosteroids on DEC 205 and MHC class II expression suggest that corticosteroid-related impairment of antigen presentation is due to changes in processing rather than to changes in the uptake of antigens.

The present data demonstrate that topically applied pimecrolimus, in contrast to corticosteroids, does not affect number and phenotype of epidermal LCs, perhaps suggesting that their function may be unaltered.

References

1 Takeda K, Arase S. Side effects of topical corticosteroids and their prevention. *Drugs* 1988; **36** (Suppl. 5): 15–23.

2 Aberer W, Romani N, Elbe A *et al.* Effects of physicochemical agents on murine epidermal Langerhans cells and Thy-1 positive dendritic epidermal cells. *J Immunol* 1986; **136**: 1210–16.

3 Belsito DV, Baer RL, Schultz JM, Thorbecke GJ. Relative lack of systemic effects of mometasone furoate on Langerhans cells of mice after topical administration as compared with other glucocorticosteroids. *J Invest Dermatol* 1988; **91**: 219–23.

4 Grabbe S, Steinbrink K, Steinert M *et al.* Removal of the majority of epidermal Langerhans cells by topical or systemic steroid application enhances the effector phase of murine contact hypersensitivity. *J Immunol* 1995; **155**: 4207–17.

5 Pakes WL, Muller HK, Schwarz MA, Marks R. Langerhans cells—a reduction in numbers and their reappearance following steroid and cytotoxic therapy in humans. *Clin Exp Dermatol* 1986; **11**: 450–9.

6 Ashworth J, Booker J, Breathnach SM. Effects of topical corticosteroid therapy on Langerhans cell antigen presenting function in human skin. *Br J Dermatol* 1988; **118**: 457–69.

7 Maurer D, Stingl G. Langerhans cells. In: *Dendritic Cells* (Lotze MT, Thomson AW, eds), 2nd edn. London: Academic Press, 2001; 35–50.

8 Girolomoni G, Caux C, Dezutter-Dambuyant C *et al.* Langerhans cells: still a fundamental paradigm for studying the immunobiology of dendritic cells. *Trends Immunol* 2002; **23**: 6–8.

9 Nickoloff BJ. Cutaneous dendritic cells in the crossfire between innate and adaptive immunity. *J Dermatol Sci* 2002; **29**: 159–64.

10 Meingassner JG, Grassberger M, Fahrngruber H *et al.* A novel anti-inflammatory drug, SDZ ASM 981, for the topical and oral treatment of skin diseases: *in vivo* pharmacology. *Br J Dermatol* 1997; **137**: 568–76.

11 Harper J, Green A, Scott G *et al.* First experience of topical SDZ ASM 981 in children with atopic dermatitis. *Br J Dermatol* 2001; **144**: 781–7.

12 Luger T, van Leent EJ, Graeber M *et al.* SDZ ASM 981: an emerging safe and effective treatment for atopic dermatitis. *Br J Dermatol* 2001; **144**: 788–94.

13 Jiang W, Swiggard WJ, Heufler C *et al.* The receptor DEC-205 expressed by dendritic cells and thymic epithelial cells is involved in antigen processing. *Nature* 1995; **375**: 151–5.

14 Juhlin L, Shelley WB. New staining techniques for the Langerhans cell. *Acta Derm Venereol (Stockh)* 1977; **57**: 289–96.

15 Chaker MB, Tharp MD, Bergstresser PR. Rodent epidermal Langerhans cells demonstrate greater histochemical specificity for ADP than for ATP and AMP. *J Invest Dermatol* 1984; **82**: 496–500.

16 Hoetzenecker W, Ecker R, Kowalsky E *et al.* Corticosteroids but not pimecrolimus induce apoptosis in murine Langerhans cells *in vivo*. *Arch Dermatol Res* 2003; **294**: 508 (Abstract).

17 Swiggard WJ, Mirza A, Nussenzweig MC *et al.* DEC-205, a 205-kDa protein abundant on mouse dendritic cells and thymic epithelium that is detected by the monoclonal antibody NLDC-145: purification, characterization, and N-terminal amino acid sequence. *Cell Immunol* 1995; **165**: 302–11.

18 Watts C. Capture and processing of exogenous antigens for presentation on MHC molecules. *Annu Rev Immunol* 1997; **15**: 821–50.