

Original article

Modulation of the atopy patch test: tacrolimus 0.1% compared with triamcinolone acetonide 0.1%

Background: The atopy patch test (APT) is an *in vivo* model to study the induction of eczema by inhalant allergens in atopic dermatitis patients. We studied the effect of pretreatment with topical tacrolimus 0.1% on APT in nonlesional skin of patients with atopic dermatitis.

Methods: Nonlesional skin of the back of patients with atopic dermatitis ($n = 8$) was treated once daily for 3 weeks with tacrolimus 0.1% ointment. Ceto-macrogol ointment (placebo) was used as a negative control and triamcinolone acetonide 0.1% ointment as positive control. Twenty-four hours after the last APT application, samples were taken from the three treated areas ($t = 0$ and 24 h) for immunohistochemical analysis.

Results: Pretreatment with tacrolimus ointment did not suppress nonlesional skin infiltrate, in contrast to triamcinolone acetonide. Furthermore, tacrolimus did not inhibit the induction of the APT macroscopically ($t = 24$ h). An equal influx of T cells, eosinophils, dendritic cells, CD64+ and FcεRI-positive cells was present compared with placebo. Only CD36+ and CD68-positive cells were inhibited compared with placebo. All cell types were significantly inhibited in triamcinolone acetonide-treated sites compared with placebo.

Conclusions: Pretreatment with tacrolimus 0.1% ointment does not inhibit the APT reaction in patients with atopic dermatitis.

J. M. Oldhoff, E. F. Knol, M. Laaper-Ertmann, C. A. F. M. Bruijnzeel-Koomen, M. S. de Bruin-Weller

Department of Dermatology/Allergology, University Medical Centre, Utrecht, the Netherlands

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Dr J. M. Oldhoff
Department of Dermatology/Allergology
UMC Utrecht
Heidelberglaan 100
G02.124, 3508 GA Utrecht
the Netherlands

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Tacrolimus is a macrolide lactone acting as an immunosuppressive agent. The mechanism of action of tacrolimus is closely related to that of cyclosporin A, i.e. inhibiting intracellular calcineurin. Calcineurin is involved in multiple processes in the body, including the immune function. After T-cell activation by antigen presentation, intracellular calcium is released and calcineurin is activated to dephosphorylate certain target proteins, including nuclear factor of activated T cells (NFAT). NFAT translocates to the nucleus and activates the cell to produce different cytokines which are necessary for the initiation of the immune response (1). By inhibiting this pathway, the systemic calcineurin inhibitor cyclosporin A has been proven to be an effective treatment for atopic dermatitis (AD) (2). Application of topical cyclosporin A was studied in AD patients, showing no clinical benefit (3). Tacrolimus in topical formulation has shown clinical

efficacy in both children and adults with mild to severe AD in a number of short- and long-term studies (4–6). This is most likely due to the difference in molecular size, tacrolimus being 30% smaller and therefore capable of penetrating lesional AD skin (1).

Recent topical treatment strategies focus on prophylactic treatment of AD predilection sites. Topical steroid treatment applied twice weekly to nonlesional predilection sites is effective in preventing eczema relapses in AD patients (7–9). The use of topical calcineurin inhibitors, such as tacrolimus or pimecrolimus would also be of interest for prevention of AD flares (10), because these new topical immune modulators cause no skin atrophy in contrast to steroid treatment (11, 12).

The atopy patch test (APT) is a model to study the induction of allergic inflammation by epicutaneous application of aeroallergens in AD patients (13, 14). Macroscopically, the APT reaction resembles AD lesions showing erythema, induration, papules and/or vesicles. Microscopically, the APT reaction is characterized by acanthosis, spongiosis and a dermal infiltrate consisting of mainly T cells, activated eosinophils and dendritic cells

Abbreviations: AD, atopic dermatitis; APT, atopy patch test; GP, grass pollen; HDM, house dust mite; LSS, Leicester Sign Score; NFAT, nuclear factor of activated T cells; TACA, triamcinolone acetonide 0.1% ointment.

(15, 16). Furthermore, the APT can be used to evaluate the potency of anti-inflammatory treatment on allergic inflammation in patients with AD (17).

This study was designed to investigate the modulating effect of pretreatment with topical tacrolimus 0.1% ointment compared with triamcinolone acetonide 0.1% ointment (TACA) on the macroscopic and microscopic APT reaction in nonlesional skin of AD patients. TACA was chosen as the topical steroid as a mid-strength glucocorticosteroid, because the anti-inflammatory potency of tacrolimus ointment is comparable with that of mid-strength corticosteroids (1). Furthermore, TACA has been demonstrated to modulate the APT reaction both macroscopically and microscopically (17).

Material and methods

Patients

Eight patients aged between 18 and 50 years with AD, diagnosed according to the criteria of Hanifin and Rajka (18), were recruited on the basis of a positive APT reaction at 24 h for house dust mite (HDM), grass pollen (GP) or both. The patient characteristics are described in Table 1. Patients with eczema lesions on the back and patients on antibiotic, antihistaminic or systemic immunosuppressant treatment were excluded. All participants in this study gave their informed consent. This study was approved by the local medical ethics committee, which follows the declaration of the Helsinki Protocol.

Study design

Three equal parts of 10 × 10 cm, at least 5 cm² apart, were marked with a skin marker on the patients' back. One part was treated with topical tacrolimus 0.1% ointment (Astellas Pharma, Leiderdorp, the Netherlands), one with cetomacrogol ointment (placebo) (Genpharma, Maarssen, the Netherlands) and one with triamcinolone acetonide 0.1% in cetomacrogol ointment (TACA) (Genpharma) once daily during 3 weeks. The study design was double-blinded. This was achieved by ointment disposal in 'A, B and C' encoded cups by a third party. Twenty-four hours after the last application, APTs were performed in duplicate in the centre of the three treated areas. APT reactions were recorded at 24 h. Skin biopsy samples for microscopic evaluation were taken from pretreated nonlesional skin at 0 h and from APT sites at 24 h from each treated skin area.

Table 1. Patient characteristics

Patient no.	Sex	Age	LSS	AA	AR	Total IgE	Specific IgE
1	M	37	43/36	+	–	1933	221 (HDM)
2	M	32	8/9	–	+	20	5.7 (GP)
3	M	38	18/30	–	+	>5000	>100 (HDM)
4	M	38	14/8	+	+	1014	>100 (HDM)
5	F	21	23/31	+	–	551	54 (HDM)
6	M	30	28/45	+	–	>5000	>100 (HDM)
7	M	39	32/22	+	+	>5000	>100 (HDM)
8	M	42	21/42	+	+	7452	>100 (HDM)

AR, allergic rhinitis; AA, allergic bronchial asthma; SPT, skin-prick test; HDM, house dust mite; GP, grass pollen; and LSS, Leicester Sign Score (severity/surface).

APT

Atopy patch tests were performed as previously described (14, 19). In short, test sites were stripped 10 times with adhesive tape (Transpor hypoallergenic tape; 3M Health Care, St. Paul, MN, USA) before application of the aqueous allergen preparation. Commercially available allergen preparation from Haarlem's Allergen Laboratorium (HAL) (Haarlem, The Netherlands) containing *Dermatophagoides pteronyssinus* (HDM) and GP in concentrations of 10 000 allergy units/ml was used. The amount of allergen used to induce the APT was 0.08 ml per leukotest patch (Beiersdorf, Hamburg, Germany).

The allergens were dissolved in HAL buffer solution consisting of a buffered salt solution. The buffer solution was applied as a negative control in all subjects. The patch test reactions were read after 24 h. Patch test reactions were recorded as positive when at least erythema and induration occurred and no reaction was observed in the control patch: 1+, erythema with slight induration; 2+, marked erythema, induration and papules; 3+, marked erythema, induration, papules and vesicles.

Biopsy specimens

Skin samples for biopsy (3 mm) were taken under local anaesthesia (xylocaine), snap-frozen in liquid nitrogen, embedded in Tissuetek® (Sakura, Torrance, CA, USA) and stored at –70°C until further handling. Sections (5 µm) were cut from the Tissuetek-embedded skin tissue and mounted on 3-aminopropyl tri-ethoxy silane-coated (Sigma-Aldrich, Deisenhofen, Germany) glass slides.

Immunohistochemistry

Antibodies. The following antibodies were used as markers for immunohistochemical staining of the frozen sections: CD3 (Leu-4, BD Biosciences, San Jose, CA, USA); AA1, recognizing mast cell tryptase (Dako, Glostrup, Denmark); EG2, recognizing eosinophilic cationic protein (ECP) (Pharmacia & Upjohn, Uppsala, Sweden); CRA1, recognizing FcεRI (clone AER-37; eBioscience, San Diego, CA, USA), CD1a (FITC-conjugated Clone NA 1/34; Dako), CD64 (FITC-conjugated, 10.1; Serotec, Oxford, UK), IgE (FITC-conjugated; Kirkegaard & Perry Laboratories, Inc. Europe, Guildford, UK), CD36 (FITC-conjugated; Immunotech, Marseille, France) and CD68 (FITC-conjugated, clone KPI; Dako).

Immunostaining. Single-staining for CD3, mast cell tryptase, ECP and FcεRI, combined with a biotinylated horse anti-mouse IgG (Vector Laboratories, Inc., Burlingame, CA, USA) were performed as described by Thepen et al. (16). The FITC-conjugated antibodies CD1a, CD64, IgE and CD68 sections were fixed with dry acetone, air-dried, and pre-incubated for 20 min in 10% normal human serum (NHuS) in phosphate-buffered saline (PBS). Primary antibodies were diluted in 1% NHuS, and incubated with the sections for 60 min. Slides were washed for three times 5 min with PBS + 0.05% Tween 20 (Sigma P-1379) and were subsequently incubated with alkaline phosphatase (AP)-conjugated sheep anti-fluorescein (Fab fragments; Boehringer Mannheim, Ingelheim, Germany) in PBS for 30 min. After incubation, slides were washed with Tris-HCl (0.1 M, pH 8.5). AP reactivity was demonstrated as previously described (16).

Quantification of staining. Skin sections were examined by light microscopy at ×400 magnification by a blinded observer. Before evaluation, sections were compared with either the isotype-control-

stained counter sections or with the nonstained counter sections. Positive cells in the dermis were counted in three different sections of 1–1.5 mm² per section and calculated as cells per mm². In fields containing sweat ducts and hair shafts, only intervening dermal regions were counted. For EG2, CD3, CD1a and FcεRI, dermal and epidermal compartments were examined separately. The number of epidermal CD1a-positive cells was graded as: no or hardly any positive cells present (0), presence of scattered positive cells (1), clear abundant presence of positive cells (2) and closed maze of positive cells (3). One of every five analyses was controlled by a second independent observer. The mean inter-observer coefficient of variation was within 10%.

Statistical analysis

Statistical analysis was performed using the program SPSS for Windows (version 10.0.5, 1999). The Wilcoxon signed-rank test was used for within-group paired comparisons. For between-group comparisons, the effects of different treatments were compared using the nonparametric Mann–Whitney test for independent samples in case of ordinal values and in case of categorical data. A *P*-value of ≤ 0.05 was considered to be significant.

Results

Comparison of macroscopic APT reactions

All patients showed a positive APT reaction (*t* = 24 h) on placebo-treated skin (Table 2). Positive reactions resembled lesional AD skin, showing erythema, induration, papules and sometimes vesicles. TACA treatment was used as a positive control and showed a complete inhibition of macroscopic APT reaction in seven of eight patients. One patient showed a positive APT after TACA treatment, which was less in magnitude compared with the placebo-treated part on the back of the same patient (patient 1; Table 2). Tacrolimus treatment did not prevent the APT reaction in seven of eight patients. Patient 3 showed a complete inhibition of the APT reaction after tacrolimus treatment, and in three of eight

patients the reaction was less in magnitude compared with placebo (patient 1, 5 and 8; Table 2).

The macroscopic APT reaction was not significantly different between tacrolimus-treated skin and placebo (*P* = 0.13). The macroscopic APT reaction in TACA-treated skin was significantly reduced compared with both placebo- and tacrolimus-treated skin (*P* < 0.05).

Cellular infiltrate of tacrolimus-treated nonlesional skin compared with placebo and TACA

A total of three biopsy samples were taken from nonlesional skin of the back treated with placebo, TACA and tacrolimus of each patient before APT application (*t* = 0). In placebo-treated skin, the dermal cell infiltrate consisted of T cells (CD3), mast cells (AA1), macrophages/dermal dendritic cells (CD68), IgE+, FcεRI+, CD36-positive cells, and dermal and epidermal dendritic cells (CD1a). In tacrolimus-treated skin, the number of dermal CD3+, AA1+, CD68+, CD1a+, CD36+, IgE+, FcεRI+, and epidermal CD1a-positive cells was not different from that of placebo treatment (Figs 1–3). In TACA-treated skin, the number of dermal CD3+, AA1+, CD1a+, CD36+, IgE+, FcεRI+ and epidermal CD1a-positive cells were significantly reduced compared with placebo. CD68+ cells, however, were not reduced significantly (*P* = 0.08).

Cellular infiltrate of the APT reaction after tacrolimus pretreatment compared with placebo and TACA

Biopsy samples were taken from APT reactions (*t* = 24 h) of each treated skin site of patients. In placebo-treated skin, there was a significant (*P* < 0.05) influx of dermal T cells (CD3), eosinophils (EG2) and macrophages/dermal dendritic cells (CD68) at 24 h compared with *t* = 0. The number of dermal and epidermal dendritic cells (CD1a) was the same compared with *t* = 0; the number of mast cells was reduced (*P* < 0.05) (Figs 1 and 3). The expression of CD36 and CD64 was significantly increased compared with *t* = 0 (*P* < 0.05). Expression of IgE and FcεRI did not differ from *t* = 0 (Fig. 2).

In tacrolimus-treated skin, the number of dermal CD3+, CD1a+, AA1+, EG2+, CD64+, IgE+, FcεRI+ and epidermal CD1a-positive cells was not different from placebo-treated skin. The influx of CD68+ and CD36-positive cells was significantly decreased after tacrolimus treatment compared with placebo (*P* < 0.05) (Figs 1–3).

In TACA-treated skin, the number of dermal EG2+, CD3+, CD1a+, CD68+, CD36+, CD64+, IgE+, FcεRI+ and epidermal CD1a-positive cells were significantly decreased compared with placebo (*P* < 0.05). The number of mast cells did not differ from placebo at *t* = 24 h. The number of CD36+ and CD68+ cells were significantly lower compared with tacrolimus-treated skin.

Table 2. Clinical outcome of the APT after 3 weeks of treatment

Patient no.	Placebo	TACA	Tacrolimus	Allergen
1	3	1	1	HDM
2	1	0	1	GP
3	2	0	0	HDM
4	1	0	1	HDM
5	2	0	1	HDM
6	1	0	1	HDM
7	1	0	1	HDM
8	3	0	2	HDM
Mean	1.8	0.1	1.0	

Treatment with tacrolimus was not able to suppress the macroscopic APT reaction significantly, in contrast with TACA (*P* < 0.05).

HDM, house dust mite; GP, grass pollen. 0 = negative reaction; 1 = erythema, induration; 2 = erythema, induration and papules; 3 = erythema, induration, papules and vesicles.

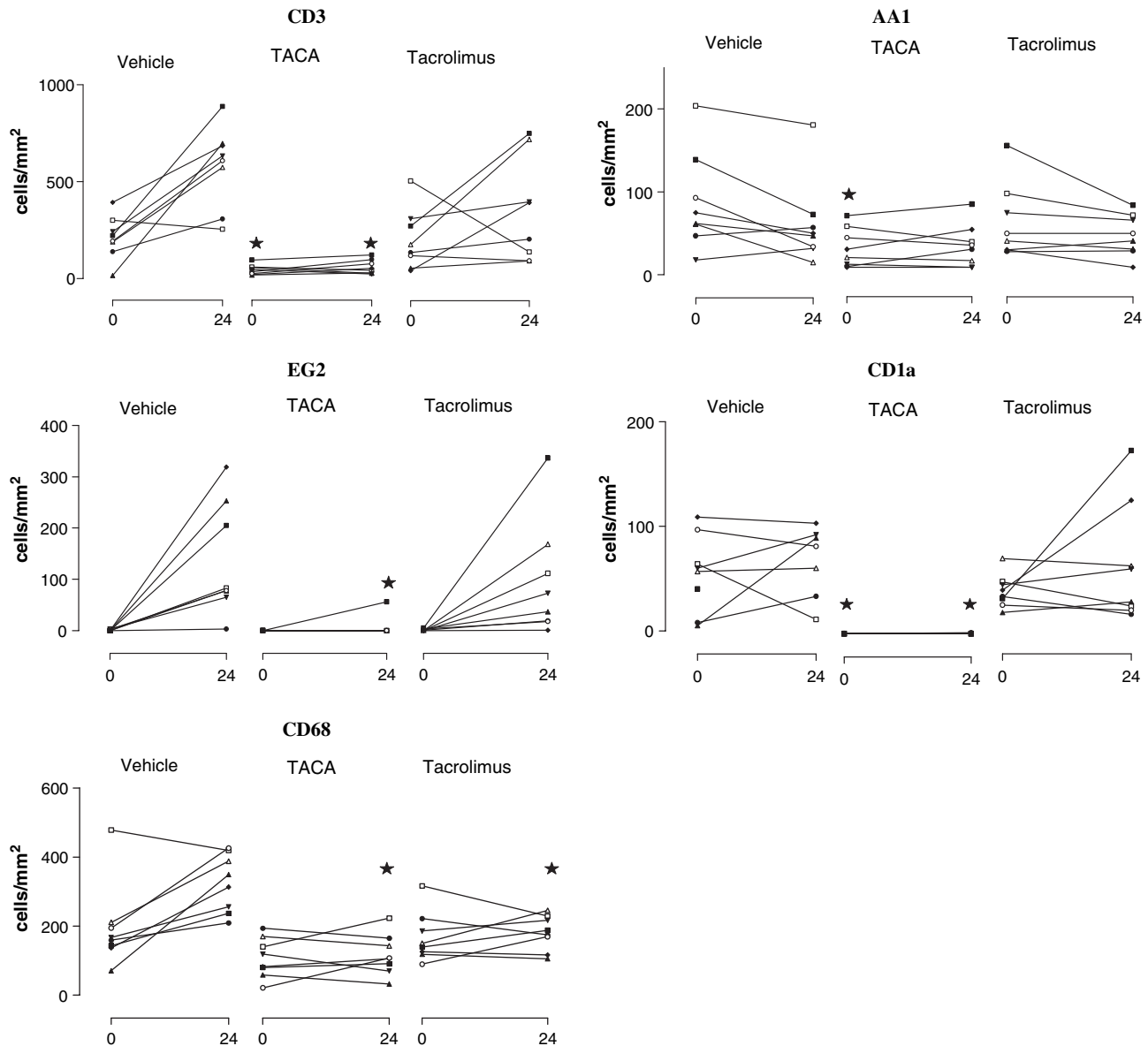


Figure 1. Cellular infiltrate before ($t = 0$) and after atopy patch testing ($t = 24$) in patients with AD, pretreated with placebo, TACA and tacrolimus. x-axes: time: 0 = nonlesional skin, 24 = APT-induced inflammation at 24 h; y-axes: cells/mm². CD3 = T cell; EG2 = eosinophil; CD1a = dendritic cell; AA1 = mast cell and CD68 = macrophage/dendritic cell staining. ★A significant difference compared with placebo.

Discussion

This study shows that treatment of nonlesional AD skin with tacrolimus ointment does not suppress the cellular infiltrate compared with TACA. Furthermore, tacrolimus does not inhibit the induction of the APT.

However, previously published data showed that tacrolimus ointment is effective in the treatment of lesional AD, showing a clinical effect comparable with mid-strength corticosteroids (4, 20, 21). Furthermore, tacrolimus was shown to reduce the cellular infiltrate of lesional

AD skin significantly (22–24). Several factors might explain why tacrolimus cannot reduce the APT reaction.

First, the application frequency; to keep the study blinded, we chose the same application frequency for TACA and tacrolimus, once daily for each treated skin part. Although the usual recommended frequency of tacrolimus application is twice daily, once daily application of tacrolimus is also effective in the treatment of atopic dermatitis (25). Therefore, it is not likely that a different application frequency will alter the current results, but this cannot be excluded.

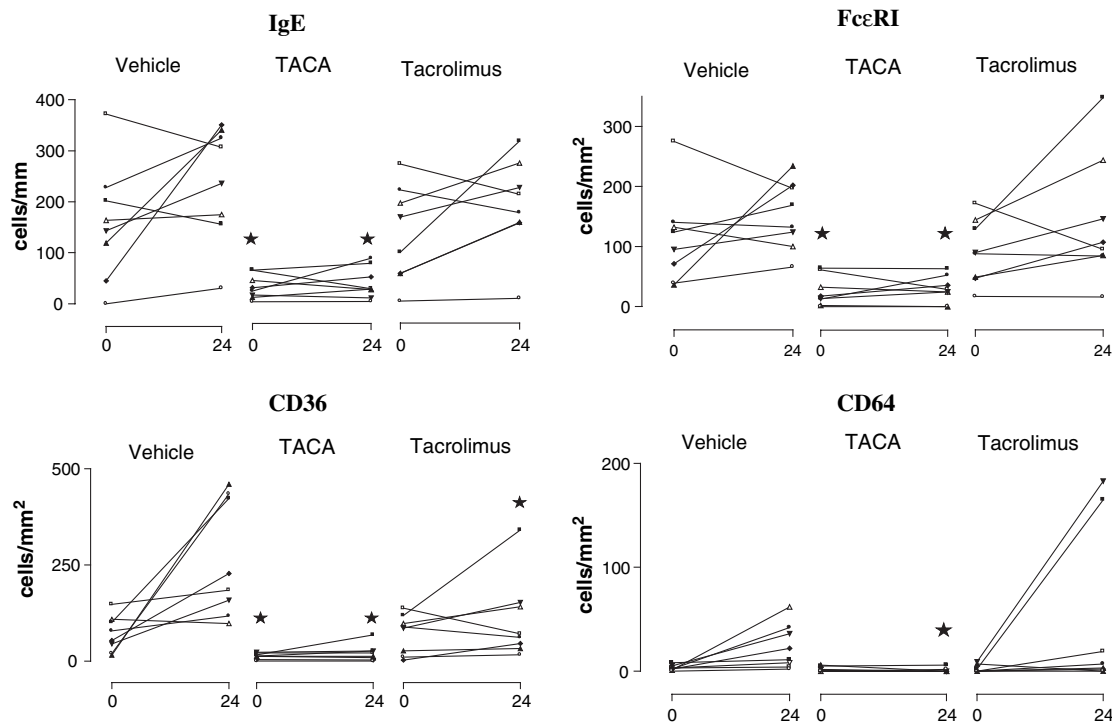


Figure 2. x-axes: time; 0 = nonlesional skin, 24 = APT-induced inflammation at $t = 24$ h. y-axes: cells/mm² expressing IgE, FcεRI, CD36 and CD64. *A significant difference compared with placebo.

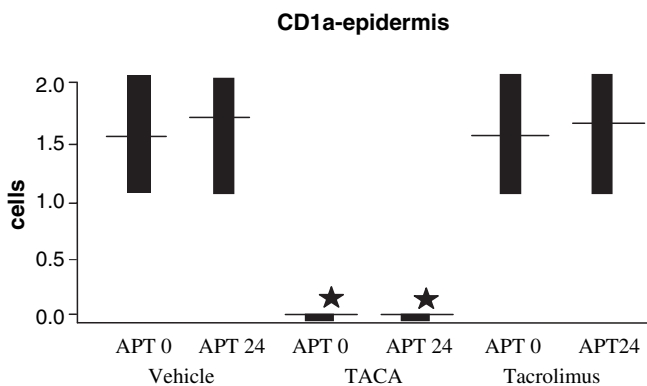


Figure 3. Number of CD1a+ cells in the epidermis before and after APT at 24 h. Average amount of cells expressed on y-axes counted semiquantitatively. 0 = no or hardly any positive cells present; 1 = presence of scattered positive cells; 2 = clear abundant presence of positive cells; 3 = closed maze of positive cells. *A significant difference compared with placebo.

Secondly, the length of the treatment period; 3 weeks was chosen, because complete inhibition of macroscopic APT was seen with TACA treatment (17). A prolonged application of tacrolimus might theoretically be needed to reach the same effect; however, this is unlikely, looking at the minor effects, even after 3 weeks of treatment.

The third, and probably the most important reason for the overall inability of tacrolimus to suppress the APT reaction, is its molecular size. Tacrolimus is a larger

molecule than TACA; 804 Da compared with 362 Da (1). The reduced ability (26) of tacrolimus to penetrate healthy skin is also one of the major advantages, preventing unwanted side effects when eczematous lesions heal (27).

Another factor influencing our data is the site of application. The current results showed hardly any effect of tacrolimus on nonlesional skin of the back of patients with AD. The back is not a predilection site of the patient with AD, so the skin barrier may differ from the flexure areas of the limbs. Pretreatment of these areas might be effective in preventing AD lesions, because tacrolimus has some capability to penetrate intact skin (26). It remains to be investigated to which extent tacrolimus can penetrate skin, and if this depends on body site.

Microscopically, the inhibition of APT induction in TACA-treated skin can be explained by the effective depletion of the epidermal dendritic cell population. Consequently, no antigen presentation and T-cell activation occurs (28). In tacrolimus pretreated skin, dendritic cells were still present in the epidermis.

In line with our results, a recent publication by Hoetzenecker et al. (29) showed that pimecrolimus, related to tacrolimus (1), does not affect the number of dendritic cells if applied on intact skin of healthy volunteers. Surprisingly, the same study also showed no effect on epidermal dendritic cells in lesional AD skin, while a reduction of the cellular infiltrate was observed in the dermis. The authors suggest that apoptosis is the

mechanism by which the cellular infiltrate in the dermis is reduced (29). More recently, even an increase in epidermal CD1a+ cells was seen after pimecrolimus treatment. At the same time, a decrease was observed in the dermal cellular infiltrate (30).

However, our study did not show a decrease in the number of T cells in the dermis. Tacrolimus pretreated skin only showed a reduction in the number of CD36+ and CD68+ cells in the dermis. CD36 has been postulated as a marker of inflammation (31). Phenotyping studies of epidermal dendritic cell subsets showed an increased expression of CD36 on the inflammatory dendritic cell subtype (IDEC) compared with the Langerhans cell subtype in lesional AD skin (31). In AD, the expression of FC ϵ RI on IDECs is high (32). As no reduction of FC ϵ RI+ cells is found after pretreatment with tacrolimus, the reduced CD36 expression observed in our study is not explained by a selective depletion of IDECs. CD36 expression is also found on CD68-positive cells (33). So, the reduced amount of CD36 can also be explained by a reduction in dermal CD68+ cells (macrophages, dendritic cells).

These results may shed some new light on flare-up prevention strategies/maintenance therapy in AD. Topical corticosteroid treatment, applied twice weekly, of nonlesional predilection sites is effective in preventing eczema relapses in AD patients (7–9). The capability to prevent AD induction is currently confirmed by the

suppressed APT in TACA pretreated skin. The APT induction is however not prevented by tacrolimus pretreatment. Therefore, tacrolimus is not expected to be effective in flare-up prevention strategies focusing on the pretreatment of nonlesional AD skin.

Another type of flare-up prevention is early intervention by treating new lesions. A study with pimecrolimus ointment showed a significant reduction in AD flares compared with placebo treatment by early intervention (34). In this type of flare-up prevention, the skin barrier is already disturbed by the starting lesion. It is expected that tacrolimus can also be effective in such types of flare-up prevention.

In conclusion, pretreatment of nonlesional AD skin with topical tacrolimus does not reduce the number of inflammatory cell presence compared with placebo. Furthermore, in contrast to topical corticosteroids, the induction of an eczematous response by the APT reaction is not prevented by tacrolimus, macroscopically as well as immuno-histochemically. Future studies are necessary to define the preventive potential of tacrolimus on nonlesional AD skin.

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