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Immunohistochemical Study of Some Chemokines Receptors in Atopic Epidermis: Before and After Treatment with Topical Tacrolimus–steroid Therapy

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Abstract

Background Tacrolimus is an immunosuppressive agent used topically, it has been found to be effective in treating moderate to severe atopic dermatitis without causing the atrophy that might occur with prolonged use of topical corticosteroids. There is a lack of studies on the effect of tacrolimus and steroid Therapy on CCR3 and CCR5 in atopic dermatitis patients.

Objective To assess expression of some chemokine receptors in the epidermis of atopic skin (chronic lesions) and to evaluate any differences in the degree and pattern of epidermal expression before and after topical tacrolimus or steroid therapy.

Methods Twenty five cases of atopic dermatitis before and after treatment by tacrolimus ointment and topical steroids were evaluated immunohistochemically for the epidermal expression pattern and intensity of some chemokine receptors namely CCR3 and CCR5 before and after treatment.

Result CCR5 and CCR3 positive epidermal cells seem to be produced in situ in higher amount before treatment compared with that after treatment. Although these cells are predominantly CCR5+.

Conclusions Enhanced expression of CCR3 and CCR5 on the surface of epidermal keratinocytes may be significant for the determination of atopic reactivity in general and also observed differences in frequencies of these activation markers before and after treatment by topical steroids-tacrolimus therapy.

Key words Atopic dermatitis, CCR3, CCR5, Immunohistochemistry, Tacrolimus

Introduction

Atopic dermatitis (AD) is a common pruritic disease that occurs primarily in infancy and childhood [(1)]. AD is characterized by itching, with patients having an individual or family history of atopic diseases in their background. Barrier dysfunction, immunological dysfunctions (type 1 and type 4 allergy) [(2)], genetic disorders and psychological factors contribute to the pathogenesis of AD. It is a chronically relapsing eczematous skin disease resulting from complex interactions between genetic and environmental factors [(3)].

In spite of controversies as regards the exact pathophysiology of eczematous lesion (and the exact type of immune reaction), three main types of cells have been confirmed to play the major role in the evolution of characteristic pathology of AD namely T- lymphocytes, Antigen Presenting Cells (APCs) and keratinocytes [(4)].

The role played by these effector cells is orchestrated by a growing list of cytokines (or chemokines), adhesion molecules and other mediators that control trafficking and action of the inflammatory cells and subsequently determine the nature, extent and duration of the inflammatory reaction [(5)].

There are many mechanisms involved in the pathogenesis of AD such as type 1 allergy [(6)], type4 allergy and barrier dysfunctions.

Moreover, in recent years, a considerable body of evidence has implicated T cells as having a major role in the pathogenesis of AD [(7)]. Increased numbers of T helper cells with a Th2-type cytokine profile are present, especially in the initial phase of skin inflammation[(8)], whereas both Th2-type cytokines Th1- type cytokines are up-regulated in chronic lesions.

Human Th1 and Th2 cells express distinct chemokine receptors (generated under the influence of IL-12 and IL-4 respectively) and are differentially recruited in response to chemokines [(9)]. Th2 cells were shown to express CCR3 preferentially and selectively migrate in response to eotaxin.CCR4 and CCR8 were shown to be expressed on Th2 cells. In contrast, CXCR3 and CCR5 were shown to be expressed on Th1 cells.

Topical tacrolimus and pimecrolimus have been developed as new non-steroidal immunomodulators [(10)]. Tacrolimus ointment 0.1% approved for use in adults; a frequently observed side effect with topical calcineurin inhibitors is a transient burning sensation of the skin. Importantly, treatment with topical calcineurin inhibitors is not associated with skin atrophy, thus they are particularly useful for the treatment of areas such as the face and intertriginous regions. So the new immunomodulators clear the rash with few side effects than do older steroids [(11)].

Methods

Twenty five patients with chronic atopic dermatitis (AD) and attended the private clinic in Baghdad during the period extended from May 2008 to September 2008, were randomly selected to participate in the study. Skin biopsy was taken from patients before tacrolimus or steroid therapy and after one month of therapy.

The diagnosis of atopic dermatitis was based on the criteria described by Hanifin and Rajika [(12)].

To investigate whether the patients were in allergic status and apart from suggestive clinical data, blood samples from all subjects were tested for total serum IgE titer and eosinophil count before and after treatment. Enzyme linked Immunosorbant Essay (ELISA) was used for the measurement of the total IgE in sera of the studied groups. Anti-Human IgE peroxidase conjugate IgG antibody was used for this purpose. The procedure of ELISA is making according to the Hunter et al 1986[(13)]. The results were expressed in IU/Ml and by cut of value were expressed as positive or negative.

These twenty patients return back to the private clinic after two weeks to one month of treatment with tacrolimus or topical and systemic steroids. The patients that re-analyzed after treatment were presented with same size and degree of severity of lesions for each patient was treated by topical steroids or topical tacrolimus. A second specimen (blood and biopsy) were taken from them.

Ten biopsies were taken before treatment (topical steroid or topical tacrolimus) and ten biopsies were taken from the same patients and the same sites after one month of treatment. After cleaning, local anaesthetic is infiltrated, an excisional biopsy is planned after considering the local anatomy. The ellipse to be excised is drawn on the skin using a marker pen. The ellipse is freed from surrounding skin, secured at one end with a skin hook and removed from the underlying fat, usually using the scalpel blade and preserved immediately in 10% formalin and subsequently embedded in paraffin blocks. Four micron sections were prepared for subsequent immunohistochemical staining.

The procedure of immunohistochemistry as following:

1. Slide baking; the slides were placed in a vertical position in incubator at 37 °C overnight then the slides were placed in a vertical position in a drying oven (hot air oven) at 65 °C for one hour.
2. Deparaffinizing the tissue sections: the slides were immersed sequentially in the following solutions at room temperature for the indicated times:

Xylene for 5 minutes, Absolute ethanol for 5 minutes, 95% ethanol for 5 min, 70% ethanol for 5 min, 50% ethanol for 5 min. and distilled water for 5 min.

3. After draining and carefully blotting around the specimen to remove any remaining liquid, the slides were placed in the humid chamber then 100 µl of protein – blocking reagent were applied onto the tissue to cover the whole specimen then incubated at room temperature for 15 minutes. Then the slides were rinsed gently with distilled water then drained and blotted as before.

4. Hundred µl of the diluted primary antibody were applied onto the tissue after the slides were placed in the humid chamber then incubated at 37 °C for 1 hour. After that, the slides were rinsed with (1X) rinse buffer for a minimum of 15 seconds then the slides were drained and blotted as before.

5. 100 µl of the diluted conjugate secondary antibodies were applied onto the tissue after the slides were placed in the humid chamber then incubated at 37 °C for 10 minutes. After that, the slides were rinsed with (1X) rinse buffer for a minimum of 15 seconds then the slides were drained and blotted as before.

6. 100 µl of DAB solution were applied to the in a dark place for 10 minutes at room temperature. The slide were washed in distilled water for 5 minutes and then drained and blotted gently.

7. The tissue was stained by 100 µl of counter (Haematoxyline) stain which was placed onto the tissue and incubated for 30 seconds at room temperature. Slides were drained gently.

8. Slides were washed in distilled water then drained and cleaned gently.

9. A drop of mounting medium (DPX) was placed onto the tissue section and then quickly covered with a cover slip and left to dry.

10. Slides were examined by light microscope at 40 X magnification. Immunostaining was scoring.

Table 1. Primary Ab and secondary Ab working dilution

Primary Ab	manufecture	Source	Working dilution	Secondary Ab	Manufacture	Source	Working dilution
Anti-human CCR3	USA Biological	Rabbit	6-32 µg/ml	Anti-Rabbit IgG	USA Biological	SHEEP	1/50-1/100
Anti-human CCR5	USA biological	Mouse	10 µg/ml	Anti-mouse IgG	USA biological	Goat	1/50

The drugs used in our study are topical corticosteroids (beta– methasone valerate), tacrolimus ointment and newer macrolide antibiotics (oral azithromycin). The details of each drug were shown in Table 2.

Table 2. The details of drugs are used in our study

Drug Name	Trade name	Name of company	Rout of administration	Duration of use	Dose concentration
Tacrolimus ointement	Talimus ointment	Ajanta pharma	Topical	2 weeks-	0.1% w/w
Betamethason ointment	Betnovate ointment	Glaxo Smith	Topical	2 weeks-	0.1%
Azithromycin	Zithroiv	Riva pharma	Oral, single dose/daily	2 weeks	250 mg

Statistical analysis

Statistical analysis was performed with the SPSS 15.01 statistical package for social sciences and also Excell 2003. Data analysis was done using paired sample t-test for tables with pre treatment and post treatment data means, independent sample t- test if we have two different groups. *P* value of ≤ 0.05 was used as the level of significance. Descriptive statistics for the clinical and laboratory results were formulated as mean and standard deviation (SD) and standard error (SE).

Cut-off value was measured by calculation the upper limit of the 99% confidence interval, which calculated by the calculation of the mean of the (OD-values) of standard readings (M) and the stander deviation (SD) and the stander error (SE). Cut-off value = M + 2.57(SD × SE).

Pearson correlation was done to explore possible association between markers involved in the study. (Al-Murrani, 2000) [13].

Slides were examined by light microscope at 40X – magnification power equipped with Image Analysis Computer System, the dark brown (homogenous) staining identified positive labeled cells. A total of 100 cells were counted to determine the percentage of reactivity of each of the tested monoclonal Abs. The percentage of positive cells calculated as following: percentage of positive cells = (No. of positive cells/ total No. of cells × 100%). Four sections per specimen have been examined; the first two sections for CCR3 marker and second two sections for CCR5 marker.

Results:

Table 3 demonstrates the clinical data of the material of the study and of the biopsied lesions.

Table 3. Clinical data of patients

	Total patients	Pre treament	Post treatment	Clinical response (topical steroid)	Clinical Response (topical tacrolimus)
Number	20	20	20	7/10	9/10
Age (year)	6-45 (27.08±11.37)	6-45 (27.08±11.37)	6-45 (27.08±11.37)	--------	--------
Site of biopsy	Arm/ (4) Leg /(10) Back/(2) Foot /(4)	Arm/ (2) Leg /(5) Back/(1) Foot/(2)	Arm /(2) Leg/(5) Back/(1) Foot/(2)	----------	---------

Table 4 shows results of estimation and comparison of eosinophil count in which a highly significant difference recorded among AD group at time of diagnosis when compared with those after treatment and was elevated in most AD patient correlating roughly with the disease severity.

Also there was significant difference between pre-treatment group and post-treatment group to evaluate the disease activity.

Table 4. Result of eosinophil count

Count		Mean	Std. Dev.	Std. Error	Sig. (2-tailed)
Eosinophil count (Percentage in blood)	pre treatment	273.256	120.687	18.405	0.000**
	post treatment	197.674	72.336	11.031	

(No. = 20)// **=statistical highly significant difference (p≤0.001).

The determination of total IgE in the serum was performed by using sandwich ELISA for all subjects and the results in Table 5 show that AD patient's serum contains significant higher level at time of diagnosis when compared with that of post-treatment group. Furthermore, there was highly significant difference between pre-treatment group and post-treatment group.

Table 5. Result of total serum IgE

	Serum IgE		Sig. (1-sided)
	Negative	Positive	
Pre treatment	18	32	0.000
Post treatment	34	9	

Immunohistochemical examination revealed significantly increased immunoreactivity for CCR-5 (Mean value= 49.500±18.922) in lesional epidermis of compared to CCR3 (Mean value= 26.5±7.8) at time of diagnosis. When evaluated separately, CCR-3, showed statistically significant difference ($p \leq 0.05$) in lesional epidermis of pre-treatment group compared to post treatment group but in lesser degree than that of CCR5+ expression (marker of Th1).
For CCR-5, statistically highly significant difference between pre and post-treatment groups ($p \leq 0.001$). (See Table 6 and Figure 1).

Table 6. Mean percentage of immunohistochemical expression of CCR3 and CCR5 pre and post-treatment.

Immunohistochemistry		Mean	Std. Dev.	Std. Error	Sig. (2-tailed)
Immunohistochemical expression of CCR-3	pre treatment	26.500	7.835	2.478	0.007*
	post treatment	17.500	7.906	2.500	
Immunohistochemical expression of CCR-5	pre treatment	49.500	18.922	5.984	0.000**
	post treatment	27.500	16.874	5.336	

*statistical significant difference ($p \leq 0.05$) **statistical highly significant difference ($p \leq 0.001$).

One month after treatment with steroid-tacrolimus therapy, the skin lesions regressed. Table 7 showed that there was no significant difference could be found between the effects of both drugs on expression of CCR-3 and CCR-5 in skin lesions before and after treatment. Also see Figure 1 and 3.

Table 7. Effect of steroid-tacrolimus on expression of CCR-3 and CCR-5 on lesional epidermal cells

Immunohistochemical expression CCR-3	Steroid Tacrolimus	10 10	24.000 29.000	8.216 7.416	3.674 3.317	0.342
Immunohistochemical expression CCR-5	Steroid Tacrolimus	10 10	49.000 50.000	18.507 21.506	8.276 9.618	0.939

As Table 8 and Figure 1 show the expression of CCR-3 and CCR-5 on lesional epidermal cells significantly correlated with each symptoms, which was the sum of three individual skin aspects (itching, skin dryness, skin condition) ($p < 0.05$).

Table 8. Relationships between different parameters and total VAS score; significant correlation, ($p < 0.05$)

		VAS (itching + skin condition)
Immunohistochemical expression CCR-5	pretreatment Post treatment	$p < 0.001$ (**) $p < 0.001$ (**)
Immunohistochemical expression CCR-3	pretreatment	$p < 0.05$ (*)

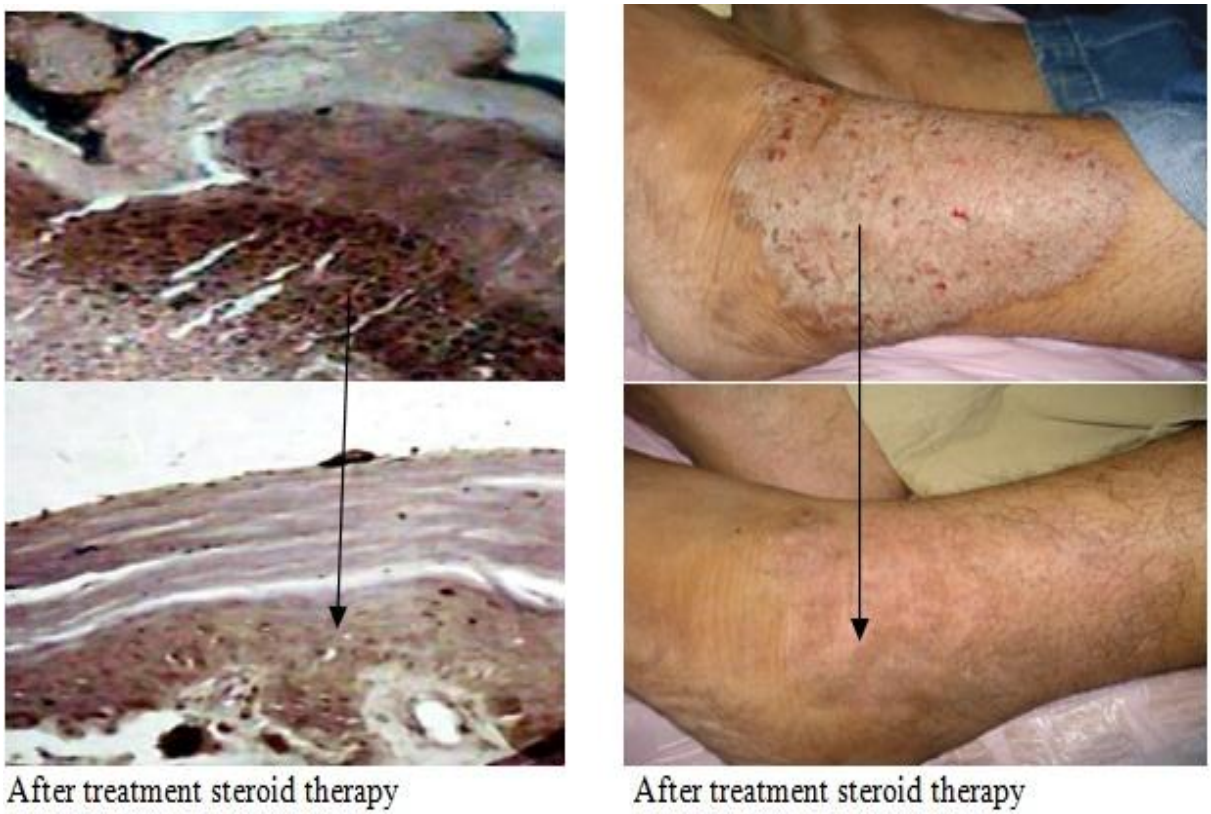

Figure 1. correlation between expression of CCR5 and skin lesion severity

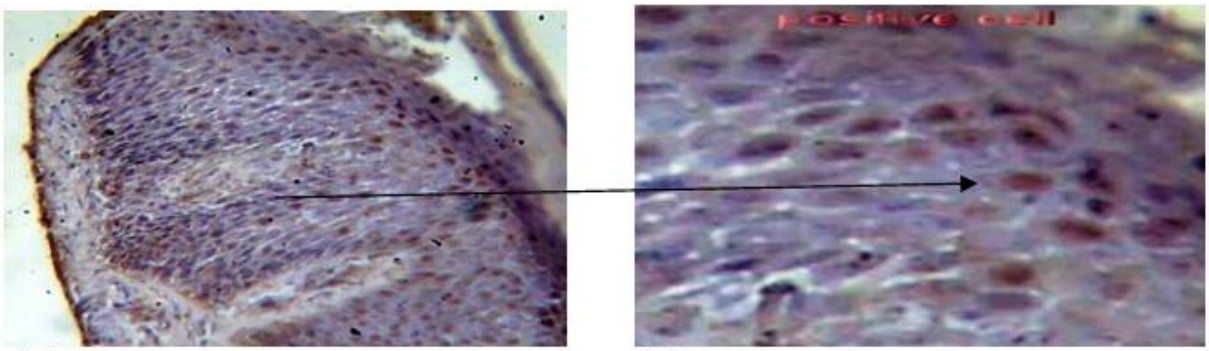

Figure 2A. Immunohistochemical staining low power magnification of 100X, 2B. Immunohistochemical staining: high power magnifications of 400X.

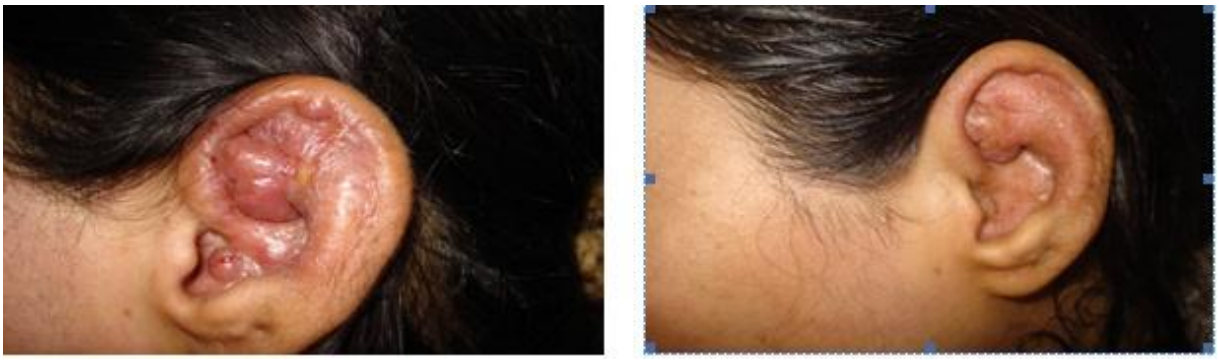

Figure 3. Ear atopic dermatitis after treatment with topical tacrolimus

Discussion

We designed this prospective study to apply this basic immunologic knowledge to confirm previous reported facts but with more practical aims, there has been a paucity of critical prospective studies on AD in which conventional laboratory tests have compared with newer biological markers. The prospective approach is a pre requisite if the prognostic or predictive features of the markers being studied are to be assessed. Also, the clinical indices must be characterized carefully. For this reason we concentrated this study on small but contrasting groups of patients with pronounced differences in the degree of disease activity (the small size of the sample of patients could be criticized). Also, we compared a group of patients at two points of time in order to monitor our putative -markers. However, such an approach will identify only the more robust markers, which in turn may become clinical useful.

In our study, blood eosinophilia is present in most patients with AD correlating roughly with the disease severity, the eosinophil count found to be high at time of diagnosis (mean value= 273.256±120.687) and the count markedly decreased after treatment (mean value= 197.674±72.336) with statistical highly significant difference ($p < 0.001$). Blood eosinophilia was described to be more pronounced in our patients if the AD was associated with respiratory allergic diseases (asthma, allergic sinusitis). As some our patients exhibit normal blood eosinophil counts despite active AD. The determination of eosinophil number in blood is not a reliable tool in establishing the diagnosis of AD but to evaluate the allergic status, this result is in agreement with Breuer, 2001[15].

The total IgE – serum level was found to be positive higher in AD patients at time of diagnosis when compared with post- treatment group. The high frequency of positive results in

pre-treatment group (64%) than in post-treatment group (19%) with statistical high significant difference ($p < 0.001$). The present study confirm a previous one done by Lilic et al 2006 who showed the relevance of total IgE levels are useful for screening for possible allergic disease but failed to establish the place of total IgE as a sensitive test as specific IgE. One of the references quoted (Sinclair and Peters, 2004) [17], although advising that total IgE should be performed as a screening test and is useful in the interpretation of specific IgE tests, because they permit the ascertainment of possible false- negative or false positive results.

The invasion of pathogenic Th2 cells into the skin tissue is critical step in the pathogenesis of acute stage of AD. However, its presently in chronic stage of AD is switching a Th2 cells to Th1 cells, with less significant role of Th2 cells in invasion to skin tissue.

Chemokines such as eotaxin and RANTES are critically involved in the migration of pathogenic T-cells into the skin tissue of AD lesions. However, for human chronic AD, there are very few data on chemokines or chemokine receptors during the course of the disease.

In another T-cell mediated immune disease, psoriasis, Th1-associated chemokine receptors (CCR5 and CXCR3) on peripheral blood lymphocytes or skin tissue have been identified as surrogate markers for the immune activity of the disease. This finding guided us to search for similar phenomena in human chronic AD since surrogate markers for the immune activity are urgently needed in chronic type AD to guide ongoing intervention trial.

These data are readily explained by switching a Th2 cells to Th1 cells with less significant role of Th2 cells. Beside this fact, the increased number of peripheral blood T cells with both T cells type (Th1 and Th2) including increased expression of CCR5+ T cells (Th1) and increased expression (less extent) of CCR3+ T cell (Th2) suggesting a mixed type of immune reaction (type 1 and type 4 allergic reactions) found in chronic AD.

In this study, the relevance of expression of chemokine receptors CCR3 and CCR5 on epidermal cells was investigated in patient with chronic AD and was correlated with disease activity or not.

Our results demonstrated that The percentages of CCR3+ and CCR5+ epidermal cells in patients were significantly higher at time of diagnosis from that post-treatment group patients (CCR3: pre-treatment mean value=26.500±7.835 Vs post-treatment mean value 17.500±7.906, $p \leq 0.05$) whereas high significant difference between two groups of CCR5: pre-treatment mean value=49.500±18.922 Vs post-treatment Mean value 27.5±16.874, $p \leq 0.001$) and was correlated positively with the total serum IgE, eosinophil number and ruption score. These results in agreement with Okazaki, 2002 [18].

In this study, to investigate the effects of tacrolimus on the expression of chemokine receptors (CCR3 and CCR5) in patients with chronic AD.

We found a striking reduction of the Th1-associated chemokine receptors (CCR5) and Th-2 associated chemokine receptors (CCR3) on lesional skin tissue after steroid-tacrolimus treatment from that at the time of diagnosis.

However presence the Th-1 and Th-2 chemokine receptors in the chronic AD which is the poorly defined nature of immune reaction in AD and which does not exactly confirm to one of the well known classic types of immune reaction (type 1, type 4 or mixed). Moreover, certain difficulties are traditionally encountered in the interpretation of any findings related to AD research.

In our study, we used the new immune-modulator topical drug (tacrolimus ointment) at the first time in Iraq in treatment of atopic dermatitis and compared with the old traditional topical steroids therapy for AD.

In our study, we investigated the effects of tacrolimus ointment on chronic AD lesions and compared with effects of topical steroids (Betamethasone valerate) clinically and immunlogically.

Immunologically; our study showed that tacrolimus and topical steroid reduced the expression of chemokine receptors (CCR3, CCR5) on epidermal keratinocytes, nearly in equal percentages and This result is in agreement with Shozo Sakuma, 2001 [19].

Tacrolimus ointment unlike some topical corticosteroids does not cause a decrease in collagen synthesis or skin thickness, nor does it produce skin abnormalities or de-pigmentation. So significant improvements in AD were observed in majority of patients treated with tacrolimus ointment and associated with few side effects unlike topical steroids (Bokersky and Fitzsimmons, 2001) [20].

In this our 2 phase study, clinically, topical steroids treated 10/20 patients showed greater improvement in AD signs and symptoms thereafter, tacrolimus ointment treated 10/20 patients showed improvement in AD signs and symptoms, there were no differences in clinical and immunological events between topical steroids and tacrolimus ointment but significantly longer time to first relapse and significantly fewer disease relapse days.

So our interesting treatment option which goes with Ehrchen, 2008 [21], for patients with stabilized moderate to severe atopic dermatitis, long term intermittent application of tacrolimus ointment to change the skin lesion to normal appearing skin and significantly more effective than steroid at maintaining disease stabilization, with safety profile and very few side effects similar to vehicle.

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