Chapter 7
In-Vivo Pharmacodynamic Studies in murine model

7.1 Introduction

Over the last decade, animal models of AD have received increasing attention. The earlier models included NC/Nga mice and a hapten-induced mouse model. More recently, other mouse models of AD have been developed using knockouts or transgenes (Shiohara et al, 2004).

Comparison for mouse models of AD

Variables	NC/Nga mouse model	Hapten-induced model	IL-18 Tg mouse model
Incidence of AD-like lesions	~50%	100%	(~0)[a] 100%
Need for additional treatment to develop AD-like lesions	Hapten application	No	Hapten application[a]
Age of onset months	8–17 weeks	Anytime 30 days after starting hapten application	6
Reproducibility	Poor–fair	Excellent	Fair
Need for particular conditions	Conventional conditions	No	No
Variations	Impossible	Possible by changing haptens or mouse strains employed	Impossible
Availability	Limited	Excellent	Limited

Hapten-induced model

During the process of developing a mouse model, it was noted that antigen specific IgE antibody is preferentially produced in mice repeatedly painted with a hapten. Repeated application of 2,4,6-trinitrochlorobenzen (TNCB) at 2-day intervals for 24 days to the same skin site results in a site-restricted shift in the time course of antigen-specific hypersensitivity responses from a typical delayed-type to an immediate type hypersensitivity followed by a late reaction, a finding often seen in skin lesions of AD patients. This shift is associated with epidermal hyperplasia, accumulation of large numbers of mast cells and CD4þ T cells beneath the epidermis, and elevated serum levels of antigen-specific IgE. Acute lesion is driven by the production of Th1 cytokines (IFN-g and IL-12) while chronic is driven by the production of Th2 cytokines IL-4 and IL-10. This model allows study of events characterizing the

progression from acute to chronic inflammation. By changing hapten and mouse strain, various types of chronic inflammation, probably reflecting heterogeneity in clinical presentation of AD, can be induced. It has significant disadvantages inherent to inducible models, such as the requirement for previous sensitization to a hapten and a concern about potential interactions between the hapten and therapeutic agents (Kitagaki et al, 1995, 1997, Thomas et al, 1978).

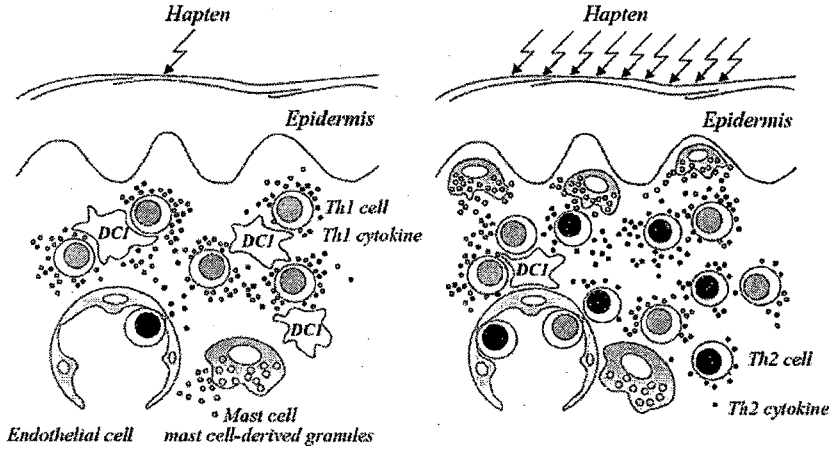


Fig. 7.1: Conversion from a Th1- (left) to a Th2-dominated (right) response upon repeated hapten application in a hapten-induced mouse model.

Animals

Mice models are widely investigated models of AD. Balb/C mice of either sex weighing between 20-30 g, 6-8 weeks of age were provided by Zydus Research Centre, Ahmedabad. The mice were housed 3-4 per cage. The mice were acclimatized in an animal room maintained at room temperature of 23-25 °C and with a 12-h light:12-h dark cycle. The mice were provided food and tap water ad libitum. The study protocol was approved by the Institutional Animal Ethical Committee and conducted in adherence to ethical guidelines.

7.2 Methods

7.2.1 Fluorescent Microscopy

To visualize the permeation of the ME based cream into the skin tissues, ME containing fluorescent dye, 6-coumarin (0.001%), was prepared with the optimized formula for HP microemulsion and Tac Microemulsion and incorporated into cetomacrogol cream base. Mice were applied the fluorescent dye containing formulations and sacrificed 6 hours later. The skin sections were observed and photographed with an Olympus fluorescence microscope (BX61, Japan). All photographs were taken with 40X objective.

7.2.2 Elicitation and Sensitization Protocol

Briefly, BALB/c mice were sensitized with 10 μL of 0.5% (w/v) 2,4,6 trinitro chlorobenzene (TNCB) solution in acetone on day 0 to the inner side of right ear, and then 10 μL of 0.5% (w/v) TNCB solution or acetone was repeatedly applied to the inner site of right ear at three times per week, through days 7-28.

7.2.3 Treatment Protocol

Formulations were applied topically twice a day for 2 weeks from the 21st day of TNCB elicitation. The drug application was 30 min after the challenge.

Test Formulations

Halobetasol Propionate

- HP ointment (marketed product)
- HP cream (marketed product),
- HPMEC 0.035%
- HPMEC 0.05%
- HP ME
- placebo

Tacrolimus

- Tac ointment
- Tac ME
- TacMEC 0.1%
- Placebo

7.2.4 Dermatitis Score

The skin symptoms were evaluated on day 0, 7, 14, 21, 28 and 35 after TNCB challenge and drug treatment according to the scoring method described previously (Ueda et al, 2006) and the following symptoms were recorded: erythema/hemorrhage; oedema; excoriation/ erosion; dryness. The symptoms were classified as follows: 0: no sign; 1: mild; 2: moderate; 3: severe. The sum of the individual scores (maximum score: 12) was taken as the dermatitis score.

7.2.5 Ear Swelling Studies

Ear thickness was measured with a dial thickness gauge. The ear thickness was measured on days 0, 7, 14, 21, 28 and 35. The ear swelling response elicited by epicutaneous application of the TNCB was expressed as the difference between ear thickness before and at 3, 24 h after each elicitation.

7.2.6 Measurement of Serum IgE

Blood was collected by cardiac puncture under ether anesthesia at 24 h after each challenge on days 0, 7, 14, 21, 28 and 35 during the elicitation phase and on day 28 and day 35 during the treatment phase. Total serum IgE levels were quantified by sandwich ELISA according to the manufacturer's protocol (Immunology Consultants Laboratory Inc., USA).

7.2.7 Histopathology

Excised skin specimens from hapten elicited/ drug treated ear of mice were fixed in 10% neutral formalin and embedded in paraffin. Sections were prepared and stained with hematoxylin and eosin, acidic toluidine blue. Toluidine blue specifically stains mast cells. Number of mast cells in 3 sites chosen at random was counted under a light microscope.

7.2.8 Cytokine Gene Expression by RT-PCR analysis

The expression of Th1 (IL-2, IFN- g) and Th2 (IL-4 and Il-10) cytokine mRNAs were semi quantitatively evaluated by means of reverse transcriptase polymerase chain reaction (RT-PCR) using β- actin as an internal reference.

RNA Isolation

Briefly, total RNA was isolated from control and hapten elicited/ drug treated tissue (ear) using Trizol reagent (Invitrogen, CA, USA). The excised ear was frozen immediately by immersing in liquid nitrogen. The tissue was minced under liquid nitrogen. It was then homogenized with Trizol reagent (RNA extraction reagent) using a tissue homogenizer at 3000 rpm for 3 cycles of 30 sec each at temperatures not exceeding 4°C. Choloroform (0.2 ml) was added to the Trizol aliquot containing the homogenized tissue. It was centrifuged at 12000 g for 15 min at 2-8°C. The upper aqueous phase was then transferred to a fresh tube and isopropyl alcohol (0.5 ml) was added to it. It was allowed to precipitate at -20°C for 15-20 min and centrifuged at 12000 g for 15 min at 2-8°C. Supernatant was removed and pellet was washed with 80% ethanol (1.0 ml) and centrifuged again at 12000 g for 15 min at 2-8°C. Ethanol was decanted and the RNA (total RNA) pellet was allowed to air-dry for 5 min. The pellet was dissolved in 50µl of DEPC treated water.

Removal of DNA contamination and quality check of isolated total RNA

The total RNA extracted was made free from any DNA contamination by DNase treatment (Turbo DNA free, Ambion Inc., Texas, USA) according to manufacturer's instruction. Briefly, the isolated RNA was mixed with DNAse enzyme and incubated at 37°C for 15 min. DNAse was then inactivated by heating the mixture at 90°C for 5 min. Quality and quantity of the total RNA was checked spectrophotometrically by measuring $A_{260/280}$. The concentration of RNA can be conveniently measured due to its ability to absorb light at 260 nm. The ratio A260:A280 is an indication of the purity of the RNA. A ratio between 1.8 - 2.0 is considered as good quality RNA and can be subsequently confirmed by gel electrophoresis. The amount of RNA is calculated from the formula:

$$A = e\,C\,l$$

Where,

A is the measured absorption at 260 nm

e is the RNA extinction coefficient (25 ul / ug / cm)

C: the RNA concentration

l is the pathlength (1 cm)

The quality is also checked by running the isolated RNA in denaturing agarose gel (2.0%) with ethidium brodium. Three distinct bands confirm the presence of RNA and no fluorescence in the well indicates removal of DNA contamination.

First strand cDNA synthesis

1ug of total RNA was subjected to first strand cDNA synthesis using Revertaid first strand cDNA synthesis Kit (MBI Fermentas, Burlington, Canada). In brief, About 1 ug of total RNA was taken and to it oligo dT primer was added and made upto volume with nuclease free water and incubated at 65°C for 5 min. Then reaction buffer, ribolock® (ribonuclease

inhibitor) and dNTP mix was added. Then reverse transcriptase (Moloney Murine Leukemia Virus: M-MuLV RT) was added and incubated at 42°C for 60 min. Finally the reaction is stopped by heating at 70°C for 10 min.

Polymerase Chain Reaction

cDNA amplification was carried out by polymerase chain reaction using gene specific primers (Table 7.1) on a thermal cycler (CG Palmcycler, Genetix, New Delhi, India). Primers were designed based on the corresponding gene and mRNA sequences from NCBI website (www.ncbi.nlm.nih.gov) Designed primers were covering exon–exon boundary sequences.

The cDNA template was mixed with forward and reverse primers, Taq buffer, dNTP mix, Taq DNA polymerase. The template was initial denaturated at 95°C for 3 min. The PCR is run for 35 cycles. Each PCR cycle comprised following steps: denaturation at 95°C for 30 sec, primer annealing at gene specific temperature for 30 sec and extension at 72°C for 1min. Finally the mixture is kept at 72°C for 10 min to allow complete extension.

The resultant amplicons from polymerse chain reaction of cDNA were checked on 2.0% agarose gel stained with ethidium bromide. The images were recorded with a Gel documentation system (Alpha Innotech Corp., CA, USA) and analysed using AlphaView software, Version 1.2.0.1. The relative amounts of cytokine mRNAs were calculated after normalization with respect to β- actin as internal control.

Table 7.1: Primer sequences and PCR conditions for genes investigated under semi quantitative mRNA expression studies.

Gene	Primer sequence	Annealing Temp. (°C)
β- actin	Forward:5′ CTAGGCACCAGGGTGTGATGGTG 3′ Reverse:5′CCACAGGATTCCATACCCAAGAAGGA3′	55
IL-2	Forward:5′ GCAGGATGGAGAATTACAGG 3′ Reverse:5′ GTCAGAGCCCTTTAGTTTTAC 3′	52
IL-4	Forward:5′ CAGGAGAAGGGACGCCATGCACG 3′ Reverse:5′ GGTGCAGCTTATCGATGAATCCAGGC 3′	62
IL-10	Forward:5′ TCAGCCAGGTGAAGACTTTCTTTCA 3′ Reverse:5′ GAAATCGATGACAGCGCCTCAG 3′	56.5
IFN-gamma	Forward:5′ TTTAACTCAAGTGGCATAGATGTGG 3′ Reverse:5′ TGGGTTGTTGACCTCAAACTTGG 3′	55

7.3 Results

7.3.1 Halobetasol Propionate

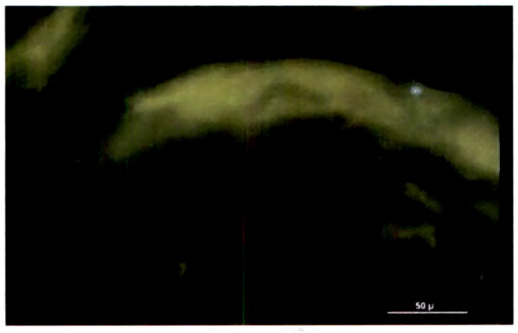


Fig. 7.2: Penetration of 6-coumarin into mice abdominal skin from HP cream

Fig. 7.3: Penetration of 6-coumarin into mice abdominal skin from HP ointment

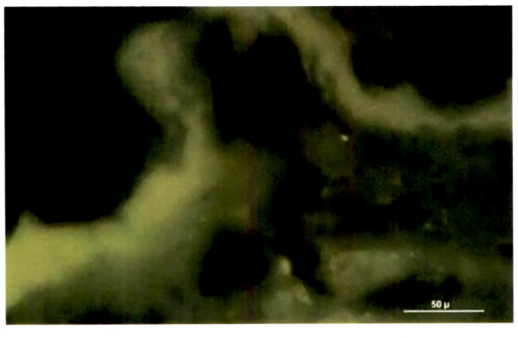


Fig. 7.4: Penetration of 6-coumarin into mice abdominal skin from HPMEC

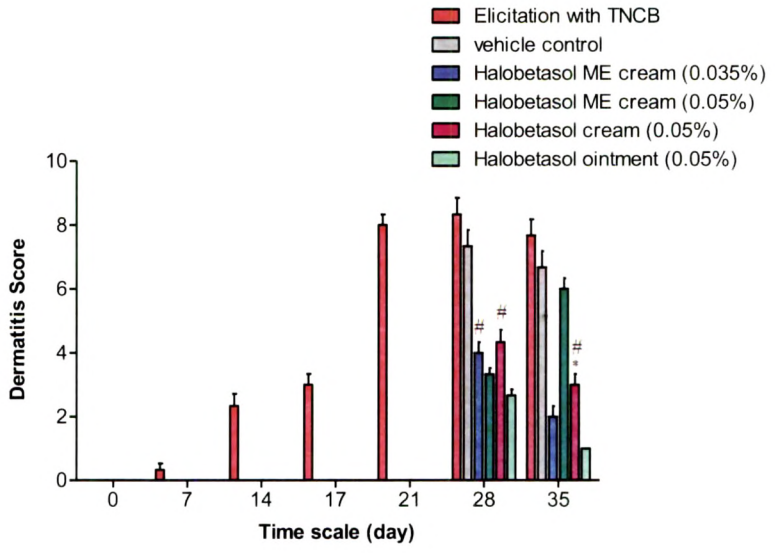


Fig. 7.5: Comparative dermatitis score in mice during the course of study after elicitation with inducing agent (TNCB) and treatment with different formulations. The columns and the error bars represent means ± SEM for n=3 animals. * ($p<0.05$) significant difference in comparison to HP ME cream (0.035%). # ($p<0.05$) significant difference in comparison to HP ointment, when evaluated by Two Way ANOVA, Bonferroni post test.

Table 7.2: Comparative dermatitis score in mice during the course of study after elicitation with inducing agent (TNCB) and treatment with different formulations.

Day	Elicitation with TNCB	Vehicle control	HPMEC (0.035%)	HPMEC (0.05%)	HP cream (0.05%)	HP ointment (0.05%)
0	0.00					
7	0.33 ± 0.33					
10	2.33 ± 0.66					
14	3.00 ± 0.57					
21	8.00 ± 0.57					
28	8.33 ± 0.88	7.33 ± 0.88	4.00 ± 0.57	3.33 ± 0.33	4.33 ± 0.66	2.66 ± 0.33
35	7.66 ± 0.88	6.66 ± 0.88	2.00 ± 0.57	6.00 ± 0.57	3.00 ± 0.57	1.00 ± 0.00

*n=3

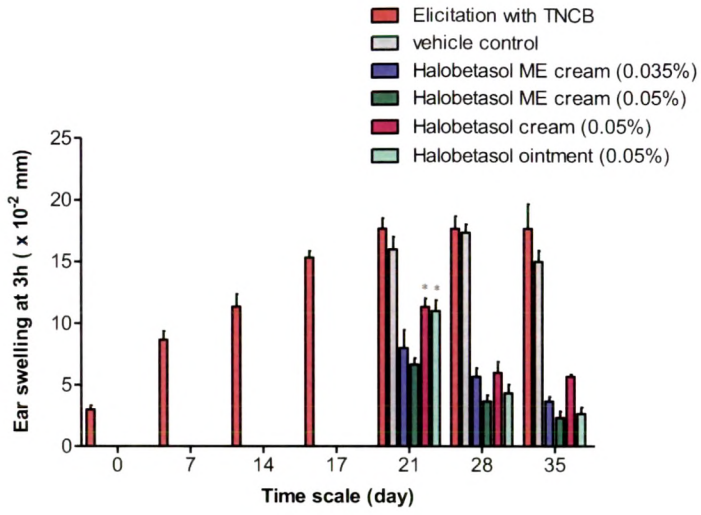


Fig. 7.6

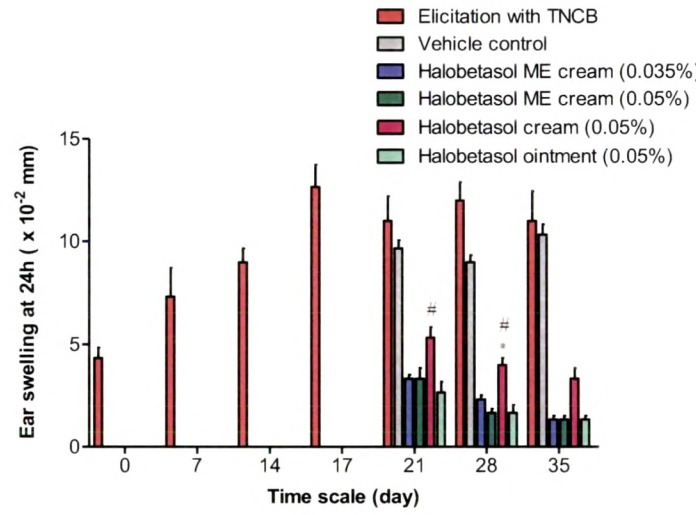


Fig. 7.7

Fig. 7.6: Ear swelling response in mice recorded 3 h post application of formulations. (The difference in ear thickness is plotted and the columns and error bars represent means ± SEM for n=3 animals). * ($p<0.05$) significant difference in comparison to HPMEC (0.035%) and (0.05%) both when evaluated by Two Way ANOVA, Bonferroni post test.

Fig. 7.7: Ear swelling response in mice recorded 24 h post application of formulations. (The difference in ear thickness is plotted and the columns and error bars represent means ± SEM for n=3 animals). * ($p<0.05$) significant difference in comparison to HPMEC (0.05%). # ($p<0.05$) significant difference in comparison to HP ointment, when evaluated by Two Way ANOVA, Bonferroni post test.

Table 7.3: Ear swelling response in mice recorded 3 h and 24 h post application of formulations

At 3h

Day	Elicitation with TNCB	Vehicle control	HPMEC (0.035%)	HPMEC (0.05%)	HP cream (0.05%)	HP ointment (0.05%)
0	3.00 ± 0.57					
7	8.67 ± 1.20					
10	11.33 ± 1.76					
14	15.33 ± 0.88					
21	17.67 ± 1.46	16.00 ± 1.73	8.00 ± 2.51	6.67 ± 0.88	11.33 ± 1.20	11.00 ± 1.52
28	17.67 ± 1.76	17.33 ± 1.20	5.66 ± 1.20	3.67 ± 0.88	6.00 ± 1.52	4.33 ± 1.20
35	17.66 ± 3.48	15.00 ± 1.52	3.67 ± 0.66	2.33 ± 0.88	5.67 ± 0.33	2.67 ± 0.88

*n=3

at 24h

Day	Elicitation with TNCB	Vehicle control	HPMEC (0.035%)	HPMEC (0.05%)	HP cream (0.05%)	HP ointment (0.05%)
0	2.33 ± 0.88					
7	7.33 ± 2.40					
10	9.00 ± 1.15					
14	12.67 ± 1.85					
21	11.00 ± 2.08	9.66 ± 0.66	3.33 ± 0.33	3.33 ± 0.88	5.33 ± 0.88	2.67 ± 0.88
28	12.00 ± 1.52	9.00 ± 0.57	2.33 ± 0.33	1.67 ± 0.33	4.00 ± 0.57	1.67 ± 0.66
35	11.00 ± 2.51	10.33 ± 0.88	1.33 ± 0.33	1.33 ± 0.33	3.33 ± 0.88	1.33 ±0.33

*n=3

Table 7.4: Comparative serum total IgE levels during the course of study after sensitization and elicitation with hapten (TNCB) and treatment with different HP formulations

Day	Elicitation with TNCB	Vehicle control	HPMEC (0.035%)	HPMEC (0.05%)	HP cream (0.05%)	HP ointment (0.05%)
0	243 ± 59					
7	1048 ± 200					
10	3658 ± 947					
14	5961 ± 1109					
21	5283 ± 1187					
28	4692 ± 1335	4365 ± 414	4886 ± 414	5261 ± 967	3790 ± 1079	5237 ± 1379
35	4765 ± 891	4179 ± 627	4925 ± 985	5240 ± 1332	4462 ± 1251	5619 ± 1371

*n=3

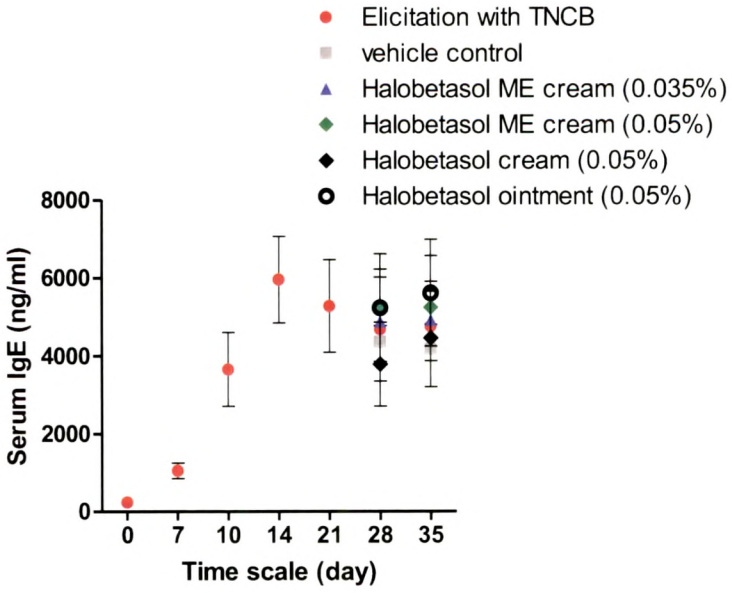
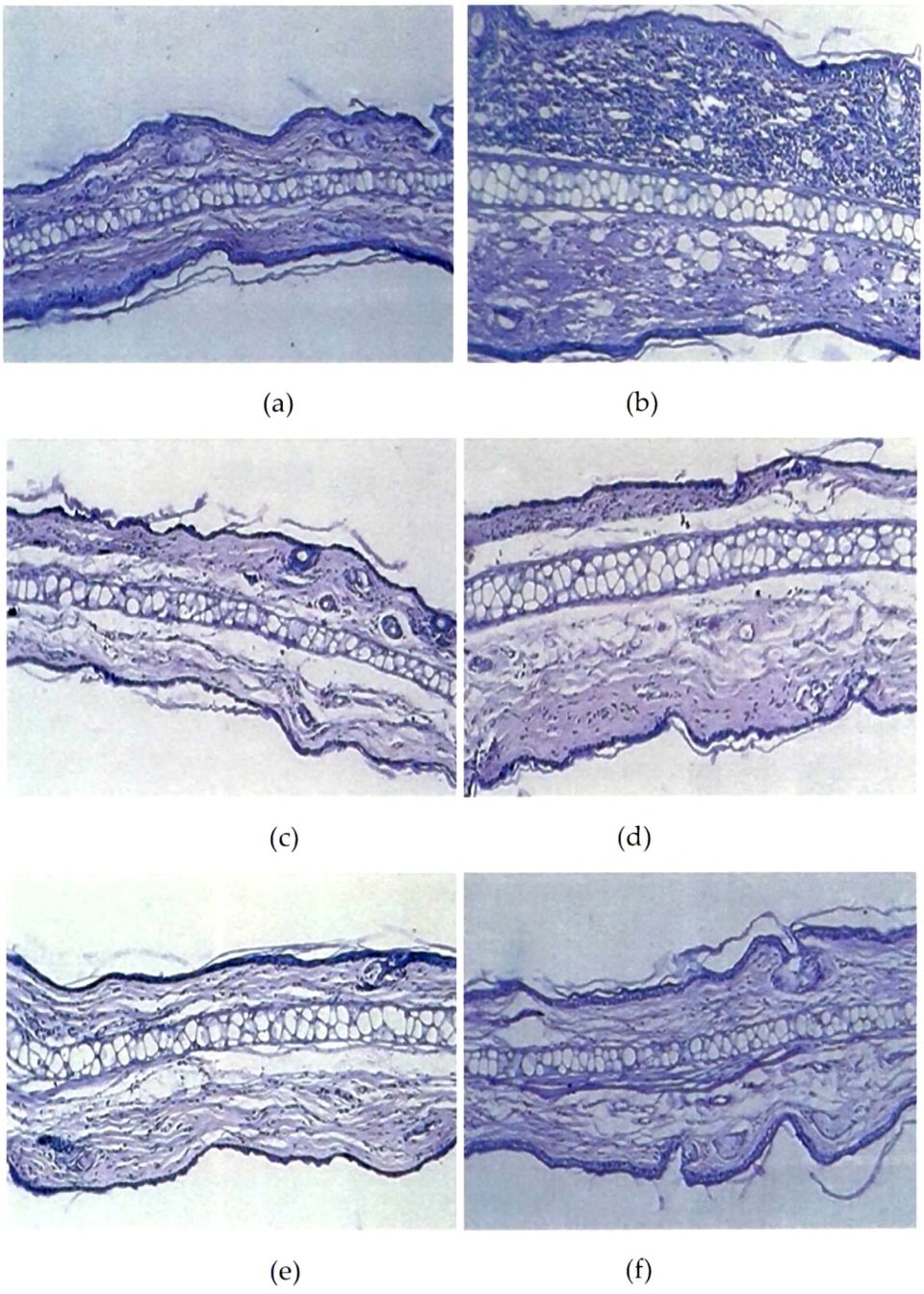


Fig. 7.8: Comparative serum total IgE levels during the course of study after sensitization and elicitation with hapten (TNCB) and treatment with different HP formulations. Values are mean ± SD for n=3 animals. Results were statistically compared using Two Way ANOVA, Bonferroni post test with $p<0.05$

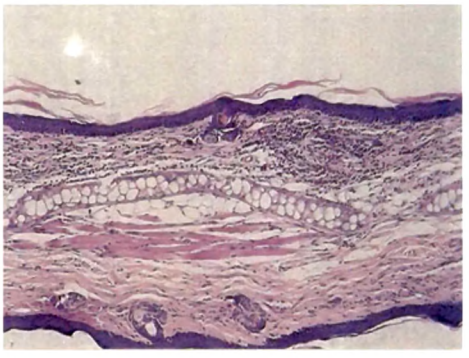
Fig. 7.9:



(a) (b)

(c) (d)

(e) (f)



(g)

Fig. 7.9 (a-g): Histopathology of ear skin lesions caused by repeated application of TNCB and after treatment with HP formulations in BALB/C mice. The skin tissues were stained with hematoxylin and eosin and magnification is 10X.

(a) On day 0

(b) After elicitation with TNCB on day 28.

(c) After treatment with HP MEC 0.05% on day 35

(d) After treatment with HP ointment (0.05%) on day 35

(e) After treatment with HPMEC 0.035% on day 35

(f) After treatment with HP cream (0.05%) on day 35

(g) After treatment with vehicle control

Table 7.5: Mast cell numbers during the course of study after sensitization and elicitation with hapten (TNCB) and treatment with different formulations

Day	Elicitation with TNCB	Vehicle control	HPMEC (0.035%)	HPMEC (0.05%)	HP cream (0.05%)	HP ointment (0.05%)
0	15.33 ± 4.33					
7	20.66 ± 5.92					
10	33.33 ± 7.53					
14	51.66 ± 9.20					
21	65.66 ± 12.71					
28	64.00 ± 6.08	69.33 ± 7.68	34.66 ± 5.36	24.33 ± 3.52	36.00 ± 6.42	25.33 ± 6.11
35	71.00 ± 3.46	61.66 ± 7.42	19.00 ± 3.05	13.66 ± 2.90	24.00 ± 2.51	12.33 ± 1.45

*n=3

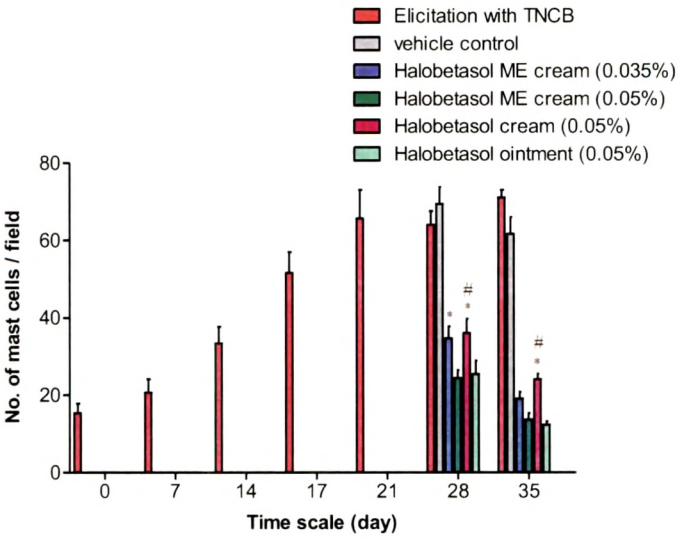


Fig. 7.10: Mast cell numbers during the course of study after sensitization and elicitation with hapten (TNCB) and treatment with different formulations. Values are mean ± SEM for n=3 animals. * ($p<0.05$) significant difference in comparison to HPMEC(0.05%). # ($p<0.05$) significant difference in comparison to HP ointment, when evaluated by Two Way ANOVA, Bonferroni post test.

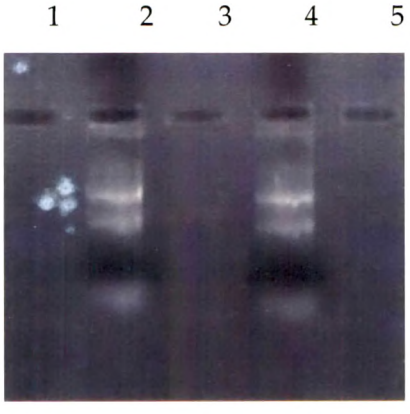


Fig. 7.11: Gel electrophoresis of total RNA extracted from mice ear (well no. 2 and 4).

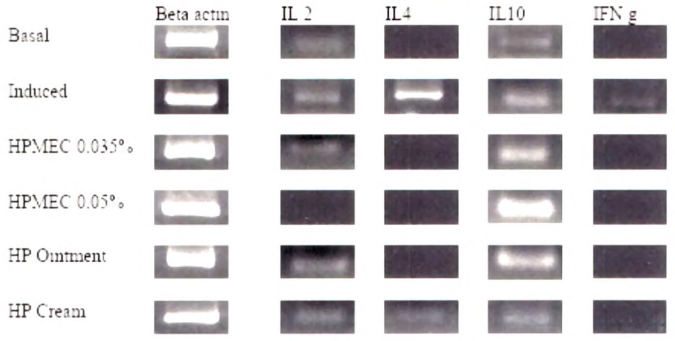


Fig. 7.12: Cytokine mRNA expression in the skin lesion excised 24 h after elicitation with TNCB painting. The figure depicts the bands of amplicons after RT-PCR as seen on 2% agarose gel with ethidium bromide staining

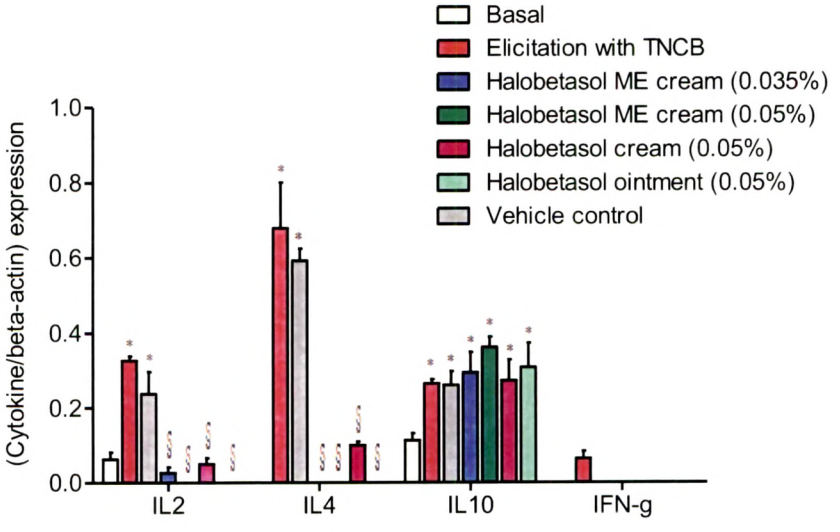


Fig. 7.13: Cytokine gene expression by RT-PCR at the end of study on day 35. The expression level is expressed as a ratio to internal reference beta-actin expression and the columns and error bars represent mean ± SEM for n=3 animals. * ($p<0.05$) significant difference in comparison to basal level. § ($p<0.05$) significant difference in comparison to elicitation with TNCB, when evaluated by Two Way ANOVA, Bonferroni post test.

Table 7.6: Cytokine gene expression by RT-PCR at the end of study on day 35. The expression level is expressed as a ratio to internal reference beta-actin expression

Gene	Basal	Elicitation with TNCB	HPMEC (0.035%)	HPMEC (0.05%)	HP cream (0.05%)	HP ointment (0.05%)	Vehicle Control
IL2	0.063 ± 0.03	0.33 ± 0.02	0.027 ± 0.027	0.000	0.050 ± 0.03	0.000	0.238 ± 0.12
IL4	0.000	0.678 ± 0.21	0.000	0.000	0.100 ± 0.017	0.000	0.593 ± 0.05
IL10	0.113 ± 0.03	0.264 ± 0.02	0.293 ± 0.094	0.362 ± 0.05	0.272 ± 0.09	0.308 ± 0.11	0.260 ± 0.06
IFN g	0.000	0.065 ± 0.03	0.000	0.000	0.000	0.000	0.000

*n=3

7.3.2 Tacrolimus

Fluorescent Microscopy

Fig. 7.14: Penetration of 6-coumarin into mice abdominal skin from Tac ointment.

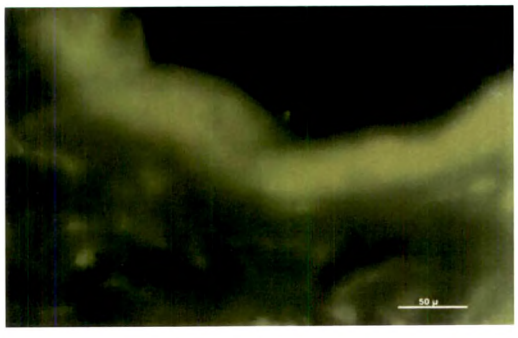


Fig. 7.15: Penetration of 6-coumarin into mice abdominal skin from Tac MEC

Table 7.7: Comparative dermatitis score in mice during the course of study after elicitation with inducing agent (TNCB) and treatment with different formulations

Day	Elicitation with TNCB	Vehicle control	TacMEC 0.1%	Tac Ointment 0.1%
0	0.00			
7	0.33 ± 0.33			
14	2.67 ± 0.88			
21	7.67 ± 0.88			
28	8.67 ± 0.88	7.67 ± 0.88	4.00 ± 0.57	5.33 ± 0.88
35	7.33 ± 0.66	6.33 ± 0.66	2.33 ± 0.33	4.00 ± 0.57

*n=3

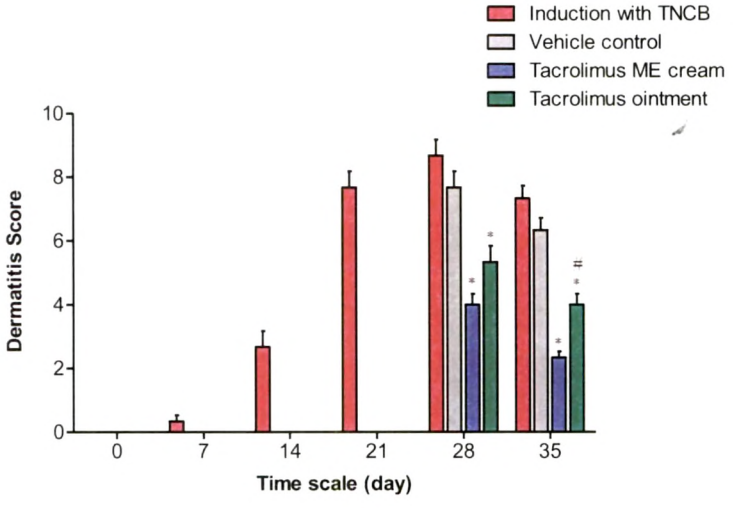


Fig. 7.16: Comparative dermatitis score in mice during the course of study after elicitation with inducing agent (TNCB) and treatment with different Tac formulations. The columns and the error bars represent means ± SEM for n=3 animals. * ($p<0.05$) significant difference in comparison elicitation with TNCB. # ($p<0.05$) significant difference in comparison to tacMEC 0.1%, when evaluated by Two Way ANOVA, Bonferroni post test.

Table 7.8: Comparative serum total IgE levels during the course of study after sensitization and elicitation with hapten (TNCB) and treatment with different formulations

Day	Elicitation with TNCB	Vehicle control	TacMEC 0.1%	Tac Ointment 0.1%
0	251 ± 73			
7	1092 ± 203			
10	3810 ± 1094			
14	5992 ± 1258			
21	5529 ± 1180			
28	4792 ± 1249	4631 ± 173	4553 ± 504	4724 ± 874
35	4469 ± 990	4002 ± 657	4592 ± 874	4573 ± 1130

*n=3

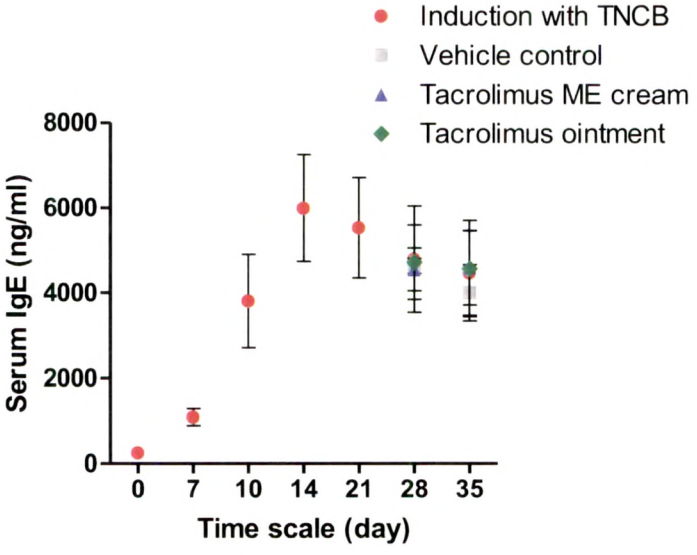


Fig. 7.17: Comparative serum total IgE levels during the course of study after sensitization and elicitation with hapten (TNCB) and treatment with different Tac formulations. Values are mean ± SD for n=3 animals. Results were statistically compared using Two Way ANOVA, Bonferroni post test with $p<0.05$.

Table 7.9: Ear swelling response in mice recorded 3 h post application of formulations

Day	Elicitation with TNCB	Vehicle control	TacMEC 0.1%	Tac Ointment 0.1%
0	3.00 ± 0.57			
7	8.67 ± 1.20			
10	11.67 ± 1.45			
14	15.00 ± 1.15			
21	17.33 ± 1.76	15.67 ± 2.02	9.67 ± 1.67	13.67 ± 0.66
28	18.33 ± 1.45	17.00 ± 1.15	7.67 ± 0.88	11.33 ± 0.88
35	17.33 ± 3.52	15.33 ± 1.45	4.00 ± 0.57	6.33 ± 0.33

*n=3

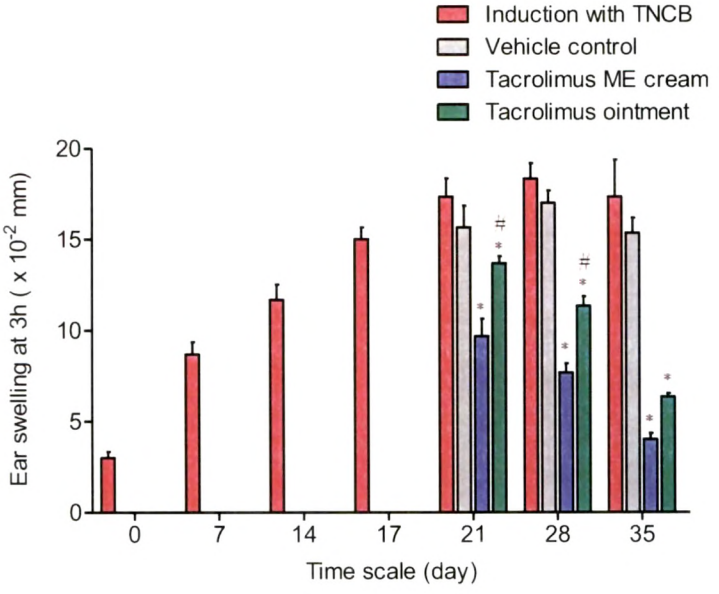


Fig. 7.18: Ear swelling response in mice recorded 3 h post application of formulations. (The difference in ear thickness is plotted and the columns and error bars represent means ± SEM for n=3 animals). * ($p<0.05$) significant difference in comparison elicitation with TNCB. # ($p<0.05$) significant difference in comparison to TacME based cream, when evaluated by Two Way ANOVA, Bonferroni post test.

Table 7.10: Ear swelling response in mice recorded 24 h post application of formulations

Day	Elicitation with TNCB	Vehicle control	TacMEC 0.1%	Tac Ointment 0.1%
0	4.33 ± 0.88			
7	7.00 ± 2.51			
10	9.33 ± 1.20			
14	13.00 ± 1.52			
21	10.66 ± 1.76	9.33 ± 0.88	4.66 ± 0.88	6.66 ± 0.88
28	11.66 ± 1.76	8.66 ± 0.88	5.33 ± 0.88	6.67 ± 1.20
35	10.66 ± 2.40	10.66 ± 1.20	1.99 ± 0.33	4.99 ± 0.66

*n=3

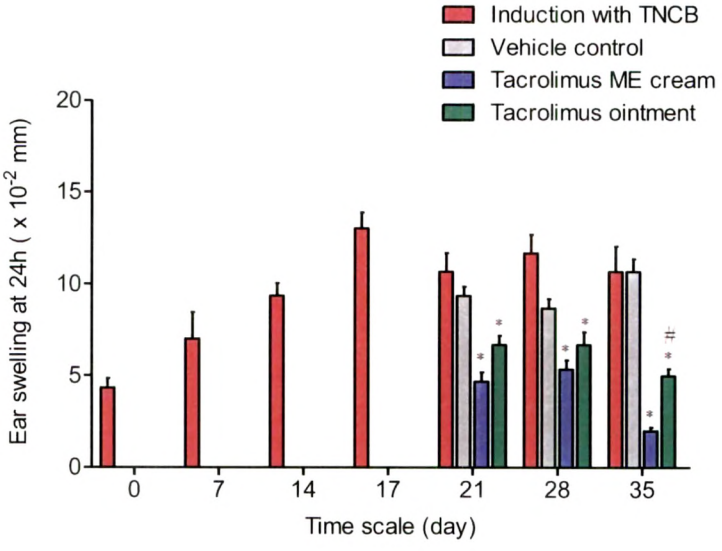


Fig. 7.19: Ear swelling response in mice recorded 24 h post application of formulations. (The difference in ear thickness is plotted and the columns and error bars represent means ± SEM for n=3 animals). * ($p<0.05$) significant difference in comparison elicitation with TNCB. # ($p<0.05$) significant difference in comparison to TacME based cream, when evaluated by Two Way ANOVA, Bonferroni post test.

Table 7.11: Mast cell numbers during the course of study after sensitization and elicitation with hapten (TNCB) and treatment with different formulations

Day	Elicitation with TNCB	Vehicle control	TacMEC 0.1%	Tac Ointment 0.1%
0	15.33 ± 4.33			
7	20.67 ± 5.92			
10	33.33 ± 7.53			
14	51.67 ± 9.20			
21	65.66 ± 12.71			
28	64.00 ± 6.08	69.33 ± 7.68	59.33 ± 2.84	58.67 ± 7.75
35	71.00 ± 3.46	61.66 ± 7.42	55.67 ± 7.05	61.67 ± 8.19

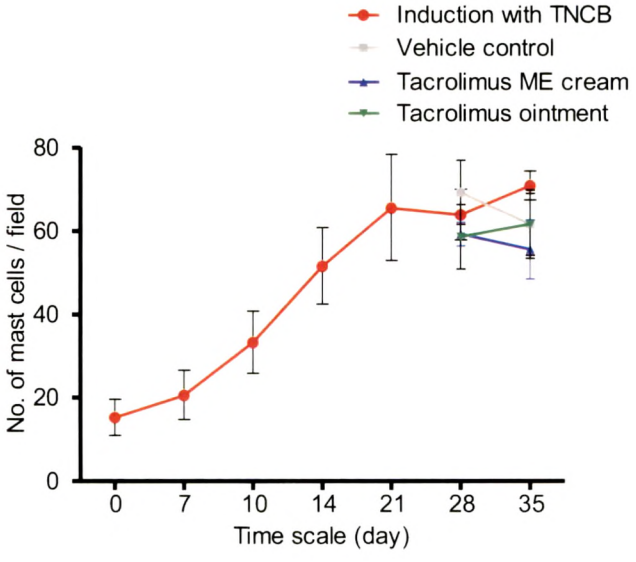
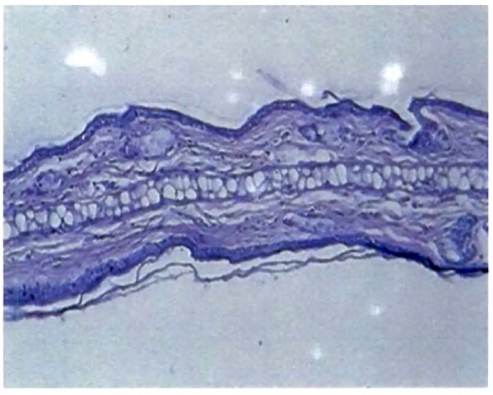
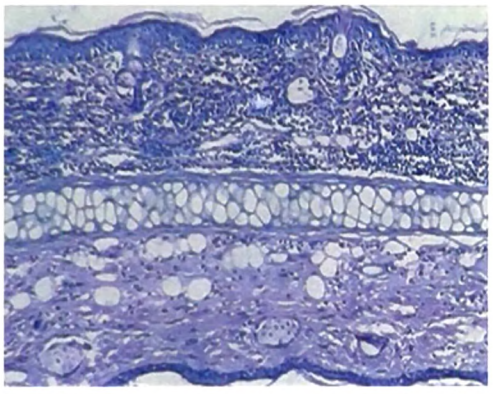


Fig. 7.20: Mast cell numbers during the course of study after sensitization and elicitation with hapten (TNCB) and treatment with different Tac formulations. Values are mean ± SEM for n=3 animals. Results were statistically compared using Two Way ANOVA, Bonferroni post test with $p<0.05$ considered as significant.

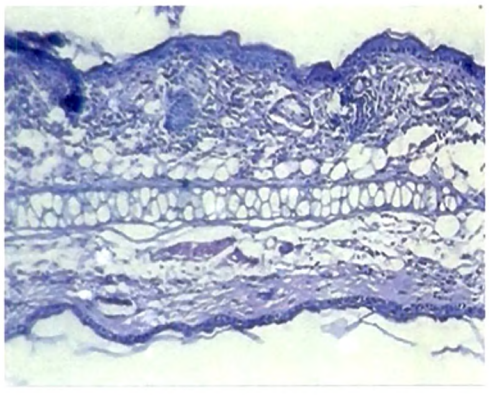
Fig. 7.21



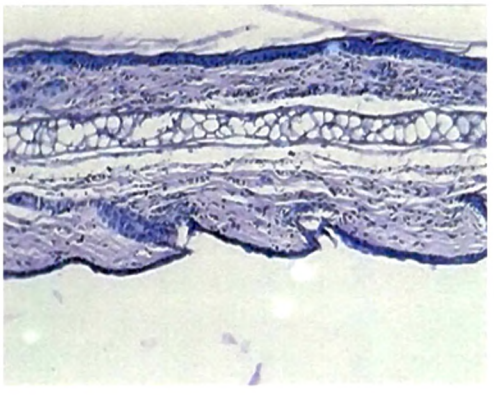
(a)



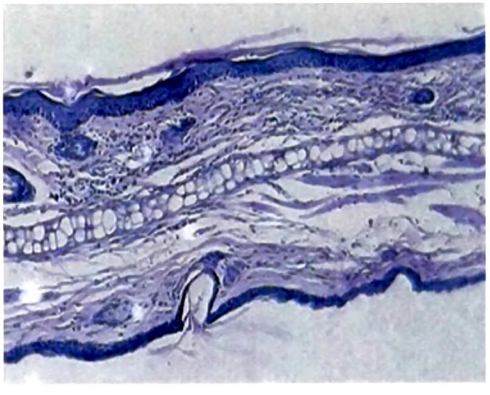
(b)



(c)



(d)



(e)

Fig. 7.21 (a-e): Histopathology of ear skin lesions caused by repeated application of TNCB and treatment with different Tac formulations in BALB/C mice. The skin tissues were stained with hematoxylin and eosin and magnification is 10X.

(a) on day 0,

(b) after elicitation with TNCB on day 28,

(c) after treatment with vehicle control on day 35

(d) after treatment with TacMEC 0.1% on day 35,

(e) after treatment with Tac ointment on day 35

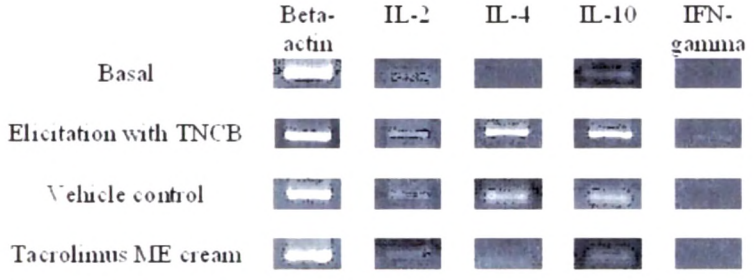


Fig. 7.22: Cytokine mRNA expression in the skin lesion excised 24 h after elicitation with TNCB painting. The figure depicts the bands of amplicons after RT-PCR as seen on 2% agarose gel with ethidium bromide staining.

Table 7.12 : Cytokine gene expression levels by RT –PCR at the end of study on day 35. The expression level is expressed as a ratio to internal reference beta-actin expression

Gene	Basal	Elicitation with TNCB	TacMEC 0.1%	Tac Ointment 0.1%	Vehicle Control
IL2	0.083 ± 0.014	0.323 ± 0.143	0.143 ± 0.037	0.143 ± 0.012	0.242 ± 0.105
IL4	0.000	0.668 ± 0.293	0.293 ± 0.120	0.293 ± 0.027	0.587 ± 0.056
IL10	0.130 ± 0.034	0.367 ± 0.167	0.167 ± 0.045	0.167 ± 0.064	0.373 ± 0.048
IFN-g	0.000	0.061 ± 0.000	0.000	0.000	0.000

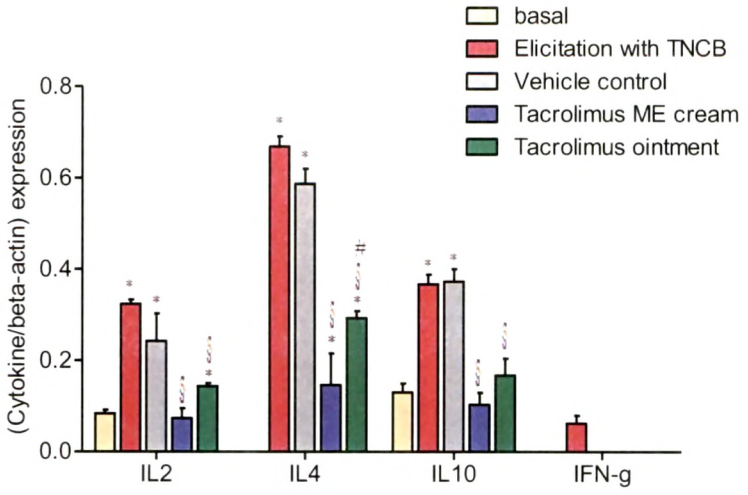


Fig. 7.23: Cytokine gene expression levels by RT –PCR at the end of study on day 35. The expression level is expressed as a ratio to internal reference beta-actin expression and the columns and error bars represent mean ± SEM for n=3 animals. * ($p<0.05$) significant difference in comparison to basal level. § ($p<0.05$) significant difference in comparison to elicitation with TNCB. # ($p<0.05$) significant difference in comparison to TacMEC 0.1%, when evaluated by Two Way ANOVA, Bonferroni post test.

7.4 Discussion

7.4.1 Halobetasol Propionate

The qualitative aspects of drug penetration in skin was also compared using fluoroscent microscopy. 6- coumarin loaded formulations were compared after 6 h of application on mice skin. It is evident that ME based cream ensured higher and deeper penetration in comparison to the marketed cream and ointment (Fig.7.2-7.4). These results are concordant with ex-vivo drug release and skin retention study results reported earlier. The fluorescent dye depicts its possible accumulation in the different layers of the skin as marker mimics drug permeation into skin (Changez et al, 2006).

The efficacy and safety of the formulations was evaluated in hapten (TNCB) induced model of dermatitis. The repeated application (sensitization and elicitation) of TNCB, results in development of an antigen-specific hypersensitivity response which is mix of immediate type hypersensitivity and delayed type response. These reactions are also characteristic of clinical cases of AD. In murine model of epicutaneous sensitization with hapten, morphological changes are accompanied by changes in histopathology like epidermal hyperplasia, increased numbers of mast cells and CD+ T cells beneath the epidermis, and elevated serum levels of antigen-specific IgE (Kitagaki et al, 1995 and Shiohara et al, 2004).

In murine model, a comprehensive dermatitis score can be calculated from the observed severity of 4 major symptoms of lesional skin. When hapten is repeatedly applied on the face of mice ear, severity of symptoms increased progressively to give a mean score of around 9 on day 21 (Table 7.2, Fig. 7.5). HP application improves the morphological changes significantly from the very first week. In the first week, the ointment was significantly better over

other formulations. The low dose ME based cream also gave similar therapeutic response as commercial cream. At the end of 2 week treatment, the reduction in dermatitis score with low dose ME based cream is equivalent to commercial cream and ointment both. It was observed that dermatitis score does not reduce significantly with HP ME based cream (0.05%). Skin atrophy is seen at the site of application with HP ME based cream (0.05%).

A greater reduction in dermatitis score with HP ointment is recorded after 1 week of treatment. The low dose HP ME based cream was also equally effective which may be interpreted as enhanced permeation of drug resulted in efficient control over symptoms. At the end of 2 week treatment, the low dose HP ME based cream had a similar therapeutic efficacy as other marketed preparations. The anti-inflammatory activity of corticosteroid is attributed to its multimodal effects. They are known to suppress the transcription of several genes encoding for inflammatory cytokines. Corticosteroids increase synthesis of lipocortin-1 which has an inhibitory effect on phospholipase A2 and reduces production of lipid mediators (Barnes, 1998). In epithelial cells, corticosteroids increases degradation of inflammatory peptides such as substance P, bradykinin and endothelin-1 implicated in scratching behavior associated with AD (Ohmurra et al, 2004). They reduce the survival of inflammatory cells like eosinophils and T cells to decrease cytokine and chemokine release.

HP ME based cream in clinical strength did not reduce the dermatitis score as it showed skin atrophy and associated erythema after 2 weeks of treatment. This may be attributed to the increased drug concentration in skin with microemulsion based cream. Skin atrophy is a common local side effect with corticosteroids which results from reduced synthesis of collagen and antiproliferative effects on fibroblasts and keratinocytes. The antinflammatory

and atrophogenic potential of a corticosteroid is usually directly proportional (Lutgemeier et al, 1987 and Schoepe et al, 2006). However, reducing the atrophogenic potential while maintaining anti-inflammatory efficacy is tricky and various strategies based on pharmacokinetics, inherent pharmacodynamic properties of molecule and optimized drug delivery may address it. We have attempted the same here with a microemulsion based cream to optimize drug delivery of a potent corticosteroid.

The anti-inflammatory responses are characterized by ear swelling studies in murine models as it is considered as a very reliable parameter for evaluation of therapeutics. The measurements are made 3h and 24h post application so as to study the effects on early and late phases of hypersensitivity response (Table 7.3). It is observed (Fig. 7.6) that HP ME based creams (0.035% and 0.05%) shows a prompt and significant reduction in ear swelling (at 3h) upon its first application on day 21 in comparison to HP cream and ointment. The anti-inflammatory response (at 3h) is comparable between the HP ME based creams (0.035% and 0.05%) and commercially available cream and ointment at the end of 2 weeks treatment. The reduction in ear swelling after 24h (Fig. 7.7) with ME based creams (0.035% and 0.05%) is comparable to commercial cream and ointment respectively. There is a significant difference between the anti-inflammatory control between commercial cream and ointment in the first week of treatment with the ointment being more effective. The ear swelling studies at 3h and 24 h post application further validate the therapeutic efficacy of HP ME based cream. The reduction in ear swelling (at 3h) on the day of first application of HP formulations showed a significant difference in ME based creams in comparison to marketed preparations. This signified a faster drug penetration into the skin by ME based creams. After 2 weeks of application, all the formulations were comparable in their response at 3h. This may be due to drug accumulation in the skin after 2 weeks of

treatment. But noteworthy is the equivalent anti-inflammatory response with low dose HP ME based cream due to enhanced permeation and skin retention of drug. Ear swelling response (at 24h) showed equivalent response of ME based creams with HP ointment. Commercial cream is comparatively less efficacious than other formulations in controlling late phase reaction. This may be due to insufficient drug localization and retention in viable epidermis and dermis. Corticosteroids inhibit vasodilatation of the microcirculation, therefore preventing the increase in blood flow and exudate formation that characterizes the initial stages of the inflammatory response (Perreti, 2000). Besides, they reduce nitric oxide to reduce local blood flow (Ahluwalia, 1998). Corticosteroids also reduce epidermal mast cell number and inhibit release of proinflammtory mediators and vasoactive amines (Finotto et al, 1997).

It is observed that total IgE levels are not suppressed with HP application. There is no significant difference between any of the groups. In the investigated animal model of AD, serum IgE levels are found to be elevated like the clinical cases of extrinsic AD. These models show a Th2 skewed response on chronic hapten application where elevated IL-4 levels are accompanied by high serum IgE titers (Harada et al, 2005). The serum IgE levels in currently used model peaks on 17 or 21 day (Table 7.4, Fig. 7.8). Corticosteroids fail to suppress IgE titers but upregulate CD40 ligand expression on T cells and induce CD40L-dependent immunoglobulin isotype switching in B cells (Barnes, 2001).

The histopathology of skin biopsy specimens are shown in Fig. 7.9 (a-g) and it is observed that epidermal hyperplasia, spongiosis and increased dermal infiltrate of inflammatory cells and mast cells is seen after chronic application of hapten. A significant reduction of epidermal thickness with all HP formulations is seen. The protective effects are with low dose ME based cream

is comparable to other formulations. Epidermal thinning is more prominent for ME based cream (0.05%). Corticosteroid started reducing mast cell numbers from the first week (Table, 7.5, Fig. 7.10). The histopathology of skin biopsy showed that HP application reduces the dermal infiltration of inflammatory cells, mast cells. Developed low dose formulation provided an equivalent control of inflammation in comparison to commercial formulations. Corticosteroids start reducing mast cell numbers from the first week, and reduces the degranulation of mast cells by crosslinking with IgE and release of inflammatory mediators and vasoactive amines like histamine (Finnotto, 1997). Eosinophil infiltration is significantly reduced, lowering the cytokine and chemokine release (Barnes, 1998). Epidermal thinning observed is attributed to reduced collagen synthesis and fibroblast and keratinocyte inhibition (Schoepe, 2006). ME based cream (0.05%) showed a more prominent epidermal thinning which may be due to higher drug deposition into skin layers.

Total RNA was isolated from excised ears at various time points during the course of study and Th1 (IL-2 and IFN-gamma) and Th2 (IL-4, IL-10) cytokine gene expression was assessed by RT-PCR as AD is thought to be a mixed Th1 and Th2 response. An equivalent concentration of total RNA isolated from mice ears was used for cDNA preparation and consequent PCR amplification using gene specific (IL-2, IL-4, IL-10 and IFN-gamma) primers. It was observed that at the end of elicitation phase, the expression of all the cytokines was significantly higher in comparison to basal level at the start of study. The increased expression of IL-4 and IL -10 in comparison to IL-2 and IFN-gamma on chronic application demonstrates a Th2 dominant response (Fig. 7.12 and 7.13). After treatment with vehicle control (placebo) and drug loaded formulations, a significant reduction of cytokine expression was observed with HP formulations. IL-4 expression is undetectable before study

and becomes highly elevated in the hapten challenged group. It shows a marked reduction in the HP treated groups, although the levels do not drop to basal level after 2 weeks of treatment with HP cream. IL-10 is also significantly upregulated in the challenged group and almost similar upregulation is observed in all the HP treated groups. In case of IL-2, there is a significant reduction with all formulations. The expression level of IFN-gamma was below detection at basal level and showed a rise after elicitation. It remains undetected in treatment groups. Vehicle control does not have any significant impact on cytokine expression (Table 7.6)

Cytokine gene expression in lesional skin was studied by RT-PCR. The increased expression of IL-4 and IL -10 in comparison to IL-2 and IFN-gamma on chronic application of hapten demonstrates a Th2 dominant response. The tissue microenvironment favors Th2 dominance over Th1 so that a more cytotoxic Th1 reaction is limited and less damaging Th2 response takes over (Kitagaki, 1997). IL-10 has been reported to be very crucial in development of hypersensitivity reaction in murine model by down regulating IL-12 production by dendritic cells so as to inhibit development of Th1 cells. It serves to down regulate pro-inflammatory cytokine expression (Huang et al, 2001). In the present investigations, an increased IL-10 expression is observed with hapten elicitation and remains upregulated after HP treatment. IL-4 is responsible for the IgE isotype switching. The increased production favors development of Th2 cells, and induction of other adhesion molecules on endothelial cells that recruit eosinophils into the lesion area (Spergel et al, 1997). In fact, IL-4 levels are reported to reflect the severity of inflammation (Webb, 1998). Thus, Th2 cells are crucial for humoral immunity. IL-4 levels increase maximally in sensitized animals and decrease significantly with HP application ensuring an effective control of inflammation by reducing its transcription through NF-κB pathway (Ahluwalia, 1998). Vehicle control does

not have any significant impact on cytokine expression but may reduce pruritus partially by alleviating xerosis.

Th1 cells are important in development of cell mediated immunity. IL-2 is important for T-cell growth and activation as it primes the naive T cells (Malek, 2003). Corticosteroids are reported to suppress IL-2 and IFN- gamma expression apart from other cytokines. IFN-gamma activates monocytes and macrophages, brings about the skin hypertrophy in AD and favors the development of Th1 cells (Boehm, 1997). The developed formulations were equally effective in suppressing Th1 cytokines and validate their potential as better alternative to existing formulation. Thus, a combined suppressive effect on multiple cytokine expression even by low dose HP cream translates into therapeutic benefit for better control of AD.

7.4.2 Tacrolimus

The drug penetration in the skin was also compared using fluoroscent microscopy. 6- coumarin loaded formulations were compared after 6 h of application on mice skin. It is evident that ME based cream ensured higher and deeper penetration in comparison to the ointment (Fig. 7.14 – 7.15) and was in agreement with the ex-vivo drug release and skin retention study results discussed earlier. The fluorescent marker mimics drug permeation into the skin layers and demonstrates its possible accumulation in the different layers of the skin (Changez, 2006).

In murine model, a 4 symptom based dermatitis scoring system is used which grades the severity of each symptom to give a comprehensive dermatitis score. When hapten is repeatedly applied to the auricular skin of mice, severity of symptoms increases progressively to give a mean score of around

9 on day 28 (Table, 7.7, Fig. 7.16). Tacrolimus application improves the morphological changes significantly from the very first week. The reduction in dermatitis score is higher with ME based cream within 7 days of application. At the end of 2 week treatment also, a significantly better control over symptoms is observed with novel formulation. A greater reduction in dermatitis score with ME based cream is recorded after 1 week of treatment. At the end of 2 week treatment a significantly better control over symptoms is observed with ME based cream formulation. Tacrolimus controls inflammation by inhibiting the various cellular mediators of inflammation like T cells, basophils, mast cells, dendritic cells through multifarious immunosuppressant mechanisms (Norris, 2005 and Simon et al, 2004). Enhanced permeation afforded by the ME based cream formulation leads to effective and rapid control over signs and symptoms of the disease.

The anti-inflammatory responses are characterized in murine models by ear swelling studies as it is considered as a very reliable parameter for evaluation of therapeutics [Inoue et al, 2002]. The measurements are made 3h and 24h post application so as to study the effects on early and late phases of hypersensitivity response (Table 7.9, 7.10). It is observed (Fig. 7.18) that ME based cream shows a prompt and significant reduction in ear swelling (at 3h) upon its first application on day 21 as well as on day 28 in comparison to Tacrolimus ointment. TacMEC also showed a significant improvement in ear swelling suppression (at 24h) on day 35 (Fig. 7.19). The anti-inflammatory response is comparable between the ME based cream and commercially available ointment at the end of 2 weeks treatment. The reduction in ear swelling was significant from the first application in comparison to ointment. Topical tacrolimus is reported to be effective upon early mast cell dependent contact hypersensitivity mediated via G-coupled C5a receptors and on late T cell production of cytokines and late IgE mediated hypersensitivity. But, it is moderately effective in early IgE dependent mast cell responses mediated

through FcεRI receptors on mast cells (Geba et al, 2001). The results reflected that the tacrolimus formulations were more effective in early stages as compared to the late phase reaction. But, the better skin permeation of tacrolimus offered by ME based cream may have lead to a faster and higher suppression of inflammatory response through both of the above mentioned pathways of inflammation control by tacrolimus. Thus, the TacMEC formulation affords an early and effective control of inflammation by virtue of faster and higher drug concentration in dermal layers.

It was observed that total IgE levels were not suppressed with Tacrolimus application. There is no significant difference between any of the groups. Elevation in serum IgE levels in the investigated animal model of AD has been reported to be similar to extrinsic AD. These models show a Th2 skewed response on chronic hapten application where elevated IL-4 levels are accompanied by high serum IgE titers (Harada et al, 2005). The serum IgE levels in currently used model peaks on 17 or 21 day (Table 7.8 and Fig. 7.17) Tacrolimusis ineffective in lowering serum IgE levels because it supresses IL-4 production by T cell but not the T cell independent IL-4 production. Hence, an effective supression in serum IgE titers is not observed with any of the formulation, which is in agreement with earlier reports.

The histopathological observations are shown in Fig. 7.21(a-e) and it is observed that epidermal hyperplasia, spongiosis and increased dermal infiltration of inflammatory cells and mast cells are seen after chronic application of hapten. The reduction in epidermal hyperplasia is more prominent with the cream as compared to ointment. However, the number of mast cells does not reduce with any of the formulations (Table 7.11, Fig. 7.20). The histopathological observations show that tacrolimus application restores the epidermal thickness, reduces the dermal infiltration of inflammatory cells.

Tacrolimus is reported to be ineffective in reducing mast cells numbers during short term application, but downregulates the FcεRI receptors on mast cells. This consequently reduces the degranulation of mast cells by crosslinking with IgE and release of inflammatory mediators and vasoactive amines like histamine (Inoue et al, 2006 and Norris, 2005). The reduction in epidermal hyperplasia is more prominent with the ME based cream as compared to ointment.

Inflammatory cytokine gene expression was assessed by RT-PCR. A fixed concentration of total RNA isolated from mice ears was used for cDNA preparation and consequent PCR amplification using gene specific (IL-2, IL-4, IL-10 and IFN-gamma) primers. Both Th1 (IL-2 and IFN-gamma) and Th2 (IL-4, IL-10) cytokines were selected for the study, as AD is thought to be a mixed Th1 and Th2 response. It was observed that at the end of elicitation phase, the expression of all the cytokines was markedly higher in comparison to basal levels at the start of study. The increased expression of IL-4 and IL -10 in comparison to IL-2 and IFN-gamma on chronic application demonstrated a Th2 dominant response (Fig. 7.22, Fig. 7.23). After treatment with tacrolimus formulations, a significant reduction of cytokine levels was observed. IL-4 is undetectable before study and becomes highly elevated in the challenged group. It shows a marked reduction in the Tacrolimus treated groups, although the levels do not drop to basal level even after 2 weeks of treatment. The ME based cream suppresses IL-4 expression significantly more than the ointment. IL-10 is also significantly upregulated in the challenged group and almost similar reduction is observed in all the tacrolimus treated groups. In case of IL-2, the ME based cream restores the cytokine expression to basal levels. There was a significant reduction with the ointment also, but to a lower extent. The expression level of IFN-gamma was below detection at basal level

and showed a rise after elicitation. It remains undetected in treatment groups. Vehicle control does not have any significant impact on cytokine expression.

The increased expression of IL-4 and IL -10 in comparison to IL-2 and IFN-gamma on chronic application of hapten demonstrates a Th2 dominant response (Shiohara et al, 2004). In the acute lesions a Th1 response dominates, but a time dependent shift to Th2 dominant response occurs. The tissue microenvironment favors Th2 dominance over Th1 so that a more cytotoxic Th1 reaction is limited and less damaging Th2 response takes over (Kitagaki, 1997). The cytokine profiles after elicitation are in agreement with the reported cytokine profiles where increased expression of IL-4 and IL-10 are observed. IL-10 has been reported to be very crucial in development of hypersensitivity reaction in murine model. It down regulates IL-12 production by dendritic cells so as to inhibit development of Th1 cells and down regulate their pro-inflammatory cytokine expression (Huang et al, 2001). In the present investigations, an increased IL-10 expression is observed with hapten elicitation and diminishes after Tacrolimus treatment with more significant suppression with cream formulation. Tacrolimus has been reported to reduce IL-10 expression in CD4+ and CD8+ cells (Simon et al, 2004). IL-4 is responsible for the IgE isotype switching. The increased expression of IL-4 favors development of Th2 cells and induction of other adhesion molecules on endothelial cells that recruit eosinophils into the lesion area. In fact, IL-4 levels are reported to reflect the severity of inflammation (Webb, 1998). Thus, Th2 cells are crucial for humoral immunity. IL-4 levels increase maximally in sensitized animals and decrease significantly with Tacrolimus application ensuring an effective control of inflammation by reducing its transcription through calcineurin mediated pathway. The cream formulation demonstrated comparatively higher suppression of IL-4 expression by virtue of higher drug concentration in dermal layers. This can

be interpreted as differential inflammation control with the two Tacrolimus formulations because IL-4 levels directly correlate with dermal inflammation (Webb, 1998). Vehicle control does not have any significant impact on cytokine profiles but may reduce pruritus partially by alleviating xerosis.

Th1 cells are important in development of cell mediated immunity. IL-2 is important for T-cell growth and activation; it primes the naive T cells (Malek, 2003). Tacrolimus is reported to exert its immunosuppressive action also through inhibition of Th1 cells suppressing IL-2 and IFN- gamma expression apart from other cytokines (Sengoku, 1999). IFN-gamma activates monocytes and macrophages, brings about the skin hypertrophy in AD and favors the development of Th1 cells (Boehm, 1997). Reduction in IFN-gamma with Tacrolimus reduces the epidermal thickening in AD but at the same time does not bring about atrophy as in the case of steroids. IL-2 expression is restored to basal level with the cream but not with the ointment. The findings with investigated formulations are in agreement with the reported mechanisms of tacrolimus where a significant reduction is seen in Th1 cytokines. Moreover, tacrolimus is reported to reduce the T cell numbers in the lesional area may be because of a reduced IL-2 and IL-4 as their reduction brings about apoptosis of activated lymphocytes (Simon et al, 2004). Thus, a combined suppressive effect on multiple cytokine expression by Tac MEC translates into therapeutic benefit for better control of AD.

7.5 References

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