the early period of life. However, few studies have actually addressed and confirmed this assumption using population-based cohort study methodology. Kohno reported that 80% of 4-month-old AD patients became symptom-free at 18-months [26]. In the present cohort, we determined the regression rate prospectively. Spontaneous regression was observed in 71.6% of AD patients during the 3-year follow-up period in the nursery school children studied here. Such spontaneous regression seemed to occur rather rapidly because 41.9% of patients no longer showed any symptoms as early as 1 year later.

Among the 795 initially symptom-free children, AD developed in 25, 14 and 5 cases, one, 2 and 3 years later, respectively. Thus, 44 of 795 children (5.5%) developed AD over the 3 years (3.67%/person year). Consistent with this finding, it was reported that 60% of 3 year-old AD patients had not shown any symptoms at 4-months [26]. Considering the spontaneous regression and *de novo* development as mentioned above, the clinical course of AD is clearly extremely diverse in nursery school children.

In our previous study, high levels of total IgE were found in only 33.3% of those children diagnosed with AD. However, IgE antibodies specific for one or more allergens were detected in 64.1% of children with AD. The total and specific IgE levels were both significantly higher in children with AD than in those without [16]. In the present cohort, we compared IgE levels in long-term AD patients with those who spontaneously regressed, developed AD *de novo*, or never had AD. The IgE levels tended to increase gradually as the children's ages increased in all of these groups. Nonetheless, a much more marked elevation of IgE was observed in the long-term AD group compared to the others. Other studies have also found that IgE levels were elevated in 80 to 85% of children who developed AD and correlated with disease severity [27, 28]. Recently, Yamamoto *et al.* showed that subcutaneous injection of culture supernatants from keratinocytes potently enhanced IgE secretion by splenocytes and increased *in vivo* IgE levels in mice [29]. Soumelis *et al.* demonstrated that thymic stromal lymphopoietin (TSLP) was highly expressed by keratinocytes from patients with AD, and that TSLP-activated dendritic cells primed naive helper T cells to produce the proallergic cytokines IL-4, IL-5, IL-13 and tumor necrosis factor-alpha, while down-regulating IL-10 and interferon-γ [30]. These results suggest that continuous atopic inflammation of the skin may enhance IgE production by stimulating the secretion of keratinocyte-derived cytokines.

In conclusion, more than 70% of AD children experienced spontaneous regression within the 3-year follow-up period, while new onset was estimated at 3.67%/person year in nursery school age children. ■

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ORIGINAL ARTICLE

Anti-CXCR3 staining is useful for detecting human cutaneous and mucosal mast cells

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ABSTRACT

Human synovial mast cells (MC) can be immunolabelled with antihuman CXCR3 antibody (Ab) (clone 49801). We have investigated whether cutaneous and mucosal MC are stained with anti-CXCR3 Ab in paraffin-embedded sections. Immunohistochemical staining and immunofluorescence double staining assays were performed with anti-CXCR3, anti-tryptase, and anti-chymase Ab using normal skin, psoriatic skin lesions, and normal colon. When compared with tryptase and chymase staining, 100% of the cutaneous and 98% of the mucosal MC were positive for CXCR3. Anti-CXCR3 staining is a useful marker for human cutaneous and mucosal MC in paraffin-embedded sections.

Key words: colon, CXCR3, immunohistochemistry, mast cell, skin.

INTRODUCTION

Mast cells (MC) are distributed ubiquitously throughout the body and are involved in the pathogenesis of inflammatory and neoplastic disorders. Immunohistological studies have shown that the number of MC are increased in the lesional tissue of asthma, atopic dermatitis, allergic rhinitis and neurofibromatosis.[1–5] MC derive from bone marrow and migrate in the peripheral tissues. Their large cytoplasm contains abundant metachromatic granules as revealed by toluidine blue staining. These cytoplasmic granules store various mediators along with proteinases such as tryptase and chymase that are released by various external stimuli such as allergens via bridging of immunoglobulin (Ig)E–IgE-Fc receptor complexes. MC are classified into two subpopulations, MC_{TC} (tryptase and chymase positive) and MC_T (tryptase positive). The MC_{TC} population predominates in the connective tissue in the skin, heart and respiratory submucosa. In contrast, 7–67% of total MC are MC_T in the intestinal and colic mucosa.[6,7] Anti-tryptase and anti-chymase immunostaining are now widely used for the detection of MC in paraffin-embedded sections after appropriate antigen retrieval procedures.

In addition to IgE-Fc receptors, MC express various chemokine receptors, which may participate in inflammatory responses. CCR3 is expressed on human-tissue-derived MC, especially on MC_{TC}, and is attracted by its ligand, eotaxin.[8] The expressions of CXCR1, CXCR2, CXCR4 and CCR5 on cord-blood-derived MC have also been reported.[9–11] In synovial tissue from patients with rheumatoid arthritis, gene expression patterns of CXCR1, CXCR2 and CXCR3 mRNAs are upregulated.[12] In addition, the CXCR3 antibody (Ab) (clone 49801) immunostains the synovial MC.[12] In this study, we tested both cutaneous and mucosal MC for their immunoreactivity with the anti-CXCR3 Ab.

MATERIALS AND METHODS

Reagents

Monoclonal anti-human CXCR3 Ab (clone 49801) and fluorescein isothiocyanate (FITC)-conjugated

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CXCR3 Ab (clone 49801) were purchased from R&D Systems (Minneapolis, MN, USA). Monoclonal mast cell tryptase Ab (clone AA1) was obtained from DAKO (Glostrup, Denmark), and monoclonal mast cell chymase Ab (clone CC1) was purchased from NeoMarkers (Fremont, CA, USA).

Immunohistochemical staining

Tissue samples consisted of normal skin ($n = 4$), normal colon tissues ($n = 4$) and lesional psoriasis skin ($n = 4$). All samples were fixed with 10% buffered formalin. The archival paraffin-embedded tissue blocks were cut into 4 μm-thick sections and mounted on silanated slides. The sections were deparaffinized, rehydrated in ethanol, and heated in 1 mmol ethylene diamine tetra acetate (EDTA) buffer (Sigma, Saint Louis, MO, USA) in an autoclave for antigen retrieval. The sections were washed in phosphate buffered saline (PBS; pH 7.6), incubated with 2% bovine serum albumin (BSA; Sigma) in PBS to block nonspecific antibody binding, and then incubated with primary Ab after dilution to an appropriate concentration. After washing in PBS, the sections were incubated with alkaline phosphatase-conjugated antimouse immunoglobulin (Nichirei, Tokyo, Japan). The reaction cascades were visualized with a New Fuchsin Substrate Kit (Nichirei). CXCR3 staining was also performed using frozen normal skin. For immunofluorescence double staining, sections were prepared, heated and incubated in BSA in the same way as for immunohistochemical staining. After incubation of sections with tryptase or chymase primary Ab, they were washed thoroughly in PBS, then incubated with phycoerythrin-conjugated affinity purified goat antimouse IgG (Rockland, Gilbertsville, PA, USA). The double labeling was performed with FITC-conjugated monoclonal CXCR3 Ab (clone 49801). Negative control stainings were performed with isotype matched control primary Ab. The total numbers of immunoreactive cells were obtained by counting and adding positives in six representative high-power fields (HPF).

Statistical analysis

Statistical differences were analyzed by a Mann–Whitney *U*-test. Data analyses were performed using a statistical software package.

RESULTS

Human cutaneous and intestinal MC are immunoreactive to anti-CXCR3 Ab

In normal human skin, the CXCR3 positive cells were readily observed in perivascular areas of upper dermis and around the eccrine glands (Fig. 1A,B). In the colon sections, the $CXCR3^+$ cells were clearly identified in the mucosal interstitial tissue (Fig. 1C). The $CXCR3^+$ cells were similarly detected in inflammatory skin conditions like psoriasis (data not shown). The $CXCR3^+$ cells were also immunoreactive to tryptase and chymase in serial sections (Fig. 2). Samples stained with isotype-matched control antibody revealed no positive signals (data not shown). MC were immunoreactive to anti-CXCR3 Ab in normal frozen skin (data not shown).

In order to further confirm that the $CXCR3^+$ cells were MC, double immunofluorescence studies were performed with anti-tryptase/CXCR3 or anti-chymase/CXCR3 Ab. Double immunofluorescence staining of CXCR3/tryptase or CXCR3/chymase in the normal skin showed that all the $tryptase^+$ cells and $chymase^+$ cells were double-positive for CXCR3 (Fig. 3 and Table 1). The majority of the $tryptase^+$ or $chymase^+$ cells expressed CXCR3; however, 2.3 ± 0.8% (mean ± SD) of the $tryptase^+$ cells and 3.0 ± 3.3% of the $chymase^+$ cells were negative for CXCR3. Of the $CXCR3^+$ cells in the normal skin, 2–3% of cells were $tryptase^-$ or $chymase^-$ cells. In the colon, the percentage of $CXCR3^+/tryptase^-$ cells increased to 3.8 ± 2.6%. The $chymase^+$ cells represented 60.4 ± 12.1% of $CXCR3^+$ cells in the colon.

$CXCR3^+$ MC in the psoriatic skin lesions

Staining of psoriatic skin with CXCR3 resulted in the clearly positive staining similar to that observed in the normal skin. The number of $CXCR3^+$ MC (105.5 ± 54.2/6 high-power field [HPF]) was increased in the psoriatic skin compared to that in the normal skin (70.3 ± 35.1/6 HPF), but not to statistically significantly different levels. In the double immunofluorescence staining for CXCR3/tryptase or CXCR3/chymase, a small percentage of the $tryptase^+$ cells (1.5 ± 1.7%) or the $chymase^+$ cells (1.9 ± 2.6%) were negative for CXCR3 in the psoriatic lesional skin.

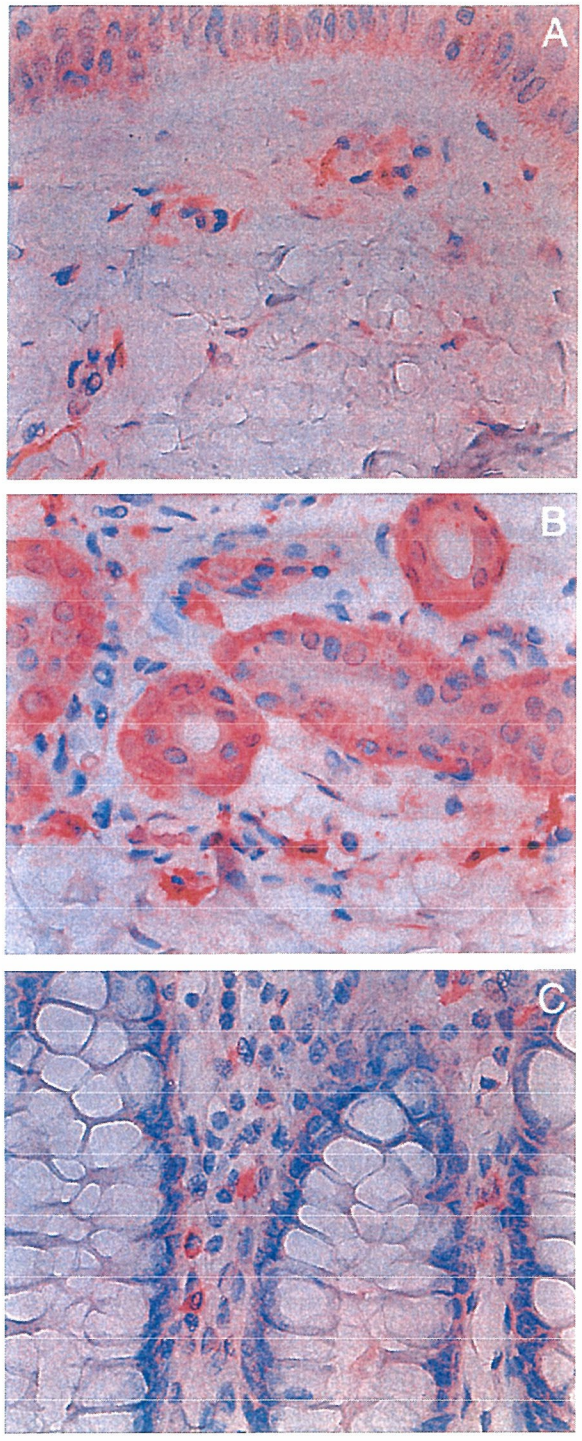

Figure 1. Immunohistochemical studies of normal skin and colon. CXCR3 staining of formalin-fixed paraffin-embedded tissue sections of (A,B) normal skin and (C) mucosa of the colon (original magnification ×400). Positive cells were seen in the perivascular area of the (A) upper dermis and (B) around the eccrine glands. In the colon, positive cells were seen in (C) mucosa.

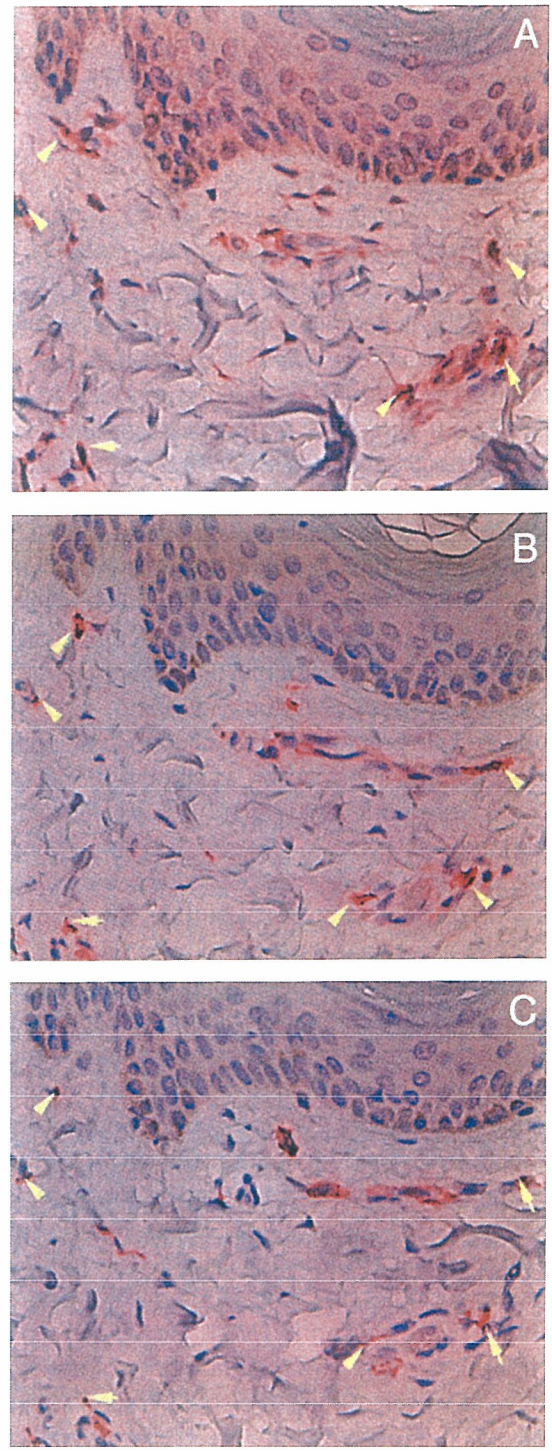

Figure 2. Immunohistochemical staining of sequential sections of normal skin. Sequential 4-μm sections were stained with (A) CXCR3, (B) tryptase, and (C) chymase monoclonal antibodies. The majority of CXCR3 positive cells were tryptase and chymase positive (arrowheads; original magnification ×400).

Table 1. Ratio of CXCR3$^+$, tryptase$^+$ or chymase$^+$ cells in the normal skin and colon detected by immunofluorescence double staining

	CXCR3/tryptase Tryptase$^+$ cells		CXCR3/chymase Chymase$^+$ cells	
	CXCR3$^+$	CXCR3$^-$	CXCR3$^+$	CXCR3$^-$
Normal skin (n = 4)	100%*	0%*	100%*	0%*
Mucosa of colon (n = 4)	97.7 ± 0.8%*	2.3 ± 0.8%*	97.0 ± 3.3%*	3.0 ± 3.3%*
	CXCR3$^+$ cells		CXCR3$^+$ cells	
	Tryptase$^+$	Tryptase$^-$	Chymase$^+$	Chymase$^-$
Normal skin (n = 4)	98.0 ± 2.7%	2.0 ± 2.7%	97.9 ± 1.6%*	2.2 ± 1.6%*
Mucosa of colon (n = 4)	96.2 ± 2.6%	3.8 ± 2.6%	60.4 ± 12.1%*	39.6 ± 12.1%*

Mean ± SD, *P < 0.05.

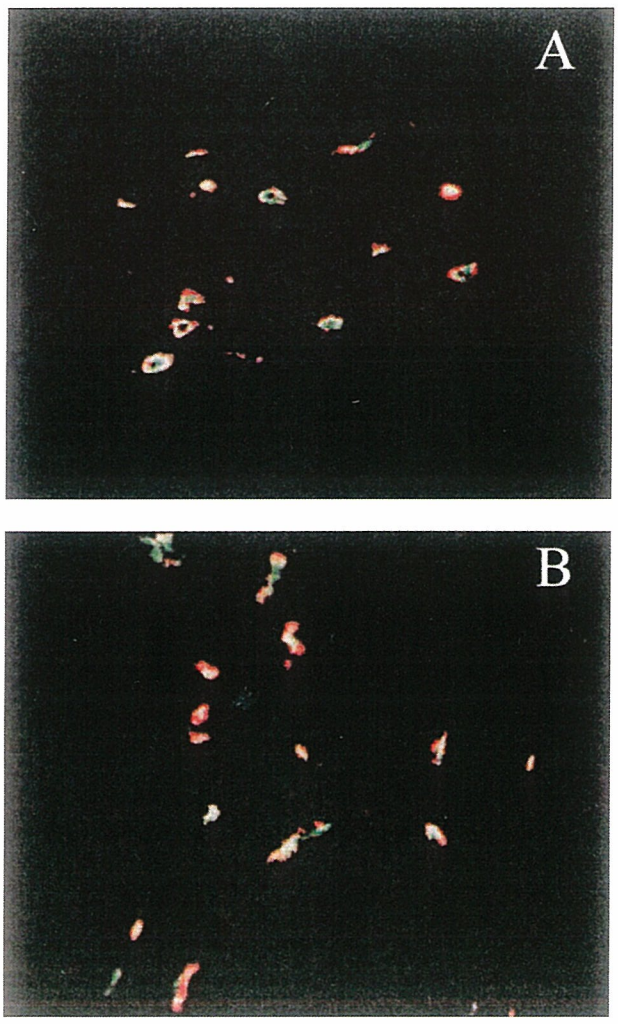

Figure 3. Immunofluorescence double staining. (A) CXCR3 (green)/tryptase (red) and (B) CXCR3 (green)/chymase (red) double staining in normal skin. The majority of the positive cells were doubly positive (yellow) as a result of staining with both sets of monoclonal antibodies (original magnification ×400).

DISCUSSION

It is well documented that MC play an important role in T-helper (Th)1- and Th2-prone disorders.[4,13–19] A recent report demonstrating the positive immunoreaction of CXCR3 Ab (clone 49801) to synovial MC[12] encouraged us to examine whether the cutaneous and intestinal MC can be detected by this method. In the present study, the anti-CXCR3 immunostaining clearly detected both the cutaneous and intestinal MC in paraffin-embedded sections. However, it should be noted that the CXCR3$^+$ cells included all the tryptase$^+$ or chymase$^+$ cells in the normal skin, whereas a very minor population of tryptase$^+$ or chymase$^+$ cells in the normal colon lack the CXCR3 immunoreactivity. Interestingly, these intestinal types of MC (CXCR3$^-$/tryptase$^+$ or CXCR3$^-$/chymase$^+$ MC) were also detected in the inflammatory skin condition of psoriasis. The CXCR3$^+$ MC were also detected in psoriasis skin lesions. The number of MC have been reported to be increased in psoriasis in some reports,[20,21] and a similar trend was also observed in the present study.

Chemokines and chemokine receptors are thought to be important for the migration of the inflammatory cells to the sites where they exert their distinct functions.[22] The human MC are known to express the chemokine receptors CCR3, CXCR1, CXCR2, CXCR4 and CCR5,[7–10] and CCR3 is closely related to Th2 reactions. Because the interaction of CXCR3 and its ligands (Mig, IP-10 and iTAC) induces the recruitment of Th1 cells, the functional properties of CXCR3 expressed on MC are also of interest. In preliminary experiments, we have examined the expression of

CXCR3 in cultured MC derived from human cord blood cells that had been incubated with stem cell factor and IL-6 for 4 weeks.[23] We were readily able to observe the tryptase$^+$ MC; however, the CXCR3 expression was negative (unpublished data). In addition, the murine MC line, MCP5 (kindly provided from Dr. H. Kawakami, La Jolla Institute for Allergy and Immunology), did not express CXCR3 even though they were activated with phorbol ester and ionomycin. Moreover, the MCP5 cells failed to migrate in response to the CXCR3 ligand chemokines (unpublished data). Further studies are necessary to reveal the importance of CXCR3 expression in MC. The anti-CXCR3 Ab (clone 49801) immunostaining is a useful tool for visualizing human MC in paraffin-embedded sections.

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Oral antihistamine therapy influences plasma tryptase levels in adult atopic dermatitis

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KEYWORDS
Antihistamines;
Atopic dermatitis;
Tryptase;
Mast cell;
Fexofenadine hydrochloride

Summary

Background: Atopic dermatitis (AD) is an allergic skin disease that follows a clinical course of 'flare-up' and remission. Histamine and tryptase are inducers of pruritus and non-sedating second-generation antihistamines, including fexofenadine, are widely used for treatment of allergic skin disorders.
Objective: We assessed the efficacy of a second-generation antihistamine in AD patients and examined its pharmacological effects on chemical mediators.
Methods: The scoring atopic dermatitis (SCORAD) instrument and visual analogue scale (VAS) for pruritus were used to assess disease severity in 349 AD patients. Twenty patients with moderate AD symptoms, who had not received any treatment for 2 weeks, were randomly assigned into two groups. Ten patients underwent fexofenadine and emollient treatment (Group 1) and 10 received fexofenadine and steroid treatment (Group 2) for 1 week. SCORAD and VAS for pruritus, and blood histamine and tryptase levels were evaluated before and after treatment.
Results: SCORAD and VAS improved in both Group 1 ($p = 0.01$ and $p = 0.006$, respectively) and Group 2 ($p < 0.001$ and $p = 0.001$, respectively). The improvement in Group 1 showed a significant correlation with the diminution rate of blood tryptase levels (SCORAD: $r = 0.83$ and $p = 0.013$, respectively; VAS: $r = 0.81$, $p = 0.015$, respectively). End-point plasma tryptase levels were significantly lower than baseline levels in Group 2 ($p = 0.046$). Histamine levels did not show any significant changes in either group.
Conclusion: These results suggest that second-generation antihistamine therapy reduces AD pruritus, resulting in the effective clinical treatment for AD. In addition,

Abbreviations: AD, atopic dermatitis; IgE, immunoglobulin E; SCORAD, scoring atopic dermatitis; VAS, visual analogue scale

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monitoring tryptase levels during antihistamine therapy in moderate AD treatment may prove to be useful in establishing treatment effects.

1. Introduction

Although topical corticosteroid treatment has been a mainstay of atopic dermatitis (AD) therapy and has generally been well tolerated, the therapeutic response of pruritus to topical corticosteroids is not always satisfactory [1,2]. Antihistamines (H_1-receptor antagonists) reduce the pruritic symptoms of AD by acting as inverse agonists of histamine at the H_1-receptors. Newer, second-generation H_1-antihistamines are a major therapeutic advancement in the treatment of allergic disorders such as urticaria and allergic rhinitis. The efficacy of the second-generation agents, combined with greatly reduced sedative and anticholinergic effects compared with the first-generation antihistamines, make this new class of H_1 antihistamines the first-line treatment in the management of urticaria and mild angioedema [3]. The pathogenesis of itch in AD, however, is different from that in urticaria. In a recent report, electrical, mechanical, chemical and heat stimuli, which induced painful sensations in normal skin, evoked itch in the eczematous skin of patients with AD [4,5]. Fexofenadine, a second-generation H_1 antihistamine, has consistently exhibited good efficacy and safety in the treatment of seasonal allergic rhinitis [6] and urticaria [7]. A recent Grade A study (large, randomized, double-blind, and placebo-controlled) further showed that fexofenadine was effective in relieving pruritus in patients with AD [8]. We have also previously reported that administration of other second-generation antihistamines appears to provide effective clinical treatment for AD, with a notable improvement in pruritus [9].

Pruritus is a serious problem for patients with AD, but the exact mediator, or mediators, inducing this symptom remain uncertain and there is little information about the pattern of mediator release that causes the dramatic signs and symptoms of AD. Mast cells, which play a key role in allergic reactions, are often found in increased numbers in patients suffering from inflammatory conditions such as AD. In the course of an allergic reaction, mast cells release a variety of mediators, including histamine and tryptase [10,11]. Unlike histamine, the measurement of serum tryptase levels selectively indicates the extent of mast-cell activation, because negligible amounts of this enzyme are present in other cell types, such as basophils [12]. Since mast cell numbers, especially tryptase-containing mast cells, are increased in AD skin lesions [13], higher tryptase concentrations could be attributed simply to the higher number of mast cells found in AD patients. Interestingly, in previous investigations, only codeine-induced tryptase release, but not histamine release, has been found to be increased in AD [14,15]. The following study was designed to assess the efficacy of a second-generation antihistamine administration in AD patients and to simultaneously investigate its pharmacological effects on chemical mediators, such as tryptase and histamine.

2. Methods

2.1. Participants

Three hundred and forty-nine patients underwent an initial examination at the St. Marianna University School of Medicine, Hospital Dermatology Department between April 2003 and June 2004. A diagnosis of AD was made in each patient by the consensus of two dermatologists according to the clinical and morphological criteria defined by Hanifin and Rajka [16]. These AD patients were then divided into three groups according to objective scoring atopic dermatitis (SCORAD) score. This index (range 0—83) is based on the sum of six individual aspects (erythema, edema or papulation, oozing or crust, excoriation, lichenification, and dryness) according to a 4-point scale: 1 = absence; 2 = mild; 3 = moderate; 4 = severe. Investigation of the extent of the disease was carried out within the six defined areas, as described previously [17]. The global baseline dermatological assessment was graded as mild, moderate and severe AD. Patients with an objective SCORAD score between 15 and 40 were defined as having moderate AD and were enrolled into the study.

Patients who had been treated with topical or systemic corticosteroids, anti-allergic agents, antihistamines, non-steroidal anti-inflammatory agents, anticholinergics, tranquilizers, hypnotics, antipsychotic drugs, cold remedies containing antihistamine agents, or any other anti-allergic or antipruritic drugs within the 14 days prior to the day of enrolment were excluded from the study. Patients were prohibited from using any experimental treatments, tranquilizers, sleeping pills and antimicrobial

drugs during the course of the study. The use of UV treatment, photochemotherapy, or cyclosporin immunosuppressive therapy was not allowed before the subject selection period. The local ethics committee approved the study protocol and all patients gave written informed consent before treatment allocation.

2.2. Study protocol

All AD patients enrolled in the study were treated orally with a non-sedating H_1 antihistamine, fexofenadine hydrochloride 60 mg, twice daily (morning, evening) for 1 week. In addition to antihistamine therapy, patients were randomly allocated to two groups: Group 1 underwent emollient (petrolatum) treatment once or twice daily for 1 week; Group 2 received topical corticosteroid (0.12% betamethasone valerate) treatment for 1 week. All interventions were administered at home. The treatment designation was randomized by assigning each patient a sealed envelope at the time of enrolment containing the details of the treatment group, with the allocation and sequence of the envelopes being completed by dermatologists not involved in the study. The intensity of AD skin lesions and pruritus were assessed at baseline and at 1 week using SCORAD and visual analogue scale (VAS) for pruritus measures [17]. Patients graded diurnal and nocturnal pruritus, as a result of eczema, on a 10 cm VAS, with 'none' on the left side of the scale and 'severe' on the right. VAS for pruritus was defined as the sum of the diurnal and nocturnal score. The SCORAD score (range 0—103) was calculated by adding the objective SCORAD score (range 0—83) to the VAS for pruritus score (range 0—20). SCORAD and VAS scores for pruritus were assessed by the same dermatologist at 1 week and compared with baseline data.

Previous studies investigating the effect of fexofenadine on pruritus associated with chronic idiopathic urticaria demonstrated a rapid effect within the first week of treatment [18,19]. Furthermore, Kawashima et al. [8] also showed a decrease in pruritus scores associated with AD that reached a plateau in the first week of treatment. Therefore, it was concluded that 1 week was sufficient time during which to estimate the effect of an antihistamine on AD.

The study protocol was based on the ethical principles of Good Clinical Practice and was approved by the St. Marianna University School of Medicine Institutional Review Board for Human Subjects Research.

2.3. Assays

Peripheral blood was drawn from patients before and after treatment. Samples were collected in plastic tubes with or without ethylenediaminetetraacetic acid, and immediately placed on ice before centrifugation at 1500 rpm for 10 min at 4 °C. Supernatants were pipetted into polypropylene tubes and the plasma and sera were stored at −20 °C until the samples were assayed. Total serum tryptase concentration was measured by an enzyme-linked immunosorbent assay (ELISA) with Anti-Tryptase immnoCAP® (UniCAP-Tryptase, Pharmacia, Sweden). The lower limit of sensitivity for the assay was 1 μg/L. Plasma histamine levels were measured by competitive radioimmunoassay after acylation of histamine (histamine radioimmunoassay kit, Immunotech, Luminy, France). The lower limit of detection of the assay was 0.2 nM (1 nM = 0.11 ng/mL). SCORAD and VAS improvement rate and tryptase diminution rate were calculated as follows:

$$\text{SCORAD (VAS) improvement rate (\%)} = \frac{\text{SCORAD (VAS) at baseline} - \text{SCORAD (VAS) at end-point}}{\text{SCORAD (VAS) at baseline}} \times 100$$

$$\text{Tryptase diminution rate (\%)} = \frac{\text{tryptase level at baseline} - \text{tryptase level at end-point}}{\text{tryptase level at baseline}} \times 100$$

2.4. Safety analyses

Any adverse events occurring throughout the study were reported. Laboratory tests, such as haematology and urinalysis, and physical examinations were performed at the first and final visits.

2.5. Statistical analysis

The statistics were analyzed by Mann—Whitney test to compare clinical scores before and after therapy; the level of significance was set at $p < 0.05$ in all cases. Correlation between SCORAD or VAS improvement rate and variation of histamine or tryptase levels in the blood was assessed by Spearman's rank correlation test. All data are expressed as mean ± S.D.

3. Results

3.1. Demographics and patient characteristics

Fig. 1 shows the trial profile. Patients were enrolled depending on accurate clinical assessment by two investigators at baseline. Therefore, it was

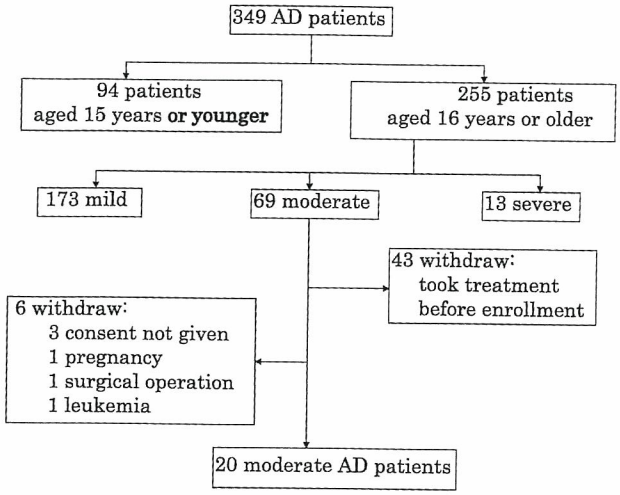
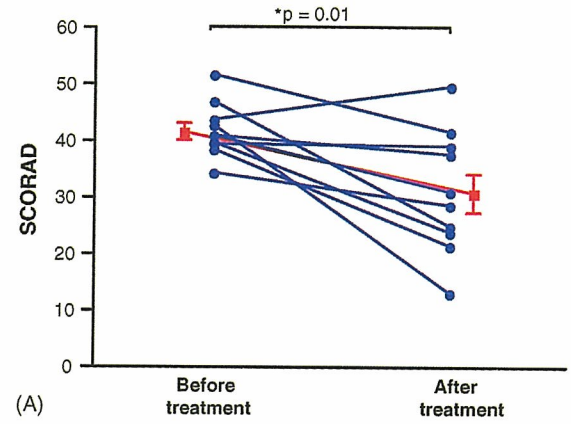


Fig. 1 Trial profile.

expected that there would be a large number of ineligible subjects. Ninety-four patients aged ≤15 years were excluded as the study protocol required adult patients. Of the 255 adult patients with AD (124 male, 131 female), 173 patients had mild symptoms, 69 had moderate symptoms and 13 had severe symptoms. Forty-nine withdrawals occurred for various reasons in the moderate group (Fig. 1), leaving a total of 20 patients with moderate AD (12 men and 8 women; aged 18–47 years) enrolled in the study. There was no statistically significant difference between the two treatment groups in terms of baseline characteristics or symptom assessments using a Mann–Whitney test (Table 1). All patients except one had a >7-year duration of AD and showed moderate or severe lichenification scores.



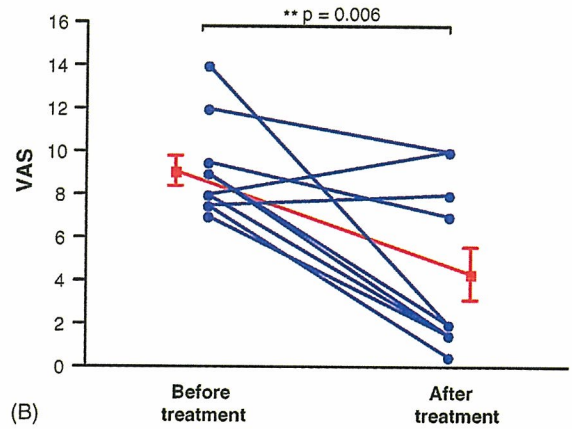


Fig. 2 Changes in SCORAD and VAS for pruritus in Group 1 patients. Ten patients were treated with antihistamines and petrolatum. After therapy, the scores of these two indices decreased significantly. (A) SCORAD, $^{*}p = 0.01$; (B) VAS, $^{**}p = 0.006$.

3.2. Efficacy assessments

SCORAD and VAS scores for pruritus improved significantly after 1 week (SCORAD, $p = 0.01$; VAS, $p = 0.006$) among Group 1 patients (Fig. 2). A similar improvement was seen in Group 2 patients (SCORAD, $p = 0.0002$; VAS, $p = 0.001$) (Fig. 3). Compared with time of enrolment, Group 1 or 2 therapy was also found to improve significantly both diurnal and

Table 1 Patient characteristics at baseline

Characteristic	Group 1	Group 2
Age (year)	27.5 ± 8.6	30.3 ± 8.3
Sex		
Male	5	7
Female	5	3
Duration of AD (year)	20.9 ± 5.9	17.7 ± 9.8
Peripheral eosinophil count	575.6 ± 106.5	582.0 ± 228.1
Serum IgE levels (IU/mL)	2521.6 ± 895.9	4055.8 ± 1416.5
Other allergies		
Asthma	4 (40%)	3 (30%)
Rhinitis	4 (30%)	4 (40%)
Conjunctivitis	4 (40%)	2 (20%)

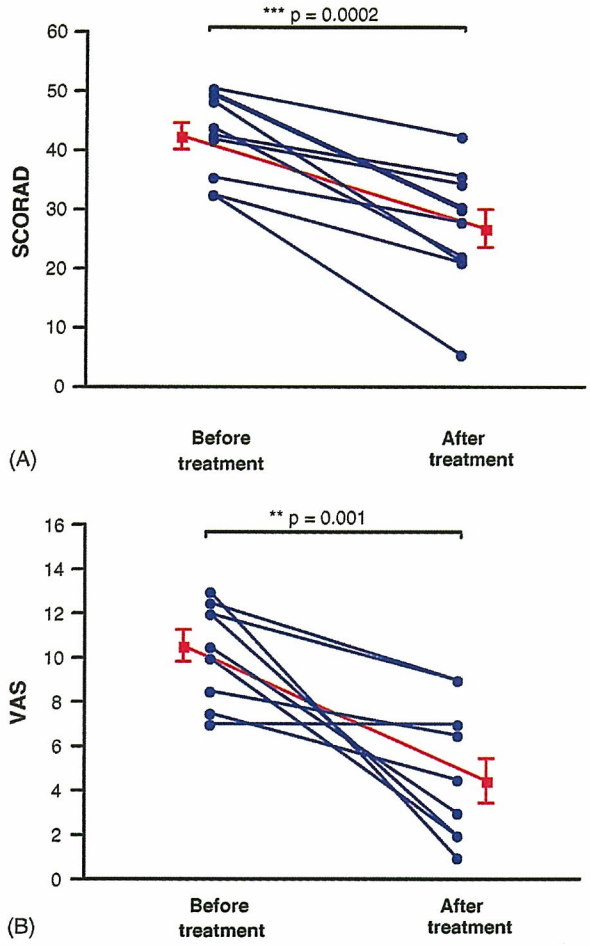


Fig. 3 Changes in SCORAD and VAS for pruritus in Group 2 patients. Ten patients were treated with antihistamines and topical corticosteroids. After the combination treatment, the scores of these two indices decreased significantly. (A) SCORAD, $^{***}p = 0.0002$; (B) VAS, $^{**}p = 0.001$.

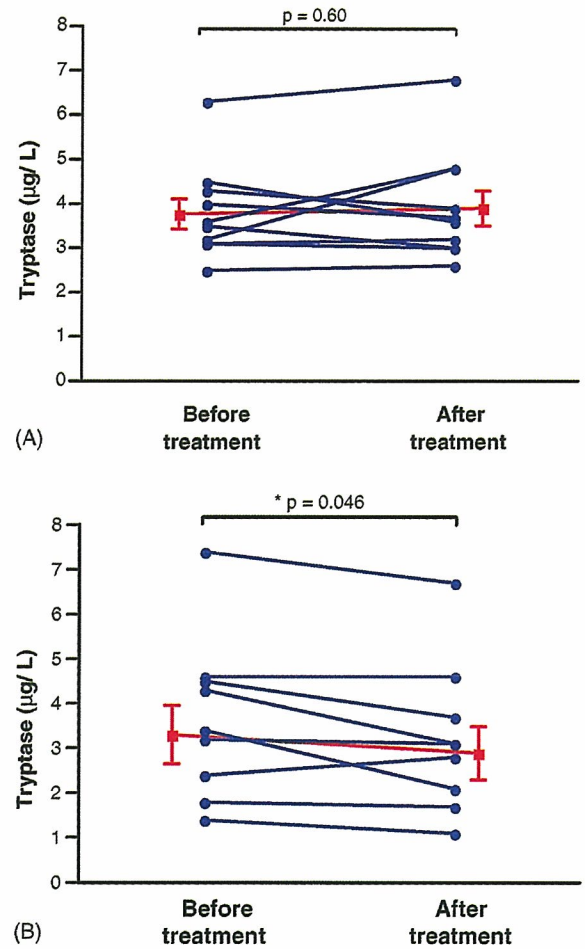


Fig. 4 Changes in plasma tryptase levels (μg/L) in Group 1 patients (A) and Group 2 patients (B). Tryptase levels were measured before and after antihistamine therapy as described in Section 2. The levels decreased significantly after 1 week compared with baseline in Group 2 ($^{*}p = 0.046$) but not Group 1.

nocturnal scores when assessed separately. In addition, a statistically significant improvement in the lichenification aspect of SCORAD was seen from baseline values in both groups (Group 1, $p = 0.026$; Group 2, $p = 0.018$). SCORAD improvement rates in Group 2 (mean: 18.4 ± 5.7%) were higher than those in Group 1 (mean: 10.8 ± 3.4%). A similar trend was seen among VAS improvement rates (Group 2 mean 6.1 ± 1.4% versus Group 1 mean 4.8 ± 1.3%). However, these changes were not significant in either group.

3.3. Mediator levels in atopic dermatitis

In Group 1, there was no overall significant difference in serum tryptase levels at study end (mean: 3.9 ± 1.2 μg/L) compared with baseline levels (mean: 3.8 ± 1.1 μg/L) (Fig. 4A). In contrast, tryptase levels decreased significantly in Group 2 patients (mean: 3.3 ± 2.1 at baseline versus 2.9 ± 1.9 μg/L at study end; $p = 0.046$, Fig. 4B). All Group 2 patients except one had a decrease in tryptase levels. Baseline plasma histamine levels in Group 1 (mean: 6.4 ± 2.2 nM) were higher than those at end-point (mean: 5.4 ± 0.1 nM). A similar trend was seen among patients in Group 2 (baseline mean 5.6 ± 2.2 nM versus end-point mean 4.4 ± 3.0 nM). However, these changes were not significant in either group. Furthermore, there was no significant correlation between additional immediate-type allergies (asthma, rhinitis and conjunctivitis) and typtase or histamine levels in either group.

3.4. Correlation of efficacy assessments and mediator levels

In Group 1, there was a significant positive correlation between the SCORAD improvement rate and the

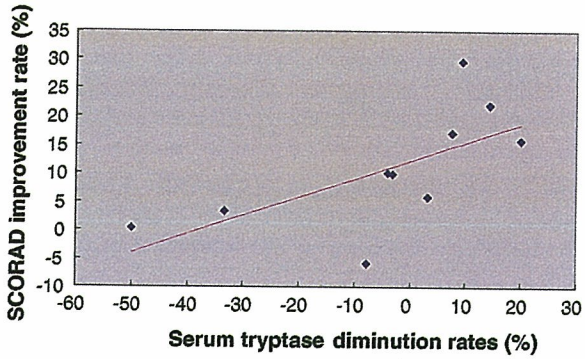


Fig. 5 Positive correlation between SCORAD improvement rate (%) and serum tryptase diminution rate (%) in Group 1. SCORAD improvement rates are shown on the ordinate, and serum tryptase diminution rates are shown on the abscissas (Spearman $r = 0.83$, $p = 0.013$).

tryptase diminution rate (Spearman $r = 0.83$, $p = 0.013$; Fig. 5). A similar correlation was also seen between the degree of diminution rate in tryptase levels and VAS improvement rate in Group 1 (Spearman $r = 0.81$, $p = 0.015$; Fig. 6). In Group 1, tryptase levels increased in five patients and decreased in five patients. Patients with decreased tryptase levels showed significantly higher SCORAD improvement rates than those with increased tryptase levels (SCORAD: 18.0 ± 8.8% versus 3.5 ± 6.7%; $p = 0.019$). Similarly, patients with decreased tryptase levels showed significantly higher VAS improvement rates than those with increased tryptase levels (VAS: 7.7 ± 2.5% versus 1.8 ± 3.4%; $p = 0.015$). The significant positive correlation between SCORAD and diminution rate in tryptase levels seen in Group 1 overall was due to the five patients who had decreased tryptase levels. Baseline peripheral eosinophil counts in the five patients with decreased tryptase levels (mean: 447.5 ± 69.8) were lower than those of patients with increased tryptase levels

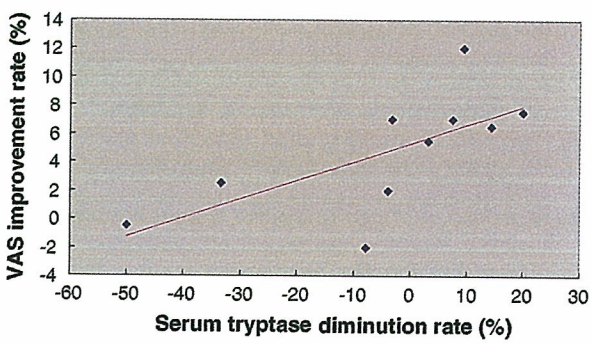


Fig. 6 Positive correlation between VAS improvement rate (%) and serum tryptase diminution rate (%) in Group 1. VAS improvement rates are shown on the ordinate, and serum tryptase diminution rates are shown on the abscissas (Spearman $r = 0.81$, $p = 0.015$).

(mean: 678.0 ± 178.9). Similar trends were seen with respect to serum IgE levels. The five patients with decreased tryptase levels had lower serum IgE levels at baseline (mean: 1633.2 ± 617.1 IU/mL) than those with increased tryptase levels (mean: 3122.0 ± 1767.9 IU/mL). However, there was no significant difference in peripheral eosinophil counts or serum IgE levels. The serum tryptase diminution rate in Group 2 did not show a significant correlation with either SCORAD or VAS improvement rate. There was a trend towards a lower tryptase level following topical corticosteroid therapy in Group 2. Furthermore, there was no significant correlation between histamine diminution rate and improvement in SCORAD or VAS for pruritus in either group.

3.5. Safety assessments

No serious systemic adverse events were noted during the course of the study. Laboratory test and physical examination results in all 20 enrolled patients were unchanged at the end of the study.

4. Discussion

Using SCORAD and VAS assessments for pruritus as indicators of AD in this study, the efficacy and mechanism of action of fexofenadine in patients with moderate AD was investigated, after a sufficient washout phase from previous therapies. A study of this nature requires careful selection of representative AD subjects. We enrolled 20 moderate AD patients who had undergone precise clinical assessment and with a definitive washout phase. Statistically significant improvements in SCORAD and VAS scores were observed for pruritus within 1 week in both Group 1 and 2 patients. Based on these findings, we believe that AD clinical improvement could be due to the effects of the antihistamine alone, irrespective of the addition of emollients or topical corticosteroids. In addition, tryptase levels, unlike histamine levels, decreased significantly in AD patients after treatment with an antihistamine and a topical corticosteroid. Some reports have suggested that mast cell mediators other than histamine could act as important itch mediators in AD [20]. Higher tryptase concentrations could be attributed simply to the higher number of mast cells found in AD patients. These results suggest that serum tryptase levels reflect the disease activity of AD better than plasma histamine levels, most likely because tryptase is more specific to mast cells than histamine, and tryptase blood levels directly reflect the degree of mast cell degranulation. Tryptase appears to play an important role

as an itch mediator in human skin, very likely by activating protease activated receptor-2 (PAR-2) [21]. PAR-2 is a sensory nerve receptor that has been implicated in the induction of pruritus during AD [22]. The existence of a histamine-independent, tryptase-dependent, and PAR-2-mediated itch pathway provides a new link that may lead to beneficial therapies for pruritus and cutaneous inflammation. In particular, serum tryptase measurement may be a useful tool in assessing the symptomology of AD.

Overall, Group 1 patients did not exhibit significant diminution of tryptase following antihistamine and petrolatum therapy since tryptase levels increased in half the patients and decreased in the other half. Among those who showed a decrease in tryptase, there was a positive correlation between tryptase diminution rate and the degree of clinical improvement. Further, the patients with decreased tryptase levels exhibited significantly higher clinical improvement rates than those with increased tryptase levels, suggesting some heterogeneity in the population. Although tryptase levels decreased significantly in patients receiving fexofenadine and a topical corticosteroid (Group 2), there was no significant correlation between the tryptase diminution rate and clinical improvement rates in this group. Mean clinical improvement rates in Group 2 were higher than those in Group 1. This may be because the rapid anti-inflammatory effect of the corticosteroid masked the onset of fexofenadine action.

The positive correlation between the tryptase diminution rate and clinical improvement rates observed in Group 1 would appear to indicate that fexofenadine induces a response in AD patients through a tryptase-dependent mechanism. Therefore, the evidence presented here suggests that tryptase levels be monitored to appropriately adjust the antihistamine therapy in the treatment of moderate AD. The regulation of tryptase activation may become a critical and promising clinical therapeutic approach. However, it is important to note that the severity of the symptoms in AD may reflect numerous pathogenic factors, including dry skin and mental state, which may vary considerably among patients.

In chronic lichenified lesions associated with AD, histological findings show an increase in the number of mast cells [23]. The release of tryptase from activated mast cells may stimulate secretion from neighbouring mast cells, thus providing an amplification signal [24]. In the present study, almost all patients demonstrated moderate to severe lichenification and showed significant improvement as a result of fexofenadine therapy. Simons et al. [25] reported that fexofenadine penetrates the skin to a significantly greater extent than the sedating H_1-receptor antagonist diphenhydramine. The authors suggested that the rapid onset and prolonged duration of action of fexofenadine could be due to its substantial distribution into the skin. These results suggest that fexofenadine blocks mast cell tryptase in the lesion by influencing the activity of mast cells, which, due to their spatial arrangement and predominance in the skin, are thought to play a pivotal role in AD. The cutaneous manifestations of AD may therefore be important in the assessment of antihistamine therapy efficacy. Further investigation of the links between antihistamine therapy, plasma tryptase levels and clinical outcomes will help provide a better treatment and assessment regimen for AD.

Acknowledgements

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CCL17 transgenic mice show an enhanced Th2-type response to both allergic and non-allergic stimuli

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CC chemokine ligand (CCL)17 is implicated in the pathogenesis of atopic dermatitis (AD). To study the effect of CCL17 produced by keratinocytes (KC) during inflammation, we created transgenic (Tg) mice in which CCL17 is overexpressed in KC. Th2-type contact hypersensitivity (CHS) was enhanced and Th1-type CHS was suppressed in these mice. Increased numbers of CC chemokine receptor (CCR)4^+ cells and mast cells infiltrated in Tg mice. Levels of IL-4 mRNA were higher and those of IFN-γ mRNA were lower in both acute and chronic CHS. Higher levels of serum IgE were observed after CHS. Numbers of $CCR4^+$ cells among PBMC were increased in Tg mice challenged acutely on the trunk. Chronic irritation with croton oil induced dermatitis and an elevation of serum IgE levels. Tg mice showed enhanced ear swelling after tape stripping. CCL17 was thought to modify the inflammation caused by sensitizing reagents as well as irritant reagents by attracting $CCR4^+$ cells into the lesional skin and creating a Th2-dominant condition. AD-like conditions such as increased number of mast cells and elevated levels of serum IgE were observed. Thus, CCL17 may participate in the pathogenesis of skin diseases such as AD by regulating both allergic and irritant inflammation.

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Introduction

Chemokines are a family of polypeptides that govern the chemotaxis and activation of different subsets of leukocytes during immune and inflammatory responses [1–3]. Recent studies have revealed that Th1 and Th2 cells differ in the chemokine receptors that they express [1–3].

Thymus and activation-regulated chemokine (TARC)/CC chemokine ligand (CCL)17 is a ligand of CC chemokine receptor (CCR) 4 [1–3], which is predominantly expressed on Th2 lymphocytes, basophils, and natural killer cells [1–4]. CCR4 is also a receptor for macrophage-derived chemokine (MDC)/

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Abbreviations: AD: atopic dermatitis · **CCL:** CC chemokine ligand · **CCR:** CC chemokine receptor · **CHS:** contact hypersensitivity · **CLA:** cutaneous lymphocyte-associated antigen · **CO:** croton oil · **HE:** hematoxylin and eosin · **hGH:** human growth hormone · **hK14:** human keratin 14 · **KC:** keratinocyte · **LC:** Langerhans cell · **OX:** oxazolone · **TARC:** thymus and activation-regulated chemokine · **UTR:** untranslated region

CCL22 [1–3]. Thus, CCL17 and CCL22 are likely to play important roles in Th2-type immune responses by selectively recruiting $CCR4^+$ Th2-polarized memory/effector T cells into inflamed tissues. Cutaneous lymphocyte-associated antigen (CLA) is expressed by the vast majority of skin- infiltrating T cells and plays an important role in leading the skin-associated T cells to inflammatory sites by interacting with the endothelial cell ligand E-selectin, the expression of which is pronounced in inflamed skin [5]. Essentially all CLA^+ skin-seeking memory effector T cells were found to express CCR4 [6, 7].

Atopic dermatitis (AD) is characterized by an expansion of the population of Th2 cells and a decrease in numbers of Th1 cells at least in the initial stages [8, 9]. CCL17, CCL22, and CCR4 are highly implicated in the pathogenesis of AD [6, 10–23]. The expression of CCL17 and CCL22 in keratinocytes (KC) is up-regulated and the number of $CCR4^+$ cells is increased in the lesional skin [10, 11, 14, 18–21], suggesting that CCL17 and CCL22 play a vital role in inducing Th2-type response by attracting $CCR4^+$ Th2 cells to the lesional skin in AD.

To clarify the contribution of CCL17 produced by KC to skin diseases such as AD, we have created a line of transgenic (Tg) mice that constitutively produce CCL17 in the epidermis under the control of the human keratin 14 (hK14) promoter. Analyses of contact hypersensitivity (CHS) reactions, mRNA expression, histopathological findings, serum levels of IgE, and response to irritation were compared between Tg mice and non-Tg mice.

Results

Establishment of CCL17 Tg mice

Generation and screening of CCL17 Tg mice

Tg mice were generated by the microinjection of a construct including murine CCL17 cDNA [24] into fertilized eggs of C57BL/6 mice as previously described [25]. This construct consisted of an hK14 promoter/enhancer, murine CCL17 cDNA, and a portion of the human growth hormone (hGH) gene with a poly A signal [26]. Only one founder was successfully bred to yield a line. Mice were screened for the transgene's presence by PCR analysis of tail genomic DNA with the primers F1 and R1 (located in the hK14 gene and in the murine CCL17 gene, respectively). A 498-bp band was detected in Tg mice but not in non-Tg mice because normal mice do not have an hK14-murine CCL17 fusion gene.

Increased expression of CCL17 mRNA in KC of Tg mice

mRNA was extracted from fresh KC and cultured KC [27]. The amount of CCL17 mRNA expressed in non-Tg mice and Tg mice was measured using RT-PCR [28]. With the primers F3 and R3 (located in the murine CCL17 gene-coding region), the fragment within the CCL17 coding region, which is included both in the transgene and in the genomic DNA was amplified. On the other hand, using F4 and R4 (located in the murine CCL17 gene-coding region and in the murine CCL17 gene 3′-untranslated region (UTR), respectively), the fragment spanning from the CCL17 cDNA region to 3′-UTR that was included only in genomic DNA was amplified. Thus, the PCR product amplified by F3 and R3 reflects mRNA transcribed from the transgene and endogenous genomic DNA while that amplified by F4 and R4 reflects endogenous CCL17 mRNA only. CCL17 mRNA expression detected with F3 and R3 was markedly increased in Tg mice compared with non-Tg mice, while mRNA expression detected with F4 and R4 in Tg mice was very weak and similar to that of non-Tg mice. The same results were obtained in fresh KC and cultured KC. This means that the endogenous CCL17 mRNA level was very low and the transgene was transcribed into mRNA at a high level in Tg mice.

Increased production and secretion of CCL17 protein in KC of Tg mice

To establish that CCL17 mRNA was being translated into the protein, immunohistochemistry, ELISA, and Western blotting were performed. Fig. 1A shows that the epidermis of Tg mice was markedly stained with anti-CCL17 antibody while immunoreactivity in the epidermis of non-Tg mice was very weak. The concentration of CCL17 in the supernatants of cultured KC [27] from Tg mice (70 382 ± 15 407 pg/mL) was markedly higher than that of KC from non-Tg mice (6.78 ± 1.45 pg/mL) ($p = 0.02$) (Fig. 1B). The CCL17 concentration was significantly higher in the serum of Tg mice (5373 ± 1723 pg/mL) than in that of non-Tg mice (108 ± 32.7 pg/mL) ($p = 0.00016$) (Fig. 1C). To confirm the molecular weight of the produced and secreted CCL17, Western blotting was performed using cell lysate of Tg KC, supernatant from cultured Tg KC, and recombinant murine CCL17 (as a control). The bands migrated as the band of recombinant murine CCL17 did. This molecular weight is iconsistent with the predicted molecular weight of murine CCL17 protein [24]. This means that murine CCL17 mRNA from the transgene was translated into protein and the production and secretion of CCL17 protein was markedly increased in Tg KC.

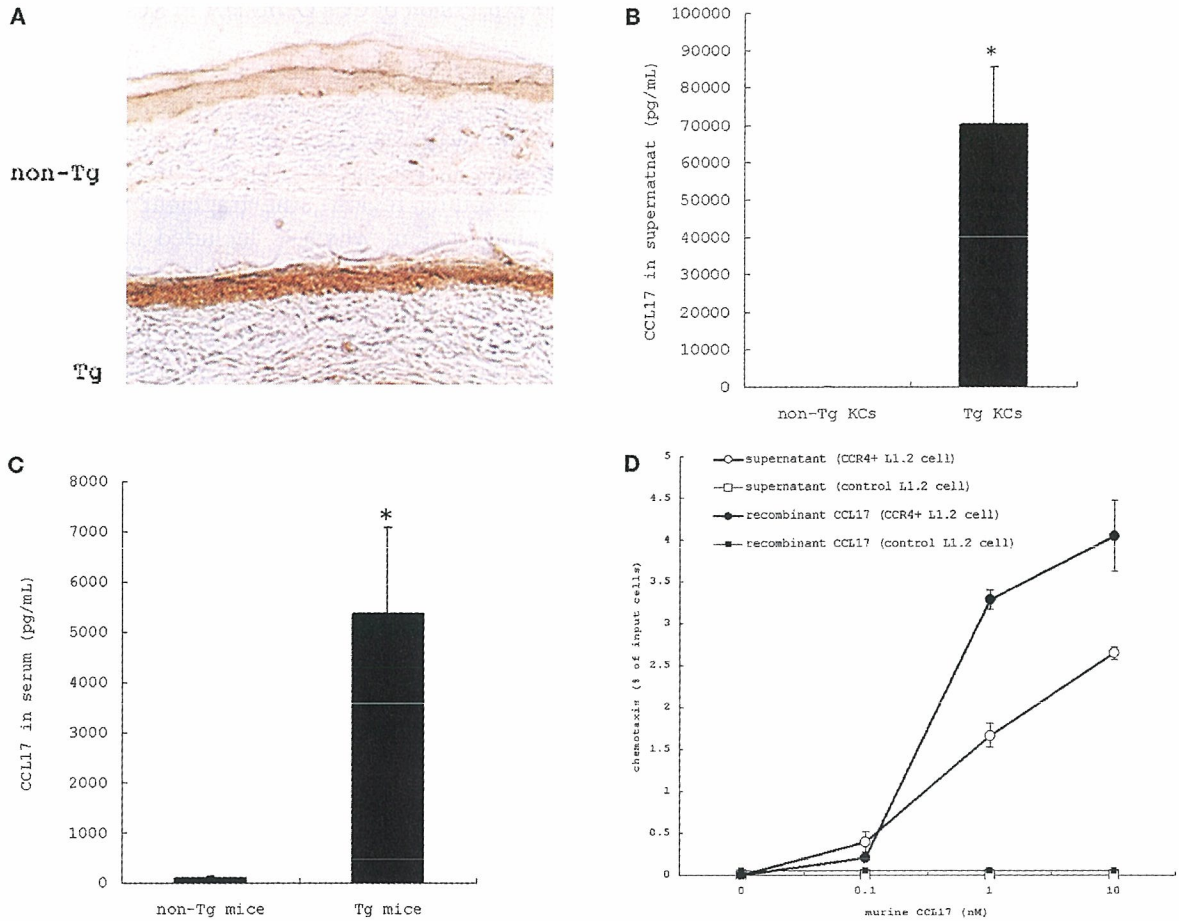

Fig. 1. Increased production and secretion of CCL17 protein with biological and functional activity by KC of Tg mice. (A) Epidermal KC in ear skin were strongly stained by anti-CCL17 antibody in Tg mice compared to non-Tg mice. (B) KC from Tg mice produced a significantly higher level of CCL17 than those from non-Tg mice. (C) A significantly higher level of CCL17 was detected in the serum from Tg mice than that from non-Tg mice. The results are presented as the mean ± SD.*$p < 0.05$. (D) The chemoattractant activity of supernatants of cultured KC from Tg mice and recombinant murine CCL17 was determined by chemotaxis assay. CCL17 secreted by KC from Tg mice showed chemotactic activity for $CCR4^+$ cells. The number of migrating cells was expressed as a percentage of the number of cells loaded in the upper chamber (mean ± SD). Supernatant and $CCR4^+$ cells: open circles, supernatant and control cells: open squares, recombinant murine CCL17 and $CCR4^+$ cells: closed circles, recombinant murine CCL17 and control cells: closed squares.

Murine CCL17 produced by Tg KC had biological and functional activity

In a chemotaxis assay [29], the supernatant of cultured Tg KC was tested for its capacity to chemoattract L1.2 cells constitutively expressing CCR4 [30] ($CCR4^+$ L1.2 cells). Supernatant of Tg KC and recombinant murine CCL17 displayed strong chemoattractant activity for $CCR4^+$ L1.2 cells, but showed no chemoattractant activity for control L1.2 cells (Fig. 1D). The chemoattraction was blocked by anti- murine CCL17 antibody (data not shown). These results indicate that bioactive murine CCL17 was produced and secreted by Tg KC.

CHS reaction in Tg mice

General remarks

CHS to oxazolone (OX) and FITC was assayed [31, 32]. The CHS reaction after a single challenge and that following repeated challenges were designated as "acute CHS" and "chronic CHS", respectively. The CHS reactions to treatment on the ear and the abdomen were designated as "CHS (ear)" and "CHS (abdomen)", respectively.

Reduced acute CHS and enhanced chronic CHS to OX in Tg mice

As the ear swelling peaked at 24 h in OX acute CHS in the preliminary experiment, we compared the ear

swelling at 24 h between non-Tg and Tg mice. Compared to non-Tg mice, Tg mice showed significantly reduced ear swelling (non-Tg: $39.4 \pm 8.9 \times 10^{-2}$ mm, Tg: $22.8 \pm 5.7 \times 10^{-2}$ mm, $p = 0.016$) (Fig. 2A). The ear swelling response peaked around day 11, then weakened slightly and reached a plateau level around day 30 in the preliminary experiment in chronic CHS. Thus, we examined the ear swelling from day 0 to day 35. Fig. 2B shows the time course of the ear swelling; a significant difference was detected after day 14 in chronic CHS.

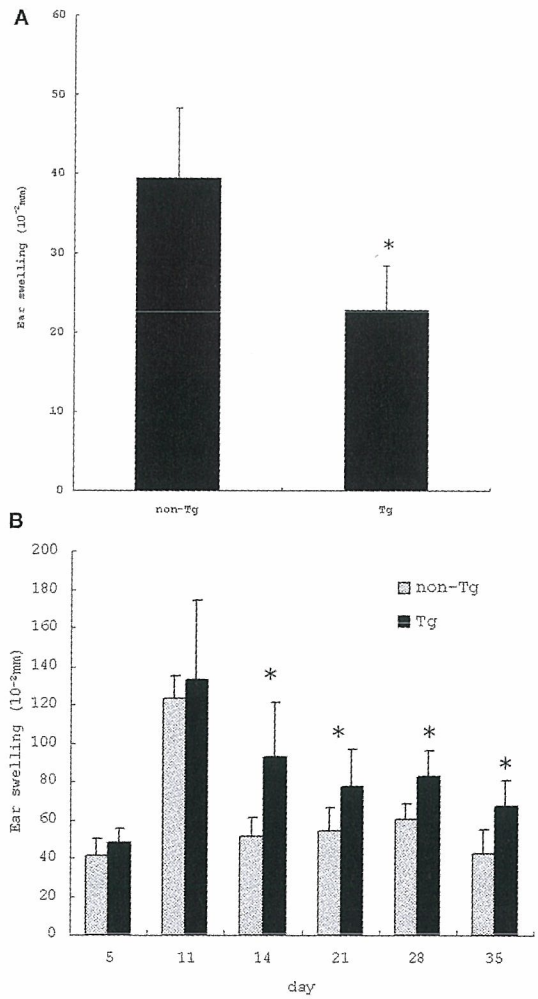

Fig. 2. Reduced OX acute CHS and enhanced OX chronic CHS in Tg mice. (A) Compared to non-Tg mice, Tg mice showed significantly reduced ear swelling in OX acute CHS. Ear swelling is presented as the mean ± SD (10^{-2} mm). (B) There was no significant difference in ear swelling between non-Tg mice and Tg mice until day 11, however, the difference became significant after day 14 in OX chronic CHS. Ear swelling responses before each challenge are shown as the mean ± SD (10^{-2} mm). *p <0.05 (*vs.* non-Tg mice).

Pathological findings on the lesional skin after acute and chronic CHS to OX

Without stimulation, there were few inflammatory cells in both non-Tg mice and Tg mice and there was no significant difference (non-Tg: 5.25 ± 1.67/field, Tg: 2.52 ± 1.2/field). In OX acute CHS, a large number of neutrophils and lymphocytes infiltrated the dermis. A significantly smaller number of cells were found in Tg mice than in non-Tg mice (non-Tg: 77.9 ± 14.5/field, Tg: 12.0 ± 7.7/field, $p = 1.4 \times 10^{-6}$) (Fig. 3A). In OX chronic CHS, large numbers of cells infiltrated the dermis and epidermis compared to numbers in acute CHS. Tg mice had significantly more infiltrating cells in the dermis and in the epidermis than non-Tg mice (non-Tg: 245.3 ± 44.0/field, Tg: 508.72 ± 116.5/field, $p = 3.2 \times 10^{-5}$) (Fig. 3B). There was an increased number of mast cells in the dermis in chronic CHS while only a few mast cells in acute CHS. Mast cell counts in chronic CHS were significantly increased in Tg mice compared to non-Tg mice (Fig. 3C) (non-Tg: 44.9 ± 8.5/field, Tg: 62.0 ± 8.5/field, $p = 1.3 \times 10^{-5}$). The number of $CCR4^+$ cells also significantly increased in Tg mice compared to non-Tg mice both in acute CHS (Fig. 3D) (non-Tg: 15.4 ± 5.4/field, Tg: 33.25 ± 7.1/field, $p = 4.3 \times 10^{-6}$) and in chronic CHS (non-Tg: 8.36 ± 4.0/field, Tg: 14.1 ± 4.8/field, $p = 0.0067$). These $CCR4^+$ cells were stained with anti-CD4 antibody but not anti-CD25 antibody (data not shown).

IL-4 mRNA expression was increased and IFN-γ mRNA expression was decreased in Tg mice in acute and chronic CHS to OX

RT-PCR was performed using the primer sets for murine IL-4 and murine IFN-γ [28] with mRNA extracted from the ear skin at 24 h in OX acute CHS and 24 h after the last challenge in chronic CHS at day 35. In the absence of stimulation, neither the mRNA of IL-4 nor that of IFN-γ was detected in non-Tg mice and Tg mice (data not shown). Representative results of mRNA expression in acute CHS are shown in Fig. 4. mRNA expression of IL-4 was increased and that of IFN-γ was reduced in Tg mice compared to non-Tg mice. The same result was obtained in chronic CHS. Tissue cytokine levels were determined by ELISA after extraction of protein from homogenized ear tissue. IL-4 but not IFN-γ was detected by ELISA in acute CHS. In chronic CHS, neither cytokine could be detected. The levels of IL-4 in acute CHS were significantly higher in Tg mice (22.3 ± 10.7 pg/mg protein) than in non-Tg mice (7.80 ± 1.73 pg/mg protein) ($p = 0.0045$).

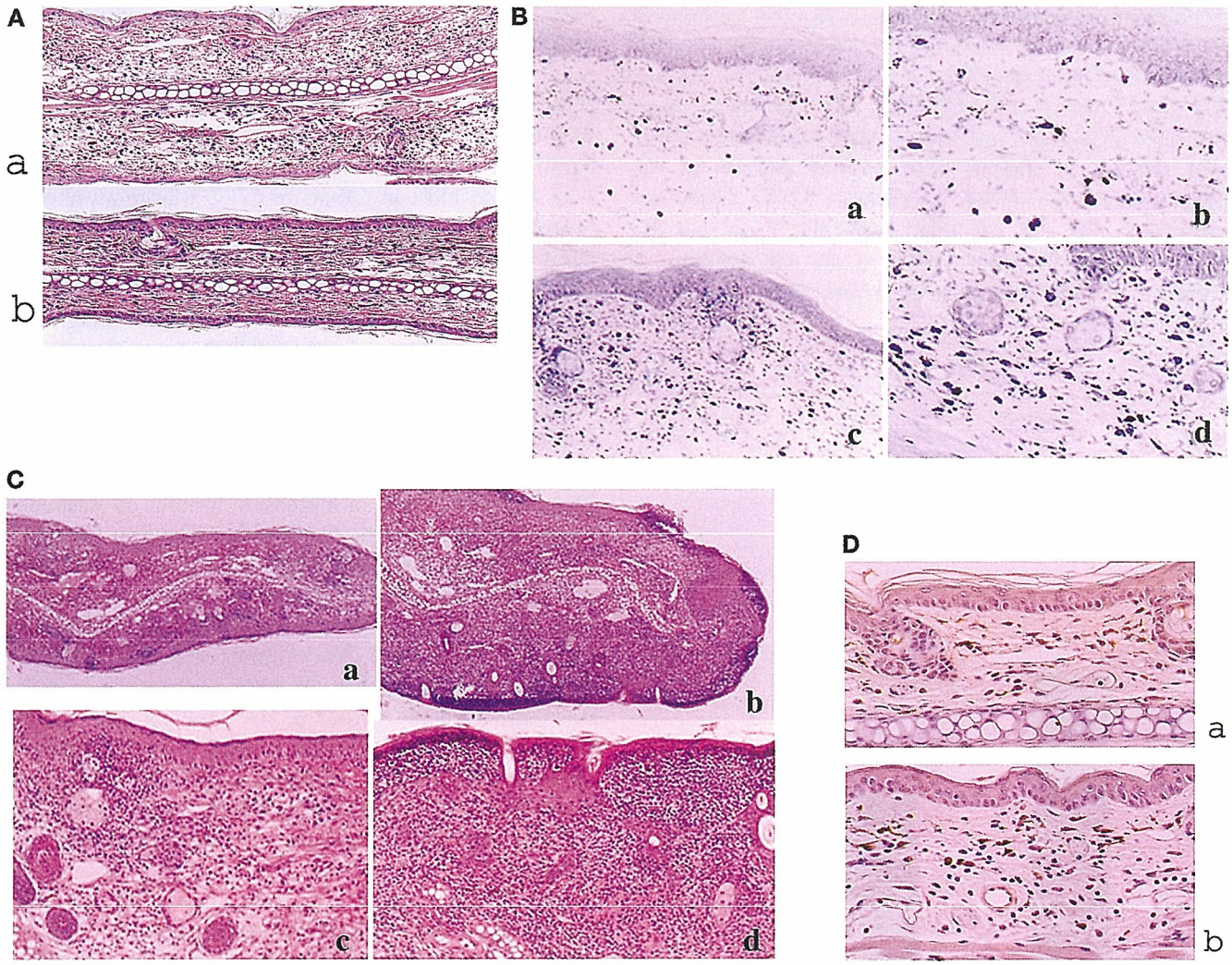

Fig. 3. Pathological findings on the lesional skin after OX acute and OX chronic CHS. (A) Many neutrophils and lymphocytes infiltrated the dermis in OX acute CHS. Significantly fewer cells were found in Tg mice than in non-Tg mice (a: non-Tg mice, b: Tg mice, HE stain, x50). (B) Tg mice showed significantly more infiltrating cells in the dermis and in the epidermis than non-Tg mice in OX chronic CHS (HE stain, a: non-Tg mice (x50), b: Tg mice (x50), c: non-Tg mice (x100), d: Tg mice (x100)). (C) Mast cell counts in OX chronic CHS were significantly increased in Tg mice compared to non-Tg mice (toluidine blue stain, a: non-Tg mice (x100), b: non-Tg mice (x400), c: Tg mice (x100), d: Tg mice (x400)). (D) $CCR4^+$ cell counts were significantly increased in Tg mice compared to non-Tg mice in OX acute CHS (a: non-Tg mice, b: Tg mice, x400).

Increased number of $CCR4^+$ lymphocytes among PBMC of Tg mice in OX acute CHS

OX was painted on the trunk of the sensitized non-Tg and Tg mice. Blood was drawn from each mouse 24 h after the first challenge for acute CHS or 24 h after the last challenge for chronic CHS, and the percentage of $CCR4^+$ lymphocytes was determined by flow cytometry. Almost all the $CCR4^+$ lymphocytes expressed CD4. Non-Tg mice and Tg mice showed a similarly low percentage of $CCR4^+$ lymphocytes (non-Tg: $0.29 \pm 0.14\%$, Tg: $0.26 \pm 0.13\%$, $p = 0.65$) when not stimulated. In both non-Tg and Tg mice, the percentage of $CCR4^+$ lymphocytes was significantly higher in acute CHS (non-Tg: $1.29 \pm 0.71\%$, $p = 0.003$, Tg: $3.05 \pm 1.30\%$, $p = 0.000085$) but not in chronic CHS (non-Tg: $0.14 \pm 0.06\%$, Tg: $0.20 \pm 0.04\%$) than without any treatment (Fig. 5). In addition, $CCR4^+$ lymphocytes significantly increased in Tg mice compared to non-Tg mice in acute CHS ($p = 0.0023$). $CD25^+CCR4^+$ lymphocytes were rarely found even in OX acute CHS in both non-Tg mice ($0.14 \pm 0.056\%$) and Tg mice ($0.15 \pm 0.073\%$).

Increased FITC acute CHS in Tg mice

As in OX CHS, we examined the acute and chronic CHS to FITC. As the ear swelling peaked at 24 h in FITC acute CHS in the preliminary experiment, we compared the ear swelling at 24 h between non-Tg and Tg mice. Tg mice showed significantly increased ear swelling

compared to non-Tg mice (non-Tg: 5.67 ± 5.29 × 10^{-2} mm, Tg: 11.8 ± 5.85 × 10^{-2} mm, $p = 0.049$) (Fig. 6). In FITC chronic CHS, the ear swelling tended to be enhanced in Tg mice compared to non-Tg mice, but the difference was not statistically significant.

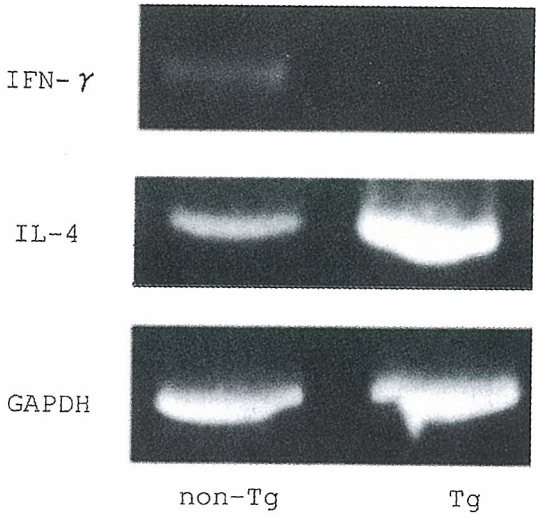

Fig. 4. Increased IL-4 mRNA expression and decreased IFN-γ mRNA expression in Tg mice in OX acute CHS. RT-PCR revealed that mRNA expression of IL-4 was increased and that of IFN-γ was reduced in ear skin of Tg mice compared to non-Tg mice in OX acute CHS.

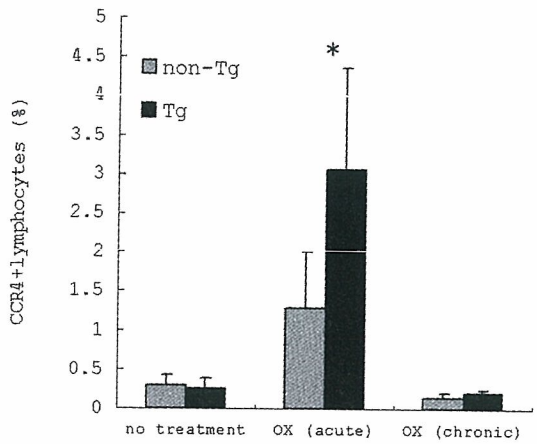

Fig. 5. Increased number of $CCR4^+$ lymphocytes among PBMC of Tg mice in OX acute CHS. The percentage of $CCR4^+$ lymphocytes was determined by flow cytometry. Non-Tg mice and Tg mice showed a similar percentage of $CCR4^+$ lymphocytes when not stimulated. In both non-Tg mice and Tg mice, the percentage of $CCR4^+$ lymphocytes was significantly higher in OX acute CHS but not in OX chronic CHS. The numbers of $CCR4^+$ lymphocytes were significantly increased in Tg mice compared to non-Tg mice in acute CHS $^*p < 0.05$ (*vs.* non-Tg mice).

Enhanced irritant dermatitis with croton oil in Tg mice

Croton oil (CO) was repeatedly painted on the trunk. Tg mice showed severe hair loss and mild erythema while non-Tg mice showed only slight erythema after day 15 (Fig. 7A). Histopathologically, hyperkeratosis, acantho-

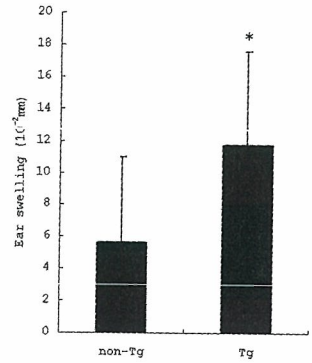

Fig. 6. Increased FITC acute CHS in Tg mice. Tg mice showed significantly increased ear swelling compared to non-Tg mice in FITC acute CHS. Ear swelling is presented as the mean ± SD (10^{-2} mm). $^*p < 0.05$.

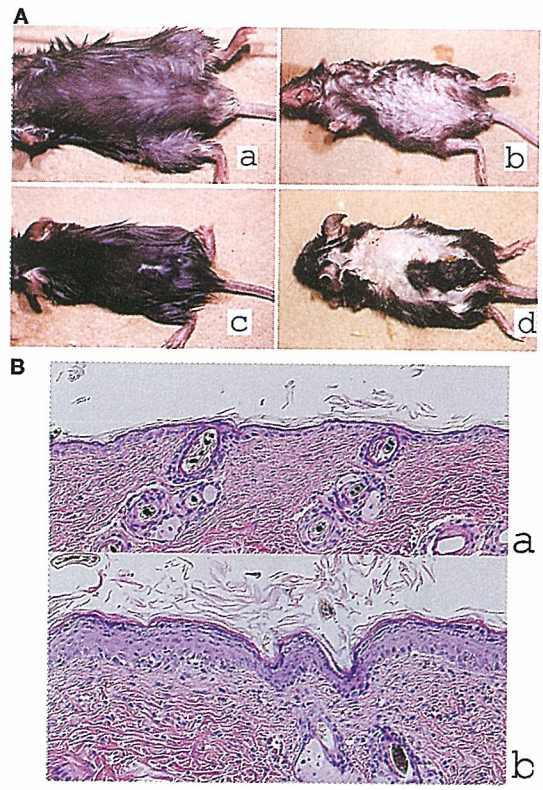

Fig. 7. Enhanced irritant dermatitis with CO in Tg mice. (A) Macroscopically, Tg mice showed mild erythema of the skin with severe hair loss on chronic irritation with CO (a: abdomen of non-Tg mice, b: abdomen of Tg mice, c: back of non-Tg mice, d: back of Tg mice). (B) Histopathologically, hyperkeratosis, acanthosis in the epidermis, and lymphocytic infiltration of the epidermis and dermis were observed in Tg mice (a: non-Tg mice, b: Tg mice; HE stain, x100).