sis in the epidermis, and lymphocytic infiltration of the epidermis and dermis were observed only in Tg mice (Fig. 7B). The number of infiltrating cells was increased in the epidermis (non-Tg: 1.83 ± 1.72/field, Tg: 12.8 ± 5.88/field, $p = 0.0039$) and in the dermis (non-Tg: 44 ± 9.2/field, Tg: 109 ± 16.0/field, $p = 0.0017$) of Tg mice.

Ear swelling was significantly increased in Tg mice by repeated tape stripping

The ear swelling after the sixth stripping is shown in Fig. 8, and was significantly increased in Tg mice (73.8 ± 18.7 × 10^{-2} mm) compared to non-Tg mice (48.6 ± 19.0 × 10^{-2} mm) ($p = 0.013$).

Increased serum IgE levels after CHS in Tg mice

Without treatment, the serum IgE concentration of Tg mice (319.6 ± 19.2 ng/mL) was significantly higher than that of non-Tg mice (233.4 ± 40.3 ng/mL) ($p = 0.0021$). After chronic CHS, the serum IgE levels were increased compared with those before the challenge in both non-Tg mice and Tg mice, and Tg mice showed significantly higher levels of serum IgE than non-Tg mice. In chronic irritation with CO on the trunk, the serum IgE levels were significantly higher in Tg mice (797.5 ± 473.3 ng/mL) than non-Tg mice (242.1 ± 45.5 ng/mL) ($p = 0.006$) (Fig. 9).

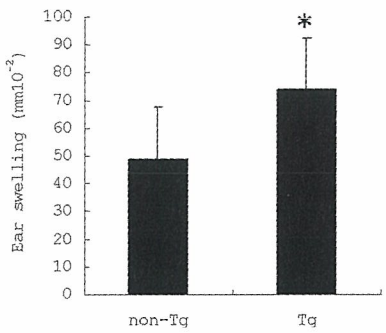

Fig. 8. Increased ear swelling in Tg mice in tape stripping. Ear swelling was significantly increased in Tg mice compared to non-Tg mice following tape stripping ($p = 0.013$).

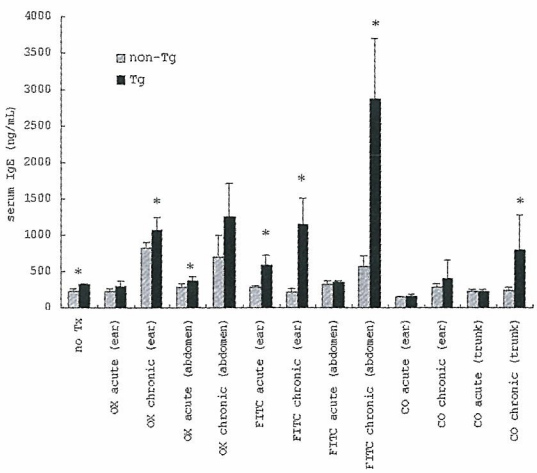

Fig. 9. Serum IgE levels before and after the challenge. In acute or chronic CHS, non-Tg and Tg mice were challenged with OX or FITC on the ear or the abdomen. In acute or chronic irritation, CO was painted on the ear or the trunk (abdomen and back). Serum IgE levels are expressed as the mean ± SD (ng/mL). *p <0.05 (*vs.* non-Tg mice).

Discussion

We created a line of Tg mice in which CCL17 expression is increased in the KC of the epidermis under the control of the hK14 promoter. It was confirmed that the transgene was integrated, mRNA for CCL17 was transcribed from the transgene, a large amount of CCL17 protein was produced and secreted by KC, the molecular weight of the protein is identical to that reported, and the CCL17 protein produced is bioactive for $CCR4^+$ cells.

In this study, CHS reactions [33] were examined. OX, a Th1-type sensitizer [34], and FITC, a Th2-type sensitizer [35, 36], were employed as sensitizers, and two types of challenge, namely acute CHS and chronic CHS, were used. In terms of cytokine levels, an initial challenge with an antigen such as OX, leads to the predominant production of Th1 cytokines like IFN-γ and IL-2, and minimal production of Th2 cytokines such as IL-4 and IL-10 in the lesional skin [28, 37, 38]. Continued exposure to antigens induces a down-regulation of the production of Th1 cytokines and an up-regulation of that of Th2 cytokines [28, 37, 38]. As Th2 cells produce IL-4 and IL-13, which is closely related to the promotion of IgE production, IgE is a reliable marker of Th2 activity [35]. Consistent with the predominant Th2 cytokine phenotype, repeated challenges induce an elevation in levels of serum IgE [28, 37]. As mentioned above, OX induces Th1-dominated inflammation in acute CHS and Th2-dominated inflammation in chronic CHS. In this study, the levels of ear swelling and the number of infiltrating inflammatory cells were reduced in OX acute CHS and increased in chronic CHS in Tg mice compared to non-Tg mice. This suggests that the Th1-type response was suppressed and the Th2-type response was enhanced in Tg mice. This was confirmed at the mRNA level. The number of $CCR4^+$ cells in the lesional skin increased in Tg mice compared to non-Tg mice in both acute and chronic CHS to OX. This suggests that CCL17 produced by the epidermal KC in Tg mice attracts $CCR4^+$ cells and these cells secrete Th2 cytokines, inducing a Th2-dominated condition in Tg mice. This Th2-dominated condition is believed to have reduced OX acute CHS, a Th1-type

inflammation and enhanced OX chronic CHS, a Th2-type inflammation. Surprisingly, chronic irritation on the trunk by CO induced erythema and hair loss macroscopically and eczematous change histopathologically in Tg mice but not in non-Tg mice. The ear swelling on tape stripping was also increased in Tg mice. These results suggest that CCL17 can modify not only an allergic reaction but also an irritant reaction. Both allergic inflammation induced by antigens and non-allergic inflammation caused by an impaired barrier function of the epidermis are important mechanisms in the pathogenesis of AD [39]. CCL17 may function in these two mechanisms. A greater number of mast cells infiltrated in chronic than acute CHS to OX, and Tg mice showed an increased number of mast cells compared to non-Tg mice. A large number of mast cells are found in chronic lesions in AD and thought to be involved in the pathogenesis of AD [40]. CCL17 might participate in the formation of chronic lesions by increasing mast cell numbers.

Katou *et al.* [41]. reported that CCR4 was expressed by epidermal Langerhans cells (LC) and dermal $CD4^+$ lymphocytes but not by dermal DC in the inflamed skin, and speculated that CCR4 is involved in the trafficking of epidermal LC at the inflamed sites. Thus, there remains a possibility that the migration of LC in the sensitization phase is altered in Tg mice. The numbers of LC in Tg mice before and after the sensitization were compared with those in non-Tg mice; no difference was found, however (data not shown), suggesting that CCL17 produced by KC does not alter the migration of LC.

Although CCL17 produced by Tg mice had chemoattractant activity for $CCR4^+$ L1.2 cells in this study, no spontaneous infiltration of inflammatory cells into the skin could be identified. However, when inflammation was provoked by CHS, CCL17 modified it. This means that CCL17 does not itself induce local inflammatory responses, but affects the inflammation caused by other stimuli. The mechanism by which lymphocytes invade tissues is a multistep process in which adhesion molecules participate [42]. Stimulation by contactants induces the production of proinflammatory cytokines from KC, which in turn induces endothelial activation and expression of adhesion molecules [33]. The lack of spontaneous inflammation in Tg mice would be due to an absence of the endothelial activation and expression of adhesion molecules. It is possible that $CCR4^+$ lymphocytes cannot infiltrate when E-selectin is not expressed even if the concentration of CCL17 is sufficient for migration, because CCR4 has been identified as being expressed preferentially on CLA^+ skin homing T cells [6, 7, 19, 20].

Simple wounds such as those caused by tape stripping are reported to induce primary cytokines [43]. Irritation by CO and tape stripping induced enhanced inflammation in Tg mice. These irritations were believed to have induced the expression of primary cytokines and adhesion molecules and, under these conditions, CCL17 prompted Th2-type inflammation.

Serum IgE levels were increased after CHS to OX and to FITC, especially after chronic CHS. Tg mice and non-Tg mice showed remarkably higher serum IgE levels, particularly in FITC chronic CHS. This is consistent with FITC being a Th2-dominant sensitizer and chronic CHS inducing Th2-type inflammation. Tg mice produced a greater amount of serum IgE than non-Tg mice in chronic CHS, suggesting that CCL17 produced by Tg KC enhanced IgE production. CCL17 might have activated $CCR4^+$ Th2 cells that, in turn, secreted IL-4 and IL-13, which are important in IgE production [44]. AD is characterized by chronic dermatitis associated with high levels of serum IgE [8, 40]. These results suggest that chronic exposure to various antigens and CCL17 produced by KC plays a role in the elevation of serum IgE levels in human AD. Chronic exposure to various irritant agents due to an impaired skin barrier function is an important factor in the pathogenesis of AD [39]. Irritation by CO on the trunk induced a rise in serum IgE levels only in Tg mice, suggesting that chronic irritation can also induce serum IgE levels to increase in the presence of a high concentration of CCL17.

Alferink *et al.* [45] reported that CCL17-deficient mice mount diminished acute CHS. They employed dinitrofluorobenzene (DNFB) and FITC as sensitizing agents. FITC acute CHS was enhanced in Tg mice in our study. These findings indicate that CCL17 promotes FITC acute CHS. This can be explained by the fact that FITC is a Th2-type sensitizer. We did not examine DNFB CHS, which is believed to be a Th1-type sensitizer. In our study, the acute CHS evoked by OX, a Th1-type agent, was reduced in Tg mice, which conflicted with the result in CCL17-deficient mice. A possible explanation is that some amount of CCL17 is necessary for Th1-type CHS [46] as well as Th2-type CHS but that excess CCL17 suppresses Th1-type responses.

The percentage of $CCR4^+$ cells among PBMC was remarkably increased in OX acute CHS on the trunk but very low without CHS and in OX chronic CHS on the trunk. This suggests that a high concentration of serum CCL17 alone is not sufficient and that other factors are necessary for mobilization of $CCR4^+$ cells. Some cytokines and chemokines produced in the skin during CHS might cooperate with CCL17 to increase number of $CCR4^+$ cells in the PBMC population. Serum CCL17 levels [11, 14–16] and percentages of $CCR4^+$ cells in PBMC [19–22] are high in AD patients. Various cytokines and chemokines are reported to be up-regulated in their expression in AD patients [8] and these factors may function with CCL17 in mobilizing $CCR4^+$ cells. The percentage of $CCR4^+$ cells among

PBMC in OX chronic CHS was similar to that without CHS and the number of $CCR4^+$ cells was reduced in OX chronic CHS compared to OX acute CHS in the inflamed skin, suggesting that $CCR4^+$ cells are more important in acute CHS than in chronic CHS. Other cells infiltrating in OX chronic CHS, such as mast cells, may function in the maintenance of the Th2-dominant condition in chronic CHS because mast cells produce Th2 cytokines.

Regulatory T (Treg) cells have been proposed to control peripheral immunological self-tolerance [47, 48]. The best-characterized population of naturally occurring Treg cells is $CD4^+CD25^+$ T cells [47, 48]. These cells are reported to express CCR4 and respond to CCL17 and CCL22 [47–49]. We studied whether $CCR4^+$ lymphocytes circulating in the blood and infiltrating the skin express CD25, the best available marker for Treg cells [48]. However, these $CCR4^+$ lymphocytes did not express CD25, indicating that they are not Treg cells. Thus, we concluded that these $CCR4^+$ lymphocytes are Th2 cells.

In summary, we studied the effect of CCL17 produced by epidermal KC in CCL17-Tg mice. Although CCL17 itself is not sufficient for the induction of inflammation, it has an effect on the inflammation caused by other factors by attracting $CCR4^+$ Th2 cells into the lesional skin and creating a Th2-dominant condition. In addition, AD-like conditions such as increased numbers of mast cells and high levels of serum IgE were observed in chronically challenged Tg mice. Tg mice also displayed an elevation of serum IgE levels and dermatitis on chronic irritation. CCL17 may participate in the pathogenesis of skin diseases such as AD by regulating both allergic and non-allergic irritant inflammation.

Materials and methods

Mice

C57BL/6 (C57BL/6J Jcl) mice were purchased from Japan Clea (Tokyo, Japan). All mice used in the experiments were 6–8 weeks old and only animals of the same sex were used in each experiment. Eight mice in both the non-Tg group and the Tg group were employed in each experiment. They were maintained under a specific pathogen-free environment unless otherwise mentioned and kept under standard conditions with a 12-h day/night rhythm and free access to food and water. All the mice received humane care and the experiments were approved by an internal ethics committee.

Generation of a transgenic construct

A DNA construct including cDNA for murine CCL17 ([24], GenBank accession no. AJ242587) was generated and microinjected as previously described [25]. The murine CCL17 cDNA coding region was inserted into the *Bam HI* site (between hK14 and hGH) of a hK14/hGH expression vector containing the hK14 promoter/enhancer and a portion of the hGH gene with a polyA signal [26]. The hK14 promoter/enhancer-CCL17-hGH fusion products were used for microinjection.

Genotyping of CCL17 Tg mice

Mice were screened for the transgene by PCR amplification of DNA from tail skin using a forward primer (F1: 5′-ACACCTCCCCCTGTGAATCA-3′) located in the hK14 gene and a reverse primer (R1: 5′-TTTCACCAATCTGATGGCCT-3′) located in the murine CCL17 gene. The murine GAPDH gene was employed as an internal control (F2 forward primer: TGAAGGTCGGTGTGAACGGATTTGGC and R2 reverse primer: CATGTAGGCCATGAGGTCCACCAC). The genomic DNA was extracted with a QIAGEN DNeasy Tissue Kit (Qiagen, Hilden, Germany).

Keratinocyte cell culture

An epidermal KC suspension was made from the ear skin as described previously [27]. These KC used just after their isolation were designated as "fresh KC." The KC were cultured and maintained on collagen type I-coated tissue culture six-well plates (Iwaki, Chiba, Japan) using Keratinocyte-SFM supplemented with 5 ng/mL of epidermal growth factor and 50 μg/mL of bovine pituitary extract (Invitrogen, CA) in humidified 5% CO_2, 95% air at 37°C. These KC were designated as "cultured KC." Supernatant was collected from KC cultures grown for 48 h at 80–90% confluence without an exchange of medium.

RT-PCR

Total RNA was extracted using standard methods [28] from fresh KC isolated from one ear, 10^6 cultured KC and whole ear excised before or after the CHS reaction. The RNA was treated with Amplification Grade DNase I (Invitrogen) to eliminate any residual genomic DNA. RT-PCR was performed as described previously [28]. The primers used in the experiments with fresh KC and cultured KC were as follows: F3 (forward primer in murine CCL17 gene-coding region): 5′-GTCACTTCA-GATGCTGCTCCT-3′, R3 (reverse primer in murine CCL17 gene-coding region): 5′-GCCTTGGGTTTTTCACCAAT-3′, F4 (forward primer in murine CCL17 gene-coding region): 5′-CAGGGATGCCATCGTGTTTCT-3′, R4 (reverse primer in murine CCL17 gene 3′-UTR): 5′-GGTCACAGGCCGCTTTATGTT-3′, F5 (forward primer in murine GAPDH gene): 5′-GAGGAGC-GAGACCCCACTAA-3′, R5 (reverse primer in murine GAPDH gene): 5′- GGCATCGAAGGTGGAAGAGT-3′. In the experiments on CHS, primer sets for murine IFN-γ and IL-4 described previously [28] were used. The samples without reverse-transcription (negative controls) yielded no bands (data not shown).

Western blot

Western blot was performed in the standard manner using the proteins extracted from cultured KC, KC culture supernatants, and a control sample of recombinant murine CCL17 (529-TR,

R & D systems, MN). The antibodies used were polyclonal goat anti- murine CCL17 antibody (AF529, R & D systems) at 0.2 μg/mL as a primary antibody and horseradish peroxidase conjugated donkey anti-goat IgG (sc-2056, Santa Cruz Biotechnology, CA) at 0.2 μg/mL as a secondary antibody.

ELISA for murine CCL17, murine IgE, murine IFN-γ and IL-4

The concentrations of murine CCL17 in the serum and the cultured KC supernatants and of serum IgE were measured with a quantitative sandwich ELISA kit: Quantikine mouse TARC/CCL17 (R & D systems) and mouse IgE kit "Yamasa" (Yamasa corporation, Tokyo, Japan), respectively. The serum was collected 24 h after the challenge in acute CHS and 48 h after the last challenge of chronic CHS. For the determination of IFN-γ and IL-4 levels in ear tissue, the ears were cut and homogenized under liquid nitrogen 24 h after the challenge in acute CHS and 48 h after the last challenge of chronic CHS. The determination of cytokine levels was performed as described previously [50] using a commercial ELISA kit Quantikine mouse IFN-γ (R & D systems) and Quantikine mouse IL-4 (R & D systems).

Chemotaxis assay

The chemotaxis assay was performed as described previously [29]. Cell migration was assayed using L1.2 cells [29] (a gift from Dr. Eugene C. Butcher, Stanford University School of Medicine) stably transfected with murine CCR4 cDNA [30] ($CCR4^+$ L1.2 cell). $CCR4^+$ L1.2 cells and control untransfected L1.2 cells were loaded in the upper wells of a Costar Transwell chamber (3 μm pore size, Corning, NY). The murine CCL17 concentration in the supernatants of cultured KC was measured by ELISA. Supernatants of cultured KC or recombinant murine CCL17 (529-TR, R & D systems) were serially diluted with RPMI 1640 medium and added to the lower wells in a volume of 0.6 mL. After 4 h at 37°C, cells in the lower chamber were counted. Values are expressed as the percentage of input cells that migrated through the filter. Polyclonal goat anti-murine CCL17 antibody (AF529, R & D systems) was used for the antibody-blocking experiment. Various dilutions of murine CCL17 (supernatants of cultured KC or recombinant murine CCL17) were incubated with 2.5 μg/mL of the antibody for 30 min at room temperature and used for the chemotaxis assay described above.

CHS

CHS to OX and FITC was assayed as described previously [31, 32]. The CHS reaction to a single challenge was designated as "acute CHS". In the experiment on the CHS reaction to repeated challenges (designated as "chronic CHS"), mice were sensitized in the same way as in acute CHS, and each reagent was repeatedly applied to the same site three times a week. These CHS reactions at the ear were designated as "acute CHS (ear)" or "chronic CHS (ear)". To examine the serum levels of IgE, elicitation at the abdomen (by painting 100 μL of each reagent) was also performed "acute CHS (abdomen)" and "chronic CHS (abdomen)". CO (0.5%, Sigma) in 4:1 acetone/olive oil was applied to the ear (20 μL) and trunk (200 μL) to assess the reaction to irritation. Acute irritation and chronic irritation caused by CO was studied as described in OX and FITC CHS.

Tape stripping

Stripping was performed by pressing adhesive tape (Transpore surgical tape, 3 M Health Care, St. Paul, MN) onto the dorsal side of the ear and pulling it off abruptly 20 times. Stripping was performed six times at 2-day intervals. The ear thickness was measured two days after the last stripping.

Flow cytometry

Heparinized blood samples were collected and placed on ice. Two-color analysis was performed by the standard method with a combination of hamster anti-mouse CCR4 mAb (2G12, to be described in detail elsewhere by Nagakubo *et al.*) and PE-conjugated rat anti-mouse CD4 mAb (RM4–5, Santa Cruz Biotechnology) or PE-conjugated rat anti-mouse CD25 mAb (PC61, BioLegend, CA). For the anti-CCR4 antibody, FITC-conjugated mouse anti-hamster IgG mAb (BD 554011, PharMingen, CA) was used as a secondary antibody. As negative controls, hamster IgG (PN IM3032, IMMUNOTECH, Marseille, France) and PE-conjugated rat IgG (IM1272, IMMUNOTECH) were used. Negative regions were aligned using the negative controls.

Histological and immunohistological analysis

Hematoxylin and eosin (HE) stain, and truisine blue stain were applied to formalin-fixed and paraffin-embedded sections. For detecting murine CCL17, CD4 and CD25, 5 μm cryostat sections were stained with polyclonal goat anti-CCL17 antibody [TARC (N-10), Sc-12271, Santa Cruz Biotechnology] diluted 1:50, rat anti-CD4 mAb (RM4–5, BioLegend) diluted 1:1000, or rat anti-CD25 mAb (PC61, BioLegend) diluted 1:1000 as the primary antibody and biotin-conjugated rabbit anti-goat IgG or biotin-conjugated rabbit anti-rat IgG as the secondary antibody using the VECTASTAIN ABC KIT (Vector Laboratories, CA). Staining was developed by adding a diaminobenzidine solution. For detecting murine CCR4, formalin-fixed and paraffin-embedded sections were stained with the primary antibody, polyclonal goat anti-mouse CCR4 (CI0122, Capralogics, MA), diluted 1:8000, as described above. Ten visual fields were picked up at random under the microscope, and averages of the counted number of cells were recorded. The infiltrating inflammatory cells and $CCR4^+$ cells were counted in fields of x400 magnification. Mast cells were counted in fields of x200 magnification.

Statistical analysis

Data were expressed as the mean ± SD. The statistical significance between two groups was evaluated using Student's *t*-test and was considered significant if $p < 0.05$.

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Inhibition of scratching behavior associated with allergic dermatitis in mice by tacrolimus, but not by dexamethasone

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Abstract

Itching is the most important problem in many allergic and inflammatory skin diseases especially in atopic dermatitis. However, animal models for allergic dermatitis useful for the study of itching have rarely been established. We established a mouse allergic dermatitis model involving frequent scratching behavior by repeated painting with 2,4-dinitrofluorobenzene (DNFB) acetone solution onto the mouse skin, and comparatively examined the effects of tacrolimus and dexamethasone on the dermatitis and associated scratching behavior. Repeated DNFB painting caused typical dermatitis accompanied by elevated serum immunoglobulin E (IgE) and frequent scratching behavior. An apparent thickening of the epidermis and dermis, and the significant accumulation of inflammatory cells were observed. Increased interferon (IFN)-γ mRNA expression and the induction of interleukin (IL)-4 and IL-5 mRNA expression were also observed in the skin lesion. The scratching behavior was inhibited by dibucaine and naloxone. Although tacrolimus reduced the increased expression of IFN-γ and IL-4 mRNA, dexamethasone potently depressed that of IFN-γ, IL-4 and IL-5 mRNA. Dexamethasone inhibited the accumulation of lymphocytes and eosinophils, although tacrolimus did not. Both drugs failed to inhibit the elevation of serum IgE levels. Tacrolimus significantly inhibited the scratching behavior that was associated with the inhibition of nerve fiber extension into the epidermis, whereas dexamethasone failed to have any effect. The mouse dermatitis model seems to be beneficial for the study of itching associated with allergic dermatitis, such as atopic dermatitis, and tacrolimus seems to exhibit an anti-itch effect through the inhibition of nerve fiber extension at least in part.

Keywords: Allergic dermatitis; Hapten; Mouse; Scratching behavior; Immunoglobulin E; Interferon-γ; Interleukin-4; Interleukin-5; Tacrolimus; Dexamethasone

1. Introduction

Atopic dermatitis is a complex eczematous skin disease accompanied by severe itching with frequently repeated episodes (Furue et al., 2004; Wahlgren, 1992). In most cases, the onset of the disease is observed in infants and is considered to be dependent on both genetic and environmental factors. The elevated serum IgE is also a characteristic feature in many patients (Hoffman et al., 1975; Sampson and Albergo, 1984). The severe itching is the most important problem because of its significant impairment of the patient's quality of life. Furthermore, induced scratching destroys the skin barrier and worsens the dermatitis, resulting in more itching (Wahlgren, 1999). The typical skin lesion in atopic dermatitis is a result of repeated scratching. Therefore, the regulation of itching and/or scratching seems to be very important and beneficial for the treatment of atopic dermatitis. Although patients suffering from atopic dermatitis, especially adult patients with severe symptoms, have been increasing recently in Japan (Furue et al., 2004), the pathogenesis of the disease, especially the itching, has yet to be elucidated.

Topical glucocorticoids are very important and a very effective remedy for the treatment of atopic dermatitis. It is well known, however, that prolonged usage with high doses of glucocorticoids frequently causes a variety of adverse effects (Barnetson and White, 1992; Leung and Barber, 2003). Furthermore, the inappropriate usage of topical glucocorticoids has been emphasised as one cause of the recent increase in the number of adult patients with severe symptoms in Japan (Furue et al., 2004). Recently, tacrolimus, an immunosuppressant, has

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been introduced for the treatment of atopic dermatitis as an ointment (Nakagawa et al., 1994; Bieber, 1998). Although the efficacy is weaker than glucocorticoids, it can be applied safely for lesions on the face and neck and its anti-itch properties have been suggested (Stander and Luger, 2003; Katoh et al., 2004).

To understand the mechanisms of the onset and development of a disease, appropriate animal models are essential. For atopic dermatitis, some artificially induced and naturally occurring animal models have been reported. In artificially induced models, passively sensitised animals with specific IgE are exposed to a corresponding allergen (Ray et al., 1983; Katayama et al., 1990) or intact animals are exposed to an allergen repeatedly to induce dermatitis (Kitagaki et al., 1997; Spergel et al., 1998; Matsuoka et al., 2003). In these models, however, itching has not been evaluated. In contrast, NC/Nga mice naturally develop an interesting form of dermatitis with some features of atopic dermatitis (Matsuda et al., 1997; Suto et al., 1999). Although the dermatitis in NC/Nga mice seems to be very interesting and scratching behaviors are frequently observed, the triggering mechanisms have not yet been defined.

In the present study, we attempted to establish a mouse allergic dermatitis model with some features of atopic dermatitis that accompany frequent scratching behavior to elucidate the mechanism of itching, and the effects of dexamethasone and tacrolimus, clinically active drugs for the treatment of atopic dermatitis, were examined comparatively.

2. Materials and methods

2.1. Animals

Male BALB/c mice, 8–10 weeks of age, obtained from Japan SLC, Inc. (Hamamatsu, Japan) were used. They were housed in an air-conditioned animal room with a temperature of 22±1 °C and a humidity of 60±5%. Experiments were undertaken following the guidelines for the care and use of experimental animals of the Japanese Association for Laboratory Animal Science (1987). The Animal Experiment Committee of our university approved the experimental protocols employed.

2.2. Antigen

2,4-Dinitrofluorobenzene (DNFB, Sigma Chemical Co., St. Louis, MO, USA) was used as an inducer of cutaneous reaction. DNFB was dissolved in acetone at a concentration of 0.15%.

2.3. Drugs

Tacrolimus (FK506, Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan), dexamethasone (Sigma), dibucaine hydrochloride (dibucaine, Nagase Chemtex Co., Osaka, Japan) and naloxone (Sigma) were used. Tacrolimus, dexamethasone and dibucaine were dissolved in ethanol at concentrations of 0.1, 0.05 and 10%, respectively, and given topically to mice at a volume of 100 μl. Naloxone was dissolved in saline and administered intraperitoneally at a dose of 3 mg/kg. The dose of each drug was determined according to our preliminary experiments.

2.4. Induction of cutaneous reaction

Mice were painted with 100 μl of 0.15% DNFB acetone solution onto the clipped abdominal skin for initial sensitisation. Cutaneous reaction was evoked in the clipped rostral part of sensitised mouse back by repeated paintings with 50 μl of 0.15% DNFB acetone solution. The DNFB challenge was repeated 2 times a week for 4 weeks, 9 times in total, from 7 days after the initial sensitisation. Vehicle mice were similarly treated with acetone without DNFB.

Blood samples for the measurement of serum IgE were occasionally obtained from mice and tissue specimens for the examination of mRNA expression and histopathological observation were excised from mice 4 h and 24 h, respectively, after the ninth DNFB painting. Mouse scratching behavior was observed after the DNFB painting. Drugs were administered topically or intraperitoneally once a day for 2 weeks from the day of the fifth DNFB painting to the day of the ninth DNFB painting.

2.5. Histopathological observation

Excised skin specimens were fixed in 10% neutral formalin or 4% paraformaldehyde and embedded in paraffin. Sections 5 μm thick were prepared and stained with hematoxylin and eosin, toluidine blue or Congo red. Toluidine blue and Congo red specifically stain mast cells and eosinophils, respectively. Inflammatory cells were counted under a light microscope. Nerve fibers were observed immunohistochemically using anti-protein gene product 9.5 (PGP9.5) antibodies (rabbit polyclonal antiserum against human ubiquitin C-terminal hydrolase, Affiniti Research Products Ltd., Exeter, UK) (Doran et al., 1983; Inoue et al., 2002), biotin-conjugated goat anti-rabbit immunoglobulin antibodies (E0432, Dako, Glostrup, Denmark) and peroxidase-conjugated streptavidin (K1016, Dako). Stained nerve fibers visualized by an enzyme reaction in the epidermis were quantitatively evaluated by LEICAQwin (Leica Microsystems Heiderberg GmbH, Mannheim, Germany) and indicated as the percentage stained area.

2.6. Measurement of serum IgE

Total serum IgE was measured by means of an ordinary sandwich enzyme-linked immunosorbent assay (ELISA) using anti-mouse IgE antibodies (monoclonal, ε-heavy chain-specific, rat IgG1, Serotec Ltd., Oxford, UK) and peroxidase-labeled anti-mouse IgE antibodies (polyclonal, ε-heavy chain-specific, goat IgG, Nordic Immunological Laboratories, Tilburg, Netherlands) (Nagai et al., 1997a,b). Dinitrophenyl (DNP)-specific IgE was measured by means of a captured ELISA (Nagai et al., 1997a; Hirano et al., 1989). In brief, the IgE in samples was trapped with anti-mouse IgE antibody (Serotec) and detected by enzyme reaction after incubating with biotinylated antigen (dinitrophenylated bovine serum albumin) and then with peroxidase-

Table 1
Primers employed for detecting mRNA

β-actin	(product size: 245 bp)	
	Sense	5′ GTG GGC CGC TAG GCA CCA 3′
	Anti-sense	5′ CGG TTG GCC TTA GGG TTC AGG GGG G 3′
IFN-γ	(product size: 405 bp)	
	Sense	5′ TAC TGC CAC GGC ACA GTC ATT GAA 3′
	Anti-sense	5′ GCA GCG ACT CCT TTT CCG CTT CCT 3′
IL-4	(product size: 279 bp)	
	Sense	5′ ACG GAG ATG GAT GTG CCA AAC GTC 3′
	Anti-sense	5′ CGA GTA ATC CAT TTG CAT GAT GC 3′
IL-5	(product size: 342 bp)	
	Sense	5′ GCC ATG GAG ATT CCC ATG AGC ACA 3′
	Anti-sense	5′ GCC TTC CAT TGC CCA CTC TGT AC 3′

conjugated streptavidin (Dako). Biotinylated antigen was prepared using a biotinylation kit (Pierce Biotechnology Inc., Rockford, IL, USA). Mouse monoclonal IgE against DNP residue (SPE-7, Sigma) was used as a standard.

2.7. Detection of cytokine mRNA expression

The expression of Th1 and Th2 cytokine mRNA was semi-quantitatively evaluated by means of reverse transcriptase-polymerase chain reaction (RT-PCR) (Nagai et al., 1997a,b). In brief, total RNA was extracted from excised skin lesions of mice using Isogen (Nippon Gene Co., Ltd., Tokyo, Japan). cDNA was prepared from the RNA by reverse transcription (Superscript II, Gibco BRL, Grand Island, NY, USA) and then amplified by PCR (denaturation: at 94 °C for 1.5 min, annealing: at 62 °C for 1.5 min, extension: at 72 °C for 1.5 min, 35 cycles) using ampliTaq DNA polymerase (Takaka Taq, Takara Holdings Inc., Kyoto, Japan) on a thermalcycler (Trio-Thermoblock, Biometra GmbH, Goettingen, Germany). The primers employed (Table 1) were obtained from Stratagene (La Jolla, CA, USA). The resultant products were electrophoresed on 2% agarose gel containing ethidium bromide. The bands were recorded with a Polaroid camera (Polaroid 665 film, Nippon Polaroid, Tokyo, Japan) and evaluated with NIH Image software.

2.8. Evaluation of scratching behavior

Mouse scratching behavior was automatically detected and objectively evaluated with an apparatus, MicroAct (Neuroscience, Inc., Tokyo, Japan) (Inagaki et al., 2002, 2003). A small magnet (1 mm in diameter, 3 mm long, coated with teflon) was inserted subcutaneously into both hind paws under ether anesthesia before the start of the experiment. It was confirmed that the operation and

Table 2
Analysis parameters of MicroAct for detecting waves corresponding to consecutive scratching behavior in mice

Threshold	0.05 V
Event gap	0.05 s
Minimum duration	0.25 s
Maximum frequency	20 Hz
Minimum frequency	5 Hz

Repeated waves of 5–20 Hz with a duration of over 0.25 s were detected. If two waves were separated by over 0.05 s, they were not considered to be consecutive.

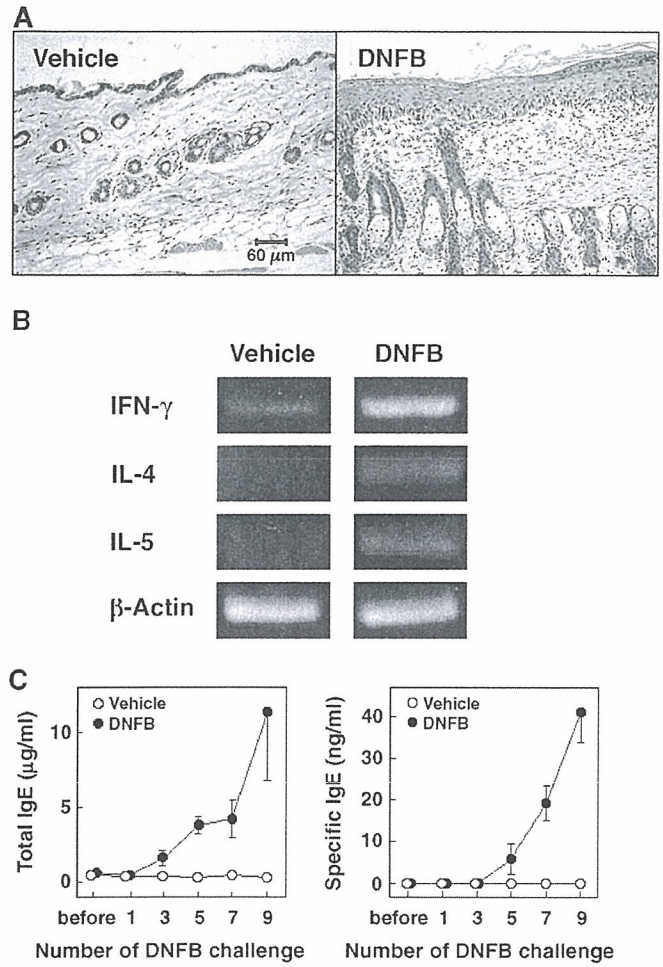

Fig. 1. Characteristics of dermatitis caused by repeated painting with DNFB in mice. (A) histopathological images for skin lesions treated with vehicle or DNFB repeatedly, 24 h after the ninth painting, hematoxylin-eosin staining. (B) cytokine mRNA expression in the skin lesion excised 4 h after the ninth DNFB painting. (C) changes in serum IgE levels, mean±SEM for 6 mice.

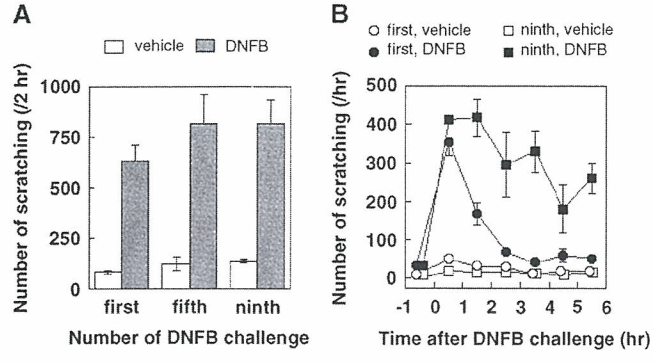

Fig. 2. Scratching behaviors caused by DNFB challenge. (A) incidence of scratching behaviors in 2 h after the first, fifth and ninth challenge. (B) changes in the incidence of scratching behaviors after the first and ninth challenge. Mean± SEM for 6 mice.

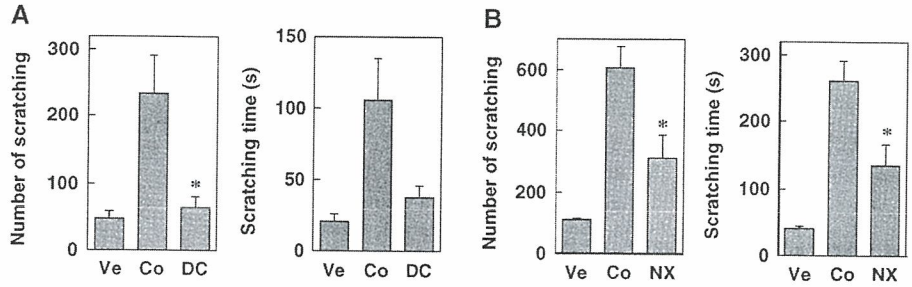

Fig. 3. Effects of dibucaine and naloxone on the scratching behaviors caused by DNFB challenge. Scratching behavior induced after the ninth challenge was evaluated for 2 h. (A) dibucaine (DC). (B) naloxone (NX). Mean±SEM for 6 mice, Ve: vehicle, Co: control, $*p<0.05$.

magnet did not affect the mouse behavior. The mouse with magnets was placed in an observation chamber (11 cm in diameter, 18 cm high), which was surrounded by a round coil. The electric current induced in the coil by the movement of magnets attached to the mouse hind paws was amplified and recorded. Then, characteristic waves corresponding to scratching behaviors were detected using a computer. Parameters for the detection of consecutive scratching behavior are shown in Table 2. Although a mouse scratches very quickly and beats by the hind paw are usually repeated several times, the apparatus was able to detect each beat. Under our experimental conditions, the apparatus detected consecutive scratching behavior consisting of 3 or more beats. The results of scratching behavior are given both as the incidence of consecutive scratching behavior and the total scratching time in an indicated period of time after DNFB painting.

2.9. Statistics

The data are expressed as the mean values with standard error. The statistical significance of the difference was evaluated with Student's or Welch's *t*-test using InStat (GraphPad Software, San Diego, CA, USA). When the *P* value was smaller than 0.05, the difference was considered to be significant.

3. Results

3.1. Characterization of dermatitis evoked by repeated challenge with DNFB

Mice were initially sensitised (abdominal skin) and then challenged 9 times repeatedly (back skin) by painting with the

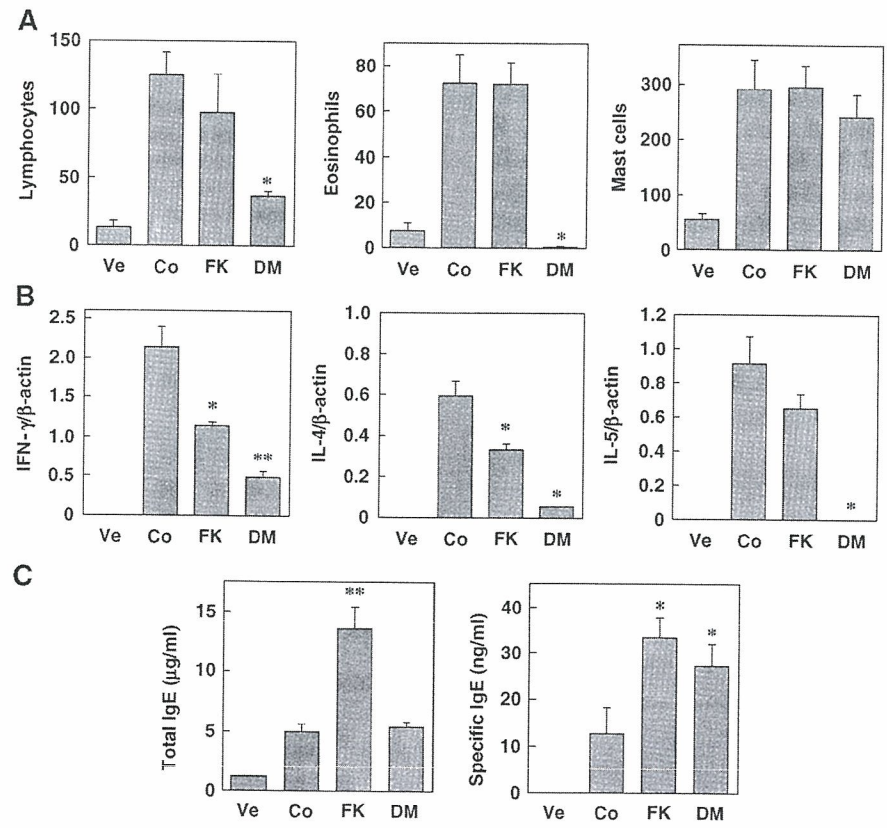

Fig. 4. Effects of tacrolimus and dexamethasone on inflammatory cell accumulation, cytokine mRNA expression, and elevation of serum IgE levels. (A) inflammatory cell counts in 0.025 mm^2, mean±SEM for 4 mice. (B) cytokine mRNA expression in the skin lesion, mean±SEM for 4 mice. (C) total and specific serum IgE levels, mean±SEM for 6–8 mice. Ve: vehicle, Co: control, FK: tacrolimus, DM: dexamethasone, $*p<0.05$, $**p<0.01$.

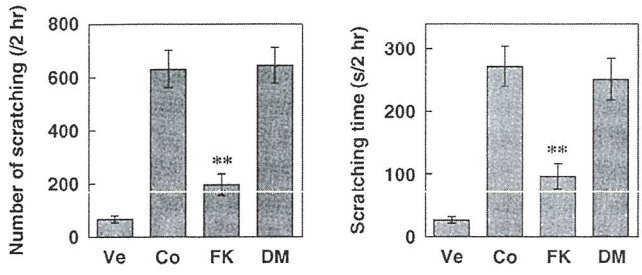

Fig. 5. Effects of tacrolimus and dexamethasone on the scratching behaviors caused by the ninth DNFB challenge. Scratching behavior induced after the ninth challenge was evaluated for 2 h. Mean±SEM for 6–8 mice, Ve: vehicle, Co: control, FK: tacrolimus, DM: dexamethasone, $^{**}p<0.01$.

DNFB acetone solution. At first, skin lesions on the mouse back were excised 24 h after the ninth DNFB challenge and observed histopathologically. The images are shown in Fig. 1A. Although repeated painting with vehicle (acetone) did not show any apparent alteration, repeated painting with DNFB solution caused potent inflammatory changes in the skin, such as the thickening of both epidermis and dermis, and the accumulation of neutrophils, eosinophils, lymphocytes and mast cells. Furthermore, the formation of scab, edema and single cell necrosis in the epidermis, and fibrosis in the dermis were also observed.

Total RNA was extracted from the skin lesions excised 4 h after the ninth DNFB challenge and the expression of cytokine mRNA was examined. As shown in Fig. 1B, although the IFN-γ mRNA expression in vehicle-treated mice was very weak, it was potentiated in DNFB-treated mice. Furthermore, although the expression of IL-4 and IL-5 mRNAs was undetectable in vehicle-treated mice, DNFB-treatment induced their expression. Changes in the serum IgE levels are shown in Fig. 1C. Elevated levels of total and specific IgE were observed in the sera of DNFB-treated mice after the second and fourth paintings, respectively, and the levels increased gradually, depending on the number of paintings.

The results of scratching behaviors are shown in Fig. 2. The first DNFB painting induced over 600 consecutive scratching behaviors for 2 h in sensitised mice (Fig. 2A) and the total scratching time reached about 250 s (data not shown). The scratching behavior increased gradually depending on the number of DNFB painting (Fig. 2A). The incidence of scratching behavior was significantly high in the first 1 h, but quickly declined thereafter at the first DNFB challenge (Fig. 2B). In contrast, after the ninth challenge, a high incidence of scratching behavior was observed in the first 2 h, and the incidence only declined slightly thereafter. Frequent scratching behaviors were still observed after 6 h in the ninth painting.

The effects of topical application of dibucaine and the intraperitoneal administration of naloxone on the scratching behavior in 2 h after the ninth DNFB painting were examined. Dibucaine inhibited the scratching behavior by 90% in incidence and 80% in time. Naloxone also inhibited the scratching behavior by 59% in incidence and 57% in time (Fig. 3).

3.2. Effects of tacrolimus and dexamethasone

Effects of tacrolimus and dexamethasone were examined by topical application as ethanol solutions. The drug treatment was performed daily for 2 weeks from the day of the fifth DNFB painting through that of the ninth painting. At first, the skin lesions obtained 24 h after the ninth painting were observed histopathologically. Although the repeated painting of DNFB caused potent inflammatory alterations as mentioned above, such as the thickening of both epidermis and dermis, and the accumulation of eosinophils and lymphocytes, dexamethasone apparently inhibited the alterations (data not shown). In contrast, the anti-inflammatory effect of tacrolimus was less apparent than in the case of dexamethasone (data not shown). Inflammatory cell counts are shown in Fig. 4A. Although dexamethasone potently inhibited the accumulation of lymphocytes and eosinophils, tacrolimus did not affect it. Both drugs failed to affect the increase in mast cell number.

As shown in Fig. 4B, dexamethasone significantly inhibited the increase in expression of IFN-γ, IL-4 and IL-5 mRNA. The expression of IL-5 mRNA was completely abrogated by dexamethasone. In contrast, tacrolimus reduced the increase in expression of IFN-γ and IL-4 mRNA, the potency was less than that of dexamethasone. The results of the IgE levels in the sera obtained 24 h after the ninth painting are shown in Fig. 4C.

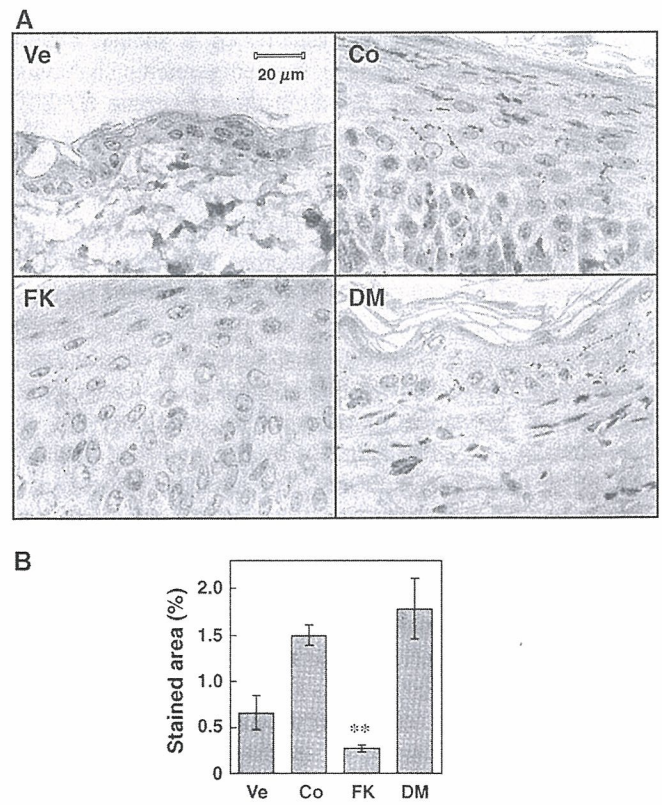

Fig. 6. Effects of tacrolimus and dexamethasone on the extension of nerve fibers in the lesions of mice repeatedly challenged with DNFB. Skin specimens were excised 24 h after the ninth challenge. (A) histological images of skin lesions immuno-stained with anti-PGP9.5 antibodies. (B) percentage stained area in the epidermis, mean±SEM for 3 mice, $^{**}p<0.01$. Ve: vehicle, Co: control, FK: tacrolimus, DM: dexamethasone.

Tacrolimus significantly elevated the levels of both total and specific IgE. In contrast, dexamethasone elevated the specific IgE levels without affecting the total IgE levels.

The results of scratching behavior induced after the ninth DNFB painting are shown in Fig. 5. DNFB painting caused over 600 scratching behaviors in 2 h and the scratching time exceeded 250 s. Treatment with tacrolimus significantly inhibited the scratching behavior in both incidence and time, although dexamethasone did not affect the scratching behavior at all.

Histological images of the skin lesion stained with anti-PGP9.5 antibodies are shown in Fig. 6A, and the results of the quantitative evaluation of stained nerve fibers in the epidermis are indicated in Fig. 6B. Although immunoreactive nerve fibers were rarely detectable in the epidermis of vehicle-treated mouse skin, potently stained nerve fibers were abundantly observed in the thickened epidermis of DNFB-treated control mouse skin. In tacrolimus-treated mice, thickening of the epidermis was apparently not inhibited, as mentioned above. However, immunoreactive nerve fibers in the epidermis were detected less frequently than in the case of control mice. In contrast, in dexamethasone-treated mice, although the thickening of the epidermis was potently depressed, immunoreactive nerve fibers were detected abundantly in the deep area of the epidermis.

4. Discussion

In the present study, we established a mouse allergic dermatitis model accompanied by frequent scratching behaviors using a contact sensitiser, 2,4-dinitrofluorobenzene (DNFB). Although a contact sensitiser primarily visualizes a Th1 response (Bellinghausen et al., 1999), repeated exposure to the agent results in a shift from the Th1 response to Th2 response or to mixed responses of Th1 and Th2 (Kitagaki et al., 1997; Nagai et al., 1997a,b). Therefore, a contact sensitiser could induce Th2 responses as well as Th1 responses (Yokozeki et al., 2000). In the present study, we painted DNFB acetone solution repeatedly, 9 times for 4 weeks, onto the clipped rostral part of sensitised mouse back. We selected the skin area for the induction of dermatitis because of its suitability for the observation of scratching behavior by the hind paw (Kuraishi et al., 1995; Inagaki et al., 2000). The induced dermatitis was characterized by a potent skin inflammation associated with inflammatory cell accumulation, expression of Th1 and Th2 cytokine mRNAs in the lesion, elevation of serum specific IgE level, and frequent scratching behavior. Furthermore, abundant nerve fibers were extended into the epidermis similar to the case of atopic dermatitis (Tobin et al., 1992; Urashima and Mihara, 1998). Therefore, the mouse dermatitis model seems to bear some characteristic features of atopic dermatitis, and to be useful for the basic study of atopic dermatitis.

Mouse scratching behavior is a useful tool for the study of itch (Kuraishi et al., 1995). In the present study, we detected and evaluated the mouse scratching behavior caused by DNFB painting automatically with an apparatus, MicroAct (Inagaki et al., 2002, 2003). The DNFB challenge caused frequent scratching behaviors in mice, and the incidence was very high immediately after the challenge. Although the incidence of scratching behavior declined rapidly after the first challenge, the decline after the ninth challenge was only gradual, and frequent scratching behaviors were still observed 6 h later. The repeated DNFB challenge seems to induce a form of dermatitis that accompanies frequent scratching behaviors with longer duration. The scratching behavior induced after the ninth challenge was inhibited by dibucaine, a local anesthetic, and naloxone, a μ-opioid receptor antagonist. In humans, itching is attenuated by local anesthetics and μ-opioid receptor antagonists (Bernstein et al., 1982; Shuttleworth et al., 1988; Metze et al., 1999), suggesting that the mouse scratching behavior is caused by a similar sense to and/or shares some mechanisms with human itching.

Topical glucocorticoids are very important and a very effective remedy for the treatment of atopic dermatitis. Recently, tacrolimus, an immunosuppressant, has been introduced for the treatment of atopic dermatitis as an ointment (Nakagawa et al., 1994; Bieber, 1998). Therefore, we examined and compared the effects of dexamethasone and tacrolimus on the dermatitis model. The drugs were dissolved in ethanol and applied topically to the skin lesion once a day for 2 weeks.

The topical application of dexamethasone at a concentration of 0.05% exhibited potent anti-inflammatory effects. Thickening of the epidermis and dermis were potently inhibited by dexamethasone. Lymphocyte accumulation in the lesion was potently reduced and the eosinophil accumulation was completely inhibited. IFN-γ, IL-4 and IL-5 mRNA expression was potently inhibited by dexamethasone. Inhibition of IFN-γ and IL-5 mRNA expression seems to contribute to the inhibition of skin inflammation and eosinophil accumulation, respectively. In spite of the potent anti-inflammatory activity of dexamethasone, however, the scratching behavior was not affected at all. In contrast, the topical application of tacrolimus at a concentration of 0.1% did not show an apparent anti-inflammatory effect. Tacrolimus exhibited inhibitory activities on IFN-γ and IL-4 mRNA expression, but the activities were less potent than those of dexamethasone. However, tacrolimus potently inhibited the scratching behavior. These results indicate that tacrolimus possesses an anti-scratching activity that is not common in glucocorticoids. Furthermore, the scratching behavior seems to be induced by a mechanism(s) independent of dexamethasone-inhibitable inflammatory mechanisms. Recently, the anti-itch property of tacrolimus has been suggested (Stander and Luger, 2003; Katoh et al., 2004). Our results coincide well with these reports. Although the direct comparison of anti-itch activities of tacrolimus and glucocorticoids has not been reported, our results strongly suggest that the anti-itch activity of tacrolimus is more potent than that of glucocorticoids.

It is well known that abundant nerve fibers are extended into the epidermis of atopic dermatitis patients (Tobin et al., 1992; Urashima and Mihara, 1998). In the present study, we confirmed that many PGP9.5-positive nerve fibers were detected in the thickened epidermis of DNFB-painted mice. It is interesting to note that the extension of nerve fibers was apparently reduced by the topical application of tacrolimus, although the inhibition of thickening of the epidermis was not

apparent. The weak inhibition of epidermal thickening may be due to the increased inflammatory cells with release of epidermal growth factors. Furthermore, the immunoreactivity detected by anti-PGP9.5 antibodies was abundantly detected in the epidermis of dexamethasone-treated mice, although thickening of the epidermis was potently suppressed. Therefore, tacrolimus seems to inhibit the nerve fiber extension, and it may contribute to its anti-scratching properties at least in part. At present, however, it is not clear whether tacrolimus simply depletes PGP9.5 in nerve fibers or destroy the fibers. Cutaneous sensory nerve fibers express vanilloid receptor subtype 1, and tacrolimus affects the receptors to activate the fibers (Senba et al., 2004; Stander et al., 2004), suggesting that the effects of tacrolimus on the nerve fibers could be exhibited directly (Stander and Luger, 2003). On the other hand, however, many reports have indicated that tacrolimus exhibits neuroprotective effects (Bavetta et al., 1999; Singleton et al., 2001) and that it enhances neurite outgrowth (Lyons et al., 1994; Varreau et al., 1997). Therefore, the inhibition of nerve fiber extension by tacrolimus observed in the present study may be an indirect action. Elucidation of the mechanisms involved in the inhibition of mouse scratching behavior by tacrolimus is now underway.

As it has been suggested that Th1/Th2 balance is skewed to Th2 in atopic diseases (Hartung et al., 2003), we examined the mRNA expression of IFN-γ, a typical Th1 cytokine, IL-4 and IL-5, important Th2 cytokines, in the present study. We indicated that repeated painting with a hapten, DNFB, resulted in the elevation of serum IgE and increased expression of IL-4 mRNA in the skin lesion. The elevated level of serum IgE is one of the typical features of atopic dermatitis patients, and the expression of IL-4 contributes to the elevation. However, both tacrolimus and dexamethasone failed to reduce the increased serum IgE levels, although they suppressed the expression of IL-4 mRNA in the skin lesion. Previously, we reported that immunosuppressants including tacrolimus do not inhibit IgE synthesis in mice (Nagai et al., 1997a). Furthermore, we confirmed that the IL-4 mRNA expression in the cervical lymph node is not inhibited by topically applied tacrolimus and dexamethasone (data not shown), suggesting that locally expressed IL-4 in the skin lesion does not seem to contribute to the systemic IgE production.

In conclusion, the mouse allergic dermatitis that accompanies frequent scratching behaviors elicited by repeated painting with DNFB seems to be beneficial for the basic study of human allergic dermatitis especially atopic dermatitis. Tacrolimus possesses an anti-itching property that is not shared by glucocorticoids. The mouse dermatitis model is also useful for the study of the anti-itching mechanisms of tacrolimus.

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ケモタキシスと治療

中村晃一郎

アトピー性皮膚炎(AD)の病変部にはT細胞，好酸球，肥満細胞などの浸潤を認め，これらの細胞がサイトカインやケモカインを産生することによって皮膚炎の増悪，かゆみの増悪を惹起していると考えられる。筆者らは，ADの病態における白血球走化因子としてのケモカインの作用が明らかにした。またADの標準治療薬として使用される副腎皮質ステロイド軟膏やタクロリムス軟膏などの免疫調節薬が，ケモカイン産生に対して抑制作用を有することを明らかにした。ADの病態におけるケモカインの作用について最近の知見を紹介した。(皮膚の科学，増*7*：*21*－*23*，*2006*)

キーワード：ケモカイン，*TARC*，*MDC*，*CTACK*，アトピー性皮膚炎

アトピー性皮膚炎とケモカイン

アトピー性皮膚炎(AD)は，増悪・寛解を繰り返し，瘙痒のある皮疹を主病変とする疾患であり，炎症の増強時には，病変部にT細胞を中心とした細胞浸潤を認め，これらが表皮での湿疹病変を形成する。この炎症の惹起，増悪には病変部より産生されるIL-10, GM-CSFなどのサイトカインや，soluble ELAM-1などの接着分子の発現が関与している。

ケモカインは，異なる細胞に特異的に遊走，活性化を誘導する低分子蛋白であり，それぞれが標的とする細胞が異なり，アレルギー反応での各免疫細胞の走化・活性を調整している。ケモカインとして代表的なものとして，IFN-γを産生するTh1細胞の活性化に作用するCXCL10/IP-10(interferon inducible protein)・CXCL9/Mig(monokine induced by interferon-γ)などがあり，IL-4，IL5を産生するTh2細胞の走化・活性化を誘導するケモカインとしてCCL17/TARC(thymus and activation regulated chemokine)，CCL22/MDC(macrophage derived chemokine)がある(**Table 1**)。筆者らは，これまでADの皮膚炎の増悪期において，病変部へのeffector T細胞，好酸球，肥満細胞の浸潤に，これらのケモカインが関与していることを明らかにしてきた。AD患者の血清には，Th2ケモカインであるCCR4陽性細胞に対して走化活性作用を有するCCL17/TARC，CCL22/MDC，また好酸球に対して走化活性を有するCCL26/eotaxin-3が上昇しており，健常人と比べて有意に高値を示している(**Fig. 1**)[1,2]。免疫組織学的にADの皮膚病変部で表皮ケラチノサイト，血管内皮細胞，浸潤細胞にCCL17発現を認めた。CCL26は好酸球の遊走を誘導するケモカインであり，末梢血好酸球数と相関していた。またCCL27/CTACK(cutaneous T cell attracting chemokine)はPSGL-1陽性T(Th1・Th2)細胞を走化活性化するケモカインであるが，AD患者でのCCL27の血中濃度は有意に増加し，組織学的に表皮ケラチノサイトに強い発現を認めた(**Fig. 2, 3**)[3]。

AD病変部におけるサイトカイン発現を検討すると，表皮ケラチノサイトにおいてGM-CSFの過剰産生があり，表皮ランゲルハンス細胞のIL-12産生調節関与している。ADの皮膚病変部におけるサイトカイン発現パターンは，急性病変でIL-5，IL-13mRNAなどのTh2サイトカインが優位であり，慢性病変部で，IFN-γ，IL-12mRNAなどのTh1サイトカインmRNAの発現が報告されており，皮膚病変の形成に，Th1・Th2サイトカイン両者の作用が重要であると考えられる。

また上述したように，表皮ケラチノサイト，浸潤細胞のCCL17，CCL22などの染色性が増強し，これらが病変部への細胞浸潤を誘導する。ケモカインの発現パターンは

Table 1 アトピー性皮膚炎の病態に関与する代表的なケモカインとその標的細胞

TARC・CCL17	CCR4陽性T細胞
MDC・CCL22	CCR4陽性T細胞
CTACK・CCL27	CCR10陽性T細胞
eotaxin-1・CCL11	好酸球
eotaxin-3・CCL26	好酸球

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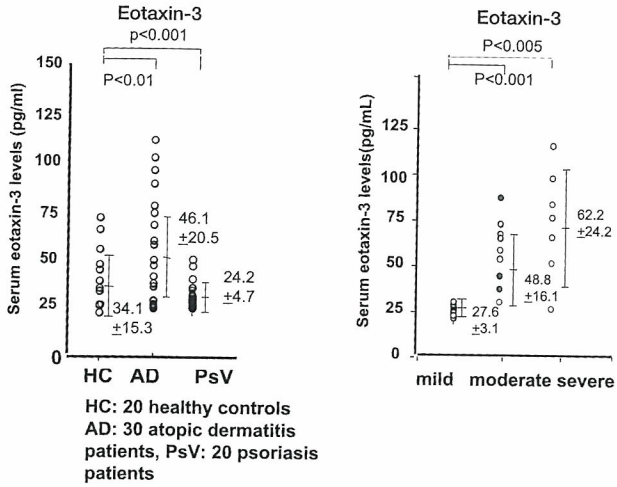

Fig. 1 アトピー性皮膚炎（AD）患者，乾癬患者，健常人における血清eotaxin-3/CCL26値。
AD患者における血清COL17値は高値であり，重症群で軽症群に比較して高値を示す。

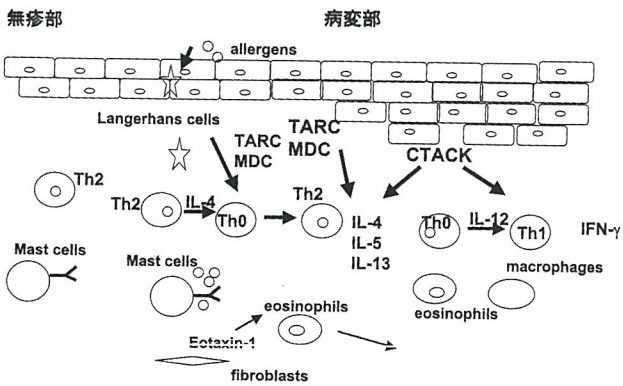

Fig. 3 アトピー性皮膚炎の炎症組織におけるケモカインの関与。

皮膚炎の増悪期，寛解期によって変化しており，IFN-γ，TGF-βなどのサイトカインによってその産生が調節されている[4]。ケモカインを産生する細胞は，表皮ケラチノサイト，T細胞，線維芽細胞，樹状細胞，肥満細胞など多彩である。

樹状細胞はそのサイトカイン産生，接着分子発現によって，Th0細胞をTh1細胞・Th2細胞に分化し，抗原をT細胞に提示し活性化する。Th1細胞の分化にはIL-12が，またTh2細胞への分化にはIL-4が必要である。筆者らはAD患者の末梢血単球を採取し，GM-CSF，IL-4刺激下で誘導した樹状細胞（MoDCs）がCCL22を大量に産生すること，さらにこのCCL22産生量がADの病勢を反映することを明らかにした。MoDCs（単球由来樹状細胞）は皮膚では真皮の樹状細胞に相当すると考えられることから，皮膚に存在している真皮樹状細胞がCCL22を産生して，ADの皮膚炎へのCCR4陽性T細胞の浸潤に関与していることが推測される。TSLP（thymic stromal lymphopoietin）は表皮より産生されるサイトカインであるが，樹状細胞のCCL17/TARC産生を増強することが報告されている。さらにTSLPをケラチン14に導入したTSLPトランスジェニックマウスでは，皮膚炎を自然発症し，病変部でCCL17，CCL22 mRNAの過剰発現が認められた[5]。このようなケモカインが，皮膚炎の発症においてきわめて重要であることが理解される。またマウスの表皮より作成したランゲルハンス細胞は，staphylococcus aureus刺激下にMIP-1αサイトカインを産生し，またCpG刺激下にIL-12産生を増強する。これらはT細胞の分化において樹状細胞の産生するケモカインが関与している可能性を示している。

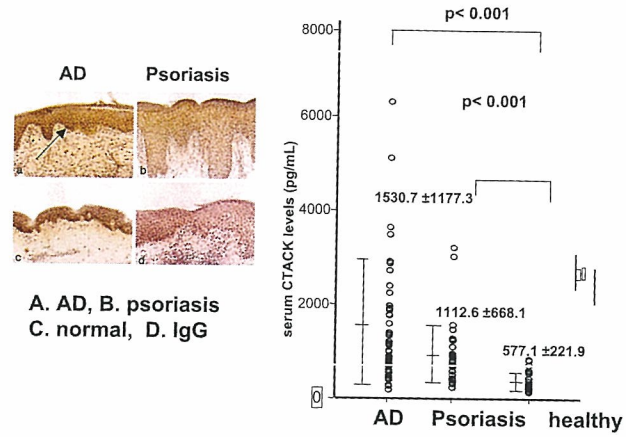

Fig. 2 アトピー性皮膚炎患者の血清CTACK/CCL27値。
AD患者における血清CCL27値は高値であり，表皮ケラチノサイトで産生される。

ADの治療とケモタキシス

ADの標準治療の主体は，乾燥に対するスキンケア，免疫調節薬を用いた薬物療法，原因抗原の除去である。このような標準治療を行なうことによって皮膚炎のコントロールが可能となる。治療薬におけるケモカインの産生能を*in vitro*で検討すると，副腎皮質ステロイド，タクロリムスは，ダニ抗原で刺激したAD患者から採取した末梢血単核球のCCL17産生能を著明に抑制することが明らかになった。また抗ヒスタミン薬にも，抗ケモカイン作用が存在することが明らかとなった。ADの増悪

Table 2 疾患における抗ケモカイン療法の試み

①抗ケモカイン療法		
eotaxin-1抗体（Bertilimumab, CAT-213）		
	アレルギー性鼻炎	第1，2相
	→鼻粘膜への好酸球が減少する	
②抗ケモカイン受容体療法		
CCR2抗体（millennium）（MLN-1202）		
	関節リウマチ	第1，2相
CCR3 antagonist（W-56750）	アトピー性皮膚炎	前臨床
CXCR3 antagonist（T-487）		
	関節リウマチ，乾癬	第1，2相
CXCR4 antagonist（AMD3100）	骨髄腫	第2相

因子として関与する抗原，反復する接触皮膚炎，ストレス，瘙痒などは，これらのサイトカイン・ケモカインによる炎症反応の増強を誘導し，皮膚炎を慢性化へ導く。副腎皮質ステロイド，タクロリムスなどの免疫調節薬は，CCL17，CCL22などのケモカイン産生を制御することによって，皮膚炎の鎮静化を誘導すると捉えることができる。このようにADの病態におけるケモカインの重要性が明らかになっており，同時にアレルギー疾患を標的とした抗ケモカイン療法なども試みられている。たとえば抗eotaxin抗体を用いたアレルギー性鼻炎の臨床試験が試行されており，アレルギー性疾患に対して治療開発が進められている(**Table 2**)。今後これらを標的とした治療法が，アレルギー疾患の治療薬として有効性が確立されることが期待される。

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Chemotaxis and Treatment of Atopic Dermatitis

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Key words：*Chemokine, TARC, MDC, CTACK, atopic dermatitis*

Atopic dermatitis (AD) is a recurrent chronic eczematous skin disease. Histoclogically AD shows a large number of T cells, eosinophils and mast cells infiltration into the lesional skin. Recently, chemokines are identified. Specific chemokines attract specific types of leukocytes in the inflammatory sites. TARC/CCL17, MDC/CCL22, CTACK/CCL27 participate in the pathogenesis of AD. High levels of serum CCL17 and CCL27 have been identified in AD patients, in accordance with the strong expression of these molecules in the lesional epidermis of AD patients. Corticosteroids and tacrolimus inhibit these productions in PBMCs of AD patients. TSLP, secreted by epidermal kertatinocytes, induce CCL17 and CCL22 mRNA expression in lesional skin in K14/TSLP transgenic mice. Future therapies targeting chemokines and chemokine receptors will be discussed.

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アレルギーをめぐるトレンド

ADにおける免疫調節薬—タクロリムス軟膏

TIM(topical immunomodulators)

タクロリムス軟膏は，カルシニューリン活性化を抑制する免疫調節薬であり，成人，小児アトピー性皮膚炎(atopic dermatitis；AD)患者に広く使用され，高い治療効果を示している．日本皮膚科学会のAD治療ガイドラインでもタクロリムス軟膏が組み入れられ，新しい治療が可能となった．海外のガイドラインでも寛解維持期における維持療法として長期間使用することが推奨されている．本薬剤はリンパ球の活性化を抑制するのみならず，皮膚免疫担当細胞のさまざまな機能を抑制することによって炎症反応を制御する．タクロリムス軟膏は分子量が大きいため健常皮膚からは吸収されず，炎症部位の皮膚のみで吸収されるなどステロイド外用薬と異なる特徴を有する．タクロリムス軟膏の有効性，安全性を十分に理解して使用することがADの治療において重要である．

カルシニューリン抑制薬の免疫学的作用

カルシニューリンは，細胞内でCaとカルモデュリンによって活性化され，転写因子NF-ATに結合して脱リン酸化したのちに核内に移行する．核内でNF-ATがサイトカイン産生を誘導し，IL-2，IL-4，IL-13などの発現を誘導する．タクロリムスは細胞内でFK binding protein(FKBP)と複合体を形成したのち，カルシニューリンに結合して，カルシニューリンの活性化を抑制する．このためNF-ATの脱リン酸化は阻害され，核内移行が抑制される．さらにタクロリムスの作用は，樹状細胞の抗原提示能の抑制，マスト細胞の脱顆粒抑制作用，好酸球脱顆粒抑制作用など多岐にわたる[1]．筆者らは，AD患者の末梢血中のNF-κB作用について検討し，タクロリムスが末梢血のNF-κB転写因子に対しても強い抑制作用を有することを認めている[2]．また，ADのアレルギー性炎症においてTh2細胞の皮膚への遊走に関与するthymus and activation-regulated chemokine(TARC)/CCL17，macrophage-derived chemokine(MDC)/CCL22などのケモカインの産生を抑制する[2]．このようにタクロリムスはTh1・Th2サイトカインに対して強い抑制作用を有し，ADの皮膚病変で炎症抑制作用を発揮する．

Trend in Allergy

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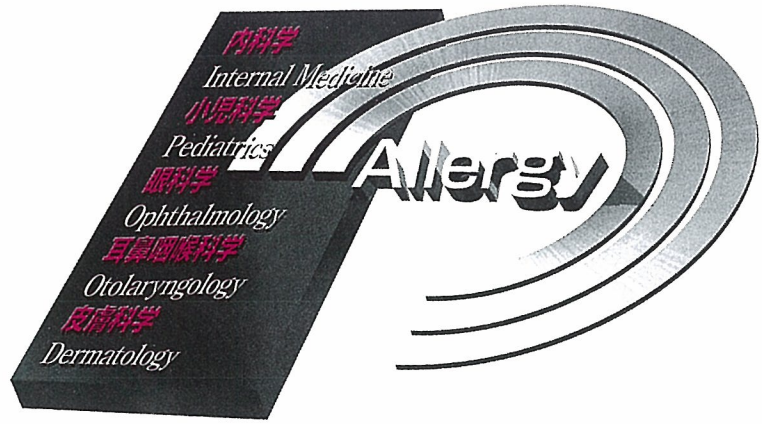

タクロリムス軟膏の効果

タクロリムス軟膏(0.1％)は1999年にわが国でADの治療薬として認可され，その後0.03％タクロリムス軟膏が小児用として承認された．本薬剤はADの紅斑，丘疹など皮膚炎，とくに顔面の紅斑に対して有効であり，短期観察のみならず長期観察でも高い治療有効性を発揮する．0.1％タクロリムス軟膏の2年間の追跡調査では，10週後で改善率が90％であり，さらに104週後で93.1％の改善率と長期間にわたる有効性が維持される．また，0.03％軟膏における小児AD患者で52週までの長期有効試験でも，90％以上の改善率であり，短期のみならず長期間でも有効性が高いエビデンスで示される[3,4]．

タクロリムス軟膏の使用法

日本皮膚科学会のタクロリムス軟膏ガイドラインでは，「対象患者はステロイド外用などの既存療法では効果が不十分または副作用によりこれらの投与ができないなど，本剤による治療がより適切と考えられるAD患者」とされる[5]．また海外でのガイドラインにおいても，タクロリムス軟膏が維持療法における治療薬として示されており，急性増悪期にステロイド軟膏を使用し，症状が持続する場合の維持療法として局所的なタクロリムス軟膏の長期投与，および間歇的なステロイド外用薬の使用が推奨されている[6]．強いランクのステロイド外用薬は，皮膚バリア機能が破壊された炎症症状が強い時期に使用し，タクロリムス軟膏は皮膚バリア機能が改善した状態で，長期間に使用することが推奨される(表1)．タクロリムス軟膏の特徴を表2にまとめる．

タクロリムス軟膏の有効持続期間についての調査を行うために，ステロイド軟膏と比較した研究では両外用薬の外用中止後，再燃するまでの期間を検討したところ，タクロリムス軟膏のほうが有意に再燃までの期間が長かったことが報告されている[7]．このため，週に数回の外用でも十分に効果が発揮できることが期待される．また顔面の皮疹に対する効果を高めるために，顔面の治療のみでなく全身における皮膚炎をコントロールしておくことが必要である．また抗アレルギー薬やTh2阻害薬などの併用によってタクロリムス軟膏の外用量を減量できることも報告されており，今後このようなエビデンスが明らかになると思われる．なお，詳細な使用方法に関しては使用ガイドラインを参照いただきたい(表3)[3]．

表1　ADの外用療法

急性増悪期の寛解導入(かゆみや炎症を早く抑える)
ステロイド外用薬 タクロリムス軟膏
寛解維持療法(長期)
長期の寛解維持にタクロリムス軟膏を使用 局所再発が生じたら，早い時期にタクロリムス軟膏を使用 間歇的にステロイド外用薬の使用
軽快(症状が出ていない)
保湿外用薬

(文献6より改変)

表2　タクロリムス軟膏の特徴

1. 短期間，長期間の投与において高い有効性を示す．
2. 強さは0.1％軟膏でステロイド外用薬のストロングクラスである．
3. 顔面の難治性紅斑に高い有効性を示す．
4. 分子量が大きく，皮膚バリア機能が破壊された時期に吸収されるが，皮膚炎が鎮静化してバリア機能が改善された時期には吸収量が減少する．
5. 血管拡張などのステロイド軟膏の長期投与によって生じる副作用をもたない．
6. タクロリムス軟膏の使用はステロイド軟膏の使用によって生じる副作用に対して副作用の頻度の減少が期待できる．

表 3　タクロリムス軟膏の使用量

0.1%タクロリムス軟膏	
16歳以上	1日1～2回，1回あたり5gまで
0.03%小児用タクロリムス軟膏	
2～5歳(20kg未満)	1日1gまで
6～12歳(20～50kg未満)	1日2～4g
13歳以上(50kg以上)	1日5gまで

タクロリムス軟膏の副作用

タクロリムス軟膏の使用において以下の副作用が報告されている．0.1％軟膏，0.03％小児軟膏の観察試験で毛嚢炎，カポジ水痘様発疹症，単純疱疹，伝染性膿痂疹などが報告されている．タクロリムス軟膏の外用回数は皮膚症状の改善に伴って減少するため，長期のコントロールにおいて，毛嚢炎，細菌感染症などの副作用の頻度は減少する[8]．また使用開始時に高頻度に灼熱感，ほてり感などの刺激感が認められるが，刺激症状は通常外用後一過性で皮疹の改善とともにまもなく発現しなくなる．刺激症状は温水など温度が高い場合に誘発されやすいことをあらかじめ患者に説明することは，治療上のコンプライアンスを高めるうえで有用である．

血中濃度に関しては，皮膚バリア機能が破壊された時期には吸収されるが，皮膚炎の改善に伴い短期間で血中濃度が検出限界以下になる．ただし Netherton 症候群などで皮膚バリア機能が破壊され長期間継続するような疾患では，長期に血中濃度の上昇が持続するため有害事象が長期に出現することがあるので注意が必要である．長期間投与による発癌性に関しては，米国アレルギー・喘息・免疫学会によれば，リンパ腫，皮膚癌の頻度は自然発症率に比較して高くなく，悪性腫瘍を増加させるエビデンスはこれまで報告されていない．通常の適切な使用量で使用すれば，長期投与による発癌性については問題ないと考えられる[8]．

まとめ

タクロリムス軟膏が国内で市販され，AD の治療における優れた治療成績が認められ，AD の標準治療における外用薬として認識されている．タクロリムス軟膏は，ステロイド軟膏と異なる抗炎症作用を有する免疫調節薬であり，AD の治療の選択肢が広がった．寛解維持期における長期投与の使用方法は，海外の AD 治療においても高いレベルでコンセンサスを得ている．このようにステロイド外用薬と使い分けを行いながら，長期維持療法で使用することによって，皮膚症状の長期コントロール，日常生活における QOL の改善が期待される．

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