

Contribution of itch-associated scratch behavior to the development of skin lesions in *Dermatophagoides farinae*-induced dermatitis model in NC/Nga mice

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Abstract Recently, we have reported that the pathophysiological features of dermatitis induced by the repeated application with *Dermatophagoides farinae* (Df) extract ointment in NC/Nga mice were similar to those observed in the patients with atopic dermatitis. In the present study, we first examined whether the application of Df in other mouse strains could induce dermatitis. The repeated application of Df body (Dfb) ointment to the barrier-disrupted back of ICR, C57BL/6, and Balb/c mice did not cause any apparent skin lesions, although transient increase in serum immunoglobulin E (IgE) levels during antigen application was observed. On the other hand, in NC/Nga mice, dermatitis scores and serum IgE levels increased remarkably, and then these changes sustained for at least 10 days after stopping of antigen elicitation. Using NC/Nga mice, we investigated the contribution of scratching behavior to the development and maintenance of Dfb-induced dermatitis. In correlation with the increase in scratching behavior, erythema, hemorrhage, edema, scarring, erosion and excoriation were

observed. Cutting off the hind toenails of mice exhibiting chronic skin lesions dramatically alleviated the dermatitis. From these findings, the onset of skin lesions and its chronically sustained course in Dfb-induced dermatitis in NC/Nga mice were closely associated with increased scratching behavior.

Keywords NC/Nga mice · *Dermatophagoides farinae* · Scratching · Itch · Atopic dermatitis

Introduction

Atopic dermatitis is a chronically relapsing inflammatory skin disease characterized by intense itching, and causes physical and emotional distress for patients and their families [9]. The prevalence of atopic dermatitis has increased steadily in recent decades [3].

We have recently established a novel animal model for atopic dermatitis using the repeated application of *Dermatophagoides farinae* (Df) extract ointment in NC/Nga mice [17]. All of the clinical, histological, and immunological features in this model are similar to the events observed in patients with atopic dermatitis. We have used NC/Nga mice to induce atopic dermatitis-like skin lesions because this mouse strain has a predisposition to spontaneously develop dermatitis in non-specific pathogen-free conventional conditions [6, 10, 12], but have not investigated the potential of other mouse strains to develop dermatitis induced by the topical application of Df. Therefore, in the present study, we examined whether the repeated application of Df body (Dfb) ointment in ICR, C57BL/6, and Balb/c mice could induce an increase in dermatitis score, histological changes in epidermis and dermis, and elevation of total and Df-specific immunoglobulin E (IgE).

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In patients with atopic dermatitis, pruritus is a major troublesome symptom, and the itch-scratch cycle is known to aggravate skin lesions [13]. In a preliminary study, we observed the scratching behavior of NC/Nga mice at the sites of antigen application even after the first challenge with Dfb ointment. Continuous scratching behavior was also observed in NC/Nga mice with chronic dermatitis. Moreover, we previously reported that the established skin lesions in the Df-induced dermatitis model in NC/Nga mice lasted for at least 2 weeks without further elicitation with the allergen [17]. Therefore, it is plausible that the onset of dermatitis and its chronically sustained course in NC/Nga mice relate to itch-associated scratching behavior. Here, we investigated the contribution of scratching behavior to the development of dermatitis by evaluating the time-dependent changes in scratching behavior and dermatitis score, and the effect of clipping toenails on the established dermatitis.

Materials and methods

Mice

Specific pathogen free female NC/Nga, ICR, C57BL/6, and Balb/c mice were purchased from Charles River Japan (Yokohama, Japan). These mice were all housed under conditions of controlled temperature (20–26°C), humidity (30–70%), lighting (lights on from 06:00 to 18:00), and room air ventilation (at a rate of 10 times/h or more through high efficiency particulate air filter). Food and tap water were provided ad libitum. All mice were 11–15 weeks old. The animal experiments in this study were approved by the Animal Care and Use Committee of Shionogi Research Laboratories.

Dermatophagoides farinae body ointment

Dermatophagoides farinae body ointment (Dfb ointment) was prepared by Biostir Inc. (Kobe, Japan). One gram of Dfb ointment contained 136.4 mg protein, 234 µg Der f 1, and 7 µg Der f 2.

Induction of dermatitis

On the first induction (Day 0), the rostral part of the back skin of the mice was clipped using an electric clipper, and residual hair was depilated using a hair removal cream under ether anesthesia. Antigen challenge was performed by topical application of 100 mg Dfb ointment or ointment base (hydrophilic petrolatum) on the dorsal shaved skin and the surface of both ears. From the second induction downward, after the removal of growing hair using an electric

clipper and an electric shaver, barrier disruption was achieved by 150 µL of 4% sodium dodecyl sulfate treatment on the shaved dorsal skin and the surface of both ears 3 h before Dfb ointment application. These procedures were repeated twice a week. Following the last antigen challenge on Day 17, the mice were killed on Day 21 or 31, and immunological and histological changes were investigated.

Scoring of dermatitis

The development of (1) erythema/hemorrhage, (2) scarring/dryness, (3) edema, (4) excoriation/erosion was scored as 0 (none), 1 (mild), 2 (moderate), and 3 (severe). The sum of the individual scores was taken as the dermatitis score.

Histological observation of the skin

Portions of the dorsal skin were fixed with 10% neutral formalin, embedded in paraffin, and sectioned at 4-µm thickness. Sections were stained with hematoxylin–eosin or toluidine blue (pH 4.1).

Measurement of serum total Immunoglobulin (Ig) E and specific IgE to Dfb

Sera were collected by drawing blood from the heart under ether anesthesia. The concentration of total IgE was measured using a mouse IgE EIA kit (Yamasa, Tokyo, Japan), according to the manufacturer's protocol. Specific IgE to Dfb was evaluated using passive cutaneous anaphylaxis (PCA). Briefly, female Wistar rats (Japan SLC, Hamamatsu, Japan), which were used as recipients, were sensitized by intradermal injection of 50-µL serially diluted sera on shaved dorsal skin. The rats were challenged 24 h later by intravenous injection of 0.5 ml saline containing 1 mg Dfb and 5 mg Evans blue. Thirty minutes later, the animals were sacrificed by exsanguination under deep anesthesia. The pigmented area was measured from the inner side of the skin. An area more than 5 mm in diameter was regarded as a positive reaction, and the antibody titer was determined.

Behavioral observation

Mice were placed in an acrylic cage, and their behavior was recorded under unmanned conditions using a video camera with an infrared lamp and a hard disc recorder. The recording was performed from 18:00 to 02:00. Scratching of the upper back and ears by the hind paws was counted. Since the mice generally showed several scratches for about 1 s, a series of the behavior was counted as one incident of scratching.

Immunohistochemical staining with anti-protein gene product (PGP) 9.5 antibody

Biopsy specimens were taken from the rostral back skin of normal mice and mice with dermatitis on Day 21. Skin samples were embedded in Tissue-Tek O.C.T. compound (Sakura, Torrence, CA, USA), and sectioned using a Cryotome (ThermoShandon Ltd, UK) at 5 µm thickness. The sections were pretreated with 0.3% H₂O₂/methanol for 15 min, and incubated with non-specific blocking reagent for 10 min. This was followed by overnight incubation with rabbit polyclonal anti-PGP9.5 antibodies (Biogenesis Ltd, UK; 1:1,000) in 0.05 M phosphate buffer (pH 7.6) with 1% BSA at 4°C. The samples were then reacted with peroxidase-labeled-dextran polymer anti-rabbit goat antiserum (Dako, Glostrup, Denmark). PGP 9.5-immunoreactive nerve fibers were visualized under an optical microscope using 3-3'-diaminobenzidine-4 HCl. Finally, the sections were counterstained with hematoxylin.

Trimming of toenails

To examine the effect of clipping of toenails on established dermatitis in NC/Nga mice, the tips of all toenails on both hind paws in mice were trimmed twice a week with nail clippers, and smoothed with a nail file under ether anesthesia.

Statistical analysis

Data were expressed as means ± S.E. The statistical significance of differences was assessed by a Student's *t* test or Dunnett's test, except for the dermatitis scores. Statistical analysis of the dermatitis scores was performed by pairwise comparisons based on repeated measurement ANOVA with a mixed-effects model. In all tests, *P* values less than 0.05 were considered statistically significant.

Results

Development of atopic dermatitis-like skin lesions in various mouse strains

To validate the usefulness of NC/Nga mice for the induction of atopic dermatitis-like skin lesions, we compared the development of dermatitis after Dfb application in NC/Nga mice to that in ICR, C57BL/6, and Balb/c mice. The dermatitis scores of NC/Nga mice increased by the repeated application of Dfb, and became remarkable 2 weeks after the first elicitation and sustained until Day 31 even 2 weeks after stopping antigen elicitation (Fig. 1). Clinical symptoms appearing in NC/Nga mice included acanthosis, erythema, hemorrhage, edema, scarring, erosion, and excoriation. On the other hand, no obvi-

ous skin lesions were observed in other mouse strains (Fig. 2). The application of ointment base alone after barrier disruption (control group) did not cause significant dermatitis in either strains of mice (data not shown).

Histological evaluation

Histological changes in the dorsal skin on Days 21 and 31 were investigated in all mouse strains. As a result, a marked thickening of the dermis and epidermis, rete ridge formation, hyperkeratosis, and parakeratosis were obvious in the lesional skin of NC/Nga mice on Day 21 (Fig. 3a). The number of dermal inflammatory cells including mast cells also increased (Fig. 3b). Moreover, the histological changes in NC/Nga mice on Day 31 were almost similar to those on Day 21. On the other hand, a mild thickening of the epidermis and mild infiltration of inflammatory cells including mast cells were observed in other mouse strains when assessed on Day 21 (Fig. 3a,b), but these changes became slight or almost returned to the normal level on Day 31 (data not shown). There were hardly histological changes in the ointment base-treated control group of each mouse strain (data not shown).

Serum total IgE and specific IgE to Dfb

Serum levels of total IgE and specific IgE to Dfb in NC/Nga, ICR, C57BL/6, and Balb/c mice on Days 21 and 31

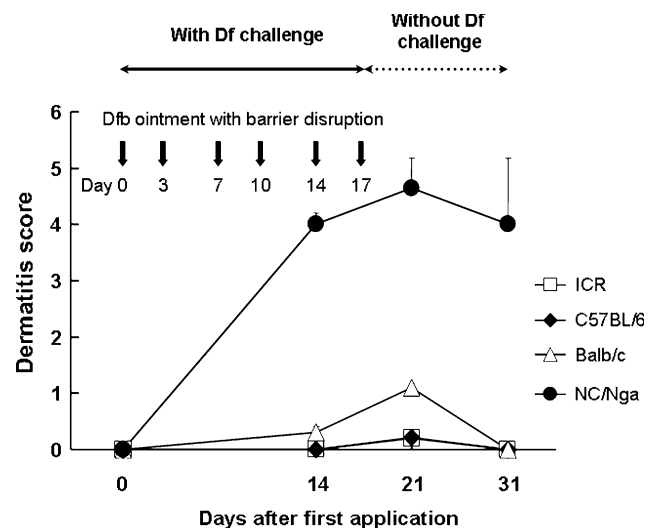


Fig. 1 Development of atopic dermatitis-like skin lesions after Dfb application in ICR (open rectangles), C57BL/6 (closed diamonds), Balb/c (open triangles), and NC/Nga (closed circles) mice. Elicitation was performed by the topical application of 100 mg Dfb ointment on the shaved dorsal skin and the surface of both ears. Barrier disruption was achieved by 150 µL of 4% sodium dodecyl sulfate treatment on the shaved dorsal skin and the surface of both ears 3 h before Dfb ointment application. These procedures were repeated on Days 0, 3, 7, 10, 14, and 17. Dermatitis scores were assessed on Days 14, 21, and 31. Data represent means ± SE of 8–10 mice

Fig. 2 Skin features of each mouse strain on Day 21

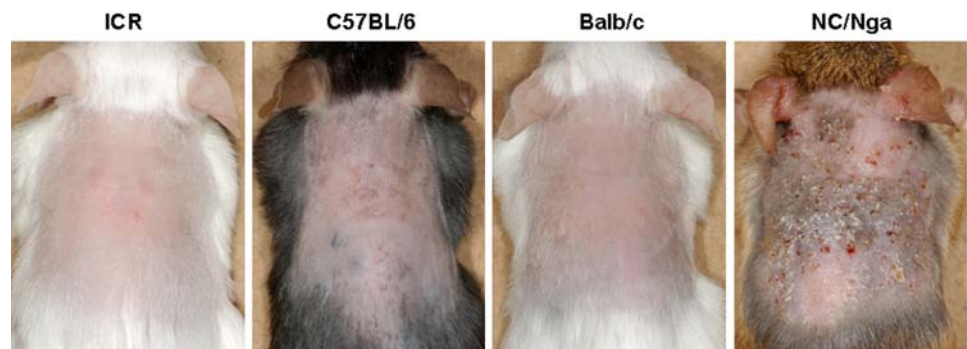
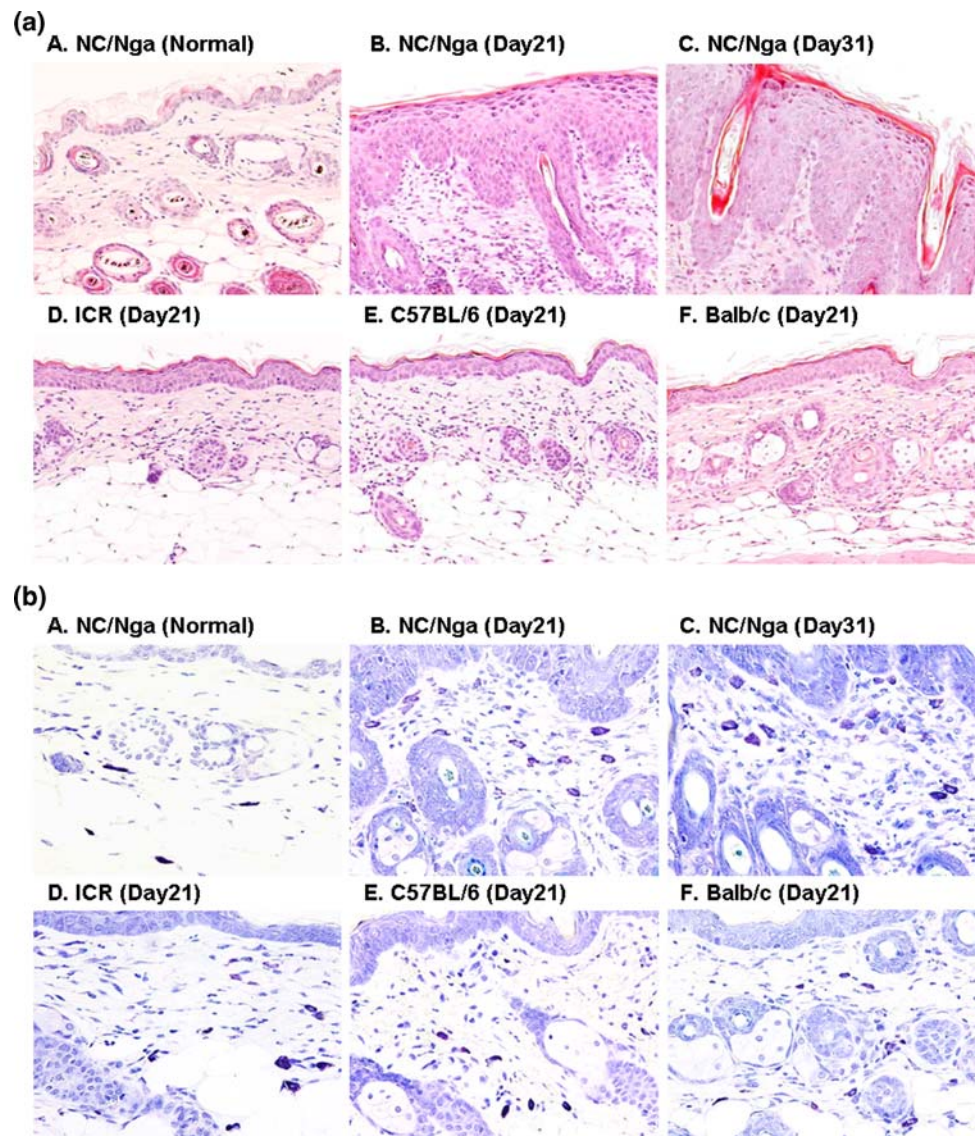


Fig. 3 Histological changes of the dorsal skin after Dfb application in ICR, C57BL/6, Balb/c, and NC/Nga mice. The dorsal skin of normal NC/Nga mice (A), Dfb ointment with barrier disruption-treated mice from each strain on Day 21 (B, D–F), Dfb ointment with barrier disruption-treated NC/Nga mice on Day 31 (C) were stained with hematoxylin–eosin (a) and toluidine blue (b). Original magnifications $\times 100$ for (a) and $\times 200$ for (b)



were examined. Total and specific IgE levels in all mouse strains treated with Dfb ointment were significantly elevated compared with normal mice and the control group, respectively, on Day 21 (Fig. 4a, b). On Day 31, total and specific IgE levels in NC/Nga mice were still high, but those in other mouse strains were clearly reduced.

Time course changes in scratching counts and dermatitis scores in NC/Nga mice

Figure 5a shows the changes in scratching behavior induced by the repeated application of Dfb ointment in NC/Nga mice. None of the skin symptoms were

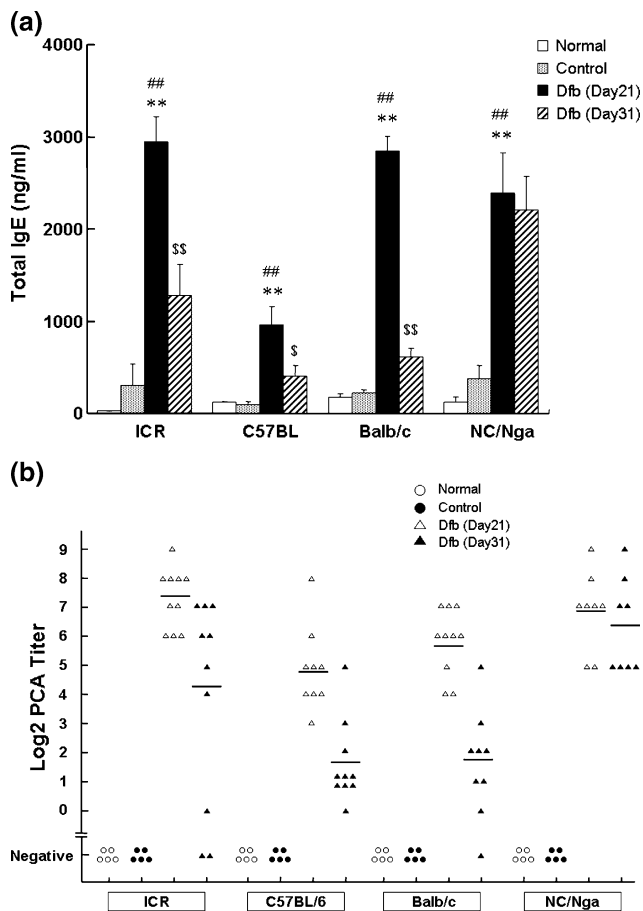


Fig. 4 Serum levels of total IgE (a) and specific IgE to Dfb (b) in normal, ointment base with barrier disruption-treated (control), and Dfb ointment with barrier disruption-treated (Dfb) ICR, C57BL/6, Balb/c, and NC/Nga mice on Days 21 and 31. Total IgE data represent means $\pm$ SE of 5–10 mice. $**P < 0.01$ compared with the normal group, $##P < 0.01$ compared with the control group, $^SP < 0.05$ and $^{SS}P < 0.01$ compared with the Dfb group on Day 21 (Student's *t* test)

observed, but scratching behavior of NC/Nga mice tended to increase compared with that of normal mice on Day 1. On Day 6, the number of scratching significantly increased, although only mild skin dryness was observed on Day 7. Thereafter, according to the increased scratching behavior, the dermatitis score gradually increased (Fig. 5b).

Maintenance of dermatitis score and scratching behavior in NC/Nga mice with established skin lesions

The dermatitis score of NC/Nga mice having demonstrated dermatitis with scores over 3 on Days 14 and 21 did not decrease at least until Day 31 even 2 weeks after stopping antigen elicitation (non-treated group in Fig. 7a). Furthermore, the increased number of scratching on Day 21 (847 ± 105 counted for 8 h) was maintained until Day 31 (828 ± 181 counted for 8 h).

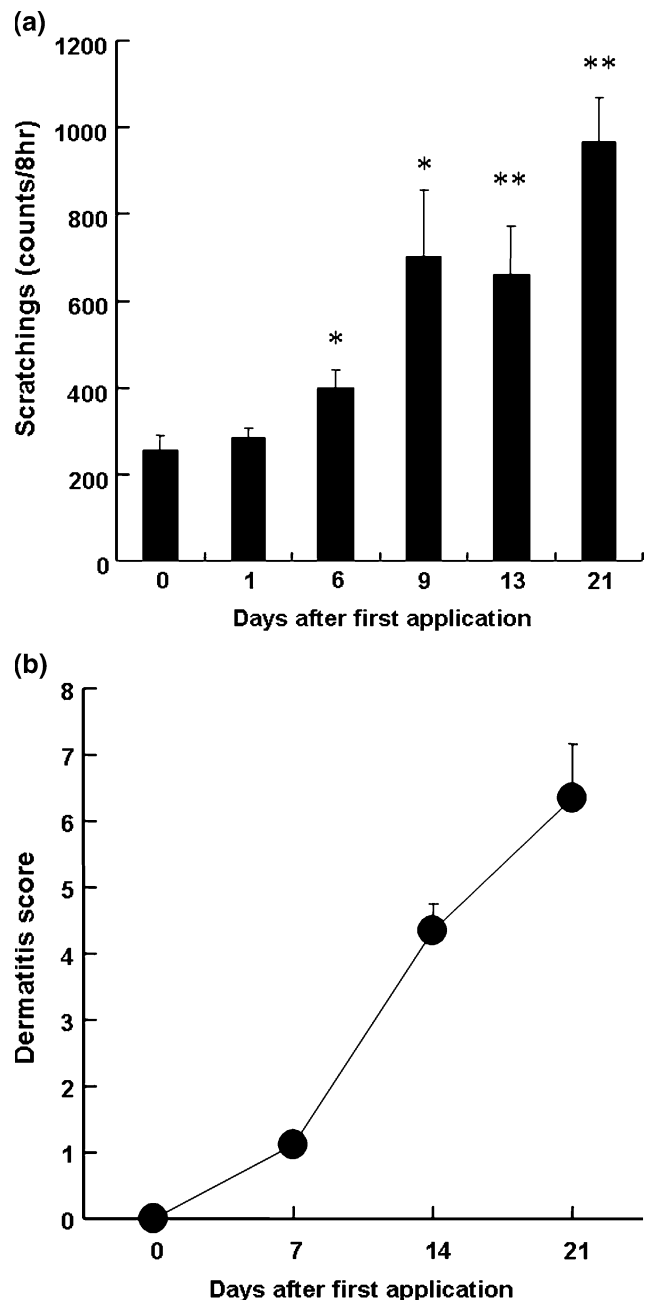
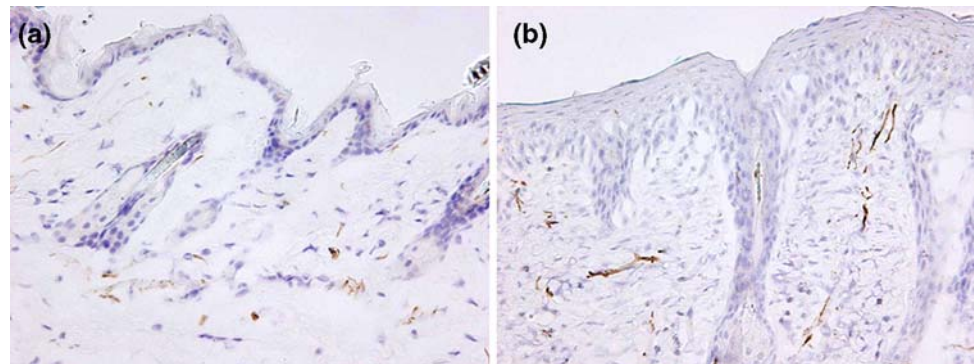


Fig. 5 Time course changes in scratching behavioral counts (a) and dermatitis scores (b) of NC/Nga mice. Total scratching counts were measured for 8 h on Days 0, 1, 6, 9, 13, and 21. Dermatitis scores were assessed on Days 0, 7, 14, and 21. Data represent means $\pm$ SE of 7–10 mice. $*P < 0.05$, $**P < 0.01$ compared with Day 0 on each day (Student's *t* test)

Immunohistochemical investigations

To investigate the distribution of nerve fibers in the skin, immunohistochemical analysis was performed using the anti-PGP9.5 antibody. In normal NC/Nga mice, some of PGP9.5-positive nerve fibers were observed in the dermis, and few were located in the epidermis (Fig. 6). On the

Fig. 6 Immunohistochemical staining of PGP9.5 in the normal skin (a) and established skin lesions on Day 21 (b) in NC/Nga mice. Original magnification $\times 200$



contrary, the number of PGP9.5-positive nerve fibers clearly increased in the dermis, especially in the upper dermis, and sprouted into the epidermis in the established skin lesions of NC/Nga mice on Day 21.

Effect of tipping toenails on established dermatitis

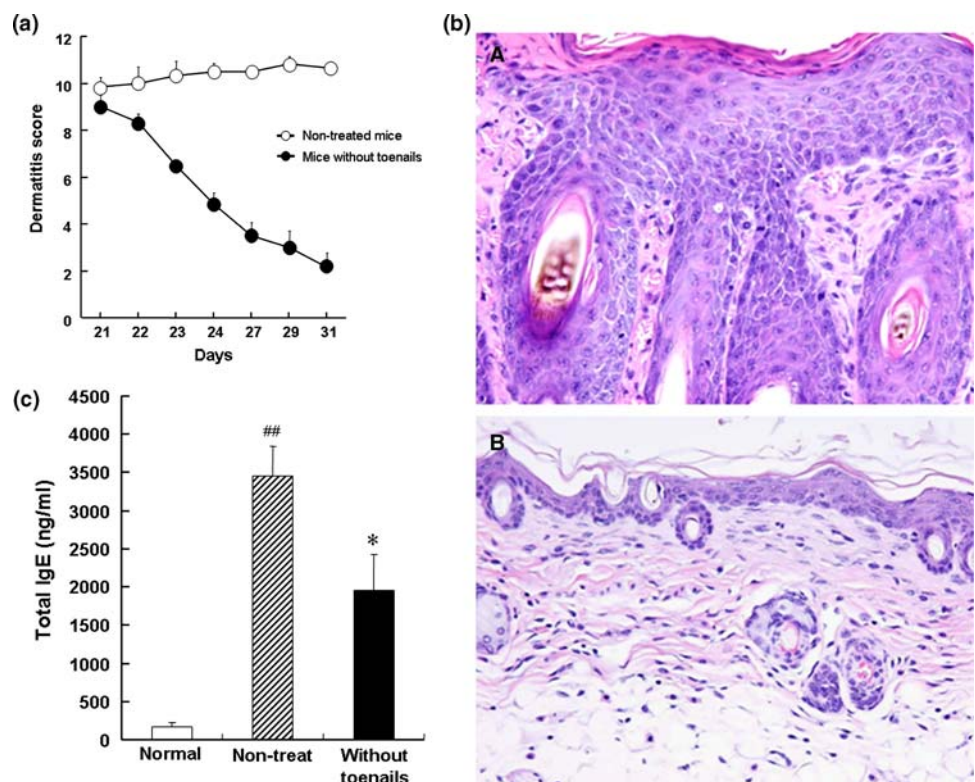
To examine the involvement of the scratching behavior in the maintenance of dermatitis without further antigen application, the hind toenails of NC/Nga mice with chronic dermatitis were cut off on Day 21. The dermatitis score clearly improved immediately after tipping the hind toenails, and continued to decrease from Day 21 to 31 (Fig. 7a). Histologically, in the group of mice without toenails, epidermal thickening and infiltration by inflammatory cells in the dermis were very slight compared with those in the non-treated

group on Day 31 (Fig. 7b). Moreover, IgE levels in serum collected on Day 31 were significantly reduced compared with the non-treated group (Fig. 7c).

Discussion

We recently developed a novel animal model for atopic dermatitis by the repeated application of Df extract ointment in NC/Nga mice [17]. All of the clinical, histological, and immunological features in this model are similar to the events observed in atopic dermatitis. We used NC/Nga mice to induce atopic dermatitis-like skin lesions, but have not investigated the potential of other strains of mice to develop dermatitis induced by the topical application of Df yet. In the present study, we demonstrated that the repeated

Fig. 7 Effect of tipping of toenails on established dermatitis in NC/Nga mice. The hind toenails of mice were trimmed on Days 21, 24, and 28 without further antigen application. **a** Time course changes in dermatitis score of mice without toenails and non-treated mice. Dermatitis scores were assessed on Days 21, 22, 23, 24, 27, 29, and 31. **b** Histological changes of the dorsal skin on Day 31. The dorsal skin of non-treated mice (A) and mice without toenails (B) was stained with hematoxylin–eosin. Original magnification $\times 400$. **c** Serum levels of total IgE in normal mice, non-treated mice, and mice without toenails on Day 31. Data represent means $\pm$ SE of 6 mice. $^{##}P < 0.01$ compared with the normal group. $^{*}P < 0.05$ compared with the non-treated group



application of Dfb ointment in ICR, C57BL/6, and Balb/c mice could hardly induce an increase in the dermatitis score and histological changes in the epidermis and dermis. Therefore, it is suggested that NC/Nga mice are appropriate for the induction of dermatitis by the repeated application of Dfb ointment.

Next, we investigated the contribution of the scratching behavior to the Dfb-induced dermatitis model in NC/Nga mice. The scratching behavior of this model significantly increased compared with that of normal mice before the development of clinical symptoms became obvious on Day 14. Thereafter, depending on the increase in the scratching behavior, erythema, hemorrhage, edema, scarring, erosion, and excoriation were observed in this model. Furthermore, the established skin lesions in the Dfb ointment-induced dermatitis model in NC/Nga mice, having demonstrated dermatitis with scores over 3 on Days 14 and 21, lasted for at least 10 days without further topical application of the allergen, and were dramatically improved by cutting off the hind toenails of mice. It is suggested that the onset of dermatitis and chronically sustained course in this model were closely associated with the increase in scratching behavior. Hashimoto et al. [4] have demonstrated that NC/Nga mice develop dermatitis in specific pathogen-free conditions when mice are kept together with NC/Nga mice with lesional skin, and the induction of scratching behavior plays the most important role for the development of dermatitis in these experimental conditions. Therefore, the development of dermatitis in NC/Nga mice could be associated with an increased scratching behavior despite the methods for induction of dermatitis [1, 15]. To confirm the critical role of the increase in scratching behavior on the onset of dermatitis, further study that examines the effect of cutting off the hind toenails on the development of skin lesions in our model is required. Interestingly, total serum IgE and specific IgE to Dfb remained high for at least 10 days in NC/Nga mice with established skin lesions by the repeated application of Dfb ointment, although both of the IgE levels in ICR, C57BL/6, and Balb/c mice were not maintained without further topical application of allergen. Moreover, by cutting off the hind toenails of NC/Nga mice with chronic dermatitis, total serum IgE decreased. It was reported that the subcutaneous injection of the soluble fraction obtained from the keratinocyte cell line, PAM 212, derived from BALB/c mice, to the same mouse strain induced an increase in serum IgE levels without the contribution of an exogenous antigen, and high level of serum IgE lasted for at least 10 weeks [18]. Moreover, the extract from PAM 212 also induced IgE production, and epsilon germ-line transcript in splenic lymphocytes of Balb/c mice under the Th2 dominant state [11]. Therefore, it is suggested that damage/stimulation of keratinocytes by scratching behavior induces the release of factors from

keratinocytes, and then these factors maintain a high level of IgE without further elicitation by the allergen. From these findings, we conclude that scratching behavior might contribute to the maintenance of not only chronic dermatitis but also sustained IgE production.

Next, we confirmed that nerve fibers were increased in the epidermis and upper dermis of lesional skin in the Dfb ointment-induced dermatitis model in NC/Nga mice. Because sprouting of intraepidermal neurons occurs in patients with atopic dermatitis, which is partly responsible for the intense itch sensations [14, 16], innervation to the epidermis and upper dermis in our model may be caused by continuous scratching behavior. In this study, repeated application of Dfb ointment in ICR, C57BL/6, or Balb/c mice could hardly induce dermatitis. Therefore, it is possible that Dfb could not induce itch sensation, and the sprouting of neurons in the other mouse strains except for NC/Nga mice. For further evaluation, we need to examine the scratching behavior and the distribution of nerve fibers in other strains.

Itching is a characteristic symptom, and is one of the major diagnostic criteria of atopic dermatitis [8]. Severe pruritus often causes sleep disruption, irritability, and generalized stress for patients and family when affected by atopic dermatitis [13]. Although antihistamines are widely prescribed to manage pruritus associated with atopic dermatitis and improve itching, their efficacy is marginal [2, 5, 7], suggesting that other known or unknown mediators than histamine are also involved in pruritus observed in atopic dermatitis. Indeed, an antihistamine did not show a significant effect on itch of our model in the preliminary study (data not shown). Thus, the precise mechanisms and mediators of itch are unclear at present. In this study, we demonstrated that the onset of dermatitis and chronically sustained course in the Dfb ointment-induced dermatitis model in NC/Nga mice were closely associated with increased scratching behavior, like itch-scratch cycle observed in human atopic dermatitis. These findings strongly suggest that our model is useful for not only elucidating the precise mechanisms and mediators of itch but also evaluating newly developed drugs for pruritus in atopic dermatitis.

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