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**Biphasic increase in scratching behavior induced by topical application of
Dermatophagoides farinae extract in NC/Nga mice**

Yoshihito Yamada¹, Yuhki Ueda¹, Aki Nakamura¹, Shoji Kanayama¹, Rie Tamura¹, Kei Hashimoto¹, Hiroko Kido¹, Tatsumi Matsumoto¹ and Ritsuko Ishii²

¹Drug Development Research Laboratories, Kyoto R&D Center, Maruho Co., Ltd., Kyoto, Japan

²Strategic Research Planning & Management department, Kyoto R&D Center, Maruho Co., Ltd., Kyoto, Japan

Correspondence: Yoshihito Yamada, Drug Development Research Laboratories, Kyoto R&D Center, Maruho Co., Ltd., 93 Awata-cho, Shimogyo-ku, Kyoto, 600-8815, Japan, Tel.: +81-75-325-3255, Fax: +81-75-325-3222, e-mail: yamada_dgc@mii.maruho.co.jp

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Abstract

Atopic dermatitis (AD) is a chronic inflammatory skin disease accompanied by severe itching and eczematous lesion. In this study, we applied an ointment containing *Dermatophagoides farinae* extract (Dfb) repeatedly on the dorsal skin of NC/Nga mice with barrier disruption to investigate the characteristics of this murine model of human AD. Following repeated topical application of Dfb ointment twice weekly for 2 weeks, the dermatitis score increased gradually, accompanied by an elevation of total immunoglobulin E level in plasma. Topical application of Dfb ointment also caused epidermal hyperplasia and accumulation of inflammatory cells in the lesional skin, and increased expression of T helper (Th) 1/Th2/Th17 cytokines in axillary lymph node cells. Furthermore, increased sprouting of intraepidermal nerve fibers was observed with an increase in the content of nerve growth factor and decrease in that of semaphorin 3A in the lesional skin. These findings suggest that the characteristics in this model were similar to those observed in patients with AD. Interestingly, it was observed for the first time that scratching behavior increased in a biphasic fashion by topical application of Dfb ointment in addition to an increase in spontaneous scratching behavior in this model. It is also suggested that further clarifying the underlying mechanisms of scratching behavior in this model leads not only to elucidating the pathogenesis of AD but also to discovering novel therapeutic drugs for AD.

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Key words: atopic dermatitis·house dust mites·nerve fibers·cytokines

Introduction

Atopic dermatitis (AD) is a common, chronic inflammatory skin disease accompanied by severe itching and eczematous lesion, and incidence of AD has been increasing gradually (1,2). The pathogenesis of AD has been attributed to a complex interaction between the skin barrier disruption caused by filaggrin mutation and the abnormal immune response to environmental allergens (3,4). Intrinsic abnormalities in skin barrier function allow the invasion of environmental allergens such as house dust mites, which are well known as major allergens in human AD, since patients with AD have high titers of specific immunoglobulin E (IgE) and show positive response on patch test to house dust mites (5-7). Immunosuppressive drugs such as topical corticosteroids and calcineurin inhibitors have been used in the treatment of AD (4). However, the use of these drugs is limited due to adverse effects such as skin atrophy and burning sensation in some AD patients (8-11). Therefore, novel therapeutic drugs without adverse effects are desired, and animal models similar to human AD are required to investigate the pharmacological effects of new drugs.

In NC/Nga mice, the skin lesions similar to human AD are spontaneously developed with scratching behavior, and IgE levels are elevated beginning at 8 weeks of age, which are accompanied by the infiltration of various inflammatory cells into the lesional skin under conventional conditions (12,13) and experimentally elicited by treatment with *Dermatophagoides* mites extract even under specific pathogen-free (SPF) conditions (14,15). These symptoms can also be induced by repeated topical application of an ointment containing *Dermatophagoides farinae* body (Dfb) extract under SPF conditions (16,17). *Dermatophagoides* species such as *Dermatophagoides farinae* are the most prevalent mites on the skin of AD patients and the source of the most potent allergens (18-20), suggesting that the AD-like murine model induced by repeated topical application of the ointment containing Dfb extract is thought to be useful to elucidate the pathogenesis of AD.

In previous reports (16,17), the dermatitis in this murine model of AD was accompanied by elevated serum IgE levels and inflammatory cells accumulation in the lesional skin. This model also showed increased expression of T helper 1 (Th1; interferon (IFN) γ) and T helper 2 (Th2; interleukin (IL) -5 and IL-13) cytokines in lymph node cells and spontaneous scratching behavior. However, production of other Th1/Th2 cytokines and time course of scratching behavior induced by the topical application of Dfb ointment remains unclear. Therefore, to clarify the characteristics of Th1/Th2/Th17 cytokine

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profiles, scratching behavior and nerve fiber elongation in this model, we examined production of Th1/Th2/Th17 cytokines (IFN- γ , IL-2, IL-4, IL-6, IL-10, IL-17A, tumor necrosis factor (TNF) - α) in axillary lymph node, the number of scratching behavior for 24 hours after topical application of Dfb ointment and changes in endogenous pruritogens and axonal guidance molecules in the lesional skin. Interestingly, we found that the number of scratching behavior increased in a biphasic fashion after topical application of Dfb ointment on the back skin, which was observed for the first time in this AD-like model.

Materials and methods

Animals

Female 7-week-old NC/Nga mice were purchased from Charles River Laboratories Japan Inc. (Kanagawa, Japan). The mice were housed in the specific pathogen free (SPF) animal facility under conditions of controlled temperature ($23 \pm 3^{\circ}\text{C}$), relative humidity ($50\% \pm 20\%$) lighting (lights for 12 hours, 7:00 to 19:00). They were given a laboratory diet (CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan) and ultraviolet sterile water *ad libitum*. In some experiments, the light/dark cycle of the animal room was reversed (lights for 12 hours, 19:00 to 7:00). All of the animal experimental procedures were approved by the Ethics Committee for Animal Experiments of Maruho Co., Ltd., and

conducted in accordance with the Guiding Principles for the Care and Use of Laboratory

Animals at Maruho Co., Ltd.

Induction of dermatitis

AD-like skin lesion was induced in NC/Nga mice as described by Yamamoto et al. (16, 17) with some minor modifications. Under isoflurane anesthesia, the back of mouse was shaved with an electric clipper, and residual hair was removed using a depilatory cream on day 0. The dorsal skin was then challenged with topical application of an ointment containing Dfb extract (Biostir® AD, Biostir Inc., Kobe, Japan) or the base (hydrophilic petrolatum, Nikko Pharmaceutical Co., Ltd., Gifu, Japan). The application of Dfb ointment was repeated twice a week for 2 weeks (day 3, 7, 10 and 14). The skin barrier was disrupted with 150 µL of 4% w/v sodium dodecyl sulfate (SDS) 2 hours before each application of the Dfb ointment. Only mice with dermatitis score of 5 or more on day 14 were used for experiments, and subjected to the final application of Dfb ointment on day 17 for histological investigation of the skin. Some mice were maintained until day 21 or 22 for measurement of the spontaneous scratching behavior.

Evaluation of dermatitis

The severity of dermatitis was evaluated prior to every barrier disruption. The developments of 1) hemorrhage, 2) dryness and 3) hyperplasia were respectively scored as 0 (none), 1 (mild), 2 (moderate) and 3 (severe). The sum of the individual scores was taken as the dermatitis score (minimum: 0, maximum: 9) in each mouse.

Measurement of plasma total IgE

Blood samples were repeatedly collected from the caudal artery on day 0, 7 and 13.

Plasma was separated by centrifugation at 10,000 rpm for 10 minutes at 4 °C and stored at -80°C until use. The concentration of total IgE was measured using a Mouse IgE enzyme-linked immunosorbent assay (ELISA) Quantitation Set (Bethyl Laboratories, Inc., Montgomery, TX, USA), according to the manufacturer's protocol. The absorbance at 450 nm was determined by using a microplate reader (Molecular Devices, LLC., Sunnyvale, CA, USA).

Histological analysis

The dorsal skins were excised 24 hours after final application of the Dfb ointment (day 18). The skin samples were fixed with 4% paraformaldehyde, embedded in paraffin and sectioned into 4 µm slices. The sections were stained with hematoxylin and eosin (HE) for measurement of the epidermal thickness, and toluidine blue (TB) for measurement of the

number of mast cells. The epidermal thickness was measured in 5 random fields, 3 sites per field, per mouse at a magnification of $\times 100$ using a cellSens imaging software (Olympus Corporation, Tokyo, Japan). The average length of the epidermal thickness was calculated from the 15 measurements in each mouse. The number of mast cells was counted in 10 random fields at a magnification of $\times 200$, and averaged for each mouse.

Measurement of scratching behavior

The number of scratching behavior was automatically measured under an unattended environment using MicroAct (Neuroscience, Inc., Tokyo, Japan). Under isoflurane anesthesia, a small magnet (1 mm in diameter, 3 mm in length) was implanted subcutaneously into both hind paws of mouse on day 8 or 9. The mice were acclimated for 1 hour in an observation chamber (11 cm in diameter, 18 cm in height) with food and water, and treated with the skin barrier disruption followed by the Dfb ointment on day 14. Then, the measurement of the number of scratching behavior (events) was started and kept for 24 hours. In another experiment, the number of spontaneous scratching behavior was measured for 24 hours from day 21 to day 22, or from day 22 to day 23. Analysis parameters of MicroAct were: threshold, 0.03 V; event gap, 0.05 s; minimum duration, 0.25 s; maximum frequency, 20 Hz; and minimum frequency, 5 Hz.

Immunohistochemical analysis

The skin samples excised on day 18 were fixed in 4% paraformaldehyde and immersed in phosphate-buffered saline (PBS) containing 30% sucrose. These samples were embedded in OCT compound (Sakura Finetek USA, Inc., Torrance, CA, USA) and sectioned using a CM1950 Cryostat (Leica Biosystems Nussloch GmbH, Wetzlar, Germany) at 20 μ m thickness. After blocking in PBS with 1% bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) and 0.3% Triton X-100 (Nacalai tesque, Inc., Kyoto, Japan), the sections were incubated with a rabbit anti-protein gene product 9.5 (PGP 9.5) antibody (1:100 dilution; UltraClone Ltd., Isle of Wight, UK) for 2 hours at room temperature. Subsequently, the sections were washed with PBS containing 0.3% Triton X-100 and incubated with an Alexa Fluor 488-conjugated anti-rabbit antibody (1:100 dilution; Molecular Probes, Inc., Eugene, OR, USA) for 1 hour at room temperature. Images of immunolabeled sections were captured with a confocal laser scanning microscopy (LSM510; Carl Zeiss MicroImaging GmbH, Jena, Germany). The area of immunoreactive nerve fibers in the epidermis was quantified using an Image-Pro Plus software (Media Cybernetics, Inc., Silver Springs, MD, USA).

Measurements of nerve growth factor (NGF) and semaphorin 3A (Sema3A) levels in the lesional skin

The skin samples of 5 mm in diameter were collected 24 hours after the final application of Dfb ointment (on day 18). The samples were homogenized in 150 μ L ice-cold PBS containing a protease inhibitor cocktail set III (Calbiochem, San Diego, CA, USA) using a ShakeMaster (Biomedical Sciences, Inc., Tokyo, Japan) and centrifuged at $10,000 \times g$ for 10 minutes at 4 °C. The concentrations of NGF and Sema3A in the supernatants were measured using a ChemiKine™ NGF Sandwich ELISA kit (Chemicon International, Inc., Temecula, CA, USA) and a mouse Sema3A ELISA kit (Cusabio, Wuhan, China), respectively.

Measurements of cell number, cell proliferation and cytokine levels in axillary lymph node cells

Axillary lymph nodes were isolated from the mice on day 14, and a single cell suspensions were prepared in RPMI 1640 (Life Technologies Corporation, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Santa Ana, CA, USA), 55 μ M β -mercaptoethanol (Life Technologies), 100 U/mL penicillin and 100 μ g/mL streptomycin (Nacalai tesque, Inc.). The number of lymph node cells was counted using a Coulter counter (Beckman Coulter, Inc., Brea, CA, USA). The lymph node cells (4×10^5 cells) were

stimulated with 10 µg/mL Dfb extract (Biostir Inc.) for 24 hours at 37°C in a 96-well flat-bottom microplate. Then, the culture supernatants were collected, and the cell proliferation was evaluated by measuring the absorbance at 450 nm after incubation with water-soluble tetrazolium salt (WST-8; Nacalai tesque, Inc.) for 2 hours. The amounts of TNF- α , IL-2, IL-4, IL-6, IL-10, IL-17A and IFN- γ in the culture supernatants were determined using a mouse Th1/Th2/Th17 cytometric bead array (CBA) kit (BD Biosciences, San Jose, CA, USA) and analyzed on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA).

Statistical analysis

Data were expressed as the mean $\pm$ standard error of mean (SEM). A significant difference between two groups was evaluated by Student's or Aspin-Welch's *t* test following F-test except for the dermatitis score, which was assessed by the Wilcoxon test. A *P*-value of less than 0.05 was considered statistically significant.

Results

Time course of dermatitis score and plasma total IgE level

Following repeated topical application of Dfb ointment for 2 weeks, the skin dryness preceded the developments of hemorrhage and hyperplasia. The dermatitis score was gradually and significantly increased in Dfb ointment-applied mice whereas it was

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hardly changed in control mice treated with hydrophilic petrolatum (Fig. 1a). Fig. 1b shows a representative gross appearance of the dorsal skin on day 17. The dermatitis scores were 0 and 7 in the control and Dfb ointment-applied mice, respectively. Total IgE level in plasma was significantly elevated on day 13 in Dfb ointment-applied mice compared to control mice (Fig. 1c).

Histological evaluation of the lesional skin

The epidermal hyperplasia and an increase in the number of dermal inflammatory cells such as lymphocytes and eosinophils were evident in the lesional skin of Dfb ointment-applied mice (Fig. 1d). A significant increase in the epidermal thickness was observed in Dfb ointment-applied mice (Fig. 1e). Moreover, the number of mast cells in the dermis was significantly increased in Dfb ointment-applied mice (Fig. 1f, g).

Time course of scratching behavior

To investigate the time-related changes in scratching behavior, the number of scratching behavior was measured for 24 hours from day 14 to day 15 after application of the Dfb ointment under a 12-hours light/dark cycle (lights on from 7:00 to 19:00). The number of scratching behavior was significantly increased in Dfb ointment-applied mice in a biphasic fashion; a transient increase in the first one hour (early phase) followed by a persistent increase lasted from about 4 to 24 hours (later phase) (Fig. 2a). The biphasic

increase in the number of scratching behavior caused by application of the Dfb ointment was also observed under the reversed light/dark cycle lights (light on from 19:00 to 7:00) (Fig. 2b). To investigate whether the scratching behavior was spontaneously elicited without application of the Dfb ointment, the number of scratching behavior was measured for 24 hours from day 21 to day 22, or from day 22 to day 23. The number of spontaneous scratching behavior in Dfb ointment-applied mice was kept at a significantly increased level during the entire period of measurement without the marked increase in the early phase, compared to control mice (Fig. 2c).

PGP9.5-positive nerve fibers and contents of expression of NGF and Sema3A in the lesional skin

In control mice, PGP9.5-positive nerve fibers were occasionally observed in the dermis, and hardly located in the epidermis (Fig. 3a). In Dfb ointment-applied mice, PGP9.5-positive nerve fibers were abundant in the dermis and sprouted into the epidermis. The area of PGP9.5-positive nerve fibers significantly increased in the epidermis of Dfb ointment-applied mice (Fig. 3b). The content of NGF, a nerve elongation factor was significantly higher whereas that of Sema3A, a nerve repulsion factor was significantly lower in the lesional skin of Dfb ointment-applied mice than in the skin of control mice (Fig. 3c, d).

Cell number, cell proliferation and cytokine levels in axillary lymph node cells

The number of cells isolated from axillary lymph nodes was significantly increased in Dfb ointment-applied mice (Fig. 4a). In the lymph node cells isolated from control mice, no significant changes in the proliferation were induced by stimulation with Dfb extract (Fig. 4b). In contrast, the proliferation of the lymph node cells isolated from Dfb ointment-applied mice was significantly enhanced by stimulation with Dfb extract. Moreover, the cell proliferation was significantly up-regulated in the lymph node cells from Dfb ointment-applied mice than in those from control mice under the condition with stimulation by Dfb extract. TNF- α , IL-2, IL-6, IL-10, IL-17A and IFN- γ levels in the culture supernatants of the lymph node cells from Dfb ointment-applied mice were dramatically elevated when stimulated with Dfb extract (Fig. 4c). IL-4 levels were undetectable in any culture supernatants of the lymph node cells from Dfb ointment-applied mice and control mice, and with or without stimulation by Dfb extract (data not shown).

Discussion

In the present study, we investigated the characteristics of the murine AD model induced by repeated topical application of Dfb ointment to the dorsal skin with barrier disruption, and it was found that all of the clinical, immunological and histological changes in this

model were similar to those of human AD. Interestingly, scratching behavior in Dfb ointment-applied mice was increased in a biphasic fashion compared to that in control mice when measured for 24 hours after topical application of Dfb ointment. To our knowledge, this is the first report showing a biphasic increase in the scratching behavior in this AD model.

AD is a chronic inflammatory skin disease accompanied by severe itching and eczematous lesion, and is one of the most common skin diseases which affects up to 20% of children and 1–3% of adults in most countries of the world (1). Although immunosuppressive drugs have been used for the treatment of AD (4), there remains a need for novel therapeutic drugs without adverse effects. However, there are some difficulties in developing new drugs for the treatment of AD due to the complicated pathogenesis of AD that involves dysfunction of the skin barrier and abnormal reaction of the immune system against environmental allergens. Recently, several animal models of human AD have been developed such as murine models induced by topical application of sensitizers, transgenic models overexpressing or lack of selective molecules, and spontaneously developed dermatitis models (21).

It has been reported that NC/Nga mice developed AD-like skin lesions spontaneously under conventional conditions or induced by repeated topical application of house dust mites extract under SPF conditions (12-17). In this study, we used an ointment

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containing Dfb extract as an environmental allergen to develop an AD model of NC/Nga mice. This model exhibited dryness of the dermal skin, followed by hemorrhage and hyperplasia as clinical features, and an increased total IgE level in plasma was also observed after the repeated topical application of Dfb ointment. Histologically, an infiltration of inflammatory cells including mast cells to dermis and an increased in the epidermal thickness were observed in the lesional skin of this model, in agreement with previous reports using this model (16,17). In AD patients, transepidermal water loss is known to be increased not only in lesional skin but also in non-lesional skin (22), and results in dry skin at all stages of the disease, which may lead to increased scratching and development of rash (4). Chronic inflammation in AD is frequently associated with elevated levels of serum IgE (23), epidermal thickening and dermal infiltration of inflammatory cells including mast cells (2,24). These observations suggest that this model has very similar clinical and histological characteristics of human AD.

In this model, the scratching behavior was increased in a biphasic fashion when measured for 24 hours after topical application of Dfb ointment on the back skin as the previous report indicating that mice with repeated application of oxazolone show severe scratching behavior for 1 hour and continuous scratching behavior for at least 8 hours after the application of oxazolone (25). Moreover, even under the reversed light/dark cycle, the biphasic increase in the number of scratching behavior was observed,

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suggesting this biphasic response is independent of circadian rhythm. Furthermore, the number of spontaneous scratching behavior was kept at an increased level during the entire period of measurement compared to control mice. These findings suggest that scratching behavior was increased chronically and boosted by the topical application of Dfb ointment in this model.

When antigens cross-link adjacent IgE antibodies bound to high-affinity receptors (FcεRI) on the surface of tissue mast cells, aggregation of FcεRI triggers a complex intracellular signaling process and induces mast cell degranulation that results in the secretion of chemical mediators such as histamine. In sensitized individuals, these immediate-type allergic reactions occur within minutes of allergen exposure (26). Elevated levels of total IgE in plasma and increase in the number of mast cells in the dermis were observed in this model. The number of scratching behavior in the early phase was increased in the first one hour, which was consistent with the response time of immediate-type allergic reactions. These results suggest that immediate-type allergic reactions may be involved in the induction of scratching behavior in the early phase in this model.

House dust mites such as *Dermatophagoides farinae* have been shown to contain several allergens with proteolytic activity (27). Mite-derived proteases are known to activate PAR-2 to produce cytokines from keratinocytes and contribute to the

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pathogenesis of AD (28). Furthermore, PAR-2 activation on keratinocytes also induces the production of thymic stromal lymphopoietin (TSLP) which acts directly on sensory neurons to trigger robust scratching behavior (29). It is therefore probable that Dfb ointment induces scratching behavior via these IgE-independent mechanisms, although further studies would be required to clarify the underlying mechanisms of scratching behavior in each phase.

Histological investigations have shown that the density of epidermal nerve fibers is higher in the skin of patients with AD than in healthy controls, which is partly responsible for itch sensation in the periphery (30-32). It has been reported that NGF is mainly produced by keratinocytes and expression of NGF is increased in the epidermis of AD-like murine model (33). Sema3A is also mainly distributed in the epidermis (34) and expressed in keratinocyte cultures (35). Therefore, skin hyperinnervation is probably caused by an imbalance between nerve elongation factors, such as NGF, and nerve repulsion factors, such as Sema3A, produced by keratinocytes (32). In this study, it was found that the increased sprouting of intraepidermal nerve fibers was accompanied by an increase in the content of NGF and decrease in that of Sema3A in the lesional skin, suggesting that nerve fiber elongation may cause abnormal itch sensations to increase the number of scratching behavior in this model.

Previous studies suggest that the changes in the expression of Th1 and Th2 cytokines produced by allergen-specific T cell responses to the environmental allergens play a role in initiating and maintaining skin inflammation in AD (36-39). Indeed, it has been reported that stimulation of peripheral blood mononuclear cells with house dust mite extract in patients with AD causes the proliferation of house dust mite-specific helper T lymphocytes, which correlates closely with the severity of symptoms in AD patients (36). Some observations in acute and chronic lesions of patients with AD indicate that although the initiation of atopic skin lesions is driven by Th2 cells, chronic lesions show either the coexistence of both Th2 and Th1 cells or Th1 dominance (4,37). An increased expression of the Th2 type cytokines IL-4 and IL-10 was reported together with increased expression of the Th1 type cytokines IFN- γ and IL-2 within identical involved skin areas of the same patients (38,39). Recently, it has been suggested that IL-17 may be involved in the pathogenesis by exacerbating and maintaining the inflammation of AD. An increase in the percentage of Th17 cells was shown in peripheral blood of AD patients that correlate with disease severity (40). Furthermore, the levels of IL-2, IL-5, IL-6, IL-10, IL-17A and IFN- γ were increased in serum from AD patients when compared with healthy controls (41).

We observed an increase in the number of axillary lymph node cells in Dfb ointment-applied mice. Axillary lymph node cells isolated from Dfb ointment-applied mice showed the enhancement of cell proliferation and elevated levels of cytokine production such as TNF- α , IL-2, IL-6, IL-10, IL-17A and IFN- γ by stimulation with Dfb extract, which are consistent with the previous report that lymph node cells showed marked elevation of IFN- γ levels from Dfb ointment-applied mice (16). These results suggest that house dust mite-specific T cells play an important role in the development and maintenance of chronic dermatitis by producing Th1, Th2 and Th17 cytokines in this model as well as in human AD.

In conclusion, this study has demonstrated that the repeated topical application of Dfb ointment induced AD-like skin lesion in NC/Nga mice. Clinical, histological and immunological characteristics in this model were similar to those observed in patients with AD. Furthermore, we found that the number of spontaneous scratching behavior increased chronically and the topical application of Dfb ointment also induced the scratching behavior in a biphasic fashion in this model. It is suggested that further clarifying the underlying mechanisms of scratching behavior in this model leads not only to elucidating the pathogenesis of AD but also to discovering novel therapeutic drugs for AD.

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Yoshihito Yamada performed the research, designed the research study, analysed the data and wrote the paper; Yuhki Ueda, Aki Nakamura, Shoji Kanayama, Rie Tamura, Kei Hashimoto and Hiroko Kido performed the research; Tatsumi Matsumoto wrote the paper; Ritsuko Ishii designed the research study and wrote the paper.

Conflict of interests

The authors have declared no conflicting interests.

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Figure legends

Figure 1. Time course of dermatitis score and plasma total IgE level, and clinical features

of the lesional skin after repeated topical application of Dfb ointment in NC/Nga mice.

Dfb ointment (100 mg) was applied topically on the dorsal skin after disruption of the

skin barrier with a depilatory cream on day 0 and with 4% SDS (150 μ L) on day 3, 7, 10,

14 and 17. Control animals (petrolatum) were treated with hydrophilic petrolatum

instead of Dfb ointment. (a) Dermatitis score. **: $P < 0.01$ by Wilcoxon test, compared to

control mice (petrolatum). (b) Representative skin features on day 17. (c) Total IgE level

in plasma measured by ELISA. The dorsal skin excised on day 18 was stained with (d)

HE and (f) TB. Bars in photographs: 50 μ m. (e) Epidermal thickness measured in

HE-stained sections at a magnification of $\times 100$. (g) The number of mast cells counted in

TB-stained sections at a magnification of $\times 200$. Each data represent the mean $\pm$ SEM

(n=5-9). **: $P < 0.01$ by F-test followed by Student's or Aspin-Welch's t -test, compared to control mice (petrolatum).

Figure 2. Time course of the number of scratching behavior in AD-like mice. The number of scratching behavior (events) was measured for 24 hours from day 14 to day 15 after topical application of Dfb ointment under (a) a 12-hours light/dark cycle (lights on from 7:00 to 19:00) and (b) the reversed light/dark cycle (lights on from 19:00 to 7:00). (c) The number of spontaneous scratching behavior (events) was measured for 24 hours from day 21 to day 22, or from day 22 to day 23 without application of the Dfb ointment. The white and black bars indicate the light and dark periods in the animal room, respectively. Each point represents the mean $\pm$ SEM (n=4-8). *: $P < 0.05$, **: $P < 0.01$ by F-test followed by Student's or Aspin-Welch's t -test, compared to control mice (petrolatum).

Figure 3. Increases in PGP9.5-positive nerve fibers and contents of NGF and Sema3A in the lesional skin of AD-like mice. (a) The dorsal skin excised on day 18 was stained with anti-PGP9.5 antibody (original magnification, $\times 200$). White broken line indicates the border between the epidermis and dermis. Bar: 50 μ m. (b) The area of PGP9.5-positive nerve fibers was quantified in the epidermis using image software. (c) NGF and (d) Sema3A levels in the skin on day 18 were measured by ELISA. Each column represents the mean $\pm$ SEM (n=5). *: $P < 0.05$, **: $P < 0.01$ by F-test followed by Student's or Aspin-Welch's t -test, compared to control mice (petrolatum).

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Figure 4. Increases in cell number, cell proliferation and cytokine levels in axillary lymph node cells isolated from AD-like mice. Axillary lymph nodes were isolated on day 14 and homogenated to make a single cell suspension. (a) The number of lymph node cells was counted using a Coulter counter. (b) The cell proliferation and (c) cytokine levels in the culture supernatant of the lymph node cells were measured 24 hours after stimulation with 10 µg/mL Dfb extract by WST-8 assay and cytometric bead assay, respectively. The cell proliferation is expressed relative to those in the lymph node cells from control mice without stimulation by Dfb extract, which are normalized as 100%. Each column represents the mean ± SEM (n=5-6). **: $P < 0.01$ by t -test, compared to control mice (petrolatum). \$: $P < 0.01$ by F-test followed by Student's or Aspin-Welch's t -test, compared to control mice (petrolatum) with Dfb stimulation. ##: $P < 0.01$ by F-test followed by Student's or Aspin-Welch's t -test, compared to Dfb ointment-applied mice without Dfb stimulation. N.D.: Not detected.

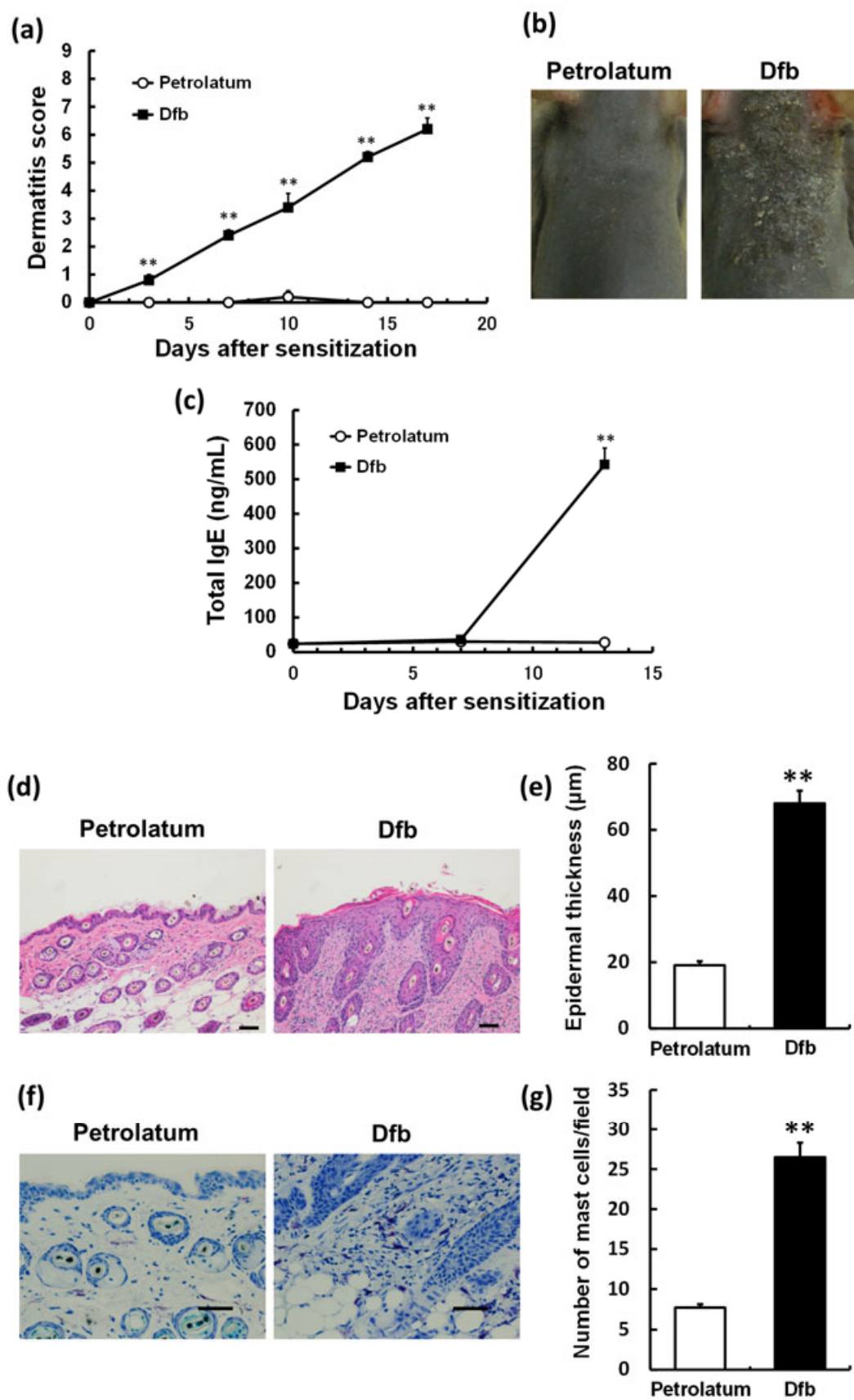


Figure 1

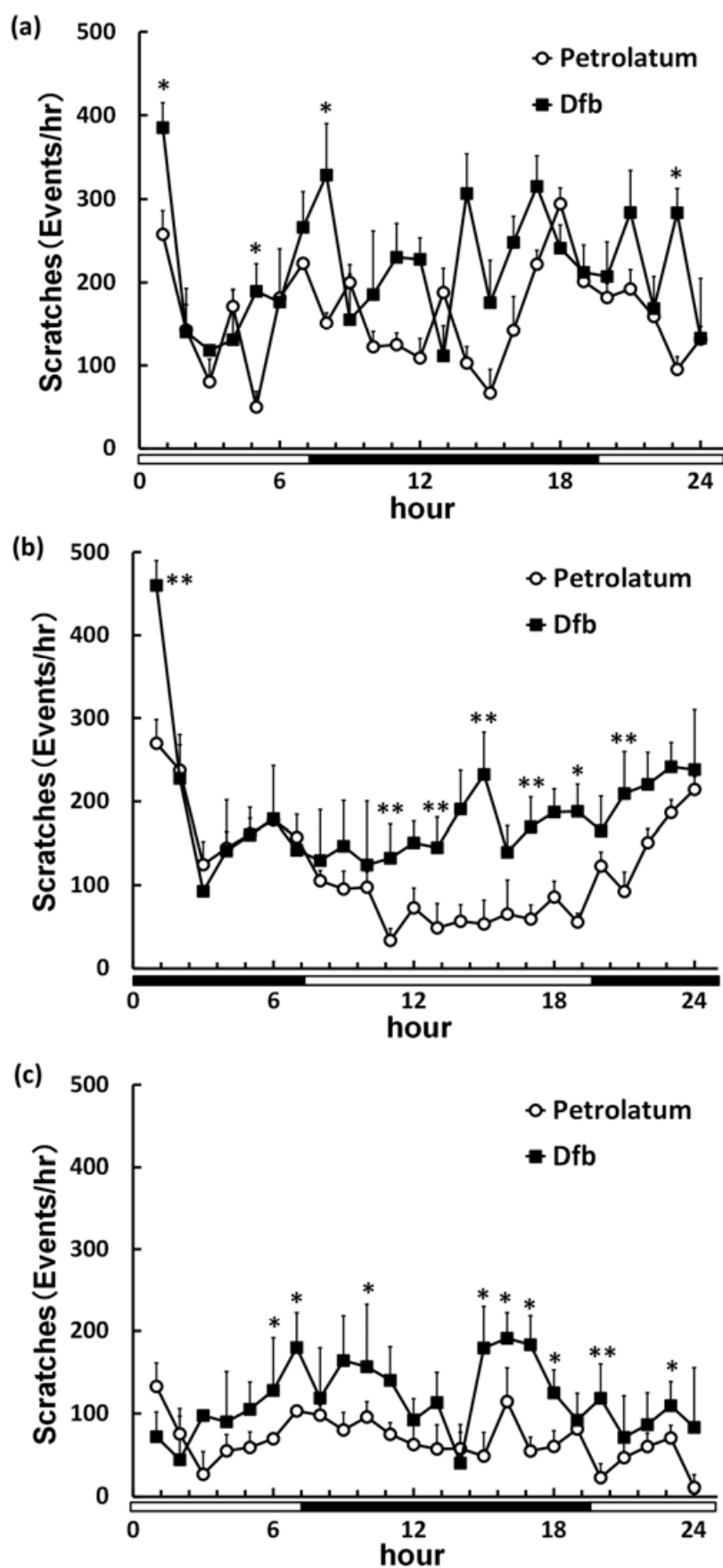


Figure 2

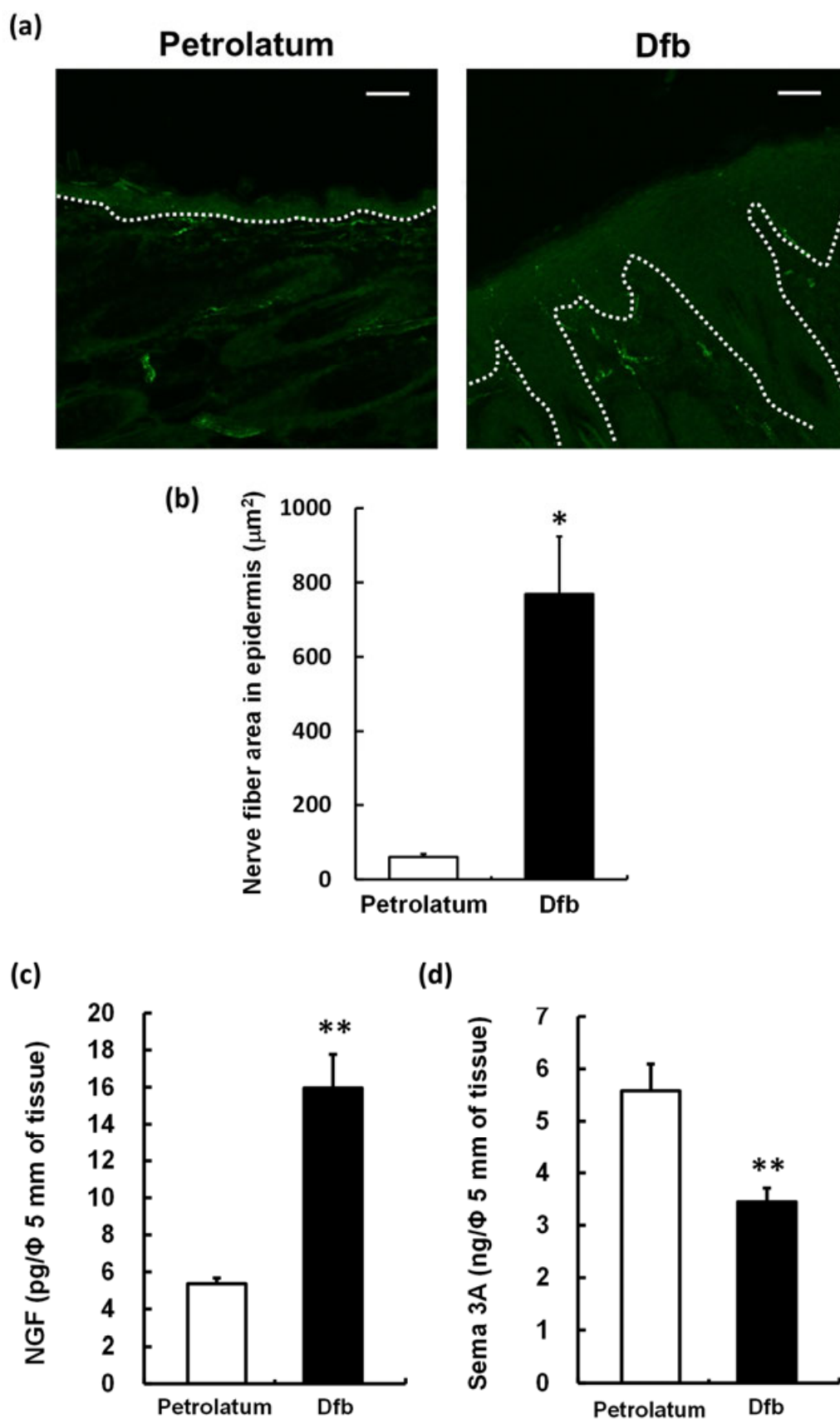


Figure 3

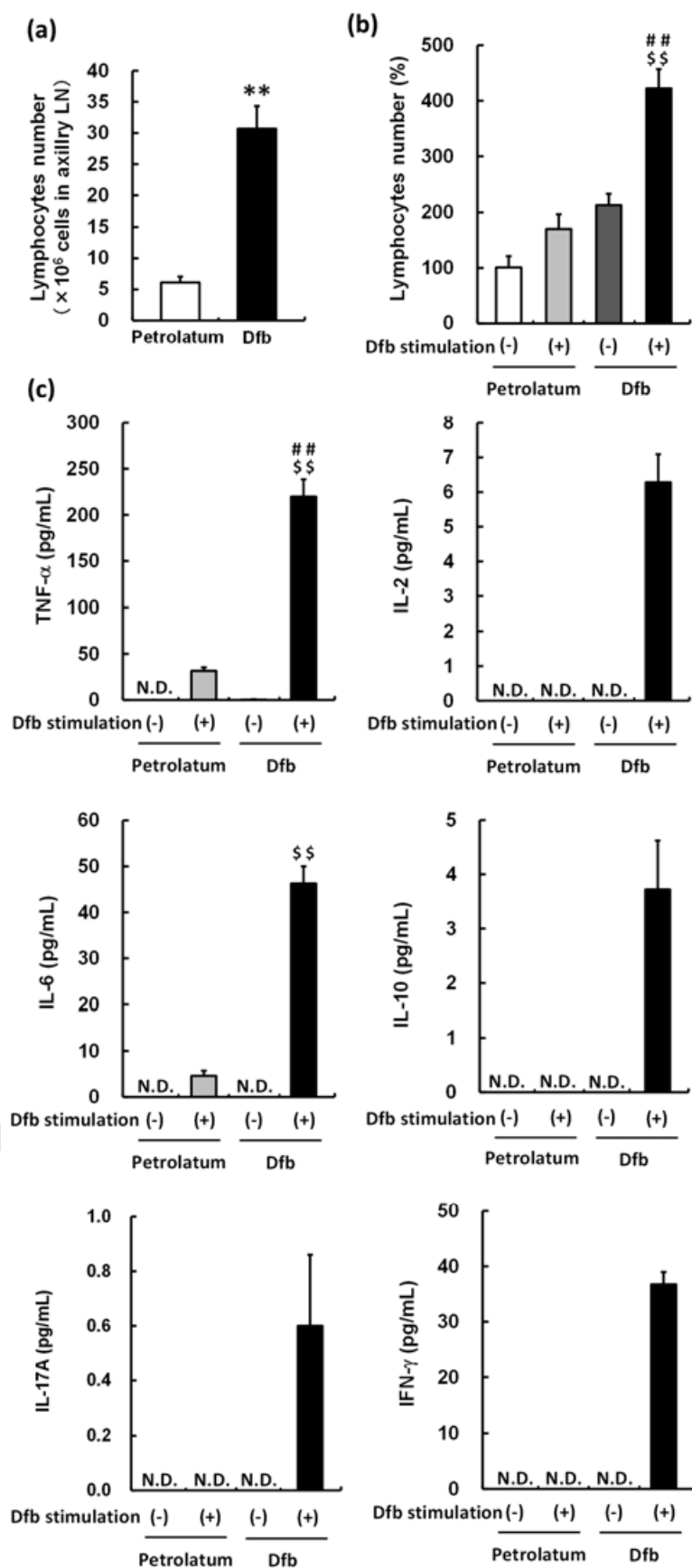


Figure 4