

Induction of atopic eczema/dermatitis syndrome-like skin lesions by repeated topical application of a crude extract of *Dermatophagoides pteronyssinus* in NC/Nga mice

Jong Soon Kang¹, Kiho Lee¹, Sang-Bae Han, Ji-Mi Ahn, Hyunju Lee, Mi Hwa Han, Yeo Dae Yoon, Won Kee Yoon, Song-Kyu Park, Hwan Mook Kim^{*}

Bioevaluation Center, Korea Research Institute of Bioscience and Biotechnology, Taejeon, 305-333, Republic of Korea

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Abstract

Mite antigen has been considered to play important roles in the development of atopic eczema/dermatitis syndrome (AEDS). In the present study, we attempted to induce an AEDS-like skin lesion in mice using *Dermatophagoides pteronyssinus* crude extract (DPE) as an antigen and performed pathophysiological evaluations. Ears of mice were tape-stripped and DPE was painted 3 times a week. Eczematous skin lesion and ear swelling were apparent in NC/Nga mice treated with DPE after 2 weeks, whereas neither skin lesion nor ear swelling were observed in BALB/c mice even after 30 days. Histological evaluation demonstrated that edema, epidermal hyperplasia and the accumulation of inflammatory cells were apparent in the ears of DPE-treated NC/Nga mice. In contrast to skin lesion and ear swelling, total serum IgE levels were increased in both NC/Nga and BALB/c mice. Treatment with DPE also increased auricular lymph node weight in both NC/Nga mice and BALB/c mice. To further characterize, we analyzed cytokine mRNA expression in ears and lymph nodes of DPE-treated NC/Nga mice. Increased expression of IL-4 and TNF- α mRNA was observed in both ears and lymph nodes of NC/Nga mice treated with DPE. Additionally, there was no change in the responsiveness of BALB/c mice to DPE treatment by adaptive transfer of serum from DPE-treated NC/Nga mice to BALB/c mice. Taken together, our results indicate that eczematous skin lesion and ear swelling caused by repeated application of DPE in NC/Nga mice has a Th2-dominant background and that inflammation is involved in this process. The animal model of AEDS established in this report may be used to investigate the pathogenesis of AEDS and evaluate the potential therapeutic agents for AEDS.

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1. Introduction

Atopic eczema/dermatitis syndrome (AEDS) is one of the most common and severe skin diseases, which is

characterized by eczematous skin lesion and IgE hyperproduction [1]. Both genetic and environmental factors are involved in the occurrence of this disease [2]. The prevalence of AEDS is increasing especially in industrialized countries because of frequent exposure to harmful environmental pollutants from industry [3,4]. Although AEDS is a severe health problem in developed countries, its pathogenesis is not clearly understood.

^{*} Corresponding author. Tel.: +82 42 860 4660; fax: +82 42 860 4605.

E-mail address: hwanmook@kribb.re.kr (H.M. Kim).

¹ These authors contributed equally to this work.

Therefore, appropriate animal models have been desired to investigate the pathogenesis of AEDS and explore new therapeutic agents for the treatment of this disease. There are several mouse models of AEDS available now. NC/Nga mouse model is a representative of the animal model of AEDS. NC/Nga mice develop AEDS-like skin lesions spontaneously in a conventional condition and shows elevated levels of serum IgE [5]. The IL-18-transgenic mouse model has been known as one of the closest transgenic mouse model of human AEDS [6]. Although each of these models has usefulness, it is reported that all of these models have significant disadvantages [7]. Therefore, the development of new animal models for atopic dermatitis has been required.

The most important allergens associated with human AEDS are house dust mite allergens [8]. *Dermatophagoides* species, including *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*, are the most common house dust mites present in the environment. The geological distribution of *Dermatophagoides* species are known to be related to the climate. *D. pteronyssinus* prefers humid climates and found abundantly in islands and coastal areas [9,10]. In contrast, *D. farinae* is more common than *D. pteronyssinus* in drier inland areas [10,11]. Although a couple of groups established mouse models of AEDS by topical application of *Dermatophagoides* extract, they all used the extract of *D. farinae* instead of *D. pteronyssinus* [1,12]. The aim of this study, therefore, was to establish a mouse model of AEDS by repeated topical application of *D. pteronyssinus* crude extract in mice and compare with previously reported AEDS model established by topical application of the extract of *D. farinae*. Here, we provide a relatively easy, fast and consistent way to develop AEDS-like skin lesion in NC/Nga mice.

2. Materials and methods

2.1. Reagents and animals

All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. *D. pteronyssinus* crude extract (DPE, Woongbee Meditech, Inc., Seoul, Korea) was used as an antigen. DPE was dissolved in phosphate buffered saline (PBS) containing 0.5% Tween 20 as described previously [1]. Five-week-old female NC/Nga and BALB/c mice were purchased from Charles River Japan (Kanagawa, Japan) and Orient Bio, Inc. (Seoul, Korea), respectively, and acclimated for 1 week before use.

2.2. Induction of AEDS-like skin lesions by repeated topical application of DPE

Induction of AEDS-like skin lesions using mite antigen was performed according to the method of Gao and coworkers

[1] with several major modifications. The dorsal surfaces of both ears were tape-stripped three times and 25 μ l of 10 mg/ml DPE solution was painted onto the surface of each ear. Tape-stripping and DPE painting were conducted three times a week for 3 weeks, and ear thickness was measured using a dial thickness gauge (Mitutoyo Corporation, Kanagawa, Japan) immediately before each tape-stripping. The vehicle solution was painted instead of DPE solution in control mice. At day 21, blood samples were collected by orbital puncture and used for the measurement of serum immunoglobulin levels. After blood collection, ears were removed and used for the evaluation of cytokine mRNA expression and histological observations. Auricular lymph nodes were also removed, weighed and used for the analysis of mRNA expression of cytokines. In adaptive transfer experiment, isolated sera were intravenously injected to the tail vein of BALB/c mice.

2.3. Detection of serum immunoglobulin levels

Immunoglobulin levels were measured using enzyme-linked immunosorbent assay kit (Bethyl Laboratories, Inc., Montgomery, TX, USA) according to the manufacturer's instructions. Briefly, capture antibodies were plated in Nunc C bottom immunoplate contained in the kit and incubated 1 h at room temperature. After washing the wells with washing solution (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0) three times, 200 μ l of blocking solution (50 mM Tris, 0.14 M NaCl, 1% BSA, pH 8.0) was plated in each well. After 30 min incubation at room temperature, the wells were washed three times with washing solution, serum samples and standards were diluted (serum samples were diluted 20-fold for IgE detection and 5000-fold for IgG, IgG₁ and IgG_{2a} detection), plated in the wells and incubated for 1 h. After washing the wells with washing solution, horseradish peroxidase-conjugated detection antibodies were diluted 5000-fold with conjugate diluent (50 mM Tris, 0.14 M NaCl, 1% BSA, 0.05% Tween 20, pH 8.0), transferred to each well and incubated for 1 h. After washing three times with washing solution, the enzyme reaction was initiated by adding substrate solution and the plate was kept for 30 min at room temperature in a dark place. The reaction was terminated by adding 2 M H₂SO₄ and the absorbance was measured.

2.4. Evaluation of cytokine mRNA expression

The ears were frozen in liquid nitrogen, grinded and suspended in TRIzol® Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). The lymph nodes were homogenized in TRIzol® Reagent using tissue tearer (Biospec Products, Inc., Bartlesville, OK, USA). Total RNA was isolated as described previously [13]. The expressions of the mRNA transcripts of IL-4 (forward primer: 5'-GAATGTACCAGGAGCCATATC-3', reverse primer: 5'-CTCAGTACTACGAGTAATCCA-3'), TNF- α (forward primer: 5'-CCTGTAGCCACGTCGTAGC-3', reverse primer: 5'-TTGACCTCAGCGCTGAGTTG-3') and β -actin (forward primer: 5'-TGGAATCCTGTGGCATCCATGAAAC-3', reverse primer: 5'-TAAAACGCAGCTCAGTAACAGTCCG-3') were

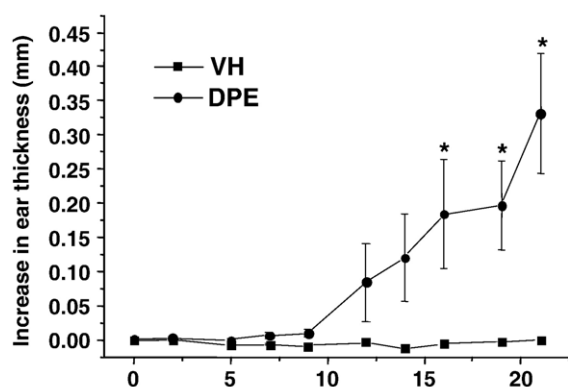
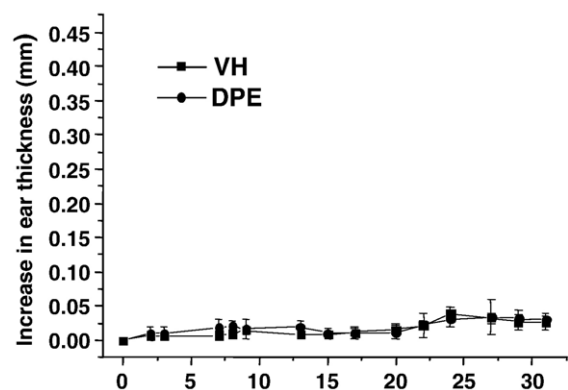
(A) NC/Nga**(B) BALB/c**

Fig. 1. Increase in ear thickness and development of eczematous skin lesion by repeated topical application of DPE in NC/Nga and BALB/c mice. Vehicle (0.5% Tween 20 in PBS) or DPE (10 mg/ml, 25 μ l/ear) was painted after tape-stripping 3 times a week onto the dorsal surface of both ears of NC/Nga (A) and BALB/c (B) mice. Ear thickness was measured immediately before DPE application and sacrifice. Each value represents the mean $\pm$ S.E.M. of changes in thickness of both ears of 5 mice ($n=10$). * $p<0.05$.

evaluated by RT-PCR as described previously [14] with slight modifications. Equal amounts of RNA were reverse transcribed into cDNA using oligo(dT)₁₅ primers. Samples were heated to 94 °C for 5 min and cycled 30 times at, 94 °C for 30 s, and 56 °C for 30 s, and 72 °C for 45 s, and this was followed by an additional extension step at 72 °C for 5 min. PCR products were electrophoresed in 3% NuSieve 3:1 gels (FMC Bioproducts, Rockland, ME, USA) and followed by ethidium bromide staining and photography.

2.5. Histopathology

The ears were fixed with 10% formaldehyde, embedded in paraffin and thin sections were made. The skin sections were stained with hematoxylin and eosin.

2.6. Statistical analysis

The results are expressed as mean $\pm$ S.E.M. Data were analyzed by analysis of variance (ANOVA), and Student's *t*-test was used for comparisons of multiple comparisons. The criterion for statistical significance was set at $p<0.05$.

3. Results**3.1. Increase in ear thickness and development of eczematous skin lesion by repeated topical application of DPE**

To induce AEDS-like skin lesion in mice, we repeatedly painted DPE onto the dorsal surfaces of both ears. Tape-stripping was conducted before DPE painting to accelerate the development of skin lesion according to the report of Gao and coworkers [1]. As shown in Fig. 1A, ear thickness started to increase after about 2 weeks and continued increasing until Day 21 in NC/Nga

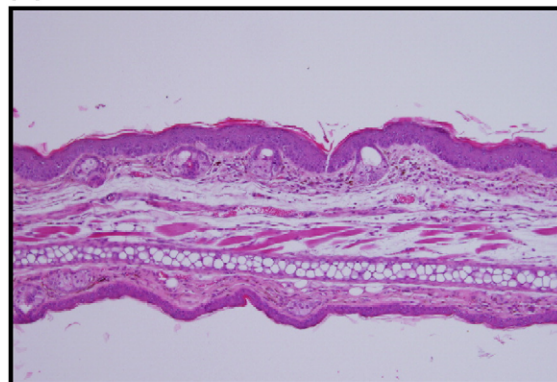
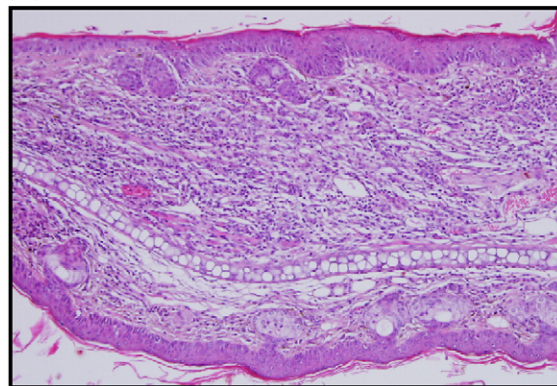
(A) VH**(B) DPE**

Fig. 2. Histological status of NC/Nga mouse ear treated repeatedly with DPE. (A) Vehicle (0.5% Tween 20 in PBS) or (B) DPE (10 mg/ml, 25 μ l/ear) was painted after tape-stripping 3 times a week for 3 weeks onto the dorsal surface of both ears of NC/Nga. The ears were excised, fixed with 10% formaldehyde, embedded in paraffin and thin sections were made. The skin sections were stained with hematoxylin and eosin.

mice. In contrast, no increase in ear thickness was observed in BALB/c mice treated with DPE until Day 31 (Fig. 1B). In accordance with the ear thickness increase, eczematous skin lesions were developed in the ears of DPE-treated NC/Nga mice, whereas no skin lesions were observed in DPE-treated BALB/c mice. We next examined hematoxylin and eosin-stained sections of ears from NC/Nga mice. As shown in Fig. 2, DPE treatment resulted in potent inflammatory changes, such as edema, epidermal hyperplasia and large number of inflammatory cell infiltration into the dermis.

3.2. Changes in serum immunoglobulin levels by DPE treatment

The hyperproduction of IgE is one of the most characteristic features of AEDS [1]. To investigate whether the IgE levels were increased in our model, we analyzed blood samples collected at Day 21. As shown in Fig. 3, repeated topical application of DPE caused a significant increase in serum IgE levels in both NC/Nga and BALB/c mice. The increase in serum IgE level in NC/Nga mice was higher than that in BALB/c mice. The level of serum IgG, IgG1 and IgG2a was not changed in DPE-treated NC/Nga mice, whereas IgG and IgG1 serum level was slightly increased in DPE-treated BALB/c mice (data not shown).

3.3. Increase in auricular lymph node weight by DPE treatment

It has been known that the weight of local lymph node increases by topical application of agents which have allergenic or sensitizing potential [15]. Moreover, the local lymph node weight was recently validated as a main endpoint of a murine local lymph node assay in a European ring study

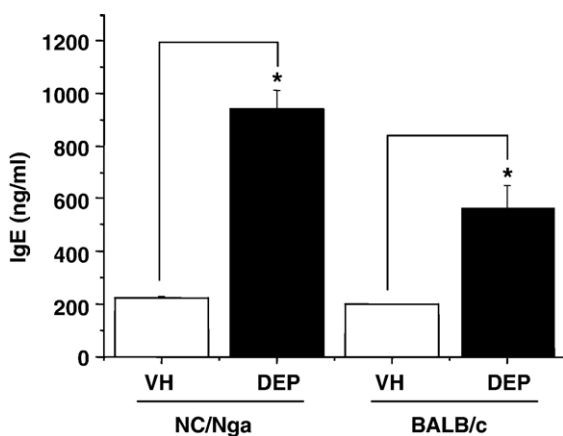


Fig. 3. Increase in total serum IgE levels in NC/Nga and BALB/c mice by repeated topical application of DPE. Vehicle (0.5% Tween 20 in PBS) or DPE (10 mg/ml, 25 μ l/ear) was painted after tape-stripping 3 times a week for 3 weeks onto the dorsal surface of both ears of NC/Nga and BALB/c mice. Blood samples were collected by orbital puncture and serum IgE was quantified by enzyme-linked immunosorbent assay. Each column shows the mean $\pm$ S.E.M. of 5 mice. * p < 0.05.

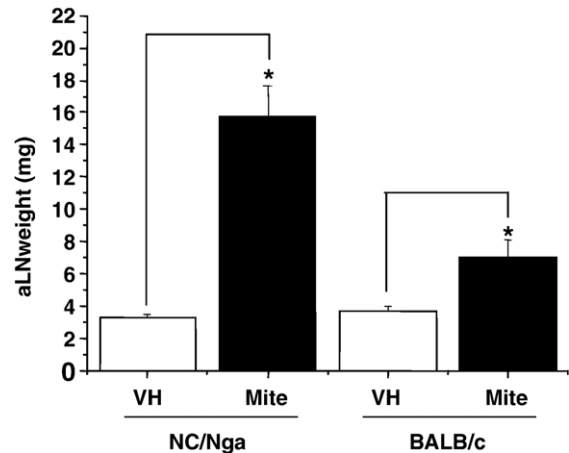


Fig. 4. Increase in auricular lymph node weight by repeated topical application of DPE. Vehicle (0.5% Tween 20 in PBS) or DPE (10 mg/ml, 25 μ l/ear) was painted after tape-stripping 3 times a week for 3 weeks onto the dorsal surface of both ears of NC/Nga and BALB/c mice. The auricular lymph node was excised and weighed. Each column shows the mean $\pm$ S.E.M. of 5 mice. * p < 0.05.

[16,17]. In the present study, we examined the increase in auricular lymph node weight to investigate the magnitude of local immune reaction by topical application of DPE in mice. As shown in Fig. 4, the auricular lymph node weight was increased in both NC/Nga and BALB/c mice. Consistent with the result of changes in IgE levels, the increase in auricular lymph node weight in NC/Nga mice was higher than that in BALB/c mice.

3.4. Changes in cytokine mRNA expression by DPE treatment

To further investigate the characteristic of AEDS established in this report, we examined the mRNA expression of IL-

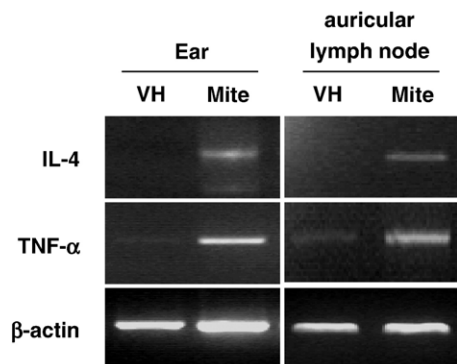


Fig. 5. Expression of IL-4 and TNF- α mRNA in ears and auricular lymph nodes of NC/Nga mice treated repeatedly with DPE. Vehicle (0.5% Tween 20 in PBS) or DPE (10 mg/ml, 25 μ l/ear) was painted after tape-stripping 3 times a week for 3 weeks onto the dorsal surface of both ears of NC/Nga mice. Expression of IL-4 and TNF- α mRNA was examined using RT-PCR as described in Materials and methods. A representative of three independent experiments is shown.

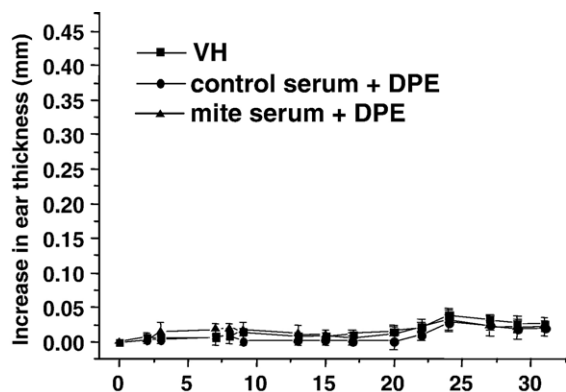


Fig. 6. Change in ear thickness by repeated topical application of DPE in BALB/c mice after adaptive transfer of serum from NC/Nga mice. Sera from control or DPE-treated NC/Nga mice were injected intravenously to tail vein of BALB/c mice. Vehicle (0.5% Tween 20 in PBS) or DPE (10 mg/ml, 25 μ l/ear) was painted after tape-stripping 3 times a week onto the dorsal surface of both ears BALB/c mice. Ear thickness was measured immediately before DPE application and sacrifice. Each value represents the mean $\pm$ S.E.M. of changes in thickness of both ears of 5 mice ($n=10$). control serum: serum from control NC/Nga mice, mite serum: serum from DPE-treated NC/Nga mice.

4 and TNF- α in ear and auricular lymph node of NC/Nga mice treated with DPE. As shown in Fig. 5, IL-4 and TNF- α mRNA expression was markedly increased in both ears and auricular lymph nodes of DPE-treated NC/Nga mice. In contrast, the mRNA expression of IL-2 and IFN- γ was not changed (data not shown).

3.5. Effect of adaptive transfer of serum isolated from DPE-treated NC/Nga mice to BALB/c mice

To investigate whether higher serum levels of IgE or unknown serum factors in DPE-treated NC/Nga mice are involved in the development of AEDS, we examined the effect of adaptive transfer of serum isolated from DPE-treated NC/Nga mice to BALB/c mice. However, as shown in Fig. 6, adaptive transfer of serum caused no effect on the responsiveness of BALB/c mice to DPE treatment.

4. Discussion

In the present study, we tried to establish an AEDS-like skin lesion in both NC/Nga and BALB/c mice by repeated topical application of DPE. However, the ear swelling and AEDS-like skin lesion were observed only in NC/Nga mice after DPE treatment. In consistent with our results, it was reported that the AEDS-like skin lesion was developed in NC/Nga mice, but not in BALB/c mice, after repeated topical application of the extract of *D. farinae* (DFE) [12]. In contrast, Gao and coworkers demonstrated that the treatment of DFE

caused inflammatory changes in both NC/Nga and BALB/c mice, although the magnitude of inflammatory changes in BALB/c mice was less severe than that in NC/Nga mice [1]. In all cases, the application of mite extracts resulted in AEDS-like skin lesions in NC/Nga mice independent of tape-stripping although tape-stripping potentiates the development of AEDS-like skin lesions. Aioi and coworkers demonstrated that the skin barrier function is genetically impaired in NC/Nga mice [18], and this explains why the development of AEDS-like skin lesions by mite extract is independent of tape-stripping in NC/Nga mice. However, it seems like that the disruption of skin barrier function by tape-stripping is necessary for the induction of AEDS-like skin lesion in BALB/c mice. Matsuoka and coworkers applied DFE on the shaved back of mice by swabbing using a cotton stick without tape-stripping and no dermatitis lesions appeared in BALB/c mice [12]. The importance of tape-stripping on the induction of AEDS-like skin lesion in BALB/c mice was clearly demonstrated in the report of Gao and coworkers, which showed that the inflammatory changes were elicited only when the ears were tape-stripped before mite antigen challenge [1]. However, in our model, AEDS-like skin lesion was not induced BALB/c mice although the ears were tape-stripped. In our opinion, this is maybe because of the difference in potency to induce AEDS-like skin lesion between DPE and DFE. Our unpublished data showed that the potency of DPE to induce AEDS-like skin lesion is weaker than that of DFE (data not shown). Therefore, the application of DPE could not induce AEDS-like skin lesion in BALB/c mice even though the ears were tape-stripped. One more thing to be considered is that there was not enough time for the induction of AEDS-like skin lesions in BALB/c mice. Although no AEDS-like skin lesion was observed in BALB/c mice until Day 31, we still cannot exclude the possibility that AEDS-like skin lesions eventually come up if we treat DPE for longer time. The exact mechanism responsible for this discrepancy needs to be further investigated.

An elevated level of serum IgE is one of the most characteristic features of AEDS. It has been known that about 70–85% of human AEDS is related to IgE hyperproduction and the extent of IgE sensitization is directly associated with the disease severity [19]. It was reported that the application of DFE resulted in the increase in serum IgE levels both in NC/Nga and BALB/c mice [12]. Moreover, Gao and coworkers showed that the level of IgE was significantly increased by repeated application of DFE in both NC/Nga and BALB/c mice independent of tape-stripping [1]. Consistent with these

reports, we also demonstrated that the treatment of DPE caused the elevation of serum IgE level in both NC/Nga and BALB/c mice. Considering that AEDS-like skin lesions were developed only in part of these animal models, these results suggest that the elevation of serum IgE level is not sufficient for the development of AEDS-like skin lesion in these mouse models. This can be further supported by the results of Tsudzuki and coworkers which show genetic analyses for AEDS-like skin lesion and IgE hyperproduction in NC/Nga mice by pairing NC/Nga and BALB/c mice [20]. They demonstrated that AEDS-like skin lesion and IgE hyperproduction were controlled by one autosomal recessive gene and two autosomal recessive genes, respectively, and these three genes are distinct genes because some of F₂ and backcross generations showed IgE hyperproduction but did not develop AEDS-like skin lesion [20]. However, all mice which develop AEDS-like skin lesion in these reports showed IgE hyperproduction. Collectively, at present, it is assumed that IgE hyperproduction is necessary, but not sufficient for the development of AEDS-like skin lesions in these mouse models. The increased serum IgE level is known to be closely related to the production of Th2 cytokines [2]. Th2 cytokines, such as IL-4, stimulate the proliferation and differentiation of B lymphocytes to produce IgE. Previously, Gao and coworkers demonstrated that DFE treatment caused the increase in IL-4 mRNA expression in ears and cervical lymph nodes of NC/Nga mice [1]. Consistent with these results, here we demonstrated that IL-4 mRNA expression was increased in ears and auricular lymph nodes of NC/Nga mice treated with DPE. Our results suggest that repeated DPE application seems to shift Th1/Th2 balance to a Th2-dominant state. The Th2-dominant state induced by DPE treatment might contribute to the increase in IgE production and the development of AEDS-like skin lesion.

Recently, the animal models of AEDS have received increasing attention. There are a couple of mouse models of AEDS available now, including traditional NC/Nga mouse model and IL-18 transgenic mouse model. However, these animal models are known to have significant disadvantages. The low incidence of AEDS-like lesions (~ 50%), late onset of disease (2–4 months) and poor reproducibility are critical disadvantages of the traditional NC/Nga mouse model [7]. IL-18 transgenic mouse model also has significant problems, such as late age of onset (~ 6 months) and limited availability [7]. The disadvantages of these animal models mentioned above, including late onset and development of disease, poor reproducibility and/or limited availability, make it hard to use them as testing tools for the development of potential therapeutic agents. Compared with these animal models, the major advantage

of our model is a shortened age of onset for development of AEDS-like skin lesions (21 days after first DPE treatment). The incidence of AEDS-like skin lesions is about 90% and the reproducibility is a lot better than the traditional NC/Nga mouse model. However, it also has a significant disadvantage. A major concern is a potential interaction between mite extract and therapeutic agents. Nevertheless, animal model of AEDS established in this study might enable us to evaluate a large number of therapeutic agents in an appropriate time with fair reproducibility.

In conclusion, animal model of AEDS established in this report seems to be similar to IgE-associated form of human AEDS. This model might be used to investigate the pathogenesis of AEDS and evaluate potential therapeutic agents for this disease.

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