

Evaluation of Eosinophilic Inflammation in a Novel Murine Atopic Keratoconjunctivitis Model Induced by Crude *Dermatophagoides farinae* Antigen

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ABSTRACT

Background: The purpose of this study is to conduct a histopathological research of the conjunctival findings and eosinophilic inflammation of novel atopic keratoconjunctivitis in a NC/Nga mouse model using crude *Dermatophagoides farinae*.

Methods: NC/Nga mice were sensitized by repeated topical applications of an ointment containing *Dermatophagoides farinae* body (Dfb). They were then divided into 4 groups depending on the following topical ophthalmic treatment: DFb group undergoing topical ophthalmic ointment containing Dfb; DFco group undergoing topical instillation of allergen extracts of *Dermatophagoides farinae*; Ba group undergoing topical ointment with substrate alone and NT group without after-topical ophthalmic treatment. At 24 hours after the last ophthalmic treatment, histopathological examination was performed. The density of the subepithelial infiltration of the eosinophils was determined. Serum total IgE and house-dust-mite (HDM)-specific IgE antibody concentrations were determined by enzyme-linked immunosorbent assay (ELISA).

Results: In the DFb group, the conjunctiva showed similar findings to those of atopic keratoconjunctivitis, i.e. intraepithelial pseudotubular formation, Torus-form infiltration due to massive lymphocytes in the palpebral conjunctiva and gelatinous hyperplasia in the limbus with subepithelial granuloma composed of lymphocytes and eosinophils. Subepithelial infiltration of eosinophil density in the DFb group [878.4 ± 399.7 cells/mm² (mean $\pm$ SD)] was significantly higher than in the other 2 groups (DFco 85.6 ± 40.1 Ba 49.2 ± 32.3) ($P < 0.001$). Total serum IgE concentration and HDM-specific serum IgE antibody concentration in the DFb group and the DFco group were significantly higher compared with those in the NT group.

Conclusions: Topical application of an ointment containing DFb to both the skin and eyes of NC/Nga mice can induce an atopic keratoconjunctivitis (AKC) model in these mice.

KEY WORDS

atopic keratoconjunctivitis, *Dermatophagoides farinae*, eosinophil, histopathology, IgE

INTRODUCTION

Atopic keratoconjunctivitis (AKC) is a clinical entity proposed by Hogan *et al.*¹ in 1952. They reported 5 patients with bilateral keratoconjunctivitis complicated with atopic dermatitis. Currently, the etiology

of AKC is thought to be primarily an involvement of type I hypersensitivity and AKC is classified together with other allergic conjunctival diseases (ACD) such as seasonal and perennial allergic conjunctivitis, vernal keratoconjunctivitis (VKC) and giant papillary conjunctivitis.² However, clinical findings of the eye

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lid and keratoconjunctival tissue in AKC are severe compared with other allergic conjunctival diseases.³⁻⁵ The difference in pathophysiology between AKC and other ACDs is not clearly understood.

The reported characteristic findings of AKC are atopic blepharitis, papillary proliferation of the upper and lower palpebral conjunctiva, limbal gelatinous hyperplasia, subepithelial conjunctival fibrosis, fornix foreshortening and corneal epitheliopathy with neovascularization.⁵ Most of these may be severe and sight threatening lesions. Severe cases undergo combined therapy of anti-allergic and corticosteroid and/or calcineurin inhibitor type immunosuppressant drugs but there may be occasional refractory cases. Therefore, a rational treatment strategy should be developed based on drug efficacy programmed in line with the pathophysiology of AKC.

A development of an AKC animal model can contribute to the investigation of that pathophysiology and to determine the effective drug regimens of AKC. Recently, spontaneous onset atopic dermatitis-like skin lesion NC/Nga mice were developed. Using this model mouse, pathophysiologic studies and the study of treatment methods of atopic dermatitis have been reported.⁶⁻⁸ We used the NC/Nga mice to study the development of atopic dermatitis-like skin lesion and AKC.

METHODS

MICE

Female NC/Nga mice, 8-week old, reared under specific pathogen-free (SPF) conditions, were purchased from Charles River Japan (Yokohama, Japan). The mice were all housed under SPF conditions. Food and tap water were provided freely. The animal experiments in this study were approved by the Animal Care and Use Committee of Nihon University School of Medicine. All the animals were treated in accordance with the Association for Research in Vision and Ophthalmology (ARVO) resolution regarding animals used in research.

DERMATOLOGIC OR OPHTHALMIC OINTMENT OF *Dermatophagoides farinae* BODY

Either a *Dermatophagoides farinae* body (Dfb) dermatologic ointment (Biostir® AD, Biostir, Kobe, Japan) at a concentration of 5 mg Dfb extract/1 g hydrophilic petrolatum or Dfb ophthalmic ointment (Biostir) containing at a concentration of 5 mg Dfb extract/1 g white petrolatum (PROPEITO®, Maruishi Pharmaceutical, Tokyo, Japan) were used in this study.

SENSITIZATION AND DEVELOPMENT METHOD OF MOUSE MODEL FOR ATOPIC KERATOCONJUNCTIVITIS

NC/Nga mice were sensitized by repeated topical applications of the Dfb dermatologic ointment. They

were then divided into 4 groups depending on the following topical ophthalmic treatment: DFb group ($n = 7$) undergoing treatment with topical Dfb ophthalmic ointment; DFco group ($n = 5$) undergoing treatment with a topical instillation of 0.01% allergen extracts of *Dermatophagoides farinae* (Torii Pharmaceutical, Tokyo, Japan); Ba group ($n = 5$) undergoing treatment with a topical ointment with substrate alone and NT group ($n = 7$) without any after-topical ophthalmic treatment.

The experimental protocol is shown in Figure 1. Atopic dermatitis-like skin lesions' onset in the mice was induced according to a previous report.⁹ For the first induction (Day 1), the hair of the back skin of the mice was clipped using an electric clipper and depilated using a hair removal cream under systemic intraperitoneal anesthesia (sodium pentobarbital, Somnopentil®; Kyoritsu Seiyaku, Tokyo, Japan). Antigen challenge was performed by topical application of 100 mg Dfb dermatologic ointment on the dorsal denuded skin and the surface of both ears. For the sensitization, the same treatment was performed repeatedly after the initial treatment. The sensitization methods from the second treatment on were as follows. The re-growing skin hairs at the initially treated dorsal skin were depilated and 150 μ l of 4% sodium dodecyl sulfate ointment was applied to the skin induce a barrier-disrupted skin and to the surface of both ears at 3 hours before each application of the Dfb dermatologic ointment. These procedures were repeated twice a week. On Day 17, atopic dermatitis-like skin lesions developed.

On Days 18 to 20, a topical ophthalmic treatment was performed once a day according to the protocol. For the DFb group, 5 mg of Dfb ophthalmic ointment was instilled. The DFco group underwent topical instillation of 5 μ l of allergen extract of *Dermatophagoides farinae* (Torii Pharmaceutical). The Ba group underwent an instillation of 5 mg of ointment substrate alone. At 24 hours after the final instillation, eyeballs, eyelids and lacrimal glands were removed in all the groups after euthanasia by overdose of pentobarbital.

MEASUREMENT OF TOTAL IgE AND HDM-SPECIFIC IgE ANTIBODY LEVELS IN THE SERUM

The concentrations for the total IgE and house-dust-mite (HDM)-specific IgE in the serum were determined by enzyme-linked immunosorbent assay (ELISA). For the ELISA, the total serum IgE levels were determined by using the mouse IgE EIA kit (Yamasa, Tokyo, Japan) according to the manufacturer's instructions. For the HDM-specific IgE antibodies, 96-well plates (EIA/RIA Stripwell™ Plate 1 X 8 Well, Flat Bottom, high bridging, type I, Corning, NY, USA) were coated with 100 μ l of 20 μ g/mL *Dermatophagoides farinae* antigen (Torii Pharmaceutical). The chemical reagent of the mouse IgE EIA kit

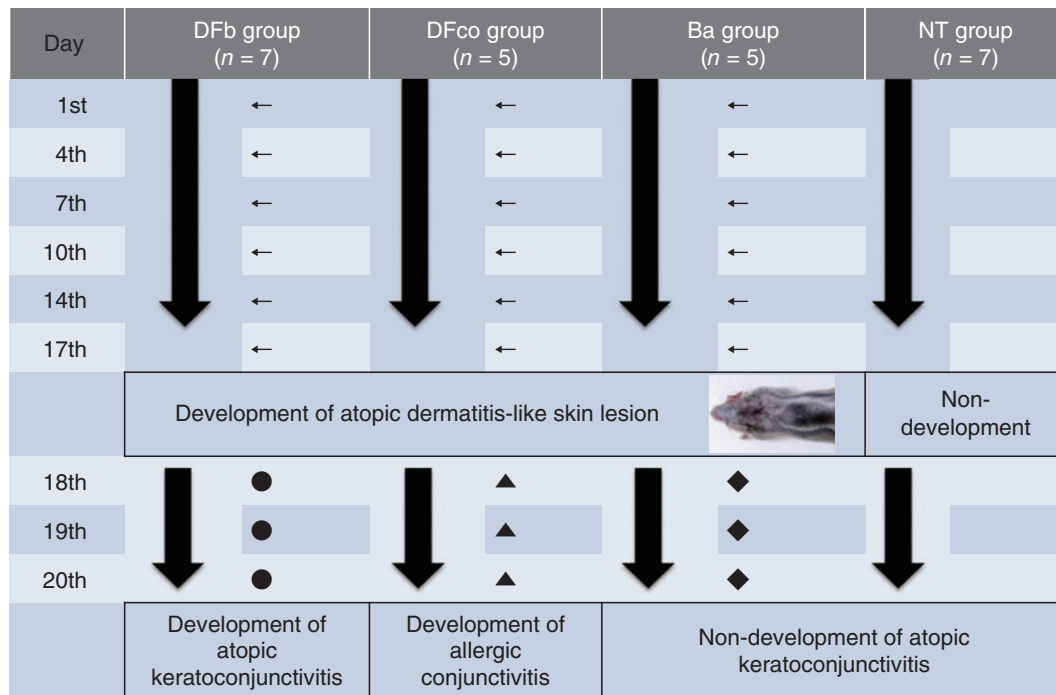


Fig. 1 Experimental protocol for atopic keratoconjunctivitis mouse model. → : Application of ointment including *Dermatophagoides farinae* body (Dfb) (biostire® AD). Black circle: Instillation of ophthalmic ointment including Dfb. Black triangle: Instillation of ophthalmic solution including allergen extracts of *Dermatophagoides farinae*. Black diamond: Instillation of ointment substrate (white petrolatum).

was used for enzyme reaction following a labeled antibody reaction that was the same as the measurement of the total IgE. After 5 min of converting substrate to a colored end product with tetramethylbenzene solution, the enzyme reaction was stopped with 1N H₂SO₄ solution, and the absorbance at 450 nm was detected on a microplate reader (TOSOH, Tokyo, Japan).

Concentration of total IgE in the samples was converted using a standard curve of absorbance for each standard IgE solution. Since purified mouse HDM-specific IgE antibody was not available for a standard calibration curve, the concentration of serum HDM-specific IgE was converted into a relative unit using a calibration curve obtained from DFb sensitized NC/Nga mice with a high anti-HDM specific IgE antibody. The serum with the highest antibody titer was set arbitrarily to 300 MIU of anti-HDM specific IgE antibody per ml. In this paper, we use a tentative unit (MIU: mouse IU) which corresponds to IU/ml in humans. Using the standard serum with 300 MIU/ml, stepwise diluted solutions ranging from 2 to 64 times dilution factor were used to make a calibration curve. Good lineal correction was obtained between these dilution factors and anti-HDM specific IgE antibody titer. ANOVA was used for statistical analysis of the results of the ELISA, and results with less than a 5% hazard ratio were considered to be significant.

HISTOLOGICAL STUDY

The removed eyeballs and eyelids were fixed in 10% neutral formalin for 2 hours and embedded in paraffin. The paraffin block was cut into about 7-μm thick sections with a microtome (HM505E, Microm International GmbH, Walldorf, Germany). The paraffin-sections were stained using the direct fast scarlet (DFS) staining method.

CELL DENSITY OF EOSINOPHILS IN CONJUNCTIVAL TISSUE

After staining for each group of conjunctiva sections, photographs were taken with a light microscope (BH 2, Olympus, Tokyo, Japan). The total area of the subconjunctival tissue in each digital image was calculated using a semi-automated image analysis method employing a computer (Apple Macintosh) with Adobe Photoshop Elements 9 software (Adobe Systems Inc., San Jose, CA, USA). The density of eosinophils (/mm²) stained with DFS staining in the subconjunctival tissue was calculated. The cell densities in 15 thin sections for each group were counted and averaged. Statistical analysis was performed using the Kruskal-Wallis H-test, and differences of less than 5% were regarded as statistically significant.

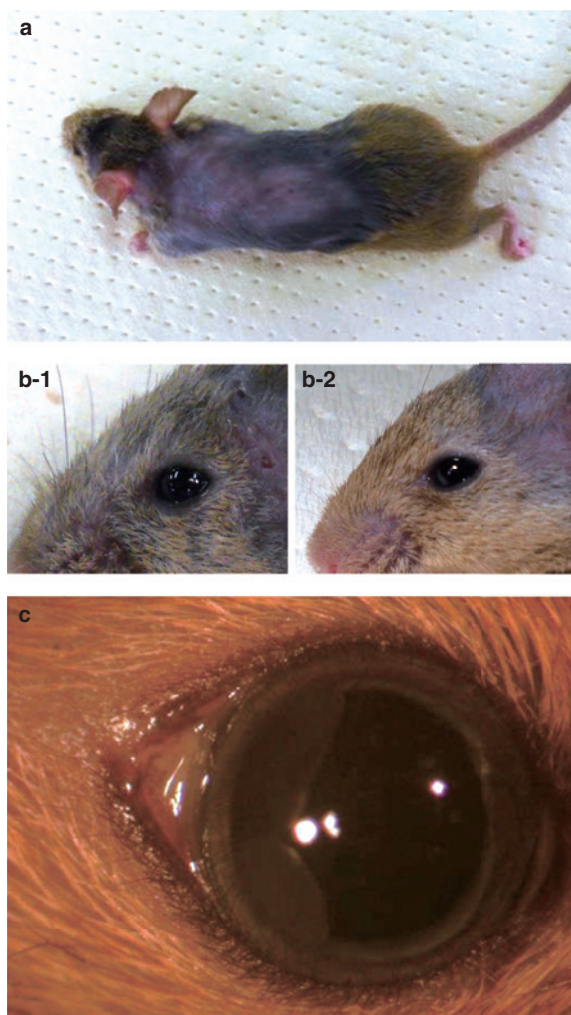


Fig. 2 Development of atopic keratoconjunctivitis-like ophthalmic lesion in NC/Nga mouse. As clinical observation in the DFb group, dermatitis-like skin lesion (**a**), blepharitis (**b-1**) and conjunctivitis (**c**) are observed. The blepharitis is observed in the DFb group (**b-1**) and is absent in the Ba group (**b-2**). For observations of the conjunctivitis, severe conjunctival hyperemia and chemosis are noted.

RESULTS

CLINICAL OBSERVATIONS

As the antigen was applied to the skin, ear and eyelid areas in the DFb group, dermatitis was observed macroscopically as shown in Figure 2a. Furthermore, blepharitis (Fig. 2b) and severe hyperemia (Fig. 2c) in bulbar conjunctiva were observed in the DFb group. In the DFco group, mild conjunctival hyperemia without blepharitis was observed.

TOTAL IgE AND HDM-SPECIFIC IgE ANTIBODY LEVELS IN SERUM

Total serum IgE concentration was 7439.2 ± 2749.2 (mean $\pm$ SD) ng/ml for the DFb group and $7000 \pm$

1615.4 for the DFco group. Both the skin sensitized DFb group and DFco group showed a significantly higher total serum IgE concentration than the non-sensitized group, the NT group, as shown in Figure 3 ($P < 0.01$, ANOVA).

The concentration for HDM-specific serum IgE antibodies in the DFb, DFco, and NT groups were 302.7 ± 260.3 (mean $\pm$ SD) MIU/ml, 335.8 ± 283.8 , 7.2 ± 2.7 ng/ml, respectively. Concentration for HDM-specific serum IgE antibodies in the DFb and DFco groups was significantly higher than those in the NT group ($P < 0.05$, ANOVA) (Fig. 4).

HISTOLOGICAL STUDY

In the DFb group, subepithelial cellular infiltration mainly by eosinophils was observed in both the bulbar and palpebral conjunctiva diffusely (Fig. 5a). The palpebral conjunctiva in the DFb group showed tubular mimic changes of the epithelium and pseudotubular formation (Fig. 5b). Torus-form infiltration due to massive lymphocytes in the palpebral conjunctiva (Fig. 5c) and dome-shaped elevation due to subepithelial granuloma composed of lymphocytes and eosinophils were found in the limbal conjunctiva (Fig. 5d). This subepithelial eosinophil infiltration in the DFb group was severer than in the DFco group and the Ba group (Fig. 6).

CELL DENSITY OF THE EOSINOPHILS IN CONJUNCTIVAL TISSUE

Cell density of the eosinophils (cells/mm²) in the subepithelial tissue was 878.4 ± 399.7 (mean $\pm$ SD) for the DFb group, 85.6 ± 40.4 for the DFco group and 49.3 ± 32.4 for the Ba group. Cell density in the DFb group was significantly higher than that in the DFco group and the Ba group (vs DFco group; $P < 0.05$, vs Ba group; $P < 0.001$, respectively) (Fig. 7).

DISCUSSION

We aimed to produce an AKC model in mice using NC/Nga mice. When reared under conventional conditions, NC/Nga mice develop spontaneous onset atopic dermatitis-like skin lesions by the time they are 8 weeks old,¹⁰ but the incidence of atopic dermatitis onset is indefinite. To overcome this unreliable factor, an atopic dermatitis model was developed using the repeated application of Dfb ointment to the barrier-disrupted back skin of NC/Nga mice reared under specific pathogen free conditions instead of conventional conditions.^{9,11} We were able to produce an AKC model in mice by the additional repeated treatment of NC/Nga mice who developed atopic dermatitis-like skin lesions with ophthalmic ointment containing Dfb. The histopathological and immunological studies confirmed that the sensitized mice showed not only atopic dermatitis-like skin lesions but also had a high serum IgE concentration and AKC compatible conjunctivitis.

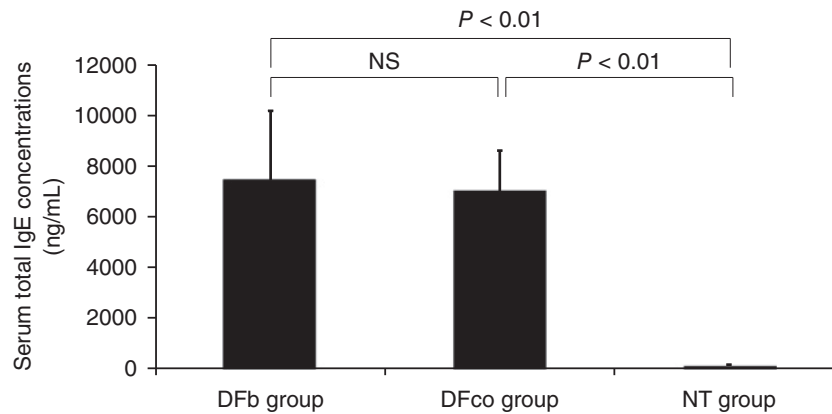


Fig. 3 Total IgE concentration in serum. DFb group ($P < 0.01$, ANOVA) and DFco group ($P < 0.01$) show significantly higher total serum IgE concentration than non-sensitized group, NT group. NS, not significant.

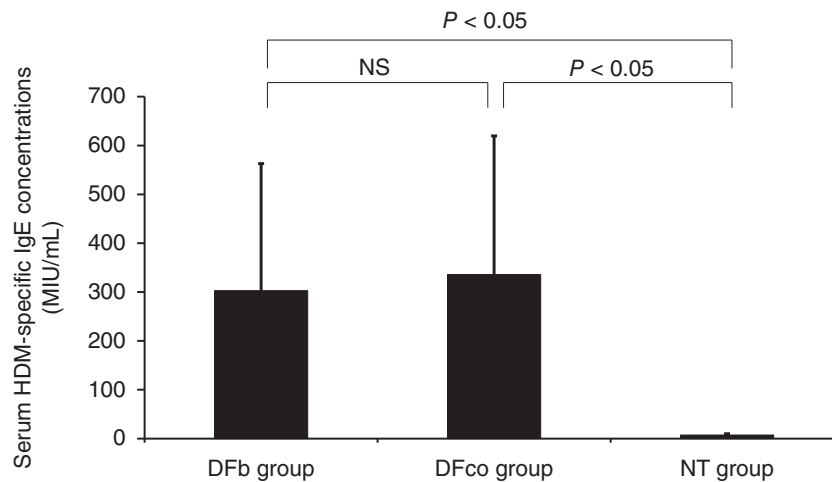


Fig. 4 HDM-specific IgE antibody concentration in serum. DFb group ($P < 0.05$, ANOVA) and DFco group ($P < 0.05$) show significantly higher HDM-specific IgE antibody concentration than non-sensitized group, NT group. NS, not significant.

After a systemic sensitization of the NC/Nga mice was performed according to a previous report,⁹ we succeeded by the additional repeated application of an ophthalmic ointment containing DFb in producing an AKC model with good reproducibility. In terms of the increased serum IgE concentrations in the AKC model, both the total IgE and the HDM-specific IgE antibodies were increased. An increase of serum IgE was induced in the DFb and DFco groups, but not in the NT group, indicating that the increase in serum IgE was caused by skin sensitization initiated by the Dfb ointment. Serum HDM-specific IgE antibody study revealed that the development of atopic dermatitis-like skin lesions can be a useful index in the finding of type I allergic sensitization.

The characteristics of the AKC mouse model can be summarized as afflicted by conjunctivitis due to skin sensitization and antigen-challenge ophthalmic

ointment. Currently reported allergic conjunctivitis animal models were induced by either passive or active sensitization.¹² In most conjunctivitis models induced by active sensitization, eye drops containing a specific antigen were instilled after systemic sensitization using an adjuvant and an antigen. In these methods, pathologic findings may differ depending on the animal species and the properties of the adjuvant. The reported conjunctivitis models were a guinea pig model induced by alum as an adjuvant and ovalbumin (OVA) as an antigen,¹³ Brown Norway rat model induced by Freund's complete adjuvant and short ragweed pollen (RW)¹⁴ and a Balb/c mouse model induced by alum and OVA.¹⁵ With the conjunctivitis model using NC/Nga mice, blepharitis and conjunctivitis¹⁶ and an increase in mast cell density in the subconjunctival tissue¹⁷ were reported with the additional complication of the development of atopic

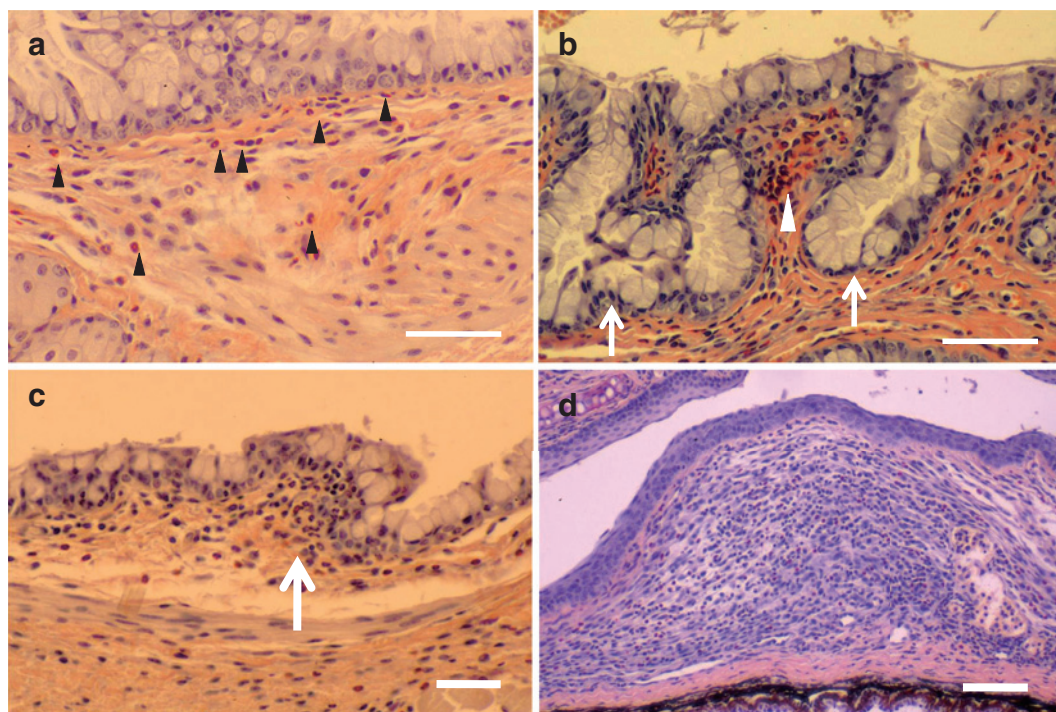


Fig. 5 Histological changes in conjunctival tissues in Dfb group. **a:** Infiltration of inflammatory cells with prominent eosinophil (arrowhead) is observed in conjunctival subepithelial tissue. Bar = 100 µm. **b:** Pseudotubular formation (forward arrow) in conjunctival epithelium and marked infiltration of eosinophil (white triangle) in subepithelial tissue can be observed. Bar = 100 µm. **c:** Torus-form infiltration due to massive lymphocytes (arrow) is found in palpebral conjunctiva. Bar = 100 µm. **d:** Dome-shaped elevation due to subepithelial granuloma composed of lymphocytes and eosinophils is found in limbal conjunctiva. Bar = 100 µm.

dermatitis-like skin lesions. In these experimental animal models, early and late phase reactions with immediate hypersensitivity are observed, but the inflammatory reaction is not so severe compared with clinical and pathological findings in AKC. In this study, AKC compatible clinical findings could be obtained. There were two major differences from the previous reports. The first one was the use of dermatologic ointment with Dfb used for skin sensitization. The other was that an adjuvant was not used.

Characteristic pathological findings in the human conjunctiva of AKC are reported to be infiltrations of mast cells and eosinophils in the epithelium, goblet cell proliferation, epithelial pseudotubular formation, prominent mast cells and eosinophils in the substantia propria and infiltration of chronic mononuclear cells in the substantia propria.¹⁸ In the present AKC model, the histological study of the conjunctiva revealed the infiltration of inflammatory cells with prominent eosinophils in the subconjunctival tissue and pseudotubular formation in the conjunctival epithelium. Furthermore, torus-form proliferation due to the infiltration of lymphocytes in the palpebral conjunctiva and dome-shape lesions with subepithelial granuloma composed of lymphocytes and eosinophils

at the limbal zone are compatible with the papillary proliferation of the palpebral conjunctiva and gelatinous infiltration at the limbus in humans. These pathological findings are thought to be proliferative conjunctival lesions of AKC and different from the conjunctival inflammatory findings in allergic conjunctivitis. Thus, we concluded that the present AKC model mouse is histologically compatible with AKC in humans.

Another breakthrough in the present study is the application of an ointment containing body of *Dermatophagoides farinae* (Dfb) rather than the extracted *Dermatophagoides farinae* (Df) antigen. The application of Dfb caused severer eosinophil infiltration than that of the Df antigen. Rather than the application of a component antigen, the application of the crude antigen plays a pivotal role in inducing human AKC compatible findings.

It is noteworthy that, in the present mouse model HDM-antigen specific IgE increased concomitantly with the total IgE in the serum, indicating that type I allergic response takes part in the etiology of AKC. But the pathological mechanism remains unclear as to why the present mouse model was induced by a crude antigen to produce a more severe infiltration of

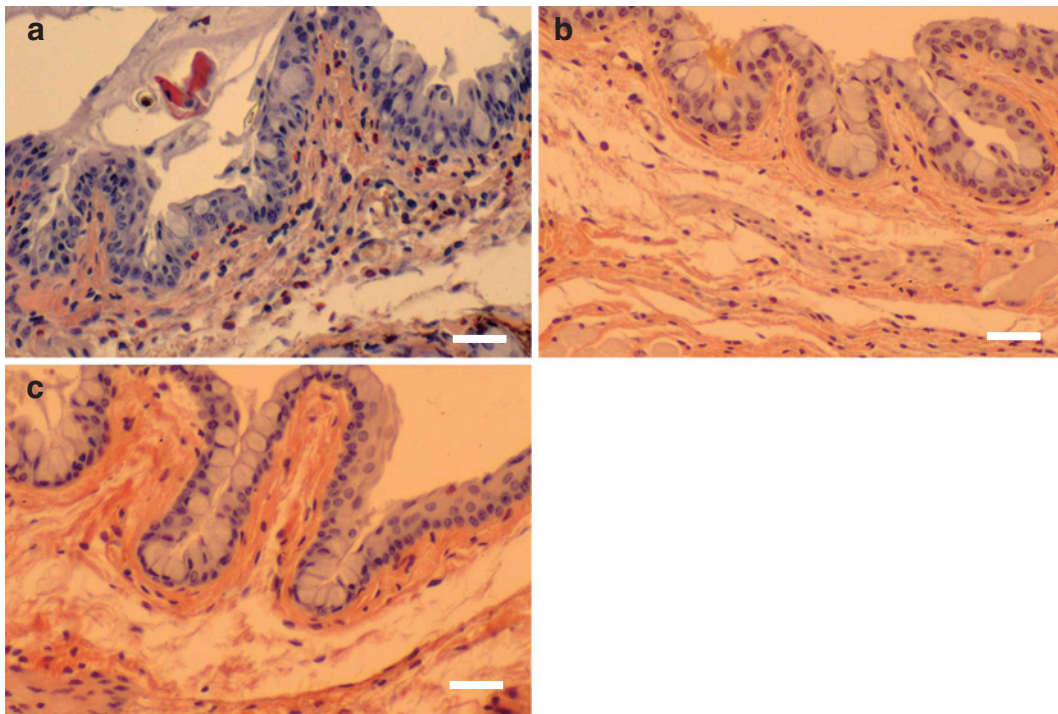


Fig. 6 Comparison of eosinophilic infiltration among the three groups. The DFb group shows more marked infiltration of eosinophils than the DFco group and Ba group. **a:** DFb group, **b:** DFco group, **c:** Ba group. Bar = 100 μ m.

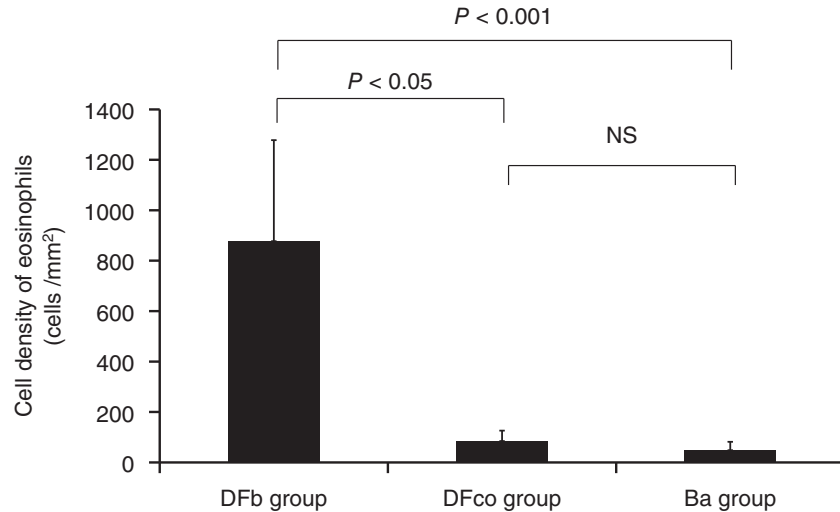


Fig. 7 Cell density of eosinophils in the subconjunctival tissues. Cell density of infiltrating eosinophils in the subconjunctival tissue in the DFb group is significantly higher than that in the DFco group and the Ba group (Kruskal-Wallis H-test). NS, not significant.

eosinophils than that by a component antigen. Further investigation regarding this pathological mechanism including allergic inflammation in the conjunctiva and the proliferative conjunctival lesion in this model mouse is needed.

The newly developed AKC mouse model can de-

velop similar clinical and pathological findings as human AKC. It is concluded that this mouse model can be useful not only in the investigation of the pathological mechanism of the proliferative etiology in allergic conjunctival disorders but can also be used to evaluate eosinophilic inflammation in AKC.

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