INTRANASAL SENSITIZATION WITH Blomia tropicalis ANTIGENS INDUCES ALLERGIC RESPONSES IN MICE CHARACTERIZED BY ELEVATED ANTIGEN-SPECIFIC AND NON-SPECIFIC SERUM IgE AND PERIPHERAL BLOOD EOSINOPHIL COUNTS

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SUMMARY

In order to evaluate the potential allergenicity of Blomia tropicalis (Bt) antigen, IgE production of both specific and non-specific for Bt antigen was monitored in BALB/c mice after exposure to the antigen by nasal route. It was evidenced that B. tropicalis contains a functional allergen in its components. The allergenic components, however, when administered intranasally without any adjuvant, did not function to induce IgE response within a short period. On the other hand, intranasal inoculation of Bt antigens augmented serum IgE responses in mice pretreated by a subcutaneous priming injection of the same antigens. Inoculation of Bt antigen without subcutaneous priming injections induced IgE antibody production only when the antigen was continuously administered for a long period of over 24 weeks. Even when the priming injection was absent, the Bt antigen inoculated with cholera toxin (CT) as a mucosal adjuvant also significantly augmented the Bt antigen-specific IgE responses depending on the dose of CT co-administered. The present study also demonstrated that Bt antigen/CT-inoculated mice showed increased non-specific serum IgE level and peripheral blood eosinophil rates without noticeable elevations of the total leukocyte counts. The immunoblot analysis demonstrated 5 main antigenic components reactive to IgE antibodies induced. These components at about 44-64 kDa position were considered to be an important candidate antigen for diagnosis of the mite-related allergy.

KEYWORDS: Mite; Blomia tropicalis; Intranasal sensitization; Mucosal adjuvant.

INTRODUCTION

Blomia tropicalis VAN BRONSWIJK et al., 197329 is a species of house dust mites which belongs to the family Glycyphagidae. The mite has been ubiquitously found in common houses in tropical and subtropical areas1,2,10,21,23. Although there have been a few reports indicating the scarce distribution of the genus Blomia in mainland of Japan18,20, the authors and other investigators have recently reported that B. tropicalis is one of the major mite species found in Okinawa, a subtropical prefecture of Japan14,26,27.

B. tropicalis, as well as Dermatophagoides pteronyssinus, has been implicated as one of the important causative agents of mite-related allergic diseases in many countries in South America and Southeast Asia, because patients with typical allergic symptoms, such as asthma, atopic dermatitis and rhinitis, in these areas are known to show positive skin responses and high levels of serum IgE against antigens of B. tropicalis3,6,8,13,15,19,25. These facts strongly suggest that B. tropicalis contains allergen(s) with the potential to induce systemic IgE responses in humans4,5. However, there were few experimental studies to demonstrate the Bt-specific IgE production by antigen exposure24. In the present study, the authors tried to demonstrate Bt antigen-specific serum IgE production in mice exposed to Bt antigen by nasal route.

MATERIALS AND METHODS

Mice: Male BALB/c mice, 4-6 weeks old, were purchased from Seac Yoshitomi Ltd. (Fukuoka, Japan) and kept in the animal care facility throughout the course of experiments, according to the guidelines for animal experimentation in the University. The protocols for the experiment were also approved by the Committee for animal care and use in the University.

Extraction of mite antigen: B. tropicalis collected from dust in an old Okinawan house was cultured to propagate in a food medium consisting of an equivalent mixture of rodent chow (Clea Rodent Diet CE-2, Clea Japan Inc., Tokyo, Japan) and dry yeast (Ebiosu, Tanabe Seiyaku Co., Osaka, Japan) at 28 °C with 85% humidity. After 2 months of cultivating, the propagated mites were collected using a modified Tullgren apparatus. The antigens were extracted from the mites essentially according to the method of STANALAND et al.25. Briefly, the mites were treated with an excess of cold diethylether for delipidization for 4...
hr in Erlenmeyer flask without agitation, and dried at room temperature after the diethyl ether was removed. The mites were then homogenized with a glass homogenizer and the homogenates were stirred to extract antigens for 24 hr at 4 °C in PBS (pH 7.2) containing 0.02% NaN₃, as a disinfectant. The extract was then centrifuged at 10,000 rpm for 30 min and the supernatant fluid was recovered as the source of Bt antigen. Protein concentration in the extract was measured by the Bradford method (Protein assay, Bio-Rad Lab., USA) and the antigen solution was stored at –85 °C until use.

The extracts from other mite species, *Dermatophagoides pteronyssinus* (Dp) and chironomid species, *Tokunagayusurika akamusi* (Ta) and *Chironomus yoshimatsui* (Cy), were also served in the present study. *D. pteronyssinus* were cultured similarly by the author but the chironomid species were kindly supplied by Dr. Matsuoka, Jichi University, Tochigi, Japan.

**Antigen sensitization of mice:** The intranasal sensitization of mice with Bt antigen was performed by dripping a drop (approximately 10 µl) of the Bt antigen solution (4.0 mg/ml) into the nasal cavity of mice. In order to prime immune responses generally, subcutaneous injections of Bt antigen emulsified in an equal volume of Freund’s complete adjuvant (Difco Lab, USA), twice at an interval of 7 days, was performed in a group of mice prior to the intranasal inoculation. The effect of mucosal adjuvant on induction of Bt-antigen specific IgE was also evaluated in another group of mice inoculated intranasally with the mixture of Bt antigens and cholera toxin (CT; Sigma Chemical Co., USA). The mice which were administered PBS, instead of Bt antigen, were served as the control for each experiment.

Mice were periodically bled by tail vein puncture or cardiac puncture 2-7 days after the final inoculation, and the sera were separated to store at –85 °C.

**Passive cutaneous anaphylaxis (PCA):** PCA was applied to detect Bt-antigen specific IgE response. Briefly, sensitized or control mouse sera were diluted five-fold in physiological saline and 50 µl of the diluted sera was injected intradermally on a shaved back of a male Wistar rat (Seac Sehleich & Schuell, Germany) for 1 hr at 60V. The blotted membrane was soaked in 1.0% BSA solution in PBS containing 0.05% Tween-20 for 1 hr at 37 °C, and the color was allowed to develop by soaking the membrane in a diaminobenzidine substrate solution (DAB; D-4418, Sigma, Germany). The polypeptides separated in the gel were transferred to a nitrocellulose membrane (BA-S 83, Schleicher & Schuell, Germany) for 1 hr at 60V. The blotted membrane was soaked for 30 min at room temperature. The wells were further filled with HRP labeled anti-mouse IgE for 30 min at room temperature. The wells were finally filled with substrates and allowed to develop colors for 30 min. The reaction was stopped by addition of a 2.0N HCl solution (100 µl for each well).

**ELISA for specific IgE measurement:** IgE antibody levels against Bt antigen were determined by indirect ELISA method as follows: Bt antigen was dissolved at a protein concentration of 100 µg/ml in 50 mM carbonate buffer, pH 9.65, and 100 µl of this was applied to each well of a polystyrene microplate (MS-8496F, Sumitomo Bakelite Co., Tokyo, Japan). The microplate was incubated first for 2 hr at 37 °C to sensitize the well surface with Bt antigen and subsequently for overnight at 4 °C. After removal of the contents, the wells were further treated with 300 µl of 2.0% bovine serum albumin (BSA; Sigma Chemicals Co., USA) in PBS for 2 hr at 37 °C to block the remaining binding sites on the well surface. After washing three times with 0.05% Tween-20 using a plate washer (Immuno Wash Model 1575, Bio-Rad Lab., Italy), the wells were filled with 100 µl of mouse test sera diluted 10-fold in 1.0% BSA-PBS, and incubated for 2 hr at 37 °C followed by overnight incubation at 4 °C. After similar washing, 100 µl of rat anti-mouse IgE monoclonal antibodies (affinity purified; IM2992, Immunotech, France), diluted in 1:300, was applied to each well. After incubation for 2 hr at 37 °C, the wells were washed and further incubated with 100 µl of rabbit anti-rat IgG antibodies conjugated with horseradish peroxidase (HRP) (SAB-200; StressGen Biotechnologies Corp., Canada), diluted in 1:300, for 2 hr at 37 °C. After washing wells, 100 µl of a 0.01%-orthophenylene-diamine solution was added to each well as substrate of HRP, and the color was allowed to develop for 30 min at room temperature. The reaction was stopped by adding 10 µl of 4.0M H₂SO₄ and absorbance (OD) was read at 490 nm using a Microplate Reader Model 550 (Bio-Rad Lab., Italy).

**Total serum IgE measurement:** Total serum IgE was measured according to the manufacturer’s instructions using the mouse IgE measurement kit (Yamasa EIA, Yamasa-syoyou Co., Tokyo, Japan). The wells of a microplate coated with anti-mouse IgE monoclonal antibodies were allowed to capture IgE in the test serum by incubation with 100 µl of: 1:24 diluted test sera for 30 min at room temperature. After washing, the wells were further incubated with HRP labeled anti-mouse IgE for 30 min at room temperature. The wells were finally filled with substrates and allowed to develop colors for 30 min. The reaction was stopped by addition of a 2.0% orthophenylendiamine solution (100 µl for each well). Mouse IgE isotype standard solutions were used to construct the standard curve (10-500 ng/ml) and total serum IgE levels were calculated from the standard curve, based on the optical density measured at 450 nm.

**Total leukocyte count and eosinophil rate:** Total leukocyte numbers in peripheral blood were counted according to the standard method using Neubauer’s hemocytometer (Erma, Tokyo, Japan). Thin blood films smeared on a glass slide were stained with Giemsa’s staining solution and the percentages of eosinophils were calculated by counting the number of eosinophil in a total of 200 leukocytes. The measurements were repeated twice and the average was calculated.

**Immunoblotting:** The antigen components reactive to anti-Bt IgE antibody were identified by Western blot analysis using the mouse sera sensitized with Bt antigen. Briefly, the crude extract from *B. tropicalis* (20 µg) was applied on a 12.5% SDS-PAGE gel and electrophoresed for 2 hr at 120V in the presence of 5% 2-ME (2-mercaptoethanol, Nakarai, Kyoto, Japan). The polypeptides separated in the gel were transferred electrophoretically onto a nitrocellulose membrane (BA-S 83, Schleicher & Schuell, Germany) for 1 hr at 60V. The blotted membrane was soaked in 1.0% BSA solution in PBS containing 0.05% Tween-20 to block binding sites on the membrane. Then, the membrane was well incubated with 25-fold diluted mouse serum for 1 hr at 37 °C. After washing three times with PBS-Tween 20, the membrane was further incubated with rat anti-mouse IgE monoclonal antibodies (affinity purified; IM2992, Immunotech, France) for 1 hr at 37 °C. After washing similarly, the membrane was treated with HRP-conjugated rabbit anti-rat IgG antibodies (SAB-200; StressGen Biotechnologies Corp., Canada) for 1 hr at 37 °C, and the color was allowed to develop by soaking the membrane in a dianinobenzidine substrate solution (DAB; D-4418, Sigma, Germany).

**Statistics:** Statistical analysis was performed by computer software (StatView-J4.11). ANOVA and Fisher’s PLSD were used to analyze differences in IgE levels, leukocyte counts and eosinophil rates. A p value less than 0.05 was considered to be statistically significant.
RESULTS

Induction of serum IgE by inoculation of Bt antigen: Results of the PCA test in mice variously exposed to Bt antigen for 50 days are summarized in Table 1. The mice inoculated intranasally with Bt antigen without priming immunization were completely unresponsive in the PCA test. However, when mice were pretreated with a subcutaneous injection of the Bt antigen, the PCA response became positive in many mice. The PCA responses after the priming subcutaneous injection were generally stronger in mice followed with a subsequent intranasal inoculation of Bt antigen, as represented in Fig. 1.

The effect of co-administration of mucosal adjuvant on the induction of Bt-specific IgE response was monitored in the other groups of mice and the results are summarized in Table 2. Co-administration of cholera toxin (CT) as a mucosal adjuvant produced strong PCA responses in mice that received Bt antigen without priming immunization, although the mice received Bt antigen or CT alone failed to respond in the PCA test (Fig. 2). When CT was administered as a mucosal adjuvant, the intranasal inoculation of Bt antigen induced specific IgE responses in a relatively short sensitization period of 35 days (5 times at an interval of 7 days).

To examine the dose-dependent effect of Bt antigen and adjuvant CT on induction of Bt antigen-specific IgE response, different dosages of Bt antigen and CT were co-administered and serum IgE responses were determined by ELISA method for the specific IgE (Fig. 3). The mice inoculated with Bt/CT mixture showed elevated levels of specific IgE on day 35 in a dose-dependent manner of Bt antigen and CT. Positive responses in PCA test were observed with 1.0-10 µg of CT inoculated with Bt antigen (20 µg), but not with 0.1 µg of CT inoculated with the same antigen (data not shown).

Finally, the production of specific IgE was monitored for a long period of 24 weeks without previous priming immunization and co-administration of CT. As shown in Fig. 4, specific serum IgE levels gradually elevated by week 16 throughout the course of inoculation, although the levels were significantly lower than the IgG levels in the sera of mice inoculated with Bt/CT mixture.

Elevation of total serum IgE by inoculation of Bt antigen: The total serum IgE levels measured in the indicated groups of mice inoculated intranasally with Bt antigen, CT and Bt/CT mixture are represented in Fig. 5. Non-specific total IgE levels also elevated significantly in the sera of mice inoculated with Bt/CT mixture on day 35.

Eosinophil count in Bt inoculated mice: Total counts of leukocyte

and ratios of differential counts of eosinophils in peripheral blood were also monitored (Fig. 6). Although leukocytes counts were not so different among the groups of mice throughout the experiments, the percentages of peripheral blood eosinophils increased significantly in the mice exposed to Bt/CT mixture.

**Immunoblot analysis on allergens in the Bt extract:** The major allergenic components in the Bt extract were analyzed for their reactivity to IgE antibodies induced by Bt/CT inoculation. As shown in Fig. 7, the immunoblot analysis showed that more than eight polypeptide
components in the extract are reactive to the IgE antibodies. Five bands at position corresponding to molecular weights 64, 60, 52, 49, and 44 kDa were relatively strong in their reactivity.

In Fig. 8, immunoblot patterns were compared between the sera from mice sensitized with Bt/CT mixture and those from mice sensitized with Bt antigen alone for a long period of over 24 weeks. The appearance of bands with serum sensitized only with Bt antigen was significantly weaker than that of bands with serum inoculated with Bt/CT mixture. Furthermore, the patterns of antigenic recognition by serum sensitized only with Bt antigen varied from those sensitized with Bt/CT mixture; some bands including major bands at the 44-52 kDa position were absent in the pattern with the serum exposed only to Bt antigen.

Fig. 9 represents SDS-PAGE patterns of extracts from Dermatophagoides pteronyssinus (Dp), Tokunagayusurika akamusi (Ta) and Chironomus yoshimatsui (Cy) and its cross-reactivity to the sera sensitized with Bt/CT mixture. Scarce but notable bands were observed only with the extract of D. pteronyssinus and mouse anti-Bt IgE antibodies induced.

**DISCUSSION**

*B. tropicalis* is a common mite species in the living environment of humans in tropical and subtropical regions. The mites and mite-related antigens have frequently been found in house dust in these areas and it has been indicated that the patients with allergic diseases, such as asthma and atopic dermatitis, respond strongly to Bt antigen in skin-prick test, suggesting the presence of allergen(s) which induces IgE antibodies specific to *B. tropicalis* antigen. The experimental study to identify
the antigen(s) having the potential as allergen(s) in the mite components may be important in developing a useful diagnostic antigen for allergic diseases in these regions.

However, there were few experimental studies with animal model to induce Bt-specific IgE antibodies by administration of Bt antigen. In their study to induce oral tolerance of IgE response to B. tropicalis, SATO et al. demonstrated that IgE levels were markedly increased in mice by subcutaneous immunization of Bt antigen. In the present study, the authors have first demonstrated that B. tropicalis-derived antigens could systematically induce allergic responses in mice by nasal route, providing the direct evidence that the mite antigens could function as potential allergens in vivo if administered properly.

A notable increase in IgE levels, specific and non-specific for Bt antigen, was recognized by intranasal inoculation of the antigen following the priming subcutaneous immunization of the same antigen or co-administration of CT as a functional mucosal adjuvant. Intranasal inoculation of the Bt antigen without priming sensitization, however, seemed to be insufficient for the induction of effective IgE responses. The production of IgE antibodies could not be confirmed when mice were exposed to Bt antigen alone for a short period. The administration of Bt antigen without priming immunization and adjuvant CT induced low-level specific IgE, only when the antigen was administered many times for a long period (24 times over 24 weeks). The strong immunostimulating effect of CT is attractive for a new mucosal vaccine strategy in which CT is expected to be an effective adjuvant to deliver vaccine antigen to the mucosal immune system. On the other hand, it has been indicated that immunization through mucosal tissue often induces an allergic response. The present study also indicates the possibility that vaccine antigen, when co-administered with CT as adjuvant, may induce an allergic response against the vaccine antigen. Induction of serum IgE by mucosal administration of antigen mixed with CT, however, may depend in part on the nature of antigen because while some antigens induce IgE production others do not (ARAKAWA, unpublished data). Studies on the nature of antigens and immune responses against the antigens through mucosal tissue are important not only for the development of effective mucosal vaccines, but also for prevention of allergic diseases through mucosa.

Immunoblotting analysis using the sera with high IgE levels detected more than 8 bands that reacted with the IgE antibodies at a relatively high molecular weight position of over 40 kDa. Among the reactive components, five components of molecular weights 64, 60, 52, 49 and 44 kDa were clearly recognized between mice sera sensitized with Bt/CT mixture. In their study using B. tropicalis extract and asthmatic patient sera, RAMOS et al. have pointed out that an important candidate allergen in the mite extract was 66kDa polypeptide component which was identical to mite paramyosin. The authors also detected a similar band at 64 kDa position and the component was found to be reactive to anti-paramyosin antibody, suggesting that the component is identical to the mite paramyosin (date not shown). The isolation and sequence of the component should be further investigated so as to establish it as a good candidate for the diagnostic antigen for the mite allergy.

Finally, the cross reaction with other species of dust mite, namely D. pteronyssinus, and chironomid species, which are known as important insect inhalant allergens were almost negligible in the present study, although a cross reaction was faintly observed between D. pteronyssinus extract and antisera sensitized with Bt/CT mixture.
In conclusion, the present study has significance as the first evidence that Bt antigen elicited specific IgE production in mice through mucosal membrane. Particularly, it is noticeable that the mucosal adjuvant CT inoculated with Bt antigen elicited stronger responses of Bt-specific IgE antibodies in a relatively short duration. The immunoblot analysis identified that the component at about 44-64 kDa position was nominated as a predominant and functional allergen in the mite extract. Using the induced IgE antibodies, further study to identify and clarify the allergenic component, in respect to its immunologic properties and identity with other mite allergens, is necessary.

REFERENCES


