

Characterization of Human IgE and Mouse IgG1 Responses to Allergens in Three Mosquito Species by Immunoblotting and ELISA

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Key Words

Allergen · Human IgE · Mouse IgG1 · Immunoblot · ELISA · *Aedes togoi* · *Culex tritaeniorhynchus* · *Culex pipiens pallens*

Abstract

Background: IgE-mediated allergic reactions caused by mosquito bites are a common problem all over the world. This study was undertaken to determine IgE levels in subjects, to elucidate human IgE and mouse IgG1 binding patterns and to investigate the cross-reactivity of salivary gland antigens with three mosquitoes. **Methods:** Mosquito larvae of *Aedes togoi*, *Culex tritaeniorhynchus* and *Culex pipiens pallens* were collected and maintained in the laboratory. Salivary gland extracts (SGE) and whole-body extracts (WBE) were prepared from female mosquitoes of each species. Mosquito-specific IgE levels in 17 subjects were measured by ELISA. Polypeptide patterns were analyzed by SDS-PAGE. Immunoblotting was performed with sensitized human and immune mouse sera, and elucidated human IgE and mouse IgG1 binding patterns to SGE. For the determination of cross-reactivity to the three types of mosquitoes, ELISA inhibition tests were performed using sera from mice sensitized by biting of *A. togoi*. **Results:** The 9 sera out of 12 with positive skin reactions to SGE of *A. togoi* by skin prick test showed significantly higher anti-mosquito SGE IgE levels than in those without skin reactions. Protein band pat-

terns of the SGE and WBE of the three species were different from one another. Specific human IgE reacted to the protein in SGE of 30.5, 33, 37 and 57.5 kD from *A. togoi*, of 38, 43 and 68 kD from *C. tritaeniorhynchus*, and of 23, 33, 34, 43, 44, 60, 74 and 93 kD from *C. pipiens pallens*. There were specific mouse IgG1 reactions to the bands of 30.5, 33, 37 and 57.5 kD in the SGE of *A. togoi*. The ELISA inhibition studies disclosed almost no cross-reactivities between *A. togoi*, *C. tritaeniorhynchus* and *C. pipiens pallens*. **Conclusions:** Immunoblot analysis disclosed that allergenic proteins in the SGE of mosquitoes and their patterns were remarkably similar between human and mouse sera to the SGE of *A. togoi*. Species-shared allergens may not exist among the three mosquito species prevalent in Korea.

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Introduction

The mouth parts of mosquitoes are modified to pierce the tissues and then suck blood from humans or other mammals [1, 2]. Mosquitoes inject saliva containing antigenic proteins into the wound [3]. Their bites can cause immediate cutaneous reactions, such as wheal and flare, delayed reactions and occasionally systemic reactions in humans [4–6].

Recently, it has been reported that mosquito saliva-specific IgE and IgG antibodies are involved in mosquito

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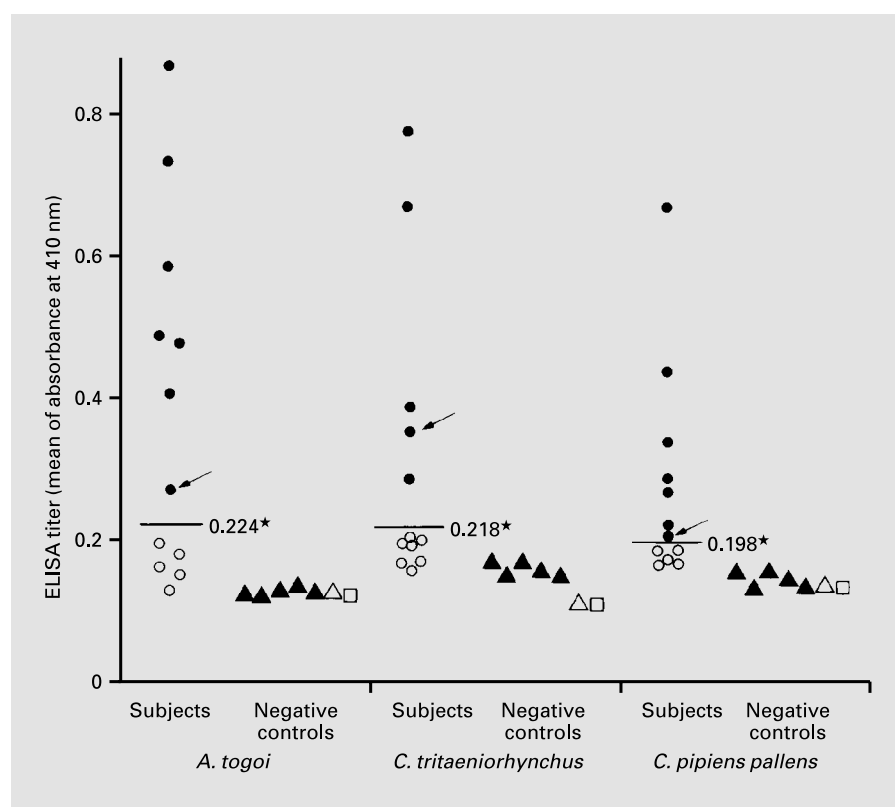
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Fig. 1. Distribution of IgE levels in 12 subjects in response to the SGE of three mosquito-to species by ELISA. Sample sera were tested individually in duplicate and the seropositive cut-off value was determined as the mean + 3 standard deviations of absorbance values in negative subjects on skin prick tests with *A. togoi* SGE. ● = Positive on skin test and seropositive; ○ = positive on skin test and seronegative; ▲ = negative subjects; △ = cord blood sera; □ = PBST. The arrows show species-specific reactions and the asterisks show the cut-off values.



bite allergy [7, 8]. In order to confirm these responses, Prausnitz-Küstner tests [9], RAST [10], ELISA [11] and immunoblot techniques [12–14] have been carried out. Previous studies have revealed both species-shared and species-specific allergens from 10 mosquito species [12].

In Korea, 9 genera and 48 species of mosquitoes have been described [15]. Of these, *Aedes togoi* and *Culex tritaeniorhynchus* transmit infectious organisms including filaria and Japanese B encephalitis virus. *Culex pipiens pallens* shows a high population density and is a direct cause of nuisance [16, 17]. The *A. togoi* species is distributed on most islands and at the seaside. *C. tritaeniorhynchus* inhabits the rural areas. *C. pipiens pallens* is found in urban environments and some rural areas. However, the allergenicity of salivary gland extracts (SGE) of Korean mosquitoes in humans and experimental mice has not been reported until now.

This study was undertaken to determine the serum IgE levels of mosquito species-specific antibodies in subjects with a skin reaction to SGE by ELISA and immunoblotting. Immunoblot analyses were performed with sensitized human and immune mouse sera and elucidated human IgE and mouse IgG1 binding patterns to SGE.

Materials and Methods

Mosquitoes and SGE

Mosquito larvae were collected from rock pools at the seaside for *A. togoi* and from ponds in rural areas for *C. tritaeniorhynchus* and *C. pipiens pallens*. The collected larvae were maintained for emergence in the laboratory and once the adults emerged, females were collected, anesthetized by CO₂ gas and identified. Salivary glands were obtained by dissection of 3- to 5-day-old adults in 0.02 M PBS, pH 7.2, under a stereomicroscope, and transferred to fresh PBS on ice. About 50 pairs of salivary glands were obtained and lyophilized. The SGE were reconstituted by dissolving the lyophilized salivary gland proteins in PBS before use. This was sonicated three times at 20 W for 15 s and centrifuged at 10,000 g for 20 min. The supernatant was filtered through a 0.45-μm Amicon filter and the protein concentrations were measured with a Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, Calif., USA).

Human Sera

The Allergy Clinic, Severance Hospital, College of Medicine, Yonsei University, Seoul, Korea, provided 17 human sera from subjects with a history of severe skin reactions to mosquito bites, and 12 positive and 5 negative sera of subjects on skin prick tests with *A. togoi* SGE (fig. 1). The skin prick test was done with SGE in 0.3% phenol saline and 50% glycine. The wheal produced by *A. togoi* was at least as large as that of the histamine-positive control (1 mg/ml) in all patients. Sera were stored at –20°C before use.

Production of Antisera in Mice

The mice were immunized by the method of Brummer-Korvenkontio et al. [18] with some modifications [1]. Briefly, 20 live female *A. togoi* per BALB/c mouse were released to inject the antigens naturally, twice a week, for 12 weeks. Serum samples were collected once every 4 weeks during the immunization period. All sera were stored at -20°C before use.

Enzyme-Linked Immunosorbent Assay

Mosquito-specific IgE levels in human serum samples were measured by ELISA. Optimal conditions for dilutions of reagents were determined by checkerboard titration. Polystyrene plates (Costar, Cambridge, Mass., USA) were coated with the SGE (0.5 $\mu\text{g/ml}$), diluted in 0.05 M carbonate buffer (pH 9.6), and incubated overnight at 4°C . After rinsing the plates three times with a washing buffer (0.05% Tween 20 in 0.01 M PBS, pH 7.2), blocking was performed with 1% bovine serum albumin (BSA) in the washing buffer for 1 h at room temperature (RT). After being washed, test samples (1/20) and cord blood serum (1/20) for negative controls were incubated overnight at 4°C . The wells were washed, and 1/500 diluted alkaline phosphatase (AP) was conjugated to anti-human IgE (Sigma, St. Louis, Mo., USA) in 0.5% liver acetone powder solution (No. L-7751, Sigma) and then incubated for 1.5 h at RT. After a final wash, the enzyme substrate (p-nitrophenylphosphate, 1 mg/ml in diethanolamine buffer, pH 9.8) was added and incubated for 10 min at RT. The reaction was then stopped by the addition of 1 N NaOH. The absorbance was read at 410 nm using an ELISA reader (Dynatech, MR-5000, Chantilly, Va., USA). Liver acetone powder was added to a final concentration of 0.5% in PBST and then incubated for 30 min at 4°C and centrifuged at 10,000 g for 10 min. The supernatant was used for assay. Sample sera were tested individually in duplicate and the positive cut-off value in ELISA was determined as the mean + 3 standard deviations of absorbance values for negative subjects on skin prick tests [19].

SDS-PAGE and Immunoblot Analyses

SDS-PAGE was performed by the method of Laemmli [20] with 12% acrylamide separating gels. SGE of mosquitoes separated by SDS-PAGE were then electrophoretically transferred to a nitrocellulose membrane by the method of Towbin et al. [21]. Membranes were incubated with pooled serum samples with positive ELISA titers and sera of controls. Nitrocellulose strips were blocked for 1 h with 3% BSA. After several washes for 30 min, the strips were incubated with 1/10 diluted human pooled antisera overnight at 4°C , and then with AP-conjugated anti-human IgE (1/10, in 1% AcPd sol.) for 1.5 h at RT. The substrate (NBT/BCIP; Promega, Wisc., USA) for AP was added and incubated for 10 min at RT. The reaction was stopped by rinsing the strips in water.

ELISA Inhibition Tests

ELISA inhibition tests were performed using sera from mice made immune with *A. togoi* bites. A serum sample (dilution 1/200) was absorbed with 10-fold serially diluted SGE of *A. togoi*, *C. tritaeniorhynchus* and *C. pipiens pallens* and whole-body extracts (WBE) of *Dermatophagoides farinae* (house dust mite) from 10 to 0.001 $\mu\text{g/ml}$ at 4°C overnight. Polystyrene plates were coated with the SGE (0.5 $\mu\text{g/ml}$) of *A. togoi*. The wells were then reacted with 1/5,000 diluted AP-conjugated rabbit anti-mouse IgG1 (Zymed, San Francisco, Calif., USA) in a 1% liver acetone powder solution. The inhibition (as a percentage) of the test sera was calculated by the formula of Klatser et al. [22].

Results

Mosquito-Specific IgE Levels by ELISA in Humans

The results of ELISA for the 12 subjects are given in figure 1. The overall 75.0% (9/12) of skin test-positive subjects showed seropositive titers with SGE of the three mosquito species. The seropositive rates against *A. togoi*, *C. tritaeniorhynchus* and *C. pipiens pallens* were 58.3% (7/12), 41.7% (5/12) and 58.3% (7/12), respectively. Of 9 seropositive subjects, 4 showed positive titers to all three species and 2 subjects showed positive titers to *A. togoi* and *C. pipiens pallens*. Three sera showed positive titers against only one species, showing species-specific reactions. All of the control sera showed very low IgE levels.

SDS-PAGE and Immunoblot Analyses

The protein band patterns in WBE and SGE of the three species are illustrated in figure 2. Up to 19 polypeptide fractions were noted in the SGE and 26–30 polypeptide fractions were noted in the WBE of the three species. Protein band patterns of the WBE and SGE of the three species were different from one another.

SGE was analyzed with the pooled serum of 9 seropositive subjects on ELISA by immunoblotting (fig. 3). There were 3–8 reactive protein bands in the SGE of the three species. Specific human IgE reacted to the proteins of 30.5, 33, 37 and 57.5 kD from *A. togoi* of 38, 43 and 68 kD from *C. tritaeniorhynchus*, and of 23, 33, 34, 43, 44, 60, 74 and 93 kD from *C. pipiens pallens*. The cord blood sera and PBST were used as negative controls.

To compare the reactive patterns between the sera from humans and immunized mice against the SGE of *A. togoi*, immunoblotting was performed with the sera of mice made immune by *A. togoi* bites. The results are shown in figure 4. The serum taken after 4 weeks of immunization reacted to the 37-kD protein band only. The immune serum taken after 8 and 12 weeks of immunization reacted to the 30.5-, 33-, 37- and 57.5-kD protein bands. The 31-, 37- and 57.5-kD proteins in the saliva were noted as main IgE-reactive proteins to anti-mouse IgG1 antibodies. Immunoblot analysis disclosed that allergenic proteins in SGE of mosquitoes and their patterns were essentially similar between human and mouse sera to the SGE of *A. togoi*.

ELISA Inhibition Test

For the determination of cross-reactivity between the SGE of the three different mosquito species, antisera from the mice immunized with *A. togoi* bites were used. The levels of inhibition in the ELISA inhibition test are shown

Fig. 2. Protein patterns of WBE and SGE of the three mosquito species by SDS-PAGE. Twelve percent gel was stained using Coomassie brilliant blue.

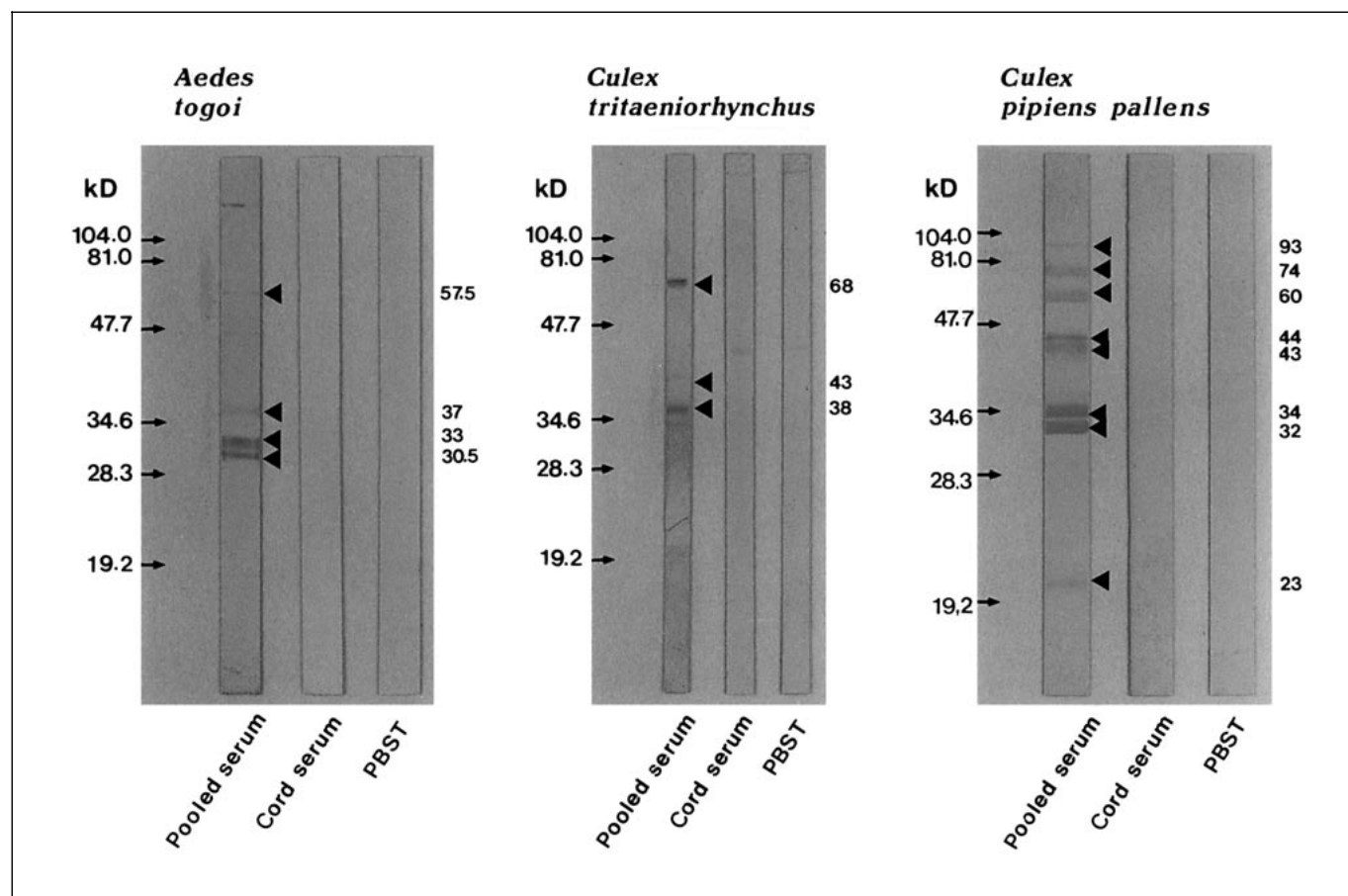
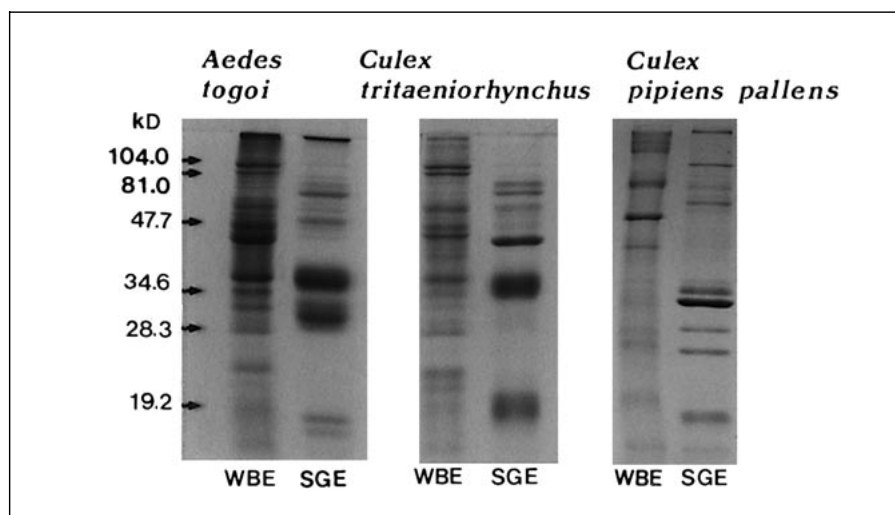


Fig. 3. Immunoblot analysis of SGE of the three different mosquito species using pooled sera. Proteins of SGE were run on 12% polyacrylamide gels in the presence of SDS and electrophoretically transferred to nitrocellulose membrane. Transferred nitrocellulose strips were incubated with pooled sera of seropositive samples by ELISA. Secondary antibody binding was determined by AP-conjugated goat anti-human IgE antibody in 1% liver acetone powder solution. Cord blood serum and PBST were used as negative controls.

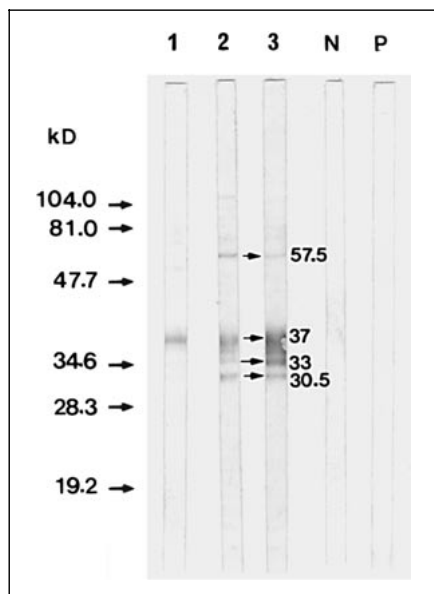


Fig. 4. Immunoblot analysis of mouse IgG1-binding SGE proteins of *A. togoi*. Twenty live female *A. togoi* per BALB/c mouse were released to inject the antigens naturally, twice a week, for 12 weeks. Lanes 1, 2 and 3 were reacted with antisera taken 4, 8 and 12 weeks after immunization, respectively. Normal serum (N) and PBST (P) were used as negative controls. Mouse immune sera showed reactive bands against salivary gland proteins of *A. togoi* at 30.5, 33, 37 and 57.5 kD.

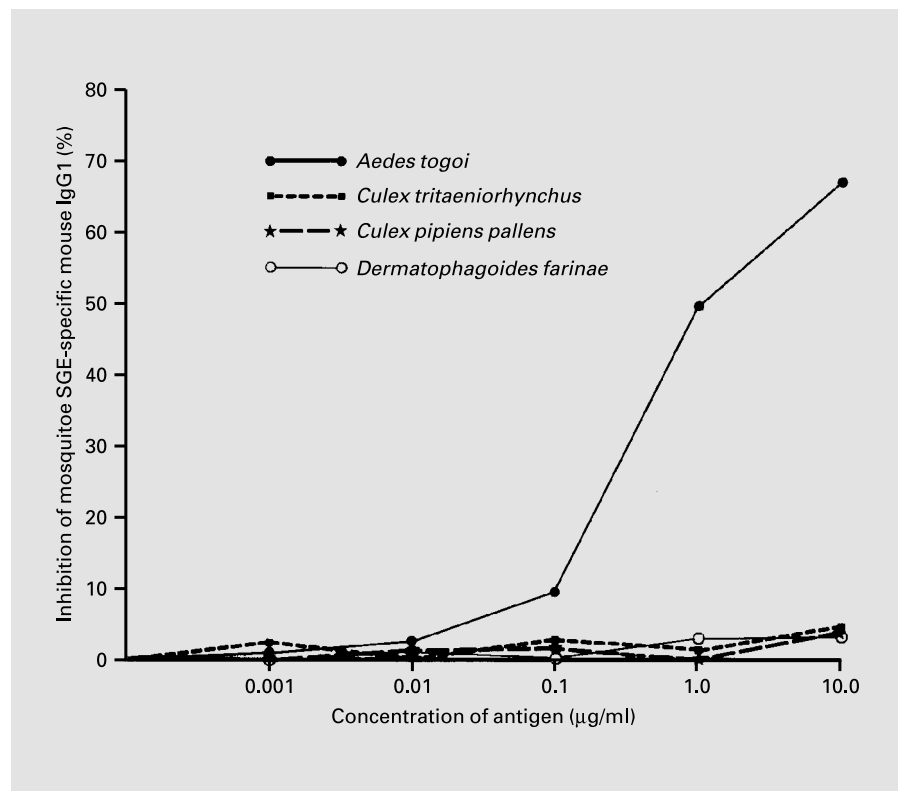


Fig. 5. ELISA inhibition of *A. togoi*-specific mouse IgG1 antibody by SGE of *A. togoi*, *C. tritaeniorhynchus* and *C. pipiens pallens* and WBE of *Dermatophagoides*. The maximum level of inhibition by *A. togoi* was 67.4%. The SGE of *C. tritaeniorhynchus* and *C. pipiens pallens* and *D. farinae* did not inhibit *A. togoi* SGE-specific mouse IgG1 antibody.

in figure 5. The absorbance of specific mouse IgG1 against SGE of *A. togoi* was inhibited dose dependently. The maximum inhibition level was 67.4% at 10 µg/ml, and the 50% inhibitory concentration was 1 µg/ml. The percentage inhibition against SGE of *C. tritaeniorhynchus* and *C. pipiens pallens* and the WBE of *D. farinae* was noted as 4.9, 4.0 and 3.2%, respectively.

Discussion

In the present study, total protein staining detected many proteins in the SGE of *A. togoi*, *C. tritaeniorhynchus* and *C. pipiens pallens*.

The mosquito bite is caused by mosquito saliva with the involvement of IgE-mediated and lymphocyte-mediated hypersensitivities [7, 8]. In the saliva of *Aedes aegypti*, a 68-kD protein was identified as apyrase, an

ATP diphosphohydrolase. The role of this enzyme in mosquitoes seems to be related to the anti-platelet aggregating activity found in their salivary secretion or salivary gland homogenates [23].

The skin reactions to mosquito bites are derived from salivary secretions and consist of an immediate and a delayed reaction [24, 25]. In the present study, 7 of the 12 subjects with immediate skin reactions to SGE of *A. togoi* using the skin prick test had significantly higher anti-mosquito IgE antibody levels than those without a skin reaction (fig. 1). These findings suggest that IgE-mediated hypersensitivity is involved in the development of mosquito allergy.

It has been demonstrated on protein patterns of mosquitoes that there are more than 20 protein fractions in the saliva of *A. togoi* using silver staining [26] and 14- to 68-kD protein fractions in the saliva of 4 *Aedes* and 3 *Culex* spp. using colloidal gold staining [13]. In the

present study, some 20 proteins were shown in the SGE and WBE of *A. togoi*, *C. tritaeniorhynchus* and *C. pipiens pallens* by Coomassie brilliant blue staining. These results are somewhat different from previous studies, which have described high-molecular-weight protein fractions of the *C. pipiens* species. These differences in the high-molecular-weight proteins between the *C. pipiens* species and *C. pipiens pallens* may reflect a different subspecies of mosquito. However, the low molecular weights of the proteins with 17, 23 and 33 kD were similar to those in the previous study [13].

The results of mosquito-specific IgE levels in humans show that three sera had positive titers against only one species (fig. 1, arrows) when the serum was diluted 1/20. It is suggested that a range of dilution used for a species-specific IgE test is useful in 1/20.

Several investigations based mainly on immunoblot analysis data have described specific allergens or IgE-reactive proteins in mosquito saliva and SGE. *A. togoi* mosquito-sensitive individuals revealed IgE-binding bands with apparent molecular weights of 18.5, 30.5, 33, 37 and 57.5 kD [12]. In the present study, data obtained from immunoblotting with the pooled serum of the 7 subjects with high anti-mosquito IgE levels to *A. togoi* were in agreement with the IgE-reactive protein fractions from 30.5 to 57.5 kD, but there was no reaction with 18.5 kD. In particular, the 30.5- and 33-kD fractions elicited strong IgE responses (fig. 3). This suggests that these two protein fractions may be major IgE-binding proteins in SGE of *A. togoi*. *C. pipiens* mosquito-sensitive individuals revealed 7 IgE-reactive bands with molecular weights of 16, 34, 38, 39, 55.5, 61.5 and 70 kD in 12 serum specimens by immunoblot analysis [12]. Using the pooled sera of the 7 subjects with high anti-mosquito IgE levels to *C. pipiens pallens*, this study showed IgE-binding bands from 23 to 93 kD. It could be found that protein fractions with relatively high molecular weights are distinctly different from those in the previous studies, which have reported protein fractions of *C. pipiens* species on immunoblot analysis [12].

On the other hand, it has been shown that IgE responses to antigen of mosquitoes are much more intense and broader in the allergic group than in the nonallergic group [12]. In our study, which was performed according to a method of Harlow and Lane [27], there were no bands in the cord blood and negative sera (fig. 3). It was mentioned by Harlow and Lane [27] that if background bands are generated due to a secondary reagent, exchange of an alternative label or adsorption of the secondary reagent with liver acetone powder is recommended. Liver acetone

powder was used to reduce background bands in this experiment.

In the mouse, interleukin-4 preferentially induces isotype switching to IgG1 and IgE [28]. A previous study showed that human IgE and IgG4 antibodies of mosquito bite-sensitive children bind to the same saliva proteins as antibodies from the immune animals [13]. As shown in this study, the immunized mice showed IgG1 antibody responses to major SGE of *A. togoi*, similar to sensitized humans (fig. 3, 4). Thus, anti-mouse IgG1 reactivity to the SGE of mosquitoes could be useful to identify allergens by immunoblot analysis.

Cross-reactivity with respect to bite reactions caused by different mosquito species has been reported previously [29].

In the present study, an ELISA inhibition experiment with sera immune to *A. togoi* revealed that the mouse IgG1 level decreases dose dependently with SGE of *A. togoi*, whereas it does not in *C. tritaeniorhynchus*, *C. pipiens pallens* and *D. farinae*. It is suggested that species-shared allergens may not exist within these three different Korean mosquitoes.

An additional study using N-terminal sequencing is needed to further characterize the degree of cross-reactivity between different mosquitoes and extend the data to the immunogenic and allergenic properties of the major human IgE and murine IgG1 binding proteins.

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