

Cross-reactivity of skin and serum specific IgE responses and allergen analysis for three mosquito species with worldwide distribution

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Background: Allergic reactions to mosquito bites are a common problem worldwide. Cross-reactive immunologic responses have not been reported.

Objective: For the three most common mosquito species, *Aedes (Ae.) vexans*, *Ae. aegypti*, and *Culex (Cx.) quinquefasciatus*, we investigated skin and serum specific IgE responses and analyzed salivary allergens.

Methods: Locally, *Ae. vexans* is a major pest, but *Ae. aegypti* and *Cx. quinquefasciatus* are not found. We studied 41 subjects living in Manitoba, using (1) skin bite tests, (2) ELISA to measure serum mosquito saliva- or salivary gland-specific IgE, (3) ELISA inhibition tests, and (4) sodium dodecylsulfate-polyacrylamide gel electrophoresis and immunoblotting. **Results:** Twenty-nine of the 41 subjects had skin reactions to *Ae. vexans* bites. Twenty-two of the 29 also reacted to *Ae. aegypti* bites. Mean serum mosquito-IgE levels to each of the three species were significantly higher in the reactive subjects than in the nonreactive subjects. Significant intercorrelations were found among skin reactions and mosquito-specific IgE levels for the three species. The *Ae. aegypti*-IgE ELISA reaction could be inhibited by addition of each mosquito extract. The serum IgE and IgG from Manitobans reacted with the antigens of all three species. A 37 kd allergen in each of the three species was recognized by the antibody against a recombinant *Ae. aegypti* saliva protein.

Conclusions: Strong cross-reactive skin and IgE responses and species-shared antigens exist among the three mosquito species studied. (J Allergy Clin Immunol 1997;100:192-8.)

Key words: Allergen, allergy, antigen, IgE, immunoblot, mosquito, *Aedes vexans*, *Aedes aegypti*, *Culex quinquefasciatus*

Allergic reactions to mosquito bites are a global problem. Although the bites commonly cause cutaneous wheals and flares and delayed papules, severe local reactions and systemic reactions may also occur.¹⁻⁴ Despite this, there are few reports in which modern immunologic techniques have been applied to the study of mosquito allergy.

Previous studies have revealed that both IgE-mediated and T-lymphocyte-mediated hypersensitivities are

Abbreviations used

<i>Ae.</i> :	<i>Aedes</i>
<i>Cx.</i> :	<i>Culex</i>
Mosquito-specific IgE:	Mosquito saliva- or salivary gland-specific IgE
PBS:	Phosphate-buffered saline
SDS-PAGE:	Sodium dodecylsulfate- polyacrylamide gel electrophoresis

involved in the development of mosquito allergy.^{2, 5-8} Analogous to immunotherapy with venoms in Hymenoptera allergy, successful subcutaneous immunotherapy with crude mosquito whole-body extract has been reported.^{3, 9, 10} To improve diagnosis and immunotherapy of mosquito allergy, purified or recombinant mosquito saliva antigens should be used.¹¹ In anticipation of this, it is important to determine whether there are any cross-reactive skin and IgE responses and species-shared antigens among various mosquito species, especially those with worldwide distribution.

Aedes (Ae.) vexans, *Ae. aegypti*, and *Culex (Cx.) quinquefasciatus* are the three most important mosquito species distributed globally. *Ae. aegypti*¹² and *Cx. quinquefasciatus*¹³ are found throughout the tropical regions of the world within 20° C isotherms; and *Ae. vexans* is found in North America, Eurasia, Asia, and Africa.¹⁴ Comparison of the human immunologic response to the three species has never been made. In Canada where *Ae. aegypti* and *Cx. quinquefasciatus* are not found, *Ae. vexans* is a major pest, representing up to 80% of the local mosquito population. To determine whether the three mosquito species have cross-reactive immunologic responses and species-shared antigens, we performed skin bite tests and evaluated serum mosquito specific IgE for the three mosquito species in 41 Manitobans who had been exposed to *Ae. vexans* bites but not to *Ae. aegypti* and *Cx. quinquefasciatus* bites. We also analyzed species-shared allergens by immunoblotting, using the sera from subjects allergic to mosquitoes and an antibody to a recombinant *Ae. aegypti* salivary protein.

METHODS

Subjects

This project was approved by The University of Manitoba Faculty Committee on the Use of Human Subjects in Research, and the participants gave written, informed consent before study entry. Forty-one healthy subjects (21 men and 20 women),

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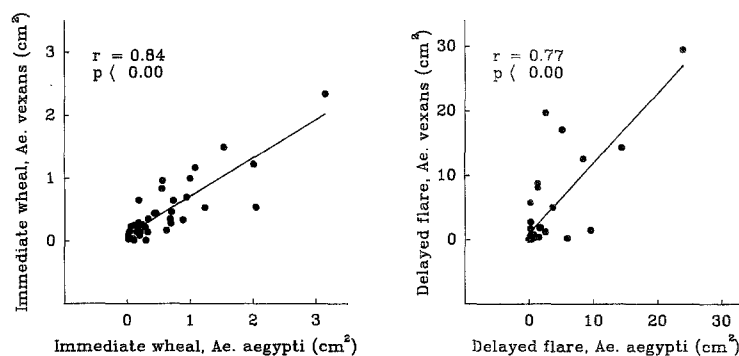


FIG. 1. Correlations of skin immediate and delayed reactions in mosquito skin bite tests between *Ae. aegypti* and *Ae. vexans*.

TABLE I. Coefficients of correlation among skin bite reactions and mosquito-specific IgE levels

	<i>Ae. aegypti</i> immediate wheal	<i>Aa. aegypti</i> delayed induration	<i>Ae. vexans</i> immediate wheal	<i>Ae. vexans</i> delayed induration	<i>Ae. vexans</i> - IgE	<i>Ae. aegypti</i> - IgE
<i>Ae. aegypti</i> delayed induration	0.43*					
<i>Ae. vexans</i> immediate wheal	0.84*	0.26				
<i>Ae. vexans</i> delayed induration	0.68*	0.77*	0.61*			
<i>Ae. vexans</i> -IgE	0.42*	0.45*	0.37*	0.45*		
<i>Ae. aegypti</i> -IgE	0.34*	0.42*	0.26	0.43*	0.60*	
<i>Cx. quinquefasciatus</i> -IgE	0.28	0.18	0.34*	0.41*	0.57*	0.35*

* $p < 0.05$.

aged 19 to 57 years, with skin reactions to mosquito bites ranging from none to strongly positive were recruited during the summer of 1993. All the subjects had lived in Canada more than 2 years, and 71% had lived in Canada (mostly in Manitoba) since birth. Antihistamines and other medications that might suppress the skin bite test response were withheld for an appropriate length of time before the study.

Mosquitoes, mosquito saliva, and salivary gland extracts

Female *Ae. vexans* mosquitoes were collected in local fields and identified by scientists in the Department of Entomology, University of Manitoba. The *Ae. aegypti* colony was obtained from the same department and maintained in our laboratory. The *Cx. quinquefasciatus* colony was imported from Dr. Robert J. Novak's laboratory (University of Illinois, Champaign) and maintained in our laboratory. Four- to twelve-day-old adult *Ae. aegypti* mosquitoes were used for the bite tests and for saliva collection. Salivary glands were dissected from 4- to 12-day-old adult *Cx. quinquefasciatus* mosquitoes.

Mosquito saliva or salivary gland extracts were prepared for use in the ELISA and immunoblot. Saliva was collected from female adult *Ae. aegypti* mosquitoes by placing the proboscis of each mosquito into a capillary tube filled with water. Salivation was induced by applying a small amount of malathion-acetone solution to the thorax.¹⁶ One hour later, the contents of capillary tubes containing saliva were collected, pooled, and lyophilized. The saliva was reconstituted before use by dissolving the lyophilized proteins in 0.02 mol/L phosphate-buffered saline (PBS).

Salivary gland extracts of *Ae. vexans* and *Cx. quinquefasciatus*

were prepared by the methods previously described⁷ from female *Ae. vexans* mosquitoes collected from the fields and female adult *Cx. quinquefasciatus* mosquitoes raised in our laboratory, respectively. The protein concentration was 0.6 mg/ml for *Ae. vexans* extract, 0.4 mg/ml for *Cx. quinquefasciatus* extract, and 0.3 mg/ml for *Ae. aegypti* extract as measured by a Bio-Rad Protein Assay kit (Bio-Rad Labs, Richmond, Calif.).

When we used the method applied to *Ae. aegypti* mosquitoes, *Cx. quinquefasciatus* mosquitoes did not salivate, and thus we had to use a salivary gland extract from this species. For *Ae. vexans*, we compared the salivary gland extract and the saliva in an ELISA and found that the salivary gland extract was better than saliva. Although both techniques are time-consuming and labor-intensive, dissection of salivary glands is relatively easier than saliva collection; therefore, we used salivary gland extract of *Ae. vexans* in the study.

Skin bite tests and blood samples

Skin bite tests were performed with one *Ae. vexans* mosquito and one *Ae. aegypti* mosquito on the volar aspect of each subject's forearm, as previously described.⁸ The wheal-and-flare circumferences were traced at 20 minutes and 24 hours after the bite by using a felt-tipped pen. All wheal-and-flare tracings were transferred to transparent paper. The area of the wheal, flare, or induration was measured with an IBM-PC digitizer and stereometric measurement software (IBM Instruments, Inc., Danbury, Conn.).¹⁵

A wheal of less than 0.3 cm² with no flare and no itching was considered to be a negative immediate reaction. An induration less than 0.3 cm² was considered to be a negative delayed reaction.

Ten milliliters of blood was obtained for measurement of

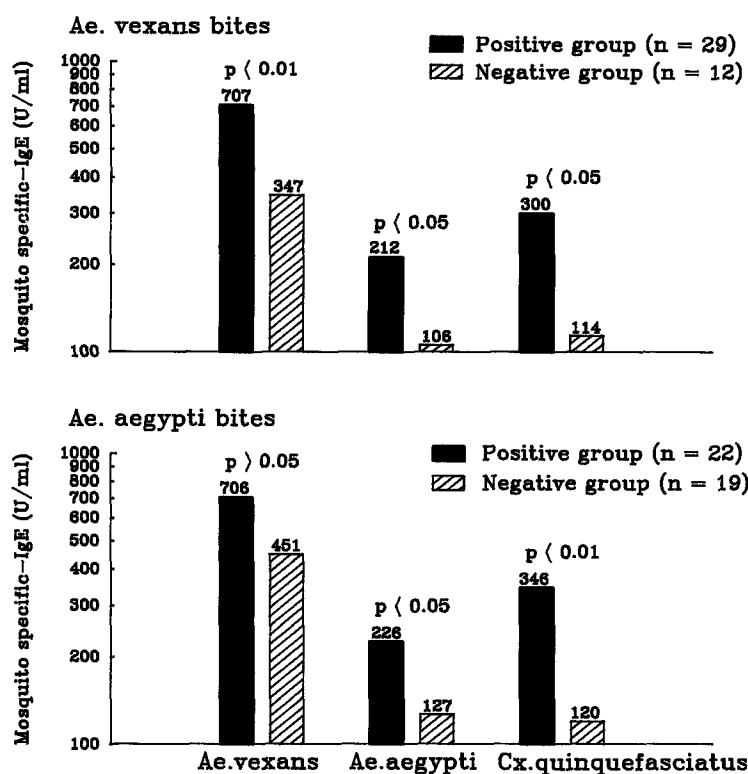


FIG. 2. Serum geometric mean mosquito-specific IgE levels to the three species in subjects with and without skin immediate reactions to *Ae. vexans* bites (top) or to *Ae. aegypti* bites (bottom).

mosquito-specific IgE to the three species. Serum was separated and stored at -70°C until use.

Measurement of mosquito-specific IgE by ELISA

Serum mosquito-specific IgE to *Ae. vexans*, *Ae. aegypti*, and *Cx. quinquefasciatus* was measured by an indirect ELISA as previously described.⁷ Optimal conditions for dilutions of the three mosquito extracts, serum samples, goat anti-human IgE, and conjugated rabbit anti-goat IgG were chosen by checkerboard titration. Standardization of ELISA results among assays and estimation of the relative amount of mosquito-specific IgE in each sample was accomplished by using reference sera. The reference serum used to measure *Ae. aegypti*-IgE and *Cx. quinquefasciatus*-IgE was obtained from a subject with systemic reactions to mosquito bites (kindly provided by Dr. Renata J. Engler, Walter Reed Army Medical Center, Washington, D.C.). Another reference serum used to measure *Ae. vexans*-IgE came from a Manitoban with severe skin reactions to mosquito bites (immediate wheal, 1.5 cm^2 ; flare, 11.6 cm^2). Both reference sera were defined as 1000 U/ml for mosquito-specific IgE. Microplates coated with mosquito saliva or salivary gland extract (0.02 to $0.05\text{ }\mu\text{g/well}$) were sequentially incubated with serum samples (1:20) or reference serum (twofold dilutions from 1:20 to 1:10,240), 1:1000 goat anti-human IgE (P.S. myeloma-affinity purified, a gift from Dr. N. Franklin Adkinson, Jr., The Johns Hopkins Allergy and Asthma Center), and 1:1000 alkaline phosphatase-conjugated rabbit anti-goat IgG (Jackson ImmunoResearch Laboratories, West Grove, Pa.). The values of mosquito-specific IgE in the tested samples were calculated by interpolation from the dilution curve of the reference serum. The sensitivity of the ELISAs was 0.8 U/ml.

ELISA inhibition tests

To study the cross-reactivity among the three species, ELISA inhibition tests were performed. A serum sample with high mosquito-specific IgE (final dilution, 1:20) was incubated with serially diluted *Ae. vexans*, *Ae. aegypti*, and *Cx. quinquefasciatus* extracts with final dilutions of 1:4 and 1:20 at room temperature for 1 hour and then at 4°C overnight. Incubation of the serum with ELISA buffer served as a positive control. These incubated materials were then assessed for *Ae. aegypti*-IgE by means of ELISA.

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis

Among the 41 subjects studied, sera from six subjects with large immediate skin wheal responses ($>1.0\text{ cm}^2$) in the bite tests and high *Ae. vexans*-specific IgE levels ($>1000\text{ U/ml}$) were pooled and used for immunoblotting.

SDS-PAGE and immunoblotting were performed according to the method previously described.¹⁷ In the analysis of IgE and IgG antigens, $2\text{ }\mu\text{g}$ of the proteins from each mosquito extract were loaded and electrophoresed in 12% acrylamide SDS-PAGE under reducing conditions. Proteins separated by SDS-PAGE were electrophoretically transferred onto nitrocellulose membranes. Free binding sites on the membranes were blocked by incubation for 2 hours with 3% bovine serum albumin in 0.02 mol/L PBS, pH 7.4, containing 0.05% (vol/vol). After washing three times with PBS-Tween-20, the membranes were incubated overnight with the pooled serum (1:10 dilution for IgE and 1:50 for IgG) and washed again. Incubation of the mem-

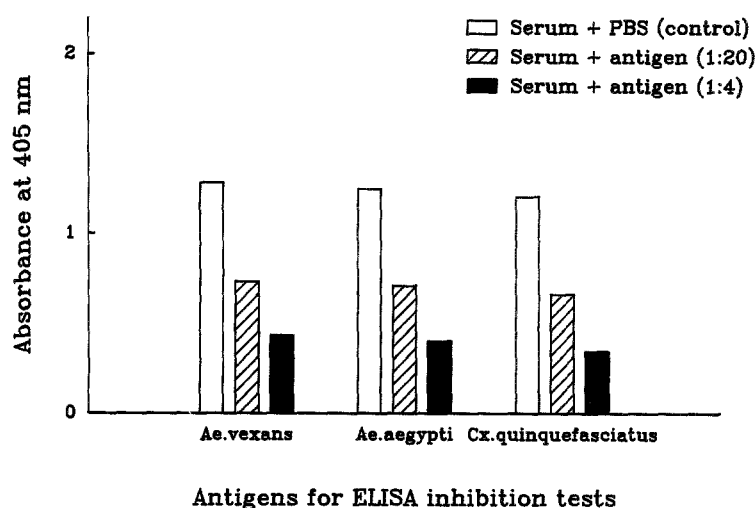


FIG. 3. ELISA inhibition tests. Pooled serum from subjects allergic to mosquitoes was incubated with *Ae. vexans* extract (left), *Ae. aegypti* extract (middle), and *Cx. quinquefasciatus* extract (right) overnight and then assayed for *Ae. aegypti*-IgE by ELISA as described in Methods.

branes with PBS-Tween-20 served as a negative control. This was followed by sequential incubations with 1:15,000 monoclonal anti-human IgE (clone 7.12, from Dr. Andrew Saxon's laboratory, University of California) or 1:15,000 monoclonal anti-human IgG (PharMingen, San Diego, Calif.), and then horseradish peroxidase-conjugated goat anti-mouse IgG (1:5000 for IgE, 1:10,000 for IgG; Calbiochem Corp., La Jolla, Calif.). After washing, the membranes were finally incubated with ECL detecting reagents (Amersham Life Science, Buckinghamshire, England) and then exposed to film (X-Omat, Kodak). Prestained SDS-PAGE standards (Bio-Rad Labs) were used to determine the relative molecular weights of the electrophoresed components.

The same method, with some modifications, was used in the study of a 37 kd recombinant *Ae. aegypti* saliva protein, which was kindly provided by Dr. Anthony James at the University of California, Irvine.¹⁸ One microliter of the baculovirus medium containing the recombinant protein and 2 μ g of each mosquito extract were loaded onto different wells and electrophoresed. Medium from cells infected with wild-type baculovirus was used as a control. The membranes containing separated proteins were then incubated with rabbit antibody to the recombinant protein (1:5000, from Dr. Anthony James) followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5000). The remaining steps were the same as those for the immunoblot with human serum.

Statistical analysis

Analysis of the data was performed by using the "Number Crunch Statistical System" software (published by Dr. Jerry L. Hintze, Kaysville, Utah). Unpaired *t* tests were used for between-group comparisons. Linear regressions were used for the analysis of correlations among skin reactions and serum mosquito-specific IgE levels.

RESULTS

Skin bite reactions

No systemic reactions were noted after the bite tests. Immediate and delayed skin reactions to mosquito bites were observed. The immediate reaction consisted of a

pruritic wheal with a surrounding flare or erythema appearing within several minutes, reaching a peak at 20 to 30 minutes and then subsiding. The delayed reaction consisted of an indurated papule, which appeared several hours later, peaked at 24 to 36 hours, and diminished over several days after the bite. In five subjects with severe delayed reactions, vesicles were found in the centers of the indurated areas, precisely in the area where the immediate wheal had been.

Twenty-nine of the 41 subjects had positive immediate skin reactions to *Ae. vexans* bites. Twenty-two of the 29 also reacted to *Ae. aegypti* bites. Seven subjects reacted to *Ae. vexans* bites only. The size of the immediate reactions ranged from 0 to 3.1 cm² for the wheal and 0 to 23.0 cm² for the flare, and the size of delayed reactions ranged from 0 to 29.5 cm².

In the immediate reaction, wheals correlated significantly with flares in both species ($r = 0.72$, $p < 0.00$ for *Ae. vexans*; $r = 0.88$, $p < 0.00$ for *Ae. aegypti*). Also, immediate wheal-and-flare reactions showed significant correlation with the delayed reactions in each species as well (r values between 0.43 and 0.61, p values < 0.00). More interestingly, significant correlations of skin reactions were found between the two species as shown in Table I and Fig. 1, especially between the immediate wheal sizes of the two species ($r = 0.84$, $p < 0.00$) and the delayed induration reactions of the two species ($r = 0.77$, $p < 0.00$).

Mosquito-specific IgE levels

As shown in Fig. 2 (top), the geometric mean *Ae. vexans*-IgE, *Ae. aegypti*-IgE, and *Cx. quinquefasciatus*-IgE levels were all significantly higher in the subjects with immediate skin reactions to *Ae. vexans* bites than in those with no immediate skin reaction to the bites ($p < 0.05$). Similar results were found for mean mosquito-

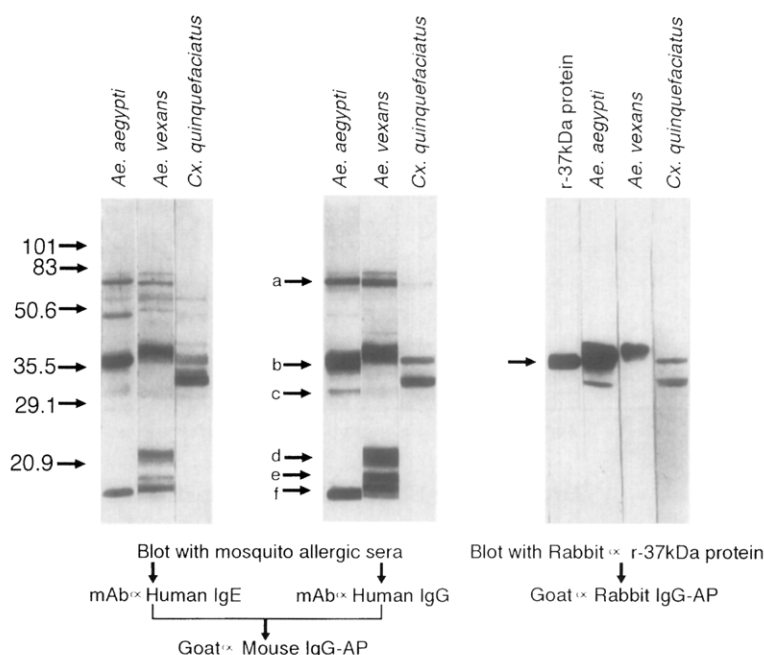


FIG. 4. Immunoblot analysis of antigens of the three mosquito species. SDS-PAGE and immunoblotting were performed as described in Methods. Proteins of saliva or salivary gland extracts separated by SDS-PAGE were transferred to nitrocellulose membranes. Membranes were sequentially incubated with pooled serum from subjects allergic to mosquito bites, monoclonal anti-human IgE (left) or monoclonal anti-human IgG (middle), and conjugated goat anti-mouse IgG. Membranes were also incubated with the rabbit antibody to a recombinant 37 kD *Ae. aegypti* saliva protein (right), followed by incubation with conjugated goat anti-rabbit IgG.

specific IgE levels of the three species in the subjects with or without immediate skin reactions to *Ae. aegypti* bites (Fig. 2, bottom). Significant correlations were also found among the IgE levels of the three species (r values between 0.35 to 0.60, p values < 0.03) (Table I).

Correlations among skin reactions and the IgE levels

Table I summarizes the intercorrelations among skin reactions and IgE levels to the three species. As expected, *Ae. vexans*-IgE values correlated significantly with skin reactions to *Ae. vexans* bites, and *Ae. aegypti*-IgE values correlated significantly with skin reactions to *Ae. aegypti* bites. *Ae. vexans*-IgE also significantly correlated with skin reactions to *Ae. aegypti* bites. The same correlation was found between *Ae. aegypti*-IgE and the delayed skin reactions to *Ae. vexans* and between *Cx. quinquefaciatus*-IgE and both immediate and delayed reactions to *Ae. vexans* bites.

ELISA inhibition tests

The cross-reactive immunologic responses among the three species were further confirmed by ELISA inhibition tests. *Ae. aegypti*-specific IgE reactions could be inhibited by incubation of the serum with all three extracts in a dose-dependent manner, supporting the existence of species-shared antigens among the extracts of the three species (Fig. 3).

SDS-PAGE and immunoblot analysis

Immunoblot analysis further revealed the existence of species-shared antigens. When the pooled serum from Manitobans allergic to mosquitoes was used, the IgE and IgG antibodies not only bound to *Ae. vexans* antigens but also to the antigens of the two species that are not found in Manitoba (Fig. 4, left and middle). There were no antigen bands found in the PBS control strips. Species-shared antigens are indicated by arrows a, b, c, and f; and *Ae. vexans*-specific antigens are indicated by arrows d and e. Further, as shown on the right of Fig. 4, two bands in *Ae. aegypti* and *Cx. quinquefaciatus* extracts (equivalent to bands b and c) and one band in *Ae. vexans* extract (equivalent to band b) were recognized by the rabbit antibody to the recombinant protein, suggesting that the 37 kD protein of *Ae. aegypti* is also present in the salivary glands of *Cx. quinquefaciatus* and *Ae. vexans*. Although the molecular weight of band b was higher in *Ae. vexans* than those in the other two species and the molecular weights of bands b and c were obviously different, they all were recognized by the rabbit antibody, suggesting that these polypeptides share some common antigenic structure.

DISCUSSION

Skin reactions to mosquito bites consisted of an immediate wheal and flare and a delayed induration. The elapsed time of the immediate reaction was compatible with that of an IgE-mediated hypersensitivity.

Further, the mean serum IgE to the three species was significantly higher in subjects with positive skin bite test results than in those with negative bite test results, and the sizes of skin immediate wheals correlated significantly with the values of mosquito-specific IgE for the same species and also with other species. Serum mosquito-specific IgE was previously identified in human beings by using the Prausnitz-Küstner test,^{19,20} ELISA,^{7,5,21} RAST,²² and Western blot techniques.^{6,17,23,24} Two studies reported that mosquito-specific IgE correlated with immediate skin reactions.^{5,8} In agreement with previous studies, our results confirm that IgE is involved in the development of skin immediate reactions in mosquito-allergic subjects.

The delayed reaction appeared to be consistent with lymphocyte-mediated hypersensitivity, which appears only after several hours and peaks between 24 and 48 hours.²⁵ In guinea pigs, delayed skin reaction to mosquito bites has been successfully transferred with spleen cells but not with serum.²⁶ In vitro studies, including our previous investigation⁸ and another study,⁵ showed that the size of the delayed papules correlated with lymphocyte proliferation response to mosquito antigens.

Another mechanism may also be involved in the early stage of the delayed reaction. Cutaneous late-phase IgE-mediated allergic reactions are characterized by erythema and induration, peaking between 4 and 8 hours²⁷ and can be transferred from a sensitized to a nonsensitized individual with serum.²⁸ In our study five subjects had a vesiculated area in the center of the delayed induration exactly mirroring the area in which the immediate wheal had occurred. This suggests that an IgE-mediated late-phase reaction may be involved in the delayed reaction. The suggestion is supported by the observation of other investigators that eosinophils and neutrophils are recruited in mosquito-bite lesions after the wheals have formed.²

Cross-reactivity with respect to bite reactions caused by different mosquito species has been reported previously.^{29,30} Sensitization of animals by mosquito bites or injection of antigenic extracts from one species can confer reactivity against another species.^{29,30} Desensitization of *Ae. aegypti*-allergic rabbits can be achieved by injections of *Ae. atropalpus*, *Ae. pionips*, or *Cx. pipiens* extracts.³¹ Although cross-reactive IgE responses have not been previously reported in mosquito allergy, cross-reactive IgE responses were reported for Hymenoptera allergy in wasps, yellow jackets, and hornets.^{32,33} Unlike other studies of cross-reactivity to insect bites or stings, we selected a specific location where *Ae. aegypti* and *Cx. quinquefasciatus* are not present, whereas *Ae. vexans* is the major pest, representing up to 80% of the indigenous mosquito population. This allowed us to exclude the sensitization caused by the other two mosquito species.

The immunologic basis for the reactive skin and IgE responses among different mosquito species is the existence of species-shared antigens, which are based on their identical protein sequences. Salivary secretions

have been demonstrated to be directly responsible for skin reactions to mosquito bites.³⁴ In this study, immunoblot analysis with saliva or salivary gland extracts, a number of species-shared antigens and several *Ae. vexans*-specific antigens were found (Fig. 4). The number of antigens and their molecular weights in the three species will be reported in a subsequent article. Both species-specific and species-shared antigens have been previously reported in various mosquito species.^{17,35,36} A 62 kd antigen was found in the salivary gland extracts of five mosquito species (*Ae. aegypti*, *Ae. taeniorhynchus*, *Cx. nigripalpus*, *Cx. quinquefasciatus*, and *Anopheles quadrimaculatus*)³⁶ and in the head and thorax extracts of two other species.¹⁷ In our previous study of antigens in *Ae. vexans*, *Cx. tarsalis*, and *Culiseta inornata*, nine antigens were shared by three species, six antigens were shared by two species, and only three antigens were species-specific.¹⁷ Whether these antigens from various species with identical molecular weights are immunologically identical remains to be confirmed.

Recently, the gene encoding a 37 kd salivary gland protein of *Ae. aegypti* has been cloned and expressed, and the specific antibody to the protein has been raised.¹⁸ In this study, using the antibody to the recombinant protein, we found the 37 kd *Ae. aegypti* saliva protein in *Ae. vexans* and *Cx. quinquefasciatus* salivary gland extracts (Fig. 4, left), confirming the existence of species-shared antigens in the three species. These species-shared antigens may well explain the cross-reactivity of skin reactions and IgE responses among different mosquito species.

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