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F. Estelle R. SimonsSection of Allergy and
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Department of Pediatrics and
Child Health,
University of Manitoba,
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IgG Binding Antigens in Extracts of
Mosquitos *Aedes vexans*,
Culex tarsalis and *Culiseta inornata*****Key Words**Allergy
Mosquito
Immunoblot
IgE
IgG
Aedes vexans
Culex tarsalis
*Culiseta inornata***Abstract**

Extracts of one globally distributed mosquito species (*Aedes vexans*) and two North American species (*Culex tarsalis* and *Culiseta inornata*) were prepared from heads and thoraxes. Proteins of the three extracts were separated by 12% SDS-PAGE and transferred to nitrocellulose membranes for immunoblotting. Immunoblotting was completed by sequential incubations of the membranes with a pooled human serum from subjects allergic to mosquito bites, monoclonal antibodies to human IgE or IgG, and goat antimouse IgG conjugate. Twelve to sixteen antigens with molecular masses ranging from 18.5 to 160 kDa were found in each extract. Nine antigens were shared by 3 species and 6 were shared by 2 species. Only 3 were species-unique. Most antigens bound to both IgE and IgG antibodies. IgE and IgG antibodies against *A. vexans* were studied by immunoblotting using individual serum from subjects with or without skin reactions to *A. vexans* bites. All 3 subjects with severe skin reactions had strong IgE and IgG antibodies to 32.5, 40, and 50 kDa proteins. The patterns and magnitudes of IgE and IgG antibodies to the antigens varied among individuals. Very faint IgE antibodies to these antigens were found in the 2 subjects with no skin reactions, suggesting that IgE plays a role in the development of mosquito allergy.

Introduction

Reactions to mosquito bites are a common problem all over the world. Although cutaneous reactions with immediate wheal and delayed papules occur most often, severe local and systematic reactions such as haemorrhagic skin lesions, angioedema, asthma and even anaphylaxis have been reported [1–3].

Reactions to mosquito bites are generally caused by immunologic mechanisms, with both type I (IgE-mediated) and type IV (cell-mediated) hypersensitivities being involved [4–7]. Serum mosquito-specific IgE has been dem-

onstrated to correlate with cutaneous mosquito bite reactions [4, 5]. Mosquito-specific IgG has also been found to correlate with skin mosquito bite reactions, suggesting that IgG may also be involved in the development of mosquito allergy [5].

Mosquito antigens have been identified by immunoblot analysis. A number of mosquito antigens with molecular masses ranging from 14 to 126 kDa have been reported in various mosquito species [8–12]. In this report, we analysed antigens using immunoblot techniques on three mosquito species not previously examined: *Aedes vexans*, a globally distributed species (and the major pest species in Manito-

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December 13, 1995
Accepted after revision
October 24, 1995

ba), and two North American species *Culex tarsalis* and *Culiseta inornata*. Species-shared and species-unique antigens, IgE and IgG binding antigens, and individual IgE and IgG responses to these antigens were found.

Materials and Methods

Subjects

This study was approved by The University of Manitoba Faculty Committee on the Use of Human Subjects in Research, and the subjects gave written, informed consent before participation. Forty-two subjects with a history of local reactions to mosquito bites were recruited during the summer of 1993. Skin mosquito *A. vexans* bite tests were performed [5] and serum mosquito (*A. vexans*) salivary gland-specific IgE and IgG antibodies (mosquito-specific IgE and IgG) were measured by ELISA [13] in all subjects. Three subjects with severe skin reactions in the bite tests who also exhibited high mosquito-specific IgE and IgG levels were selected for this study. Two subjects with no skin reactions to the bites and with low mosquito-specific antibody levels were included as negative controls.

Skin reactions in the bite tests and antibody levels in the 5 subjects studied are listed in table 1. The reactive subjects had very strong skin immediate reactions which consisted of a pruritic wheal (≥ 1 cm) with surrounding flare appearing within several minutes, reaching a peak at 30 min and then subsiding. The immediate reaction was followed by a local pruritic, indurated papule and erythema (≥ 7 cm) which appeared several hours later, reaching a peak 24 h after the bite. The papule usually lasted several days.

A pooled serum from reactive subjects was used in the analysis of antigens in the three mosquito species.

Mosquito Head and Thorax Extracts

Extracts prepared from mosquito heads and thoraxes were used in the study. Female mosquitos of the three species (*A. vexans*, *C. tarsalis*, *C. inornata*) were collected and identified by the Insect Control Branch, Parks and Recreation Department, City of Winnipeg, and then stored at -70°C . After the abdomens, wings and legs were removed, the heads and thoraxes were placed in cold 0.02 M phosphate-buffered saline (PBS), pH 7.2, homogenized on ice for 1 min using a PCU 11 homogenizer (Kinematica, Switzerland), centrifuged at 8,820 g for 30 min, filtered through a 0.45- μm Amicon filter, and then stored at -70°C . The protein concentrations of the antigen preparations were 1.48 mg/ml for *A. vexans*, 6.13 mg/ml for *C. tarsalis* and 5.34 mg/ml for *C. inornata*, as measured by the Protein Assay Dye Reagent kit (Bio-Rad Laboratories, Richmond, Calif., USA).

SDS-PAGE and Immunoblot Analysis for IgE and IgG Binding Antigens

The proteins in the mosquito extracts were separated by SDS-PAGE in a discontinuous system according to Laemmli [14] using a Bio-Rad slab gel apparatus. For each mosquito extract, 15 μg of proteins prepared in a reducing buffer were loaded onto each well and separated by electrophoresis in 12% SDS-PAGE. These proteins were then electrotransferred onto nitrocellulose membranes. Free binding sites on the membranes were blocked by incubation with 3% bovine serum albumin (Sigma, St. Louis, Mo., USA) dissolved in 0.02 M PBS for 2 h. Immunoblotting was completed by incubation of the mem-

Table 1. Skin bite tests and serum mosquito (*A. vexans*) salivary gland-specific IgE and IgG levels of the 5 subjects used in this study

Subjects No.	Skin bite test, cm^2		Mosquito specific-IgE U/ml	Mosquito specific-IgG U/ml
	immediate wheal	delayed papule		
1 (MZ)	1.5	11.6	1,026	576
2 (MD)	1.0	9.6	4,341	3,285
3 (DD)	2.0	7.2	1,067	1,054
4 (GL)	0.0	0	277	140
5 (WW)	0.1	0	112	80

branes with a pooled serum exhibiting high mosquito-specific IgE and IgG (1:10 dilution for IgE and 1:50 for IgG) over night. This was followed by incubation with monoclonal antihuman IgE (1:15,000; ascites, clone No. 7.12, a gift from Dr. A. Saxon, University of California) or monoclonal antihuman IgG (1:15,000; PharMingen, San Diego, Calif., USA) for 1.5 h. After washing 3 times with PBS containing 0.05% (v/v) Tween 20, the membranes were incubated with horseradish peroxidase-conjugated goat antimouse IgG (1:5,000 dilution for IgE and 1:10,000 for IgG; Calbiochem, La Jolla, Calif., USA) for 1.5 h. After washing, the membranes were incubated in ECL detecting reagents (Amersham Life Science, Buckinghamshire, UK) and then exposed to Kodak film (X-Omat, Kodak).

PBS and umbilical cord serum were used as controls. Prestained SDS-PAGE standards (Bio-Rad, Richmond, Calif., USA) were used to determine the relative molecular weights of the electrophoresed components.

Results

Inhibition Test

To examine the specificity of the immunoblot analysis, an antigen inhibition test was performed. Prior to immunoblotting, the pooled serum with high mosquito-specific IgE and IgG was incubated with *A. vexans* head and thorax extract with a final dilution of 1:2, 1:20 or 1:200 at 4°C overnight. Incubation of the serum with PBS served as a positive control. SDS-PAGE and immunoblotting for IgE and IgG binding antigens were then performed with these preincubated serum samples. As shown in figure 1, after incubation with mosquito extract, both IgE and IgG binding bands were significantly reduced (lanes 2 to 4) compared to the positive control (lane 1). This inhibition exhibited dose-dependency, thus indicating that the immunoblot analysis of IgE and IgG binding antigens is specific to mosquito antigens.

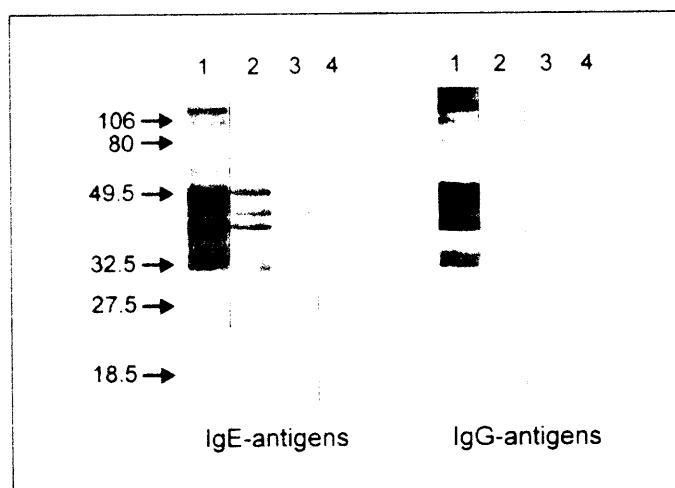


Fig. 1. Immunoblot inhibition. To facilitate inhibition, prior to immunoblotting, the pooled serum with high mosquito-specific IgE and IgG was preincubated with *A. vexans* extract at 4°C overnight. SDS-PAGE and immunoblot was then performed as described in the Methods. 1 = Serum preincubated with buffer as a control; 2 = serum preincubated with 1:200 extract; 3 = serum preincubated with 1:20 extract; 4 = serum preincubated with 1:2 extract.

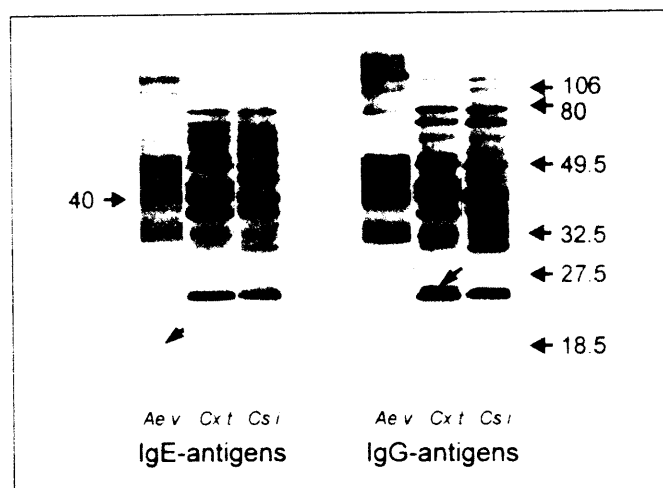


Fig. 2. SDS-PAGE and immunoblot analysis of IgE and IgG binding antigens in *A. vexans* (*Ae v*), *C. tarsalis* (*Cxt*) and *C. inornata* (*Cs i*). Proteins of the three mosquito species separated by SDS-PAGE were transferred to nitrocellulose membranes. Immunoblot was performed as described in the Methods. Left represents IgE binding antigens, and right IgG binding antigens. Most antigens were species-shared antigens. A 40-kDa major species-shared antigen and three species-unique antigens are indicated by arrows.

Table 2. Molecular masses of mosquito antigens in *A. vexans*, *C. tarsalis* and *C. inornata* (kDa).

<i>A. vexans</i> binding to		<i>C. tarsalis</i> binding to		<i>C. inornata</i> binding to	
IgE (n = 12)	IgG (n = 12)	IgE (n = 15)	IgG (n = 16)	IgE (n = 13)	IgG (n = 14)
160	160	160	160	160	160
110	110	110	110	110	110
—	—	85	85	85	85
80	80	—	—	—	—
—	—	70	70	—	70
65	65	65	—	65	—
62	62	62	62	62	62
—	—	56	56	56	56
50	50	50	50	50	50
46	46	46	46	46	46
43	43	43	43	—	—
40	40	40	40	40	40
—	—	37	37	37	37
32.5	32.5	32.5	32.5	32.5	32.5
—	—	—	—	30	30
—	—	—	28	—	28
—	—	—	25	—	—
24	24	24	24	24	24
—	—	22	22	—	—
18.5	18.5	—	—	—	—

n represents the total number of antigens.

Antigens in the Three Mosquito Species

IgE and IgG antibodies bound to various mosquito antigens in the three species (fig. 2). Twelve antigens in *A. vexans*, 16 antigens in *C. tarsalis*, and 14 antigens in *C. inornata*, with molecular masses ranging from 18.5 to 160 kDa, were found by immunoblot analysis (fig. 2, table 2). Most of the antigens bound to both IgE and IgG, and were shared by species. Nine antigens (24, 32.5, 40, 46, 50, 62, 65, 110, 160 kDa) were shared by three species, especially the 40-kDa antigen in figure 2. Six antigens (28, 37, 43, 56, 70, 85 kDa) were shared by two species. Only 3 antigens were species-unique: the 18.5-kDa in *A. vexans*, the 25-kDa in *C. tarsalis*, and the 30-kDa in *C. inornata* (fig. 2).

IgE and IgG Antibody Responses to *A. vexans* Antigens in 5 Subjects with or without Skin Reactions to Mosquito Bites

Figure 3 shows the IgE and IgG responses to the antigens of *A. vexans* in 3 subjects with severe skin reactions to the bites (strip 1–3) and 2 subjects without skin reactions to the bites (strips 4 and 5). In the 5 subjects, the patterns (spectra) of IgE and IgG responses to the antigens were similar, but the magnitudes of the antibody responses varied suggestively. Significantly stronger IgE and IgG responses to the antigens, especially to the 32.5-, 40-, 43-, and 50-kDa antigens, were found in the 3 reactive subjects (1–3), while only

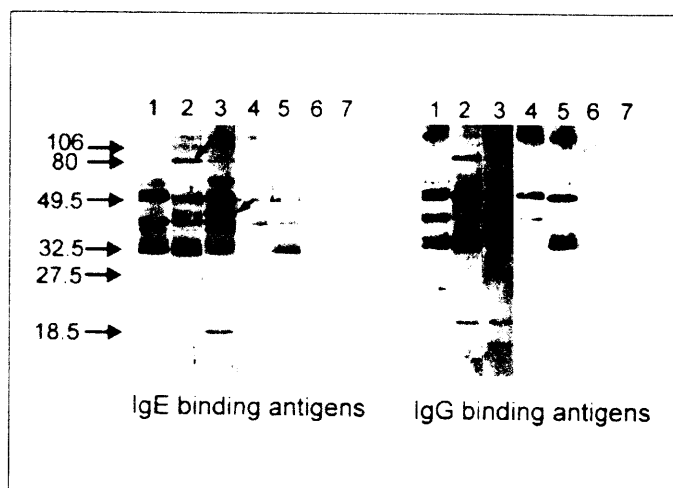


Fig. 3. Patterns of IgE and IgG responses to *A. vexans* in 5 subjects with or without skin reactions to mosquito bites. SDS-PAGE and immunoblot was performed as described in the Methods using the sera of 3 subjects with severe skin reactions to the bites (strips 1-3), 2 subjects without skin reactions to the bites (strips 4 and 5), and a cord serum (strip 6). Strip 7 is PBS control. The 2 IgE binding antigens indicated by arrows show different antibody responses in individuals.

very faint IgE antibody responses were observed in the 2 nonreactive subjects (4, 5), and very faint IgG responses to the antigens in 1 nonreactive subject (5).

The patterns of IgE and IgG responses to the antigens varied slightly among the 3 reactive subjects. Some individuals reacted to certain antigens while others reacted to different antigens, e.g. only subject 3's IgE bound to the 43-kDa antigen, and the IgE of subject 2 was the only one to bind to the 80-kDa antigens (fig. 3).

No antibody binding was found when the antigen containing membranes were incubated with either the cord serum (6) or PBS (7).

Comparison of IgE and IgG Responses in 3 Subjects

In order to define any differences between IgE and IgG responses to mosquito antigens in the same individual, the immunoblot results displaying IgE and IgG binding antigens were placed side by side for the 3 subjects with high mosquito-specific IgE and IgG antibodies (fig. 4). Although the patterns of antibody responses were slightly different among the 3 subjects, all the antigens induced both IgE and IgG responses.

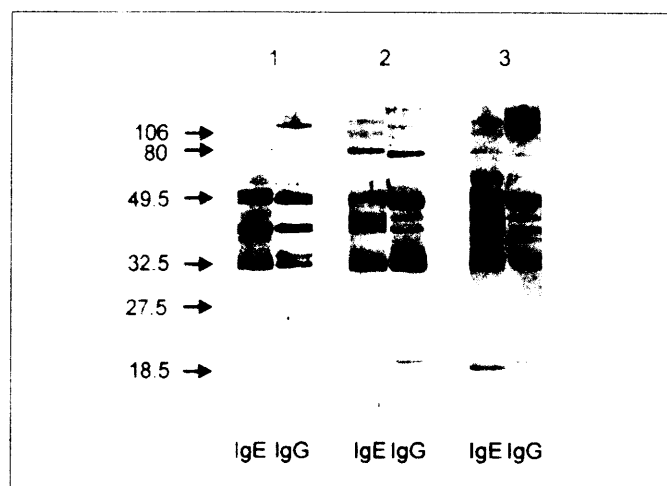


Fig. 4. Comparison of IgE and IgG responses to *A. vexans* by SDS-PAGE and immunoblot in 3 subjects with severe skin reactions to mosquito bites.

Discussion

The binding of mosquito antigens to human IgE and/or IgG in whole body extracts of six species *Aedes aegypti*, *Aedes albopictus*, *Aedes togoi*, *Armigeres subalbatus*, *Culex quinquefasciatus* and *Culex quequectates* [9, 10], salivary gland extracts of five species *Culex nigripalpus*, *Culex quinquefasciatus*, *Aedes taeniorhynchus*, *Aedes aegypti* and *Anopheles quadrimaculatus* [8], and saliva extracts of two species, *Aedes aegypti* [15] and *Aedes communis* [12] have been studied. Here, we report similar studies using *A. vexans*, globally distributed species, and *C. tarsalis* and *C. inornata*, North American species.

Salivary secretions are directly responsible for skin reactions to mosquito bites [16]. Although it would be preferable to use pure mosquito saliva in immunoblotting, it is not feasible due to the difficulty involved in obtaining saliva from several mosquito species. Also, in our experience, some mosquito species, e.g. *Culex quinquefasciatus* and *Culex pipiens*, do not salivate by the conventional method applied to *Aedes communis* [12]. Studies with whole body extracts have provided useful information [9, 10]. Salivary glands extend from the head into the thorax. Our data demonstrate that head and thorax extract contained fewer proteins than whole body extract [17]. On the other hand, the antibodies to mosquito antigens in human sera used are elic-

ited by mosquito oral secretions (i.e. saliva) during biting. These antibodies are specific to antigens in mosquito saliva. Using the specific antibodies in the sera from subjects with strong cutaneous reactions to mosquito bites, mosquito antigens in the extracts prepared by heads and thoraces can be specifically identified.

Our study demonstrated that 12–16 antigens were found in the three mosquito species, *A. vexans*, *C. tarsalis*, and *C. inornata*. The molecular masses of the antigens from the three species were widely disparate, ranging from a minimum of 18.5 kDa to a maximum of 160 kDa. These results are consistent with the report by Penneys et al. [8] in which salivary gland antigens in five mosquito species measured 14–126 kDa, and contained both species-unique and species-shared antigens.

Cross-reactivities with respect to bite reactions from different mosquito species have been reported [18–20]. Sensitization of animals by injection of antigenic extracts from one species can confer reactivity against another species. The immunologic basis for the cross-reactivity among different mosquito species is the existence of species-shared antigens. In our study, 9 antigens were shared by three species, and 6 antigens were shared by two species. These shared antigens explain cross-reactivity to different mosquito species.

Recently, the gene expressing a 37-kDa protein from *Aedes aegypti* salivary gland has been cloned [21]. A 62-kDa antigen, found in our study and shared by the three mosquito species, was also present in five other mosquito species salivary gland extracts [8]. Other antigens with similar molecular masses found in our study were reported in previous studies as well [8–12]. There are more than 3,000 species of mosquitos worldwide, so it is not surprising that there are shared antigens among many of these species. Whether these antigens found in our study are immunologically identical to antigens of the similar molecular weights described in other studies remains to be confirmed.

Successful immunotherapy with crude mosquito extracts has been reported in adults [1, 3] and in children [22, 23]. For immunologic diagnosis and potential immunotherapy of subjects sensitive to multiple mosquito species, study of species-shared antigens is more important than that of species-unique antigens.

Our immunoblotting results with mosquito head and thorax extracts showed more antigen bands than immunoblotting using mosquito saliva [12]. These additional antigen bands may be induced by inhalation of insect particles, or by being bitten by other insects which cross-react with mosquito body antigens. A 37-kDa IgE binding antigen has been found to be shared by five Diptera species [24]. Cross-

reactivity has also been reported between mosquito larvae and shrimp [25].

Our study showed that mosquito antigens induced both IgE and IgG responses (fig. 4). Previous studies also reported that the mosquito antigens recognized by IgE antibodies react with IgG [9] or IgG4 [7]. Previously by ELISA we found that both mosquito-specific IgE and mosquito-specific IgG were increased in subjects with skin reactions to bites, and that the two classes of specific antibody levels correlated significantly with skin bite reactions [5]. This suggests that IgG may be involved in the development of sensitivity to mosquito bites. However, in the present study, 1 nonreactive subject (fig. 3, right 5) seemed to have strong IgG reactions to 2 major antigens. The role of specific IgG in mosquito allergy needs to be further elucidated.

We found that individual IgE and IgG responses to these mosquito antigens varied slightly. This phenomenon was also noted in two previous reports [8, 9]. There is evidence of the existence of genetic controls in humans for specific immunoresponses and IgE production [26]. The unique pattern of IgE and IgG responses in each subject may reflect genetic variation among individuals. It could also reflect individual exposure to different mosquito populations with species-shared and species-unique antigens.

In summary, immunoblot analysis with SDS-PAGE has revealed a number of antigen components in the three mosquito species which have not previously been studied. These data are important for characterization of mosquito antigens, especially species-shared antigens, which will be useful in future studies of the diagnosis and immunotherapy of mosquito allergy.

Acknowledgements

We thank Dr. R. Brust, University of Manitoba, for advice, Dr. R. M. Gadawski and Mrs. Linda E. Glowacki, the Insect Control Branch, Parks and Recreation Department, The City of Winnipeg, for collection and identification of mosquitos.

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