
Immunologic mechanisms in mosquito allergy: correlation of skin reactions with specific IgE and IgG antibodies and lymphocyte proliferation response to mosquito antigens

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Background: Allergic reactions to mosquito bites are a common problem. Although IgE-mediated hypersensitivity has been reported, other immunologic mechanisms may be involved.

Objectives: To study the relationship between skin bite reactions and immunologic parameters.

Methods: Forty-one subjects were experimentally exposed to mosquito (*Aedes vexans*) bites. Immediate and delayed skin reactions were traced at 20 minutes and 24 hours, respectively, after the bites. Sera were analyzed for mosquito salivary gland-specific IgE (mosquito-IgE) and IgG (mosquito-IgG) by ELISA. Lymphocyte proliferation assays with mosquito extract were also performed.

Results: One of 41 subjects had only a delayed skin reaction to the bite, 23 had both immediate and delayed reactions, 6 had only immediate reactions, and 11 had no reaction. The mean mosquito-IgE and -IgG concentrations were higher in the subjects with immediate reactions than in those without immediate reactions ($P < .007$). The mean lymphocyte proliferation stimulation index was higher in the subjects with delayed reactions than in those without delayed reactions ($P < .015$). Further, both mosquito-IgE and -IgG levels correlated with skin immediate and delayed reactions ($P < .04$), while lymphocyte proliferation indices only correlated with skin delayed reactions ($P < .006$).

Inverse correlations were found between the size of skin reactions and the number of years lived in Canada ($P < .04$), but not with age.

Conclusion: These results indicate that IgE-, lymphocyte- and, probably, local IgG immune-complex-mediated hypersensitivities are involved in mosquito allergy. Naturally acquired desensitization to mosquito bites occurs during long-term exposure.

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INTRODUCTION

Mosquito bites are a common problem globally. Skin reactions consisting of an immediate wheal and flare and delayed induration are very common. Severe systemic reactions^{1,2} to the bites,

including anaphylaxis,³ have been reported.

Reactions to mosquito bites are generally considered immunologic in nature with both IgE-mediated and cell-mediated hypersensitivities involved.^{4–7} Reagin to mosquito extract was identified as early as 1938 by the Prausnitz-Küstner (P-K) test.⁸ After IgE was identified as a reaginic antibody, serum mosquito-specific-IgE was reported in several studies.^{4,5,9–15} In one study, serum mosquito-specific-IgE levels were compared with skin bite reactions using a laboratory bred clone, *Aedes albopictus*.⁴ Although lymphocyte proliferation assays were

performed in this study, measurement of mosquito-specific IgG was not included. Mosquito-specific IgG has been found in human serum^{5,10–18} but its role in the development of sensitivity to the bites is unclear.

We have developed sensitive, reproducible, and specific ELISA assays for measurement of mosquito salivary gland-specific IgE (mosquito-IgE) and IgG (mosquito-IgG).¹⁵ In this study we investigated levels of serum mosquito-IgE and -IgG, and lymphocyte proliferation responses to mosquito extract. We compared these immunologic results with skin reactions to *Aedes vexans* bites. Reactions to *Aedes vexans*, the most common human biting mosquito species in North America, Eurasia, and Africa, have never been studied previously.

MATERIALS AND 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 entering the study. Forty-one healthy subjects (21 men and 20 women), age 19 to 57 years, with various skin reactions to mosquito bites were selected from people who responded to advertisements during the summer of 1993. We specifically included some subjects highly sensitive to mosquito bites and some without reactions to mosquito bites, to ensure that these subjects represent various grades of reactions. We also included 12 subjects who were born outside North America and had lived in Manitoba between 2 and 15

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years (nine for 2 to 5 years and three for 7 to 15 years) to study the relationship between skin reactions and years of exposure or age. The remaining 29 subjects had lived in Canada, mostly in Manitoba, since birth. None of the subjects had a history of systemic reactions or papular urticaria followed by ulceration from mosquito bites, nor had any subject taken astemizole in the previous 6 weeks or other antihistamines within 1 week before the skin bite test.

Mosquitoes and Skin Bite Test

Female *Aedes vexans* mosquitoes were collected and identified in the Department of Entomology, University of Manitoba. All skin bite tests were performed between July 21 and August 5, 1993. In this test, a mosquito was aspirated into a glass aspirator, then transferred to a 15 × 3 × 3 cm³ plastic and screen cage. The screened side of the cage was placed on the volar aspect of the subject's forearm and the mosquito was allowed to feed until it was satiated and left the biting site on its own inclination.

The wheal and flare sizes were traced at 20 minutes and 24 hours after the bite using a felt-tipped pen. All tests and tracings of wheal and flare circumferences were made, transferred to transparent paper, and measured by the same investigator using an IBM-PC (IBM Instruments, Inc, Danbury, CT) digitizer and stereometric measurement software.¹⁹ This method calculates the area of the wheal, flare or induration, and is more accurate than the measurement of diameters.

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

Preparation of Mosquito Extracts

Mosquito salivary gland extract was used for the ELISA assay. Female mosquitoes (*Aedes vexans*), collected and identified by the Department of Entomology, were anesthetized by chilling them at 4 °C in a refrigerator. Salivary glands were dissected from female *Aedes vexans* in 0.02 M phosphate buffered saline, pH 7.2, under a binocular microscope and immediately transferred to 1 mL of phosphate buffered saline on ice. Approximately 370 pairs of salivary gland were gathered in 1 mL of phosphate buffered saline, ultrasounded for 30 seconds, and centrifuged at 8,820 *g* for 15 minutes. The supernatant was collected, aliquotted, and stored at -70 °C. The protein concentration of the antigen preparation was 0.6 mg/mL as measured by the Lowry method.

Mosquito (*Aedes vexans*) head and thorax extract was used for the lymphocyte proliferation assay. Female mosquitoes (*Aedes vexans*) were collected, identified, and then stored at -70 °C. Heads and thoraces were dissected from the rest of the bodies. These materials were placed in cold phosphate buffered saline, homogenized on ice for one minute using a PCU 11 homogenizer (Kinematic, Switzerland), centrifuged at 8,820 *g*

for 30 minutes, filtered through a 0.45-μm Amicon filter and stored at -70 °C. The protein concentration of this antigen preparation was 2.41 mg/mL.

Measurement of Mosquito-IgE and -IgG Antibodies by ELISA

Serum mosquito salivary gland-specific IgE (mosquito-IgE) and -IgG (mosquito-IgG) were measured by an indirect ELISA as described previously.¹⁵ Standardization of ELISA results between assays and the estimation of the relative amount of mosquito-IgE and -IgG in each sample was accomplished by using reference IgE and IgG sera. These sera were obtained from one subject with a high value of mosquito-IgE and another subject with a high value of mosquito-IgG and were defined as 1000 U/mL for mosquito-IgE and -IgG, respectively. Microplates coated with mosquito salivary gland extract were sequentially incubated with serum samples or reference serum, goat antibody to human IgE (P.S. myeloma-affinity purified, a gift from Dr. N.F. Adkinson, Jr, The Johns Hopkins Allergy and Asthma Center, USA) or to human IgG (Jackson ImmunoResearch Laboratories, Inc, PA, USA), enzyme-conjugated rabbit anti-goat IgG (Jackson ImmunoResearch Laboratories, Inc), and finally, the substrate. The values of mosquito-IgE or -IgG in the tested samples were calculated by interpolation from the dilution curve of the reference serum. Each IgE or IgG determination was performed on two dilutions of serum, with each dilution duplicated.

Table I. Coefficients of Correlation Among Years Lived in Canada, Immediate and Delayed Skin Reactions, Mosquito-IgE and -IgG Levels, and Lymphocyte Proliferation Assay (LPA) Indices

	Years Lived In Canada	Immediate Reaction Wheal	Immediate Reaction Flare	Delayed Reaction Induration	Mosquito-IgE	Mosquito-IgG
Immediate wheal reaction	-0.32*	—				
Immediate flare reaction	-0.14	0.72*	—			
Delayed reaction	-0.39*	0.61*	0.44*	—		
Mosquito-IgE	0.018	0.46*	0.39*	0.51*	—	
Mosquito-IgG	0.072	0.33*	0.24	0.48*	0.82*	—
LPA	-0.112	0.19	0.06	0.43*	0.21	0.17

* *P* < .05.

Lymphocyte Proliferation Assay

Lymphocyte proliferation in the presence of mosquito antigen was carried out by the standard procedure.²⁰ After titration of optimal conditions, lymphocytes were separated from heparinized blood by centrifugation on Ficoll-Hypaque (Pharmacia) gradients and washed twice with phosphate buffered saline and once with a complete culture media, RPMI-1640 (Gibco) supplemented with 2 mmol/L of L-glutamine, 5×10^{-5} mM 2-mercaptoethanol, 100 U/mL of penicillin G, 100 μ g/mL of streptomycin (Sigma), and 10% fetal bovine serum (Gibco). The cells were then resuspended at a concentration of 3×10^6 cells/mL in the culture media. One hundred microliters of the cell suspension was pipetted into each well of a flat-bottom tissue culture plate. As a lymphocyte stimulant, 100 μ L of the mosquito head and thorax extract (0.2 μ g/mL, diluted with a complete culture media) was added into each well. Phytohemagglutinin (PHA) at 10 μ g/mL and culture media were used as positive and negative controls, respectively. All assays were performed in triplicate. Cells were cultured in 5% CO₂ at 37 °C for four days. [³H]-Thymidine was added at 1 μ Ci per well 16 hours before harvest. The cells were then collected on glass fibre filters with an automatic harvester (Flow Laboratories). Radioisotope incorporation was determined in a β -counter (Beckman Liquid Scintillation Counter). Results were expressed as a stimulation index, the mean counts per minute (cpm) of antigen-stimulated cultures/the mean cpm of control cultures.

Statistical Analysis

Analysis of the data was performed using the Number Crunch Statistical System software. Unpaired *t* tests were used between group comparisons. Linear regressions were used for the analysis of correlations among skin reactions, serum mosquito antibodies, and lymphocyte proliferation indexes.

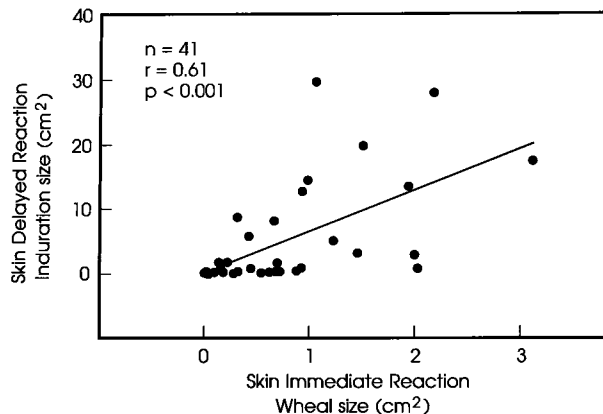


Figure 1. Correlation between skin immediate and delayed reactions to mosquito bites.

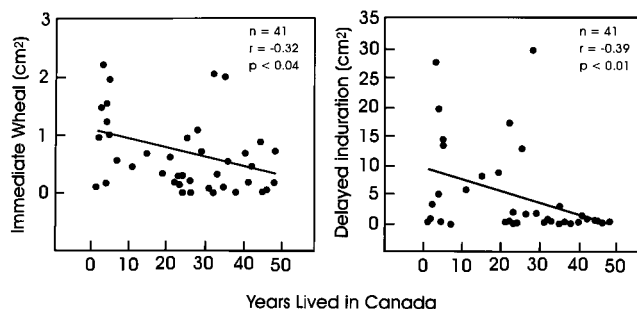


Figure 2. Correlation between the number of years lived in Canada and skin immediate and delayed reactions to mosquito bites.

RESULTS

Skin Bite Reactions

No systemic reactions were noted after the bite tests. Immediate and delayed skin reactions to mosquito bites were observed. One of 41 subjects had only a delayed skin reaction to the bites, 23 had both immediate and delayed reactions, 6 had only an immediate reaction, and 11 had no reaction.

In total, 29 of 41 subjects had positive immediate skin reactions to the bites. The immediate reaction consisted of a pruritic wheal with a surrounding flare or erythema appearing within several minutes, reaching a peak at 30 minutes and then subsiding. Twenty-four of 41 subjects had positive delayed reactions to the bites. The delayed reaction consisted of an indurated papule and erythema that appeared several hours later, reaching a peak 24 hours after the bite. In five subjects with severe delayed reactions,

vesiculated areas were found in the centers of the indurated areas, precisely in the area where the immediate wheal had been. The size of the immediate reactions ranged from nonreactive up to 3.1 cm² for the wheal and up to 23.0 cm² for the flare, and that of delayed reactions ranged from non-reactive up to 29.5 cm².

Immediate reaction wheal sizes correlated with flare sizes ($r = .72$, $P < .001$) (Table 1). Both immediate wheal and flare reactions showed significant correlation with the delayed reactions ($r = .61$, $P < .001$ for the wheal areas; $r = .44$, $P < .02$ for the flare areas) (Table 1, Fig 1). The immediate wheal and the induration sizes correlated inversely with the number of years lived in Canada ($r = -.32$, $P < .04$ for the immediate wheal sizes; $r = -.39$, $P < .01$ for the delayed induration sizes) (Table 1, Fig 2), but there was no cor-

relation with the age of the subjects ($P > .05$).

Mosquito-IgE and -IgG Levels

The mean mosquito-IgE and -IgG were higher in the subjects with an immediate reaction (707 and 746 U/mL, respectively) than in those with no immediate reaction (347 and 281 U/mL, respectively) ($p < .007$) (Fig 3). A significant correlation ($r = .82$, $P < .000$) was found between mosquito-IgE and -IgG levels (Fig 4). The two antibody levels also correlated with skin immediate wheal and delayed reactions ($r = .33$ to $.51$, $P < .036$), but not with lymphocyte proliferation indices ($P > .05$) (Table 1).

Lymphocyte Proliferation Assay

The mean lymphocyte proliferation stimulation index was significantly higher in the subjects with delayed reactions (8.5) than in those with no delayed reactions (4.0) ($P < .015$) (Fig 5). There was a significant correlation between lymphocyte proliferation stimulation indices and skin delayed reactions ($r = .43$, $P < .006$), but no correlation between lymphocyte proliferation indices and other immunologic parameters (Table 1).

DISCUSSION

Skin reactions to mosquito bites consisted of an immediate wheal and flare and delayed induration. The elapsed time of the immediate reaction was compatible with that of IgE-mediated hypersensitivity, while that of the delayed reaction appeared to be consistent with lymphocyte-mediated hypersensitivity, possibly also involving cutaneous late phase allergic reaction and local immune-complex-mediated hypersensitivity. These immunologic mechanisms will be discussed below.

The participation of reaginic antibody in mosquito allergy has been demonstrated by the successful passive transfer of immediate skin reactivity to mosquito bites or extracts in P-K tests.^{8,9,21,22} Serum mosquito-specific IgE has also been detected by Western blot,^{10,11,13,14} RAST,^{5,9} ELISA,^{4,12,15} and histamine release from basophils.²² Significant correlation between imme-

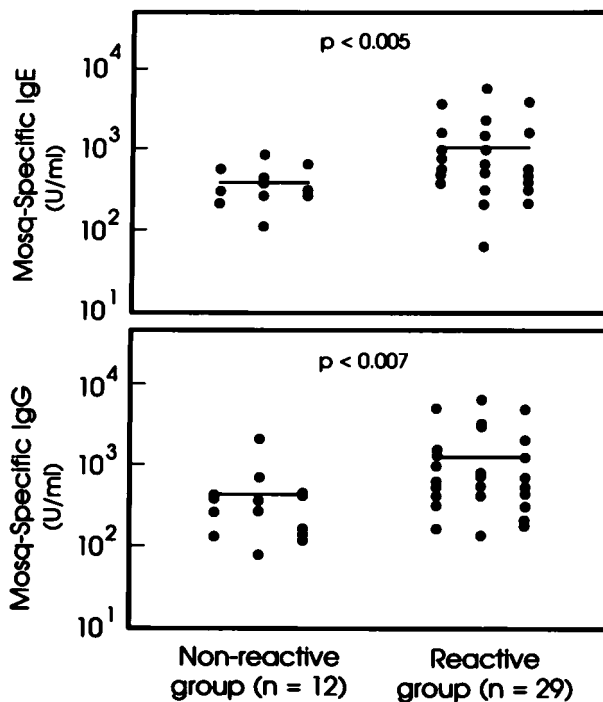


Figure 3. Mosquito-IgE and -IgG levels in subjects with or without skin immediate reactions to mosquito bites.

diate skin reactions and serum mosquito-specific IgE levels was reported in a previous study using *Aedes albopictus*.⁴ In our study using *Aedes vexans*, the major species in North America, immediate skin reactions significantly correlated with mosquito-IgE levels (Table 1), and the mean serum mosquito-IgE was also significantly higher in the subjects with immediate reactions than in those with no immediate reaction (Fig 3). These results further

confirm that IgE-mediated hypersensitivity is involved in the development of mosquito allergy.

Several investigators^{4,6,7,23} have suggested that cell-mediated hypersensitivity may play a role in delayed reactions to mosquito bites similar to the role it plays in Hymenoptera allergy.²⁴ Delayed skin reaction to mosquito bites has been successfully transferred with spleen cells in guinea pigs.²³ In vitro study of lymphocyte proliferation

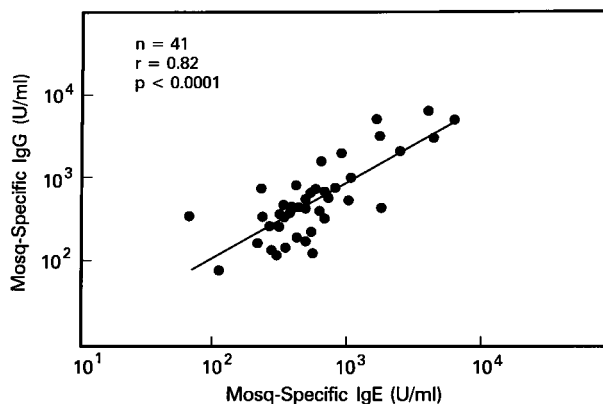


Figure 4. Correlations between mosquito-IgE and -IgG levels.

assays with mosquito extracts has shown that lymphocyte stimulation indices with mosquito antigen correlate significantly with delayed skin reactions.⁴ In our study, the lymphocyte proliferation indices correlated significantly with only the delayed skin reactions ($r = .43$, $P < .006$) and not with immediate skin reactions or mosquito-IgE or -IgG levels (Table 1). This suggests the involvement of lymphocyte-mediated hypersensitivity in mosquito allergy.

IgE-mediated reactions may be followed by late phase reactions. Cutaneous late phase allergic reactions are characterized by erythema and induration peaking at four to eight hours²⁵ and can be transferred from a sensitized to a nonsensitized individual with serum.²⁶ The cell-mediated hypersensitivity appears only after several hours, reaches a maximum at 24 to 48 hours²⁷ and can be transferred with lymphocytes^{23,28} but not with serum. Although different mechanisms are involved, it is difficult to isolate the cutaneous late phase allergic reactions from the cell-mediated delayed reactions by appearance and thus we were not able to delineate the exact nature of the late phase reactions. In our study, the vesiculated area in the centre of the delayed induration exactly mirrored the area in which the immediate wheal had occurred, suggesting that the accelerated skin damage is caused by the IgE-dependent late phase reaction as well as by the lymphocyte-dependent delayed reaction.

Mosquito-specific IgG has been previously identified in human sera using ELISA,^{12,15,16} Western immunoblot,^{10,11,13,14,17,18} and immunohistochemical methods²⁹ and is considered to consist mainly of the IgG4 and IgG1 subclasses.^{5,13,14} The role of mosquito-specific IgG is unclear. Some investigators found mosquito-specific IgG or precipitating antibodies to be related to the exposure to mosquito bites¹⁶ or the development of skin reactions,³⁰ while others found that subjects with extensive exposure to mosquito bites tended to have little or no mosquito-specific IgG.²⁹

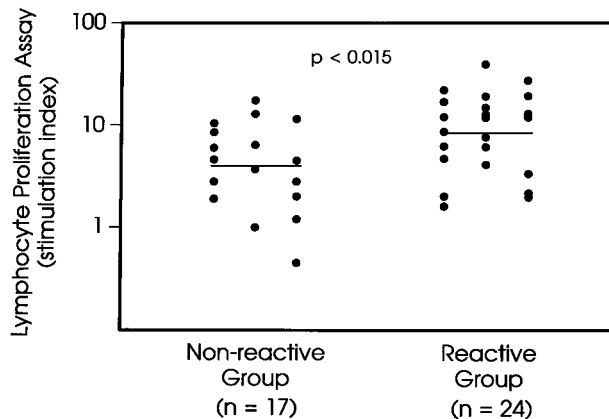


Figure 5. Lymphocyte proliferation indices (log scale) in subjects with or without skin delayed reactions to mosquito bites.

Arthus phenomenon with necrosis, immune-complex-mediated hypersensitivity, has been reported in mosquito allergy.^{8,31} The cutaneous manifestations of Arthus-type reactions can be small or large vesicular pustular, hemorrhagic or necrotic lesions.⁵ The elevated mosquito-specific IgG may form local immune-complexes with mosquito antigens. The binding of the immune-complexes to complement elicits inflammatory reactions. This lesion is characterized by an erythematous and edematous reaction reaching a peak at three to eight hours and then usually waning.²⁷ It is very likely that the elevated mosquito-IgG participates in the early stage of the delayed skin reactions. In our study, mosquito-IgG levels were significantly increased in the subjects with positive skin reactions (Fig 3) and correlated well with the sizes of both immediate and delayed skin reactions (Table 1) and with mosquito-IgE (Fig 4). Although in Hymenoptera allergy there is controversy as to whether specific IgG acts as a "blocking" antibody³²⁻³⁴ or not,^{35,36} our study strongly suggests that specific IgG may be involved in the development of mosquito allergy probably through a local mosquito antigen-IgG immune-complex-mediated hypersensitivity mechanism. A further study using the immunofluorescent staining of biopsies with conjugated anti-immunoglobulins and anti-C3 is suggested to confirm the involvement of im-

mune-complex-mediated hypersensitivity in mosquito allergy.

Human skin reactions to mosquito bites have been classified into five stages.^{37,38} In these studies, in the people who had never been exposed to a specific species of mosquito, the initial bites did not cause reaction (stage I), but subsequent bites resulted in the appearance of delayed cutaneous lesions (stage II). After repeated bites an immediate wheal developed (stage III). With further exposure, the delayed reaction disappeared and there was only an immediate wheal (stage IV). Individuals repeatedly exposed to thousands of bites from the same species of mosquito eventually lost their immediate reactions (stage V).^{37,38} In our study, all subjects had been previously exposed to mosquito bites and were classified from stage II to V. One was in stage II—delayed reaction only; 23 in stage III—immediate and delayed reactions; 6 in stage IV—immediate reaction only; and 11 in stage V—no reaction. Different stages involve different immunologic mechanisms and some mechanisms may overlap.

In one previous study, skin delayed reaction to mosquito bites was not related to age,³⁹ while another study reported this reaction decreased with age.⁴⁰ The subjects in the second study⁴⁰ had always lived in the same area, and age was related to years of exposure (equivalent to the years lived in Canada in the present study). In

order to determine if reduction of skin reactions to mosquito bites is age-dependent, as is also seen in the morbidity of atopic disorders,⁴¹ we included 12 subjects who were born outside of Canada where *Aedes vexans* is not known to be indigenous, and had lived in Canada from 2 to 15 years. We cannot establish how many bites the subjects, living in an area where *Aedes vexans* is a major pest each summer, have received, but we can assume that the more years you live in Canada, the more bites of *Aedes vexans* you receive. We have shown that the immediate wheal and the induration sizes correlated inversely with the number of years lived in Canada (Table 1, Fig 2), but there was no correlation with the age of the subjects (Table 1) suggesting that during natural exposure, desensitization to the bites eventually occurs and is exposure-dependent rather than age-dependent.

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