

Mosquito Allergy: Immune Mechanisms and Recombinant Salivary Allergens

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Key Words

Mosquito allergy · Recombinant salivary allergene · IgE

Abstract

Reactions to mosquito bites are immunological in nature, with the involvement of IgE-, IgG- and T lymphocyte-mediated hypersensitivities. Acquired desensitization to mosquito saliva may occur during childhood and adolescence or during long-term exposure to mosquito bites. Due to the lack of salivary preparations, allergic reactions to mosquito bites are underdiagnosed and undertreated. Recombinant mosquito saliva allergens with biological activity are being developed. These recombinant allergens will significantly improve diagnosis of mosquito allergy and eventually will also improve specific immunotherapy for patients with systemic reactions to mosquito bites.

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Introduction

Around the world, allergic reactions to mosquito bites are an increasing clinical concern and decrease the quality of life for many people [1]. Severe local reactions such as 'Skeeter syndrome' and systemic reactions including generalized urticaria and anaphylaxis have been reported.

These reactions are underdiagnosed due to the lack of availability of mosquito salivary preparations, which are required for accurate diagnosis. For the same reason, allergen immunotherapy is not widely used for systemic reactions to mosquito bites, and the immune mechanisms associated with the development of allergic reactions and subsequent natural desensitization after exposure to mosquito bites are not yet completely understood. Utilization of molecular biology techniques to produce pure mosquito salivary allergens has made it possible to investigate the immune responses involved and diagnose mosquito allergy accurately, and eventually will make it possible to treat people who have systemic allergic reactions to mosquito bites with standardized high-quality and specific allergen immunotherapy. In this review, we describe clinical manifestations, the immune mechanisms involved, the development of sensitization and subsequent natural desensitization, and the current status of natural and recombinant mosquito salivary allergens in mosquito allergy.

Clinical Manifestations

Typical local cutaneous reactions to mosquito bites consist of immediate wheals and flares peaking at 20 min and delayed pruritic indurated papules peaking at 24–36 h, then diminishing over several days or weeks [1–3]. Large local reactions to mosquito bites consist of itchy,

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Table 1. Coefficients of correlation among immediate and delayed skin reactions in a mosquito bite test, serum saliva-specific IgE and IgG levels, lymphocyte proliferation assay indices to mosquito allergens, and years lived in Canada in 41 healthy volunteers with various skin reactions ranging from no reaction to severe reactions to mosquito bites [2]

	Years in Canada	Immediate wheal	Immediate flare	Delayed induration	Mosquito saliva-IgE	Mosquito saliva-IgG
Immediate wheal	-0.32*	–				
Immediate flare	-0.14	0.72*	–			
Delayed induration	-0.39*	0.61*	0.44*	–		
Mosquito saliva, IgE	0.018	0.46*	0.39*	0.51*	–	
Mosquito saliva, IgG	0.072	0.33*	0.24	0.48*	0.82*	–
Lymphocyte proliferation	-0.112	0.19	0.06	0.43*	0.21	0.17

* $p < 0.05$.

red, warm swellings appearing with minutes of mosquito bites, and itchy papules, ecchymotic, vesiculated, blistering, bullous or even Authus-type reactions, appearing 2–6 h after the bites and persisting for days or weeks [4–7, 25]. Only those reactions which cause large or atypical (ecchymotic or vesiculated) local reactions or systemic reactions such as anaphylaxis, angio-oedema, generalized urticaria, or wheezing are considered to be ‘mosquito allergy’ [1, 4–12]. ‘Skeeter syndrome’ is defined as mosquito-induced large local inflammatory reaction sometimes accompanied by low grade fever, often misdiagnosed as cellulitis and treated as such. Individuals at increased risk of severe reactions to mosquito bites include those with: (1) a high level of exposure, for example, civilian or military outdoor workers [10]; (2) low or absent natural immunity, for example, infants and young children [5, 13]; (3) no previous exposure to indigenous mosquitoes, for example, immigrants or visitors, and (4) primary or secondary immune deficiency such as AIDS, malignancies [14] or Epstein-Barr virus-associated lymphoproliferative diseases [15].

Immune Mechanisms

Reactions to mosquito bites are immunological in nature. They are due to specific sensitization to the mosquito salivary proteins, because initial exposure to species to which an individual has not been previously exposed causes no reaction [3, 16]. Mosquito saliva-specific IgE and IgG antibodies as well as lymphocyte proliferation appear to be involved in the pathogenesis of allergic reactions to mosquito bites.

IgE

The time frame of the immediate reaction to mosquito bites is compatible with that of IgE-mediated type I hypersensitivity. Mosquito bite-induced immediate wheals and flares correlate well with mosquito salivary gland-specific IgE levels (table 1) [2, 17]. The development of skin sensitization to mosquito bites also parallels the levels of saliva-specific IgE antibodies [3]. The participation of IgE in mosquito allergy has been demonstrated by the successful passive transfer of immediate skin reactivity to mosquito bites in Prausnitz-Küstner tests [18, 19]. Elevated serum mosquito saliva-specific IgE has also been detected by immunosorbent assays which include enzyme-linked immunoassays (ELISA) (fig. 1) [2, 5, 20], RAST [18], and the UniCap test [21], Western blot [22, 23], and histamine release from basophils [19] in mosquito-allergic or mosquito bite test-positive subjects. There is no doubt that IgE is involved in the pathogenesis of mosquito allergy. In individuals with systemic reactions, saliva-specific IgE, but not IgG, levels are significantly increased [12].

IgE-mediated immediate local reactions to mosquito bites may be followed by late phase reactions. Cutaneous late phase allergic reactions are characterized by erythema and induration peaking at 4–8 h and can be passively transferred from a sensitized to a non-sensitized individual using serum [24].

IgG

Mosquito saliva-specific IgG antibodies, consisting mainly of the IgG4 and IgG1 subclasses, have been found to be significantly elevated in individuals with positive mosquito bite tests [2] and in individuals with severe local reactions, but not systemic reactions, to mosquito bites

Fig. 1. Serum levels of *Ae. vexans* saliva-specific IgE and IgG subclasses in children with Skeeter syndrome and age-matched control children [5].

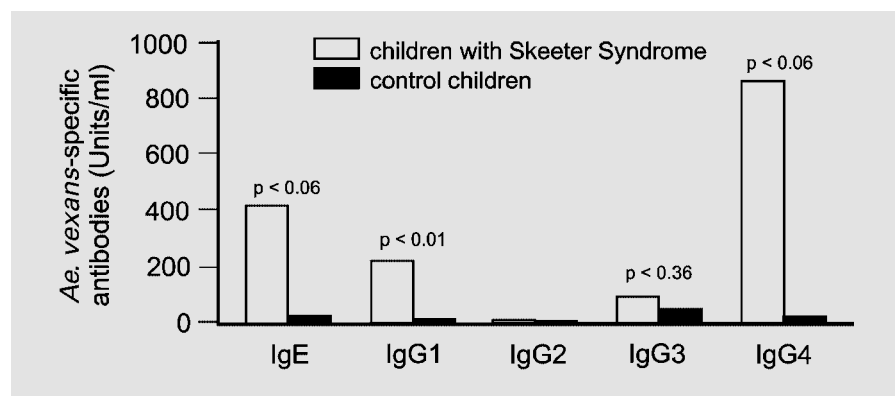
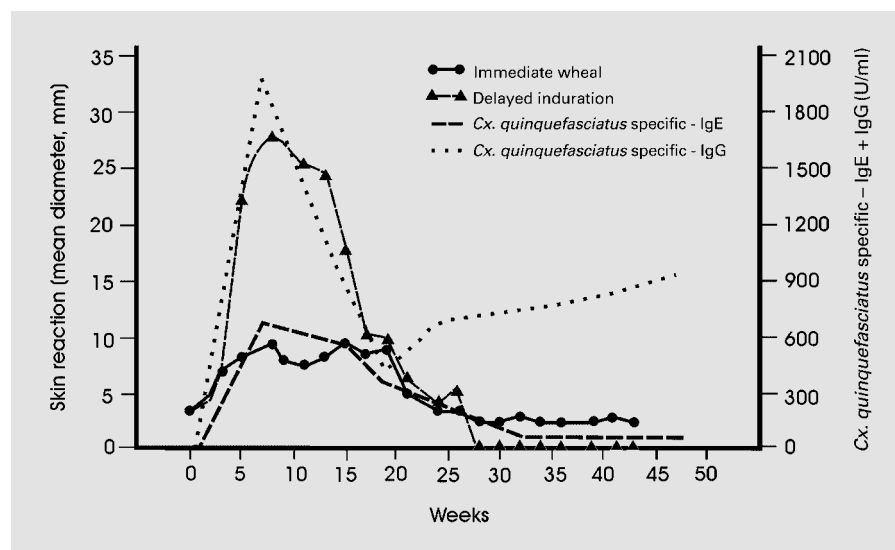


Fig. 2. Naturally acquired sensitization and subsequent desensitization to mosquito bites [3]. A man received 100 mosquito (*Cx. quinquefasciatus*) bites, to which he had never been previously exposed, every 2 weeks for 48 weeks. Skin immediate wheals (average diameter recorded 20 min after a bite test) and delayed indurations (average diameter recorded 24 h later) were monitored and serum *Cx. quinquefasciatus* saliva-specific IgE and IgG were measured using an indirect ELISA.



(fig. 1) [5, 23]. The levels of mosquito saliva-specific IgG correlate well with the sizes of both immediate and delayed skin reactions and with saliva-specific IgE levels (table 1) [2]. In a longitudinal study, mosquito saliva-specific IgG levels parallel the development of both skin immediate and delayed reactions (fig. 2) [3]. Arthus phenomenon with necrosis, IgG immune complex-mediated type III hypersensitivity, has also been reported in mosquito allergy [7, 25, 26]. The cutaneous manifestations of Arthus-type reactions can be small or large vesicular pustular, haemorrhagic or necrotic lesions. The elevated mosquito-specific IgG may form local immune complexes with mosquito antigens, which bind to the complement and elicit inflammatory reactions characterized by erythema and oedema, peaking at 3–8 h. It is very likely that the elevated mosquito saliva-specific IgG participates in the early stage of the delayed skin reactions. Studies suggest that saliva-specific IgG may be involved in the develop-

ment of mosquito allergy, probably through a local mosquito antigen-IgG immune complex-mediated type III hypersensitivity mechanism. Additional investigations using immunofluorescent staining of biopsies with conjugated anti-immunoglobulins and anti-C3 are needed to confirm the involvement of immune-complex-mediated hypersensitivity in mosquito allergy.

T Cells

Several investigators have suggested that T cell-mediated hypersensitivity may play a role in delayed reactions to mosquito bites, similar to the role they play in hymenoptera allergy [2, 17, 27]. Delayed skin reactions to mosquito bites have been successfully transferred with spleen cells, not serum, in guinea pig experiments [27]. In vitro studies using lymphocyte proliferation assays with mosquito extracts have shown that lymphocyte responses to mosquito allergens significantly correlate with delayed,

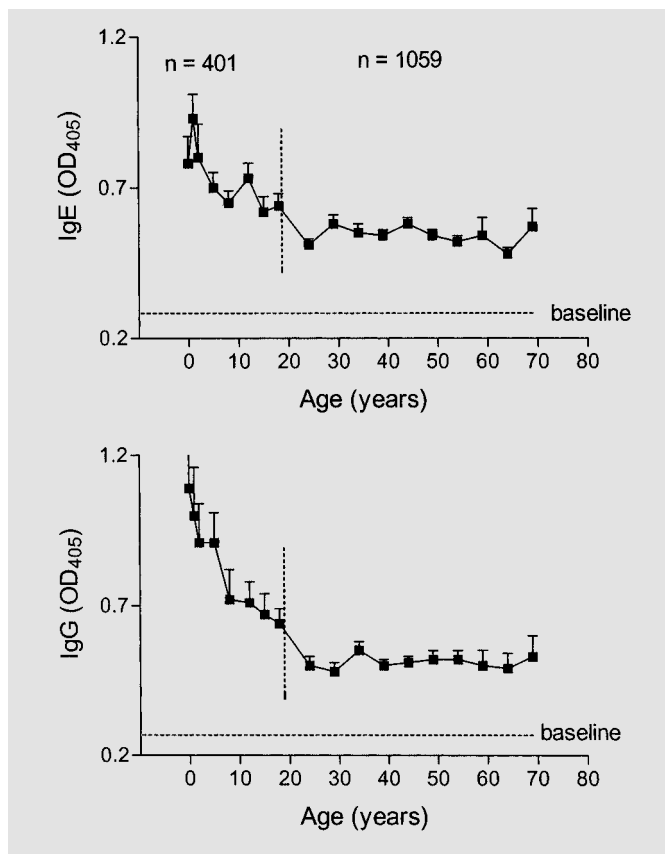


Fig. 3. Serum levels of *Ae. vexans* saliva-specific IgE and IgG in 1,460 individuals in different age groups [13, 29]. Four-hundred and one samples from children ranging in age from 1 month to 18 years were sera left over from clinical chemistry tests in a children's hospital laboratory. One thousand and fifty-two samples were from healthy Canadian blood donors aged between 17 and 70 years. The baseline was established using the mean level in 23 infants (negative controls) who had never been exposed to mosquitoes.

not immediate, skin reactions (table 1) [2, 17], suggesting the involvement of lymphocyte-mediated type IV hypersensitivity in mosquito allergy. In addition, the delayed reaction to mosquito bites may also involve cutaneous late phase allergic reactions and IgG-mediated type III hypersensitivity.

Development of Sensitization and Subsequent Natural Desensitization

Based on clinical observations, the natural history of sensitization and subsequent desensitization to the saliva injected by mosquito bites has been classified into 5 stages [16, 28]. In individuals who have never been exposed to a

specific species of mosquito, the initial bites do not cause a reaction (stage I), but subsequent bites result in the appearance of delayed cutaneous lesions (stage II). After repeated bites, immediate wheals develop (stage III). With further exposure, delayed reactions are no longer observed, and only immediate wheals are noted (stage IV). Individuals repeatedly exposed to thousands of bites from the same species of mosquito eventually lose their immediate reactions (stage V). These 5 stages have been reproduced in a prospective study, in which stages II and IV appeared to be transient (fig. 2) [3]. In this study, every 2 weeks for 48 weeks, an individual received 100 mosquito bites from a laboratory-reared species to which he had never been previously exposed. Skin immediate responses and saliva-specific IgE and IgG antibody responses were monitored prospectively. Initial bites caused no skin reactions. Delayed and immediate skin reactions developed at week 3, peaked at weeks 5–19, and disappeared by week 26. The IgE and IgG responses closely paralleled the immediate and delayed skin responses. The entire sensitization and desensitization process took approximately 26 weeks; however, in real-life, natural desensitization may take years to occur or may never occur, since mosquito-allergic individuals generally try to avoid getting bitten by mosquitoes.

Naturally acquired desensitization to mosquito saliva has also been confirmed in several cross-sectional studies. In 41 healthy volunteers with skin reactions to mosquito bites ranging from none to severe, the immediate wheal and the induration correlated inversely with the number of years lived in Canada (table 1). In another study of 401 infants, children, and adolescents ranging in age from 1 month to 18 years, both saliva-specific IgE and IgG antibodies gradually declined with age, and there was a significant inverse correlation between age and IgE and IgG levels [13]. In the age group of 16–18 years, the mean levels of IgE and IgG antibodies were similar to those reported in 1,059 adults [29] by the same authors using the same immunoassay. The results from these two studies, as shown in figure 3, suggest that natural desensitization probably occurs during childhood and adolescence and that infants and young children may be at increased risk of having severe reactions to mosquito bites.

Based on the above observations, immunotherapy with injections of gradually increasing doses of mosquito saliva proteins would be expected to be useful in preventing reactions to mosquito bites, analogous to the use of venom immunotherapy in the prophylactic treatment of systemic reactions to bee, wasp, hornet or yellow jacket stings. Indeed, immunotherapy using injections of mos-

Table 2. *Ae. aegypti* salivary proteins

Protein name	Allergen name	Molecular weight (KD)	cDNA sequenced	Biological functions	References
α -Amylase 1	?	81.5	yes	unknown	38
Apyrase	Aed a 1	68	yes	antiplatelet	67, 70, 87
α -Glucosidase (maltase-like 1)	Aed a 4	67	yes	sugar digestion	37, 68, 73
Esterase	?	65	?	unknown	88
Anticoagulant-factor Xa	?	54	yes	anticoagulant	89
Aed a X ₁	Aed a X ₁ ?	44	?	unknown	90
Aed a X ₂	Aed a X ₂ ?	37	?	unknown	90
Female-specific protein, D7	Aed a 2	37	yes	unknown	50, 63
–	Aed a 3	30	yes	unknown	64
Sialokinins	?	1.4	yes	vasodilator	91–93
Anti-tumour necrosis factor	?	unknown	?	anti-tumour necrosis factor	94
Lysozyme	?	unknown	?	bacteriolysis	39

quito whole body extracts has been reported to prevent allergic reactions to subsequent bites [6, 10]. However, because currently commercially available mosquito whole body extracts contain few mosquito saliva proteins and many nonsalivary proteins which are ineffective and may cause additional sensitization [30], this therapy is neither well studied nor widely used in mosquito allergy.

Mosquito Salivary Allergens and Commercial Extracts

It is the proteins in mosquito saliva to which people develop local or systemic allergic reactions. If the main ducts of the mosquito salivary glands are transected, the mosquito can still feed and develop eggs, but it is unable to cause skin reactions in humans [31].

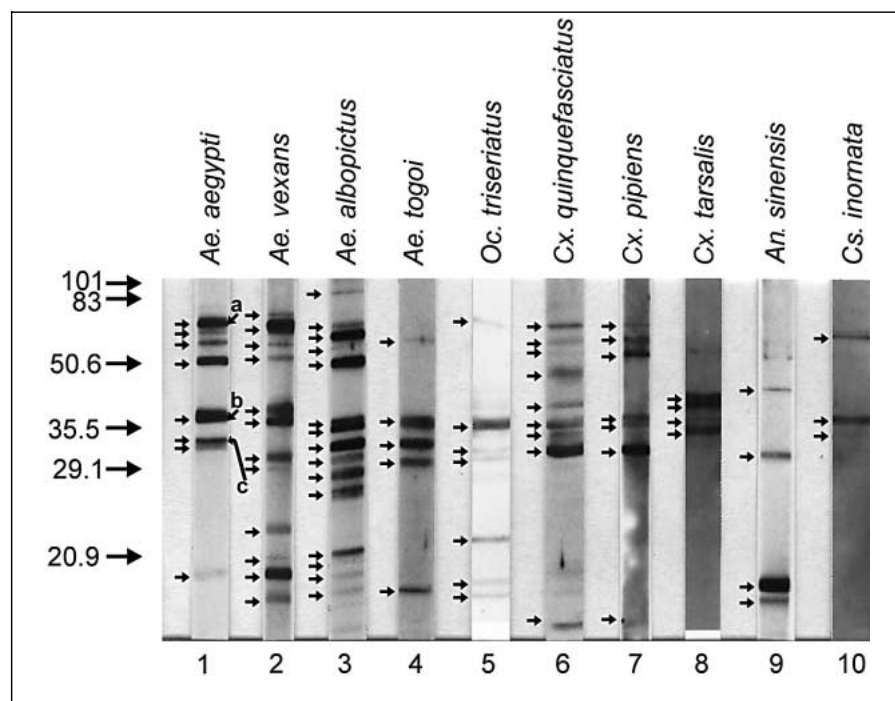
Mosquito saliva contains many biological materials which can be classified according to their functions as follows: (1) ant clotting and antiplatelet factors and vasodilators which presumably increase the speed at which blood from the host is imbibed [32, 33], (2) substances that affect parasite transmission by arthropod vectors [34–36], (3) enzymes which are associated with sugar feeding [37, 38], (4) lysozyme, which may help to control bacterial growth in the sugar meal stored in the mosquito crop [39], and (5) immunomodulators [40–44]. Some of these substances in mosquito saliva have been identified as allergens [45–47].

There are more than 40 mosquito genera and more than 3,000 mosquito species world-wide. *Ae. aegypti* sali-

va contains at least 31 proteins, the cDNA sequences of which have been deposited in the Genbank [48]. However, protein visualization techniques using gel electrophoresis and silver staining have only revealed about 20 peptides in the saliva of adult *Ae. aegypti* [49]. These include α -amylase, anticoagulants, anti-tumour necrosis factor, apyrase, esterase, D7, α -glucosidase, and sialokinins [50]. Using SDS-PAGE and immunoblot techniques, at least 8 of these proteins have been identified as allergens which bind to the IgE of individuals who have large local allergic reactions to mosquito bites [47]. Table 2 summarizes the salivary proteins in *Ae. aegypti*. In addition to the thirty-one salivary cDNAs from *Ae. aegypti* [48], thirty-three full-length salivary cDNA sequences have been reported from a full-length cDNA library of *An. stephens* [51] and twenty-one cDNAs from *An. gambiae* [52]. Most sequences match known proteins, although some do not [48, 51, 52].

Most of the proteins in mosquito saliva are allergenic in humans [23, 46, 47, 53–55]. Using immunoblot analyses with a pooled serum from individuals allergic to mosquito bites, allergens in the saliva or salivary gland extracts from 10 world-wide distributed mosquito species were studied [47] (fig. 4). Three to sixteen salivary allergens are found in each species, with molecular masses ranging from 16 to 95 kD. Both species-shared and species-specific allergens exist. Among the 10 species studied, *Ae. albopictus*' saliva contains at least 16 allergens, *Ae. vexans* contains 12 allergens, and *Ae. aegypti* and *Cx. quinquefasciatus* contain 8–9 allergens (fig. 4). In a recent study, serum saliva-specific IgE to 5 mosquito species

Fig. 4. Salivary allergens of 10 mosquito species [22]. Proteins in saliva or salivary gland extracts of 10 mosquito species were separated by SDS-PAGE and then transferred to nitrocellulose membranes. The membranes were immunoblotted with a serum pooled from mosquito-allergic individuals and then incubated with monoclonal anti-human IgE antibodies. a = Aed a 1 and Aed a 4; b = Aed a 2; c = Aed a 3.



were measured in 14 individuals who had systemic reactions to mosquito bites [12]. Eleven of these individuals had a positive IgE response to *Ae. albopictus*' saliva which contains the highest number of allergens in the 10 species previously studied [47], while 5–8 individuals had a positive IgE response to each of the remaining 4 species (*Ae. vexans*, *Ae. aegypti*, *Cx. quinquefasciatus* and *An. sinornata*), suggesting that *Ae. albopictus* has the highest number of species-shared allergens. In addition, mosquito salivary allergens may cross-react with allergens from other biting insects and from crustaceans [56–59]. Moreover, there are rare reports of cross-reactivity with allergens in hymenoptera venom [11].

Currently, commercially available mosquito allergen preparations are made from whole body extracts which contain little salivary proteins and many non-salivary proteins that may cause new sensitization and other side effects [30]. In a recent study, the UniCap test, in which a mosquito whole body extract was used as the capture antigen, was compared to a mosquito saliva capture ELISA in the diagnosis of individuals with systemic or severe local reactions to mosquito bites [21]. The mosquito saliva capture ELISA was more sensitive and specific (less likely to give false-negative and false-positive results) than the UniCap test in the diagnosis of mosquito allergy. The positivity rate was 56% for the saliva capture ELISA and

25% for the UniCap test in mosquito-allergic individuals, versus 5% in the negative controls. The results suggest that mosquito saliva proteins only should be used in the diagnosis of mosquito allergy.

Recombinant Mosquito Salivary Allergens

Mosquito saliva can be obtained only by dissecting the salivary glands from the heads and thoraxes of individual mosquitoes or by direct collection from living female mosquitoes [47, 60]. In the latter process, a living female mosquito is confined to the wall of a box by placing Vaseline on its legs and wings. The proboscis is then inserted into a capillary tube filled with water and salivation is induced by applying a small amount of malathion to the thorax. One hour later, the contents of capillary tubes are collected, pooled and lyophilized. In our experience, each salivation of *Ae. vexans* contains only 0.2 µg of proteins. Both processes are extremely tedious and therefore not practical for widespread use in diagnosis and immunotherapy of mosquito allergy.

Molecular cloning of mosquito saliva proteins has provided a solution to this problem. Utilization of this technique to produce pure mosquito salivary allergens on a large scale serves as a powerful research tool for investiga-

tion of the mechanisms involved, and will eventually facilitate diagnosis and treatment of mosquito allergy with standardized, effective, and safe allergens [61].

Ae. aegypti is the most important mosquito pest in the world, and shares many salivary allergens with other species including *Cx. quinquefasciatus* and *Ae. vexans*, another common pest mosquito indigenous to North America [47, 62]. To date, four recombinant salivary proteins of *Ae. aegypti*, a 68-kD apyrase, a 37-kD D7 protein, a 30-kD Aed a 3, and a 67-kD α -glucosidase, have been identified as allergens [63–68] (table 2). According to current allergen nomenclature [69] we have named the apyrase Aed a 1, the D7 Aed a 2, and the α -glucosidase Aed a 4, and their recombinant forms rAed a 1, rAed a 2, rAed a 3, and rAed a 4.

Biological Functions

Aed a 1, apyrase, is an adenosine triphosphate diphosphohydrolase that facilitates platelet anti-aggregation by destroying adenosine di- and triphosphates released from injured cells or by activated platelets [70]. Aed a 2, the D7 protein, is the original protein that defines a large family of D7-related proteins [71]. A large number of cDNAs corresponding with the D7-related proteins have been isolated and characterized from the salivary gland-specific libraries of a variety of haematophagous insects [72]. Therefore, it is not surprising that rAed a2 cross-reacts with saliva or salivary gland extracts from many other mosquito species [22] (fig. 5). The biological function of Aed a 3 is currently unknown, because the cDNA coding Aed a 3 was directly cloned from the *Ae. aegypti* salivary gland cDNA library using mouse anti-*Ae. aegypti* saliva serum [64]. Aed a 4, α -glucosidase, is a soluble glycoprotein that is secreted when mosquitoes ingest sugar and blood meals [37, 73].

Allergenicity and Biological Activity

These 4 recombinant allergens have been expressed using a baculovirus/insect cell system, and purified on a large scale [63–68]. The baculovirus/insect cell system has been used extensively for the production of large amounts of recombinant proteins with biological activity [67, 74–76]. The ability of the insect cells to perform many of the posttranslational protein modifications found in eukaryotic cells has a major advantage over prokaryotic expression systems, since proteins expressed using this system can be used in both in vitro and in vivo tests [74, 77]. In contrast, proteins expressed using prokaryotic expression systems such as *Escherichia coli* have no carbohydrates and are generally used only in in vitro assays [78–

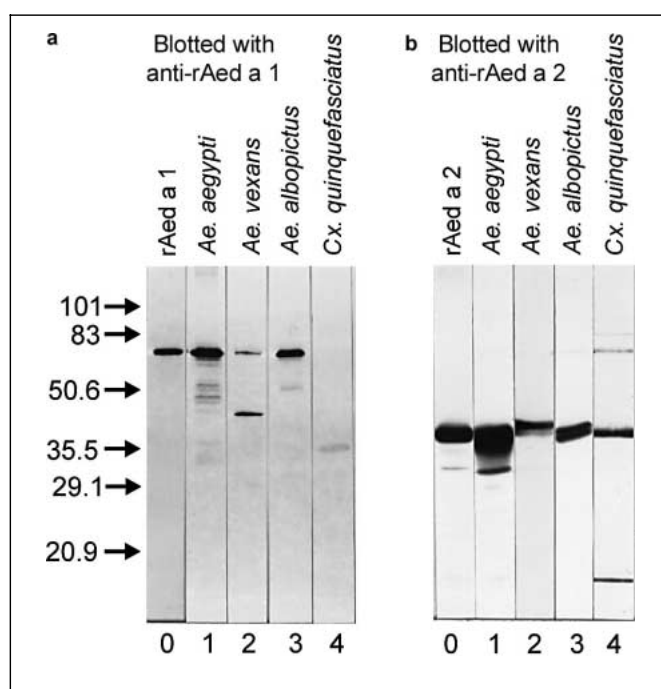


Fig. 5. Examination of the presence of Aed a 1 (apyrase) and Aed a 2 (the D7 protein) in the salivary preparations of 4 mosquito species [47]. Proteins in the saliva or salivary gland preparations were separated by SDS-PAGE and then transferred to nitrocellulose membranes. The membranes were probed for the presence of Aed a 1 or Aed a 2 using rabbit anti-rAed a 1 or anti-rAed a 2.

80]. For example, the biological activity of recombinant honey bee venom hyaluronidase expressed using baculovirus/insect system is comparable to that of the natural enzyme, while the hyaluronidase expressed in *E. coli* reaches only 20–30% of the activity of the natural enzyme [74].

The 4 recombinant salivary allergens have been demonstrated to bind to serum IgE as measured by using ELISAs (fig. 6) and Western blot [63–65, 67, 68] and to induce histamine release from basophils (previously unpubl. data) (fig. 7) in individuals with allergic reactions to mosquito bites. The recombinant allergens also induce skin immediate and delayed reactions in mosquito bite test-positive individuals [63, 66, 67] (fig. 8). Furthermore, the binding of IgE to the recombinant allergen in the ELISA can be inhibited by the addition of natural salivary preparations in a dose-dependent manner, and the sizes of recombinant mosquito salivary allergen-induced skin flare significantly correlate with those of mosquito bite-induced flares [66, 67] (fig. 9). In the immunoblot analysis, both

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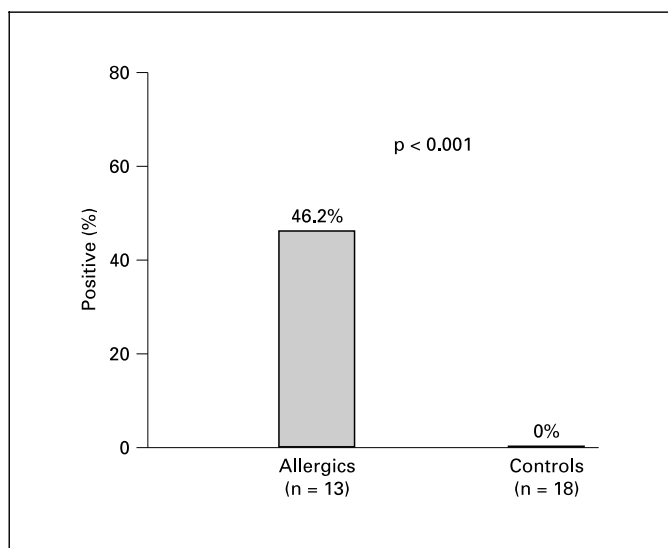
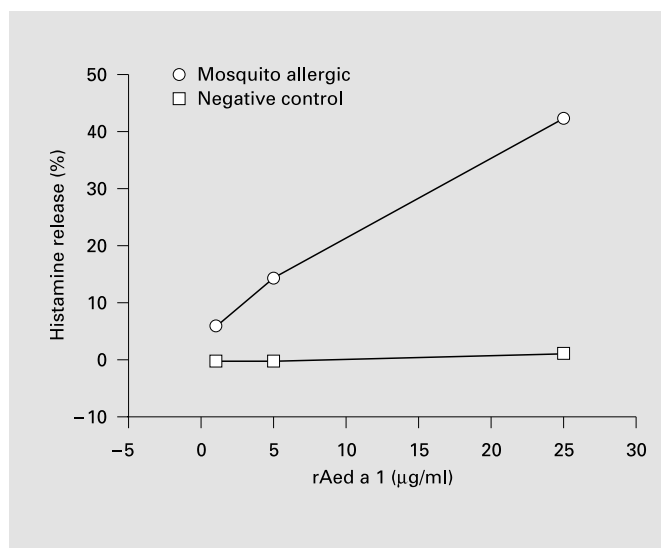


Fig. 6. rAed a 4-specific IgE in the diagnosis of mosquito allergy. rAed a 4-specific IgE was measured using an ELISA in 13 individuals with several local reactions or systemic reactions to mosquito bites. Eighteen individuals with a negative *Ae. vexans* bite test served as controls. The mean of the IgE levels in the controls plus 2 SD was used as a cutoff level [68].

Fig. 7. rAed a 1-induced histamine release from basophils of mosquito-allergic and non-allergic individuals. Basophil histamine release was performed using a modification of a method previously reported [95]. Blood was loaded on the surface of Ficoll-Hypaque (density 1.085 g/ml) and centrifuged at 1,400 g for 20 min. Basophils were collected from the interface of the two liquids, washed, counted and



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resuspended in culture medium at a concentration of 2×10^6 cells/ml. 100 μl of the basophil solution were mixed with an equal volume of rAed a 1 at different concentrations in the wells of a 96-cell culture plate in duplicate. A mixture of 100 μl of the basophil solution with PBS served as maximum for basic histamine release. The plate was incubated at 37°C for 1 h and the supernatants were collected. The level of histamine in the supernatants was determined by using an enzyme immunoassay kit (Immunotech, France). The results are expressed as a percentage of total histamine content. Spontaneous histamine release from the cells was less than 3% and was subtracted from the calculated histamine release. The sensitivity of this assay was 0.2 nM.

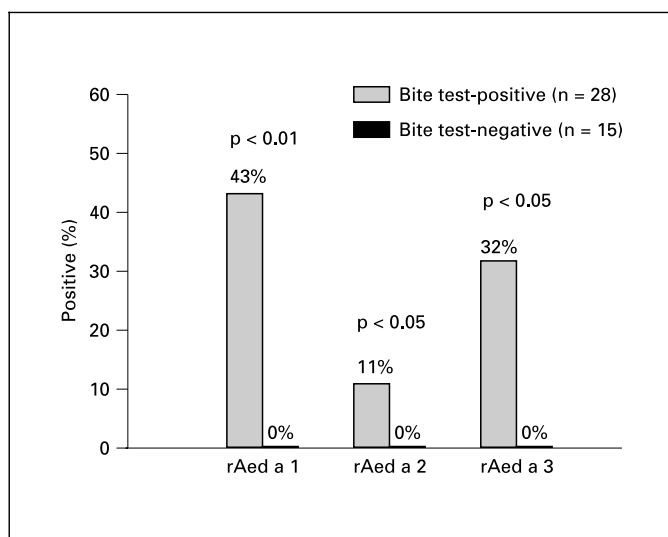


Fig. 8. Skin prick tests with rAed a 1, rAed a 2 and rAed a 3 in mosquito bite test-positive (immediate wheal >0.3 cm²) and negative healthy volunteers [63, 67]. Immediate reactions were read at 20 min after the test.

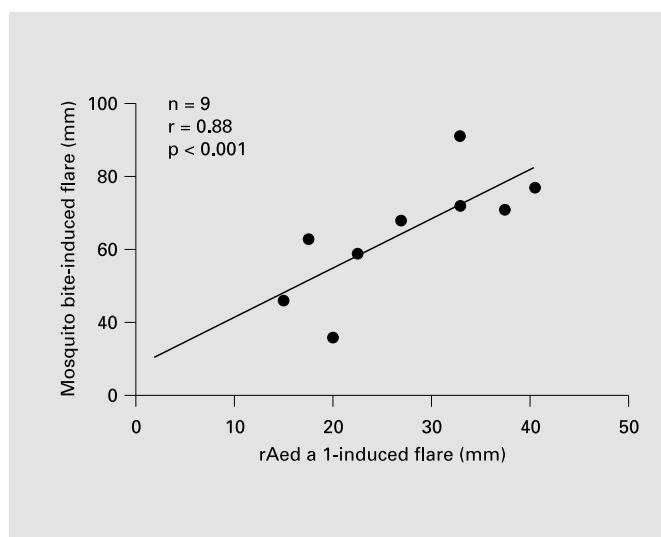


Fig. 9. Correlation between mosquito bite-induced and rAed a 1-induced skin immediate reactions [67].

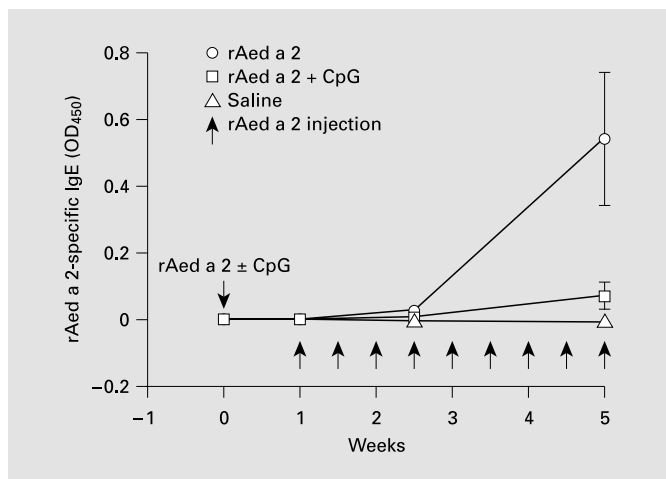


Fig. 10. The effect of vaccination with rAed a 2 and CpG ODN on the prevention of subsequent rAed a 2-induced IgE responses [85]. BALB/c mice were injected intradermally with rAed a 2 or a mixture of rAed a 2 and CpG ODN at week 0. One week later (at week 1), the mice were sensitized with rAed a 2 (i.d.) twice weekly for 9 times as indicated by arrows. Control mice were treated with saline. Serum rAed a 2-specific IgE levels were measured by ELISA.

native Aed a 1 in the saliva and rAed a 1 reacted with the IgE in mosquito-allergic human serum and with a rabbit antibody against rAed a 1 [67]. Since these recombinant allergens are expressed using a baculovirus/insect cell system, they have not only identical immunogenicity to their natural forms present in the saliva, but also have similar biological activities as shown by eliciting both skin immediate and delayed reactions and histamine release, inhibiting platelet aggregation (rAed a 1) [81], and digesting sugar (rAed a 4) [68].

Clinical Relevance

To investigate the clinical relevance of the recombinant allergens, skin prick tests with rAed a 1, rAed a 2, and rAed a 3 as well as mosquito bite tests were performed in healthy volunteers with skin reactivity ranging from none to strong skin reactions to mosquito bites [63, 67]. In all mosquito bite tests, laboratory-reared, pathogen-free mosquitoes were used. Using the mean of the mosquito bite test-negative individuals plus 2 SD as cutoff levels, 29–43% of mosquito bite test-positive individuals had a positive skin test to rAed a 1, 10% to rAed a 2, and 32% to rAed a 3, and none of the individuals with negative bite tests had a positive skin test to any of the three recombinant allergens [63, 67]. After normalization among the studies, the positivity rates of the 3 recombi-

nant allergens are shown in figure 8, in which rAed a 1 is shown to be the strongest allergen. Furthermore, sensitive and specific indirect ELISAs using the recombinant allergens are being developed to measure serum allergen-specific IgE levels. In the ELISAs, the recombinant salivary allergens are coated on microplates. rAed a 4-specific IgE levels were measured using the ELISA on 13 mosquito-allergic individuals. Mosquito bite test-negative individuals served as controls. Forty-seven percent of the mosquito-allergic individuals had a positive rAed a 4-specific IgE level, and none of the negative controls had a positive IgE (fig. 6) [68]. In a recent study, serum rAed a 1-, rAed a 2-, rAed a 3-, and *Ae. aegypti* saliva-specific IgE and IgG levels were measured in 26 mosquito-allergic individuals and 20 negative controls. The positivity rates of specific IgE were 65% for rAed a 1, 32% for rAed a 2 and rAed a 3, and 81% for saliva in the allergic group, and 0–10% in the negative controls ($p < 0.05$) [82]. The positivity rates of rAed a 1-, rAed a 3-, and saliva-specific IgG were also significantly increased in the allergic group in comparison with the controls ($p < 0.05$) [82]. These results indicate that sensitive and specific in vitro immunoassays can be developed by using recombinant mosquito salivary allergens. ELISAs with a cocktail of recombinant mosquito salivary allergens are being developed to further improve the sensitivity of the diagnosis of mosquito allergy.

In vivo Studies in Mice

The allergenicity in vivo of rAed a 2 protein has also been investigated in mice [83]. rAed a 2 induced a significant increase in IgE and IgG1 (Th2-driving antibodies), but not IgG2a (Th1-driving antibody) in BALB/c and C57BL/6 mice. In all sensitized mice, a positive immediate skin reaction was apparent. In immunoblot analysis, the mouse IgE antibodies elicited by rAed a 2 bound to not only rAed a 2 but also to naturally occurring Aed a 2 in mosquito saliva.

In a mouse model of mosquito allergy, using a new immunostimulatory-sequence DNA, oligodeoxynucleotides containing CpG motifs (CpG ODN), as an adjuvant, recombinant mosquito salivary allergens have successfully prevented subsequent antigen-specific IgE production. In this study, mice were sensitized by subcutaneous injections with rAed a 2 allergen twice weekly. Administration of rAed a 2 along with CpG ODN once 1 week before the commencement of sensitization, significantly inhibited subsequent rAed a 2-specific IgE production (fig. 10) [84, 85], which further suggests the possibility of preventing mosquito saliva-specific IgE-mediated allergic reactions by administering recombinant mosquito salivary aller-

gens along with CpG ODN to individuals with severe reactions to mosquito bites.

Cross-Reactivity

As discussed previously, cross-reactivity exists among the saliva allergens of different mosquito species [22, 62]. This enhances diagnostic sensitivity in mosquito allergy and suggests that immunotherapy in patients with systemic reactions to mosquito bites using the saliva allergen from one species may protect them against reactions triggered by another species.

In immunoblot analysis with a rabbit anti-rAed a 1 antibody, a 68-kD protein was found in 3 of the 4 species studied, namely *Ae. aegypti*, *Ae. vexans*, and *Ae. albopictus*, while using a rabbit anti-rAed a 2, a 37-kD protein was found in all 4 species studied (fig. 5) [47]. In another

study, using the same method with a rabbit anti-rAed a 2, a 37-kD protein was found in 7 of the 11 mosquito species studied (*Ochlerotatus triseriatus*, *Cx. quinquefasciatus*, and all 5 *Aedes* species – *Ae. communis*, *Ae. aegypti*, *Ae. vexans*, *Ae. albopictus*, *Ae. togoi*), but not in the remaining 4 species (*Cx. pipiens*, *Cx. tarsalis*, *An. sinensis* and *Cs. inornata*) (unpubl. data). These data indicate that the polypeptides in other mosquito species, especially in *Aedes* genus, share common antigenic structures with Aed a 1 and Aed a 2 of *Ae. aegypti*. Aed a 4, α -glucosidase, may also be a cross-reactive allergen, since α -glucosidase was found in other mosquito species [86]. Cross-reactivity of the recombinant salivary allergens is supported by studies performed in our laboratory in which all 4 recombinant allergens bind to the IgE of mosquito-allergic subjects living in a region where *Ae. aegypti* is not found [63–68].

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