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Mosquito Allergy and Mosquito Salivary Allergens

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Abstract: Allergic reactions to mosquito bites are caused by allergens in mosquito saliva. In this review, allergic reactions to mosquito salivary allergens, and characteristics of salivary allergens and their recombinant forms are described. The use of the recombinant allergens in the diagnosis of mosquito allergy is discussed.

Keywords: Mosquito allergy, recombinant allergens, antigens, salivary proteins.

INTRODUCTION

Around the world, allergic reactions to mosquito bites are a clinical concern, and decrease the quality of life for many people [1, 2]. Severe local reactions such as "Skeeter Syndrome"[3] and systemic reactions including generalized urticaria and anaphylaxis have been reported [4, 5]. Due to the lack of availability of mosquito salivary preparations, which are required for accurate diagnosis and immunotherapy, these reactions are under-recognized and allergen immunotherapy is seldom used for systemic reactions to mosquito bites. For the same reason, the immune mechanisms associated with the development of allergic reactions and subsequent natural desensitisation 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 to 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, specific allergen immunotherapy. In this review, we describe clinical manifestations in mosquito allergy, the immune mechanisms involved, and the current status of natural and recombinant mosquito salivary allergens.

CLINICAL MANIFESTATIONS

Typical local cutaneous reactions to mosquito bites consist of small immediate wheals and flares peaking at 20 minutes and delayed pruritic indurated papules peaking at 24 - 36 hours, then diminishing over several days or weeks [6, 7]. Large local reactions to mosquito bites consist of itchy, red, warm swellings appearing with minutes of the bites, and itchy papules, ecchymotic, vesiculated, blistering, bullous or even Authus reactions, appearing 2 - 6 hours after the bites and persisting for days or weeks [3, 8-12]. Only those reactions which cause large or atypical (ecchymotic or vesiculated) local reactions or systemic reactions are considered to be "mosquito allergy"[2]. "Skeeter Syndrome" is defined as

a mosquito-induced large local inflammatory reaction sometimes accompanied by low grade fever that is often misdiagnosed as cellulitis. Systemic reactions following mosquito bites includes angioedema, generalized urticaria, nausea, vomiting, wheezing and other anaphylaxis manifestations [4, 5, 13-15].

Individuals at increased risk of severe reactions to mosquito bites include those with: a) a high level of exposure, for example, outdoor workers [4], b) weak natural immunity, for example, infants and young children [3, 16], c) no previous exposure to indigenous mosquitoes, for example, immigrants or visitors, and d) primary or secondary immune deficiency such as AIDS, malignancies [17] or Epstein-Barr virus-associated lymphoproliferative diseases [18].

DEVELOPMENT OF SENSITIZATION AND SUBSEQUENT DESENSITIZATION

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 [7, 19]. 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 [6].

Based on clinical observations, the natural history of sensitization and subsequent desensitization to the saliva injected by mosquito bites has been classified into five stages [19, 20]. Individuals who have never been exposed to a specific species of mosquito, do not get a reaction from the initial bites (stage I), but following subsequent bites, delayed cutaneous lesions appear (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 the immediate reactions (stage V). These 5 stages have also been documented in a prospective study, in which stages II and IV appeared to be transient [7]. In this study, every two 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

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responses were monitored prospectively. The initial bites caused no skin reactions. Delayed and immediate skin reactions developed at week 3, peaked at weeks 5 to 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. 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 [1,2,16]. Based on the above observations, in individuals with systemic reactions to mosquito bites, immunotherapy with injections of gradually increasing doses of mosquito saliva proteins would be expected to be useful in preventing subsequent reactions to mosquito bites, analogous to the use of venom immunotherapy in the treatment of systemic reactions to bee, wasp, hornet or yellow jacket stings. Indeed, immunotherapy using injections of mosquito whole body extracts has been reported to prevent allergic reactions to subsequent bites [4, 10, 21]. However, because currently commercially available mosquito whole body extracts contain few mosquito saliva proteins and many non-salivary proteins which are ineffective in downregulating the specific immune responses to mosquito salivary allergens and may cause additional sensitization [22], 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 either natural desensitization, or large local reactions or systemic allergic reactions. If the main ducts of the mosquito salivary glands are transected, the mosquito can still feed, but it is unable to cause skin reactions in humans [23].

Mosquito saliva contains many biological materials which can be classified according to their functions, as follows: (a) anticlotting and antiplatelet factors and vasodilators which presumably increase the speed at which blood from the host is imbibed [24, 25]; (b) substances that affect parasite transmission by arthropod vectors [26-36]; (c) enzymes which are associated with sugar feeding [27, 28]; (d) lysozyme, which may help to control bacterial growth in the sugar meal stored in the mosquito crop [29]; and (e) immunomodulators [30, 31]. Most of these substances in mosquito saliva have been identified as allergens [32-34].

There are more than 40 mosquito genera and more than 3,000 mosquito species worldwide. *Aedes aegypti* saliva contains at least 31 proteins, the cDNA sequences of which have been deposited into the Genbank [35]. However, protein visualization techniques using gel electrophoresis have only revealed about 20 polypeptides in the saliva of adult *Aedes aegypti* [32, 36] 9 polypeptides in *Armigeres subalbatus* [37]. These include α -amylase, anticoagulants, anti-tumor necrosis factor, apyrase, esterase, D7, α -glucosidase, and sialokinins [38]. Table 1 summarizes major salivary proteins in *Aedes aegypti*. In addition to the thirty-one salivary cDNAs from *Aedes aegypti* [35], thirty-three full-length salivary cDNA sequences have been reported from a full-length cDNA library of *Anopheles gambiae* [39] and twenty-one

cDNAs from *Anopheles gambiae* [40]. Most sequences match known proteins, although some do not [35, 39, 40].

Most of the proteins in mosquito saliva are allergenic in humans [32, 34, 41-45]. Using SDS-PAGE and immunoblot techniques, up to 19 allergens have been found in mosquito salivary gland extracts [41] and at least 8 proteins in the saliva of *Aedes aegypti* have been identified as allergens which bind to the IgE of individuals who have large local allergic reactions to mosquito bites [32]. Using immunoblot analyses with a pooled serum from individuals allergic to mosquito bites, allergens in the saliva or salivary gland extracts from 10 worldwide-distributed mosquito species have been studied as shown in Fig. (1). Three to sixteen salivary allergens are found in each species, with molecular masses ranging from 16 to 95 kDa. Both species-shared and species-specific allergens exist. Among the 10 species studied, *Aedes albopictus* saliva contains at least 16 allergens, *Aedes vexans* contains 12 allergens, and *Aedes aegypti* and *Culex quinquefasciatus* contain 8 - 9 allergens [32] (Fig. 1). In a recent study, serum saliva-specific IgE to 5 mosquito species were measured in 14 individuals who had systemic reactions to mosquito bites [5]. Eleven of these individuals had a positive IgE response to *Aedes albopictus* saliva, which contains the highest number of allergens in the 10 species previously studied [32], while 5 - 8 individuals had a positive IgE response to saliva from each of the remaining 4 species (*Aedes vexans*, *Aedes aegypti*, *Culex quinquefasciatus* and *Anopheles sinornata*), suggesting that *Aedes albopictus* has the highest number of species-shared allergens. IgE antibodies against 35.5-, 32.5-, and 22.5-kDa allergens in *Culex quinquefasciatus* saliva were found in 22 (41%), 15(28%), and 13(24%) of 54 individuals with a history of mosquito bite allergy, respectively. The 22.5 kDa allergen was found mainly in children [46]. In addition, mosquito salivary allergens may cross-react with allergens from other biting insects and from crustaceans [14, 47-49]. Moreover, there are rare reports of cross-reactivity with allergens in Hymenoptera venom [15].

Currently, commercially available mosquito allergen preparations are made from whole body extracts which contain little salivary protein and many non-salivary proteins that may cause new sensitization and other side effects [22]. 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 [50]. The mosquito saliva-capture ELISA was more sensitive and specific (less likely to give false negative and false positive results) than the UniCap test (Pharmacia and Upjohn Diagnostics) 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 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

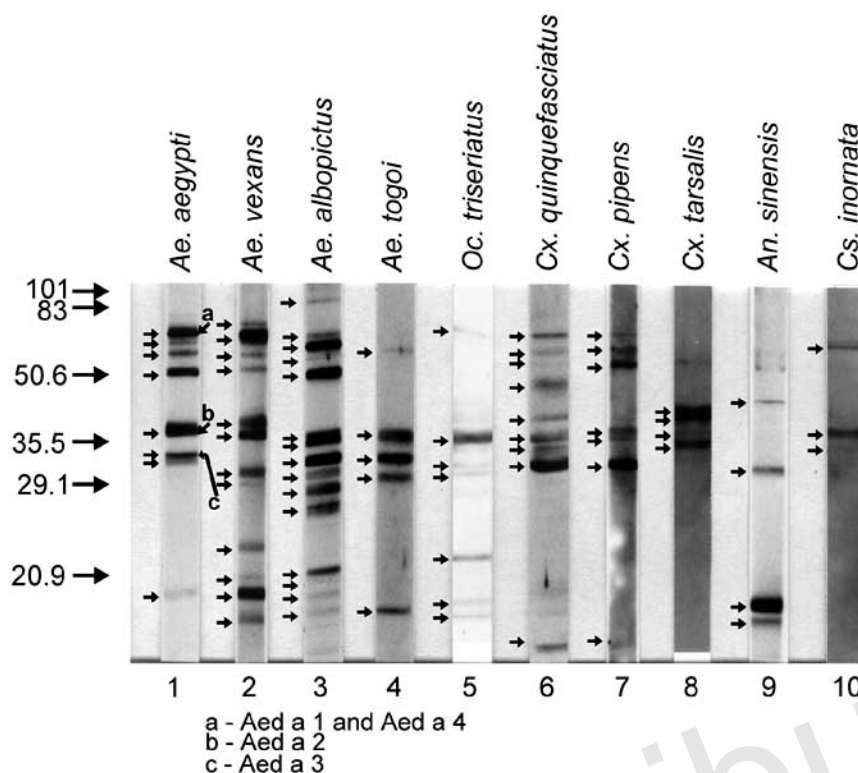


Figure 1. Salivary allergens of 10 mosquito species [32]. Proteins in saliva or salivary gland extracts of 10 mosquito species were separated by SDS-PAGE and then transferred to nitrocellulose membranes. The membranes were immunoblotted with a serum pooled from mosquito-allergic individuals and then incubated with monoclonal anti-human IgE antibodies.

mosquitoes or by direct collection from living female mosquitoes [32, 51]. In the latter process, a 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 *Aedes 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 investigation of the mechanisms involved, and will eventually facilitate diagnosis and treatment of mosquito allergy with standardized, effective, and safe allergens [52, 53].

Aedes aegypti is the most important mosquito pest in the world, and shares many salivary allergens with other species including *Culex quinquefasciatus* and *Aedes vexans*, another common pest mosquito indigenous to North America [32, 54]. To date, four recombinant salivary proteins of *Aedes aegypti*, a 68 kDa apyrase, a 37 kDa D7 protein, a 30 kDa Aed a 3, and a 67 kDa α -glucosidase, have been expressed and identified as allergens [55-59] (Table 1). According to current allergen nomenclature [60] 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 tri-phosphates released from injured cells or by activated platelets [61, 62]. Aed a 2, the D7 protein, is the original protein that defined a large family of D7-related proteins [56, 63]. 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 hematophagous insects [64]. Therefore, it is not surprising that rAed a 2 cross-reacts with saliva or salivary gland extracts from many other mosquito species [32] (Fig. 2). The biological function of Aed a 3 is currently unknown, because the cDNA coding Aed a 3 was directly cloned from the *Aedes aegypti* salivary gland cDNA library using mouse anti-*Aedes aegypti* saliva serum [65]. Aed a 4, α -glucosidase, is a soluble glycoprotein that is secreted when mosquitoes ingest sugar and blood meals [27, 66].

Allergenicity and Biological Activity

These four recombinant allergens have been expressed using a baculovirus/insect cell system, and purified on a large scale [55-57, 59, 65]. The baculovirus/insect cell system has been used extensively for the production of large amounts of recombinant proteins with biological activity [56, 67, 68]. The ability of the insect cells to perform many of the post-translational 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 [67, 69]. In contrast, proteins

Table 1. *Aedes aegypti* Salivary Proteins [1,2]

Protein Name	Allergen Name	Molecular Weight	cDNA Sequenced	Biological Functions	References
α -amylase 1	-	81.5 kDa	yes	unknown	[28]
apyrase	Aed a 1	68 kDa	yes	antiplatelet	[55, 56, 61, 79]
α -glucosidase (mal-tase-like 1)	Aed a 4	67 kDa	yes	sugar digestion	[27, 59, 66]
Esterase	-	65 kDa	-	unknown	[80]
Anticoagulant-factor Xa	-	54 kDa	yes	anticoagulant	[81]
Aed a X ₁	Aed a X ₁ ?	44 kDa	-	unknown	[82]
Aed a X ₂	Aed a X ₂ ?	37 kDa	-	unknown	[82]
Female-specific protein, D7	Aed a 2	37 kDa	yes	unknown	[38, 56]
-	Aed a 3	30 kDa	yes	unknown	[65]
Sialokinins	-	1.4 kDa	yes	vasodilator	[83-85]
Anti-tumour necrosis factor	-	unknown	-	anti-tumour necrosis factor	[86]
Lysozyme	-	unknown	-	bacteriolysis	[29]

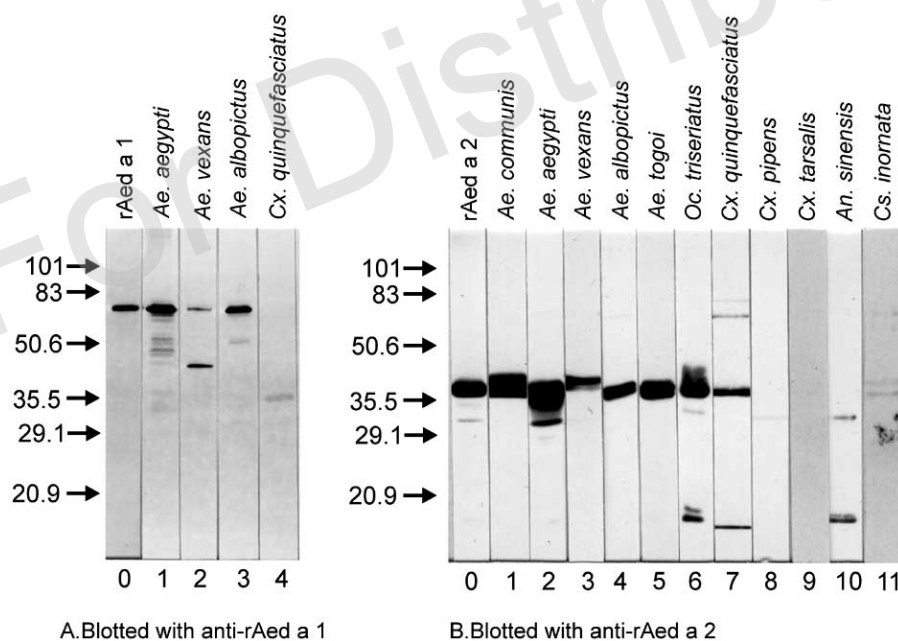


Figure 2. Examination of the presence of Aed a 1 (apyrase) and Aed a 2 (the D7 protein) in the salivary preparations of 4 mosquito species [32, 56]. Proteins in the saliva or salivary gland preparations were separated by SDS-PAGE and then transferred to nitrocellulose membranes. The membranes were probed for the presence of Aed a 1 or Aed a 2 using rabbit anti-rAed a 1 or -rAed a 2.

expressed using prokaryotic expression systems such as *E. coli* have no carbohydrates and are generally used only in *in vitro* assays [70-72]. 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% to 30% of the activity of the natural enzyme [67].

The recombinant allergens have been demonstrated to bind to serum IgE as measured by using ELISAs and Western blotting [55, 57, 59, 65, 73] and to induce histamine release from basophils (previously unpublished data) in individuals with allergic reactions to mosquito bites. The recombinant allergens also induce skin immediate and delayed reactions in mosquito-bite test positive individuals [55, 58, 73] (Fig. 3). Furthermore, the binding of IgE to the recom-

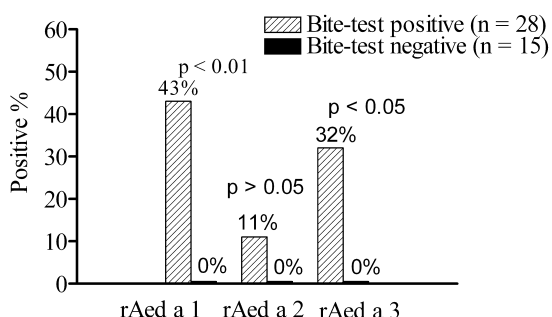


Figure 3. Skin prick tests with recombinant Aed a 1, Aed a 2 and Aed a 3 in mosquito-bite test positive (immediate wheal > 0.3 cm²) and negative healthy volunteers [55, 58, 73]. Immediate reactions were read at 20 minutes after the test.

binant 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 [55] (Fig. 4). In the immunoblot analysis, both native Aed a 1 in the saliva and recombinant Aed a 1 reacted with the IgE in mosquito-allergic human serum, and with a rabbit antibody against rAed a 1 [55]. Since these recombinant allergens are expressed using a baculovirus/insect cell system, they not only have 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 (Fig. 3), histamine release from basophils, and other biological functions. For example, rAed a 1 (apyrase) is also able to inhibit ADP-, collagen- and thrombin-induced human platelet aggregation in a dose-dependent manner, indicating that the recombinant apyrase retaining nucleotidase activity in a whole cell system (Fig. 5) [62]; rAed a 4 (α -glucosidase) is able to digest sugar in the functional hydrolase assay [59].

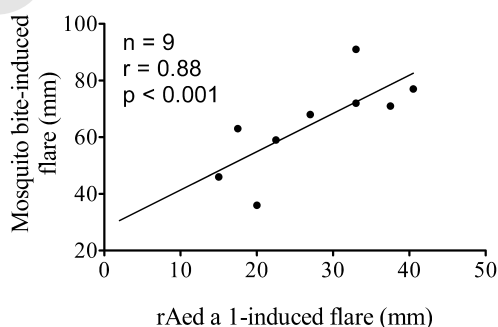


Figure 4. Correlation between mosquito bite-induced and rAed a 1-induced skin immediate reactions [55].

Clinical Diagnosis

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

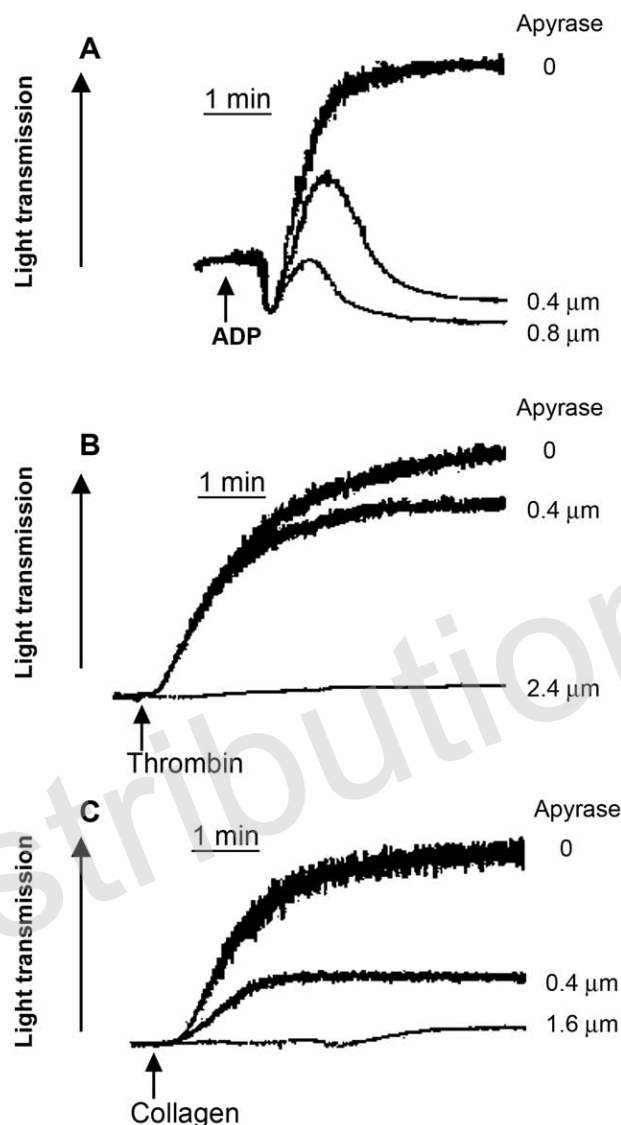


Figure 5. The effect of recombinant apyrase on inhibition of human platelet aggregation [62]. Washed human platelets pre-incubated with various concentrations, 0, 0.4, 0.8, 1.6 and 2.4 μ M, of recombinant apyrase before addition of a) 30 μ M of ADP, b) 0.1 units/mL of thrombin or c) 1.8 μ g/mL of collagen. Platelet aggregation was monitored continuously as described in the "Methods".

volunteers with skin reactivity ranging from none to strong local reactions to mosquito bites [55, 58, 73]. 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 a cut-off 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 these three recombinant allergens [55, 58, 73]. After normalization among the studies, the positivity rates of these three recombinant allergens are shown in Fig. (3), in which rAed a 1 is shown to be the strongest allergen. Moreover, sensitive and specific indirect enzyme-linked immunosorbent assays (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. 47 % of the mosquito-allergic individuals had a positive rAed a 4-specific IgE level, and none of the negative controls had a positive IgE [59]. In a recent study, serum rAed a 1-, rAed a 2-, rAed a 3-, and *Aedes 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 's < 0.05) [74]. The positivity rates of rAed a 1-, rAed a 3-, and saliva-specific IgG were also significantly increased in the allergic individuals in comparison with the controls (p 's < 0.05) [75]. 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 proteins has also been also investigated in mice [76]. 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 not only to 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, 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, the mice were sensitized with rAed a 2 intradermally twice weekly for 9 times. Control mice were treated with saline.

The results showed that administration of rAed a 2 along with CpG ODN on one occasion one week before the commencement of sensitization, significantly inhibited subsequent rAed a 2-specific IgE production [77].

This suggests the possibility of preventing mosquito saliva-specific IgE mediated allergic reactions by administration of recombinant mosquito salivary allergens along with CpG ODN to individuals with severe reactions to mosquito bites.

Cross-Reactivity

As discussed previously, cross-reactivity exists among the salivary allergens of different mosquito species [32, 54]. This enhances diagnostic sensitivity in mosquito allergy and suggests that immunotherapy in patients with systemic reactions to mosquito bites using the salivary allergen from one mosquito species may protect them against reactions triggered by another species.

In immunoblot analysis with a rabbit anti-rAed a 1 antibody, a 68 kDa protein was found in three of the four species studied, namely *Aedes aegypti*, *Aedes vexans*, and *Aedes albopictus*, while using a rabbit anti-rAed a 2, a 37 kDa protein was found in all *Aedes* species studied, *Ochlerotatus triseriatus* and *Culex quinquefasciatus* (Fig. 2) [32, 56]. In another study, using the same method with a rabbit anti-rAed a 2, a 37 kDa protein was found in seven of the eleven mosquito species studied (*Ochlerotatus triseriatus*, *Culex quinquefasciatus*, and all five *Aedes* species- *Aedes communis*, *Aedes aegypti*, *Aedes vexans*, *Aedes albopictus*, *Aedes togoi*), but not in the remaining four species (*Culex pipiens*, *Culex tarsalis*, *Anopheles sinensis* and *Culiseta inornata*) (unpublished data). These data indicate that the polypeptides in other mosquito species, especially in the *Aedes* genus, share common antigenic structures with Aed a 1 and Aed a 2 of *Aedes aegypti*. Aed a 4, α -glucosidase, may also be a cross-reactive allergen, since α -glucosidase was found in other mosquito species [78]. Cross-reactivity of 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 Manitoba, Canada, a region where *Aedes aegypti* is not found [55, 57-59, 65, 73].

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