

Expression, purification, characterization and clinical relevance of rAed a 1—a 68-kDa recombinant mosquito *Aedes aegypti* salivary allergen

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Abstract

Accurate diagnosis of mosquito allergy has been precluded by the difficulty of obtaining salivary allergens. In this study, we expressed, purified, characterized and investigated the clinical relevance of a recombinant *Aedes aegypti* salivary allergen, rAed a 1. Two cDNA segments were ligated together to form the full-length Aed a 1 gene. rAed a 1 was expressed using a baculovirus/insect cell system, and purified using a combination of anion-exchange and gel-filtration chromatography. The purified rAed a 1 bound to human IgE, as detected by ELISA, ELISA inhibition tests and immunoblot analyses. Epicutaneous tests with rAed a 1 and a commercial whole-body *Ae. aegypti* extract, and *Ae. aegypti* bite tests were performed in 48 subjects. Nine of 31 (29%) of the subjects with positive immediate bite tests also had a positive rAed a 1 immediate skin reaction and 32% had a positive immediate test to the commercial extract. Six of 33 (18%) of the subjects with positive delayed bite tests also had a positive rAed a 1 delayed skin reaction and 6% had a positive delayed test to the commercial extract. Furthermore, rAed a 1-induced flare sizes significantly correlated with mosquito bite-induced flare sizes. None of the subjects with negative bite tests had a positive skin test to rAed a 1 or to commercial extract. We conclude that the rAed a 1 has identical antigenicity and biological activity to native Aed a 1, can be used in the *in vitro* and *in vivo* diagnosis of mosquito allergy, and is more sensitive than mosquito whole-body extract for detecting delayed skin reactions.

Introduction

Allergic reactions to mosquito bites, an increasing clinical concern, involve both IgE-mediated and lymphocyte-mediated hypersensitivity (1–3). Cutaneous reactions, including large local reactions, are common and systemic reactions including angioedema, generalized urticaria, asthma or even anaphylactic shock have also been reported (1–8). These reactions are caused by the proteins in the mosquito saliva (9).

Purification of each salivary protein is required for accurate diagnosis of mosquito allergy. Obtaining a sufficient amount of highly purified saliva proteins for clinical use is almost impossible, as collection of mosquito saliva is extremely time consuming and labor intensive. Although mosquito whole-

body extracts are available from different commercial sources, these unstandardized products vary greatly in both quality and biological activity, with some lacking salivary proteins entirely (10). Moreover, all whole-body extracts contain extraneous proteins that are not present in mosquito saliva (10) and may cause adverse effects.

In the past 10 years, recombinant DNA technologies have provided a large number of cDNAs coding for allergens and have yielded many important recombinant allergens. These recombinant allergens are beginning to play a major role in the diagnosis of allergies and will also likely be used in immunotherapy (11–19). Utilization of molecular biological

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techniques to produce pure mosquito salivary allergens will greatly enhance our understanding of the mechanisms involved and facilitate diagnosis of mosquito allergy.

Aedes aegypti is the most important and widely distributed pest mosquito species in the world. The saliva of adult female *Ae. aegypti* contains up to 20 proteins (20). Immunological studies using SDS-PAGE and immunoblotting techniques have revealed that at least eight of these salivary proteins are allergens that bind to the IgE of subjects with severe skin reactions to mosquito bites (21). To date, the cDNAs encoding for seven salivary proteins of *Ae. aegypti* have been cloned and sequenced (22–28). One of these proteins is a 68-kDa apyrase, an ATP diphosphohydrolase, that functions as a platelet anti-aggregator by destroying adenosine di- and tri-phosphates released from injured cells or by activated platelets (29). In accordance with allergen nomenclature (30), we have named the 68-kDa apyrase Aed a 1. In our previous experiments, 11 of 12 mosquito-allergic subjects had Aed a 1-specific IgE as detected by immunoblotting (21), indicating that this protein elicits predominant IgE responses in mosquito-allergic subjects and is therefore a major salivary allergen of *A. aegypti*. Aed a 1 is also a species-shared allergen among world-wide distributed mosquito species *Aedes vexans*, *Aedes albopictus* and *Culex quinquefasciatus* (21).

We have previously expressed and identified the major portion of this allergen, Aed a 1 3' (54 kDa, containing 150–562 amino acid residues) using a baculovirus/insect cell system (31). Although rAed a 1 3' binds to IgE and IgG in mosquito-allergic sera, its antigenicity and biological activity are weak. In this study, we ligated two cDNA segments to form the full-length Aed a 1 gene and expressed the intact protein using the baculovirus/insect cell system. Following purification, we analyzed the physical, immunological and biological properties of the full-length rAed a 1, and investigated its clinical relevance in 48 subjects with and without reactions to mosquito bites.

Methods

Materials

A 1.2-kb 3' end cDNA clone λ SGG12 and a 0.47-kb 5' end cDNA clone λ SGG12-5, encoding the *Ae. aegypti* salivary protein Aed a 1, and rabbit anti-Aed a 1 were prepared as described previously (23,32). Restriction enzymes and T4 DNA ligase were purchased from Promega (Madison, WI). DNA sequence kits were obtained from US Biochemical (Cleveland, OH). PCR kits and the pBluescriptII SK[−] plasmid were purchased from Boehringer Mannheim (Laval, Quebec, Canada) and Stratagene (La Jolla, CA) respectively. pBlueBacHis C expression vector, *S. frugiperda* (Sf9) and High-Five insect cell lines, and Grace's insect media were obtained from Invitrogen (San Diego, CA).

Alkaline phosphatase-conjugated goat anti-rabbit IgG and peroxidase-conjugated goat anti-mouse IgG were purchased from Jackson ImmunoResearch (West Grove, PA) and Calbiochem-Novabiochem (La Jolla, CA) respectively. Monoclonal anti-human IgE (clone 7.12) was provided by Dr Andrew Saxon (University of California, Los Angeles). All

the chemicals were purchased from Sigma (St Louis, MO) or BioRad (Richmond, CA), unless otherwise specified.

Ae. aegypti saliva (0.25mg/ml) and salivary gland extracts (0.22 mg/ml) were prepared as described previously (2,10). The commercial mosquito whole-body extract was purchased from Hollister-Stier (Miles Canada, Etobicoke, Ontario, Canada).

Participants

This project was approved by the University Faculty Committee for the Use of Human Subjects in Research and participants gave written, informed consent before entering the study. Forty-eight healthy adults with various skin reactions to mosquito bites were selected from people who responded to advertisements during the summer of 1996. In order to ensure that a full spectrum of reactions was represented, we included specifically some participants who were highly sensitive to mosquito bites and some who were not reactive to the bites. None of the participants had a history of systemic reactions from mosquito bites nor had any of them taken astemizole for 6 weeks or any other antihistamine for 1 week prior to the skin tests.

Plasmid construction and generation of recombinant baculovirus

The full-length Aed a 1 cDNA was constructed from two cDNA segments and then cloned into the baculovirus expression vector pBlueBacHis C as shown in Fig. 1. In brief, the Aed a 1 3' cDNA fragment (1.2 kb) excised from the λ SGG12 clone was cloned into the *EcoRI* site of pBluescriptII SK[−]. Clones carrying the insert with *BamHI* at the 5' end and *HindIII* at the 3' end were selected and identified by restriction enzyme analysis of the unique *Csp451* site at the 3' end. The *EcoRI* site at the 3' end of the resultant plasmid was deleted by digestion, fill-in and re-ligation. Briefly, pBluescriptII SK[−]/Aed a 1 3' was partially digested with *EcoRI*. The largest 4.1-kb fragment was collected and filled in by Klenow polymerase, and ligated by T4 DNA ligase. Clones with an unique *EcoRI* site at the 5' end were screened by *HindIII* and *BamHI* dual-digestion analysis.

The Aed a 1 5' cDNA fragment (0.47 kb) cleaved from the λ SGG12-5 clone was inserted into the *EcoRI* site of the pBluescriptII SK[−]/Aed a 1 3', resulting in the ligation of the two cDNA segments. The full-length Aed a 1 cDNA clones were selected by analysis of the unique *NarI* site within the 5' fragment and confirmed by DNA sequence analysis. The *BamHI*–*HindIII* fragment derived from pBluescriptII SK[−]/Aed a 1 was cloned into the *BamHI*–*HindIII* site of pBlueBacHis C. The recombinant plasmids were identified by restriction enzyme analysis and DNA sequence analysis, purified by CsCl gradient centrifugation and used for transfection experiments.

Recombinant baculoviruses carrying the pBlueBacHis C/Aed a 1 were generated by co-transfection of Sf9 cells with the recombinant transfer vector together with linearized baculovirus AcMNPV DNA using the cationic liposome method and pure recombinant viral stocks were prepared as described previously (31).

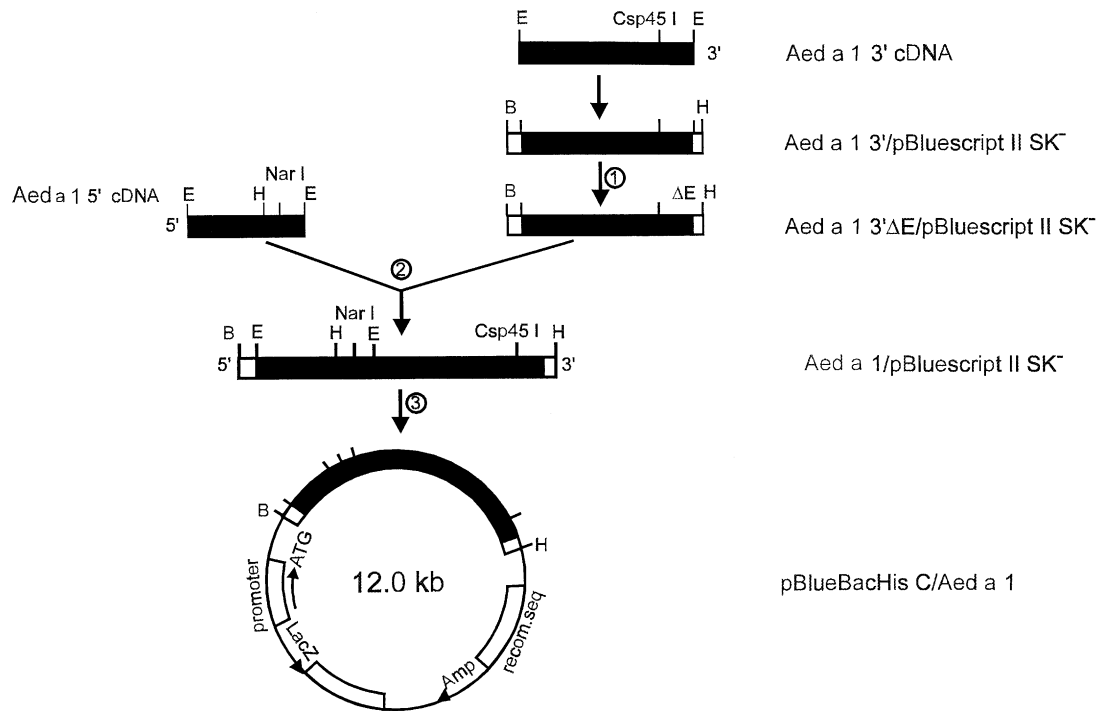


Fig. 1. Construction of the BlueBacHis C/Aed a 1 expression vector. Two DNA fragments coding for the 3' and 5' ends of rAed a 1 were excised from clones selected from a mosquito salivary gland cDNA library as described in Methods. The 3' end fragment was then inserted into another vector, pBluescriptII SK⁻ (step 1). The 5' fragment was then ligated to the pBluescriptII SK⁻/Aed a 1 3' vector in order to form the full-length cDNA coding for Aed a 1 (step 2). Finally, the vector pBluescript II SK⁻ plasmid containing the full-length Aed a 1 cDNA was inserted into pBlueBacHis C (step 3) for infection of Sf9 or High-Five cells.

Expression

To increase the yield of expressed products, Sf9 cells were replaced by High-Five cells that were grown in Ex-cell 405 serum-free medium in spinner culture maintained at 27°C and 100 r.p.m. High-Five cells were infected with each chosen virus clone at a multiplicity of infection (MOI) of 10. After 48 h of infection both cell lysates and culture media were tested for rAed a 1 expression by immunoblotting.

To assess the time course of rAed a 1 expression, a 50-ml suspension culture of High-Five cells at 1.0×10^6 cells/ml in a 125-ml spinner flask was infected with recombinant virus at an MOI of 10. At various times after infection, 1 ml aliquots of the suspension culture were collected and centrifuged at 13,000 *g* for 10 min, and the supernatants were assayed for rAed a 1 by immunoblotting. For the production of rAed a 1, 200 ml of suspension culture was infected with Aed a 1 recombinant baculovirus in a 500-ml spinner flask at an MOI of 10. The culture was maintained at 27°C, and after 96 h the medium was harvested by centrifuging at 2000 *g* for 20 min and 20,000 *g* for 20 min. The supernatant was stored at -70°C until purification could take place.

Purification and determination of isoelectric point

Purification of the rAed a 1 was performed by a combination of ion exchange and gel filtration. After concentrating and dialyzing against Tris buffer (10 mM Tris and 0.1 mM EDTA, pH 7.4), the culture supernatant was loaded onto a DEAE-Sephacel column, and the column was sequentially washed

with Tris buffers containing 0.1, 0.2 and 0.4 M NaCl. Fractions were monitored for protein concentration at OD₂₈₀ using a Uvicord S spectrophotometer (LKB, Bromma, Sweden), and for rAed a 1 content by ELISA and SDS-PAGE and silver stain. In the ELISA, microplates coated with 1:10 diluted fractions were incubated with rabbit anti-Aed a 1, followed by incubations with alkaline phosphatase-conjugated goat anti-rabbit IgG and then substrate as described previously (33). The effluent, in which rAed a 1 was identified, was concentrated using a Centriprep-30 concentrator (Amicon) and subsequently loaded onto a Sephacryl S-100HR column previously equilibrated with 10 mM Tris buffer, pH 7.4. The column was washed with Tris buffer, and fractions were collected, monitored for protein concentration at OD₂₈₀ and assayed for rAed a 1 by ELISA. The fractions containing rAed a 1 were pooled, desalted by passing through a Sephadex G-25 column, lyophilized, reconstituted in a small volume of PBS and stored at -70°C.

The isoelectric point of purified rAed a 1 was determined using a model III mini-isoelectric focussing cell (BioRad) according to the manufacturer's instruction.

Immunoblot analyses

Immunoblot analyses were performed as described previously (31). Proteins in the samples were separated by 10% SDS-PAGE under reducing conditions and then either stained with silver stain for protein visualization or transferred to nitrocellulose membranes for immunoblotting. The mem-

branes were sequentially incubated with rabbit anti-Aed a 1, then alkaline phosphatase-conjugated goat anti-rabbit IgG and finally the substrate.

To detect the binding of *Ae. aegypti* saliva proteins or rAed a 1 to the IgE in human sera, the membranes were immunoblotted with mosquito-allergic human sera followed by incubations with monoclonal anti-human IgE and then peroxidase-conjugated goat anti-mouse IgG as described previously (31).

ELISA and ELISA inhibition tests

An ELISA was developed to detect rAed a 1-specific IgE in human sera using the technique described previously (33). In brief, microtiter plates were coated with 0.5 µg/well of purified rAed a 1 protein. After blocking the free binding sites, the plates were incubated overnight at 4°C with human sera. The bound serum Aed a 1-specific IgE was detected by sequentially incubation of the plates with goat anti-human IgE, then alkaline phosphatase-conjugated rabbit anti-goat IgG and finally the enzyme substrate solution. Optical absorbance at 410 nm was read using a THERMO-max microplate reader (Molecular Devices, Sunnyvale, CA).

An ELISA inhibition test was performed to determine if the binding of human IgE to rAed a 1 could be inhibited by natural Aed a 1 in the saliva and to determine the specificity of the rAed a 1-specific IgE assay. The mosquito salivary gland extract was diluted 10-fold. Each dilution was mixed with an equal volume of a 1:25 diluted mosquito-allergic serum. Serum without mosquito extract served as a positive control. After incubation at 4°C overnight, the rAed a 1-specific IgE contained in the serum dilutions was measured by ELISA as described above.

Mosquito bite tests

Mosquito bite tests were performed using *Ae. aegypti* as described previously (2). The immediate wheal and flare sizes were read at 20 min and the delayed induration sizes were read 24 h after the bite by measuring the largest and orthogonal diameters. A sum of the two diameters ≥8 mm for immediate wheal and/or ≥30 mm for immediate flare was considered a positive immediate reaction, while a sum of the two diameters ≥8 mm was taken as a positive delayed reaction.

Epicutaneous tests

Epicutaneous tests (prick through drop method) were performed with the following materials: (i) purified rAed a 1 (1 mg/ml) in 50% glycerin/PBS (v/v) with 0.4% phenol (w/v), (ii) a commercial *Ae. aegypti* whole-body extract (3.16 mg/ml) in 50% glycerin, (iii) 1 mg/ml histamine phosphate as a positive control and (iv) 50% glycerin/PBS as a negative control. The immediate wheal and flare responses were read at 20 min, and the delayed induration sizes were read 24 h after the test as described for the mosquito bite tests. After subtracting the area of the wheal or induration induced by the negative control, a sum of the two diameters ≥6 mm for immediate wheal and/or ≥20 mm for immediate flare was considered a positive immediate reaction, while a sum of the two diameters ≥6 mm was taken as a positive delayed reaction.

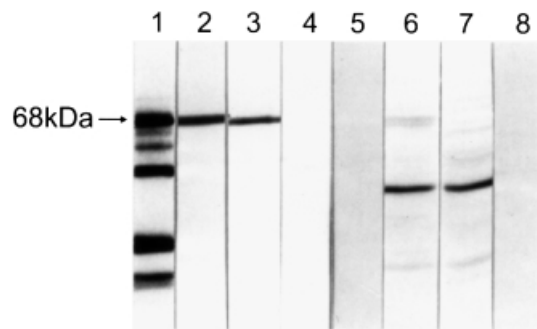


Fig. 2. Immunoblot analysis of expressed rAed a 1. The following materials were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes: *A. aegypti* saliva as a positive control (lane 1), culture medium (lanes 2 and 3) and cell lysates (lane 6) of Sf9 cells infected with recombinant baculovirus, culture medium (lane 4) and cell lysates (lane 7) of Sf9 cells infected with wild-type baculovirus, culture medium (lane 5) and lysates (lane 8) of mock infected Sf9 cells. Lanes 1 and 2 were immunoblotted with mosquito-allergic human serum, and lanes 3–8 were immunoblotted with rabbit anti-Aed a 1 as described in Methods. The 68-kDa native Aed a 1 was only found in the culture medium of Sf9 cells infected with recombinant baculovirus (lanes 2 and 3), not in the cell lysates (lane 6) and other negative control media (lanes 4 and 5) or negative control cell lysates (lanes 7, 8). Both native Aed a 1 in the saliva (lane 1) and rAed a 1 in the medium (lane 2) reacted with the IgE in mosquito-allergic human serum.

Statistical analyses

Analyses of the data were performed using the Number Cruncher Statistical Systems software. The χ^2 -test was used to compare the positive percentages between groups. Linear regressions were used to determine correlations.

Results

Generation of recombinant baculovirus

The 5' end cDNA was ligated with the 3' end fragment forming a full-length Aed a 1 cDNA (Fig. 1) that was confirmed by restriction enzyme analysis and sequence analysis (data not shown). The full-length cDNA clone of Aed a 1 was subsequently inserted into the pBlueBacHis C vector and the resultant vector was co-transfected with wild-type AcMNPV DNA into Sf9 insect cells. Five putative recombinant virus clones were selected, plaque-purified and evaluated by PCR. All five of the clones were found to contain the full-length Aed a 1 cDNA (data not shown).

Expression, identification and time course

Expression of rAed a 1 from the five recombinant baculoviruses in Sf9 cells was confirmed by immunoblot using rabbit anti-Aed a 1. With wild-type virus-infected Sf9 cells and uninfected cells as negative controls, all of the baculovirus clones expressed rAed a 1 in approximately equal amount. The 68-kDa recombinant protein was only found in the culture medium (Fig. 2, lanes 2 and 3) and not in the cell lysates (Fig. 2, lane 6). Also, the 68-kDa rAed a 1 in the culture medium bound to the IgE in mosquito-allergic human serum (Fig. 2, lanes 2 and 3), suggesting that the rAed a 1 is recognized by the IgE antibody induced by mosquito saliva

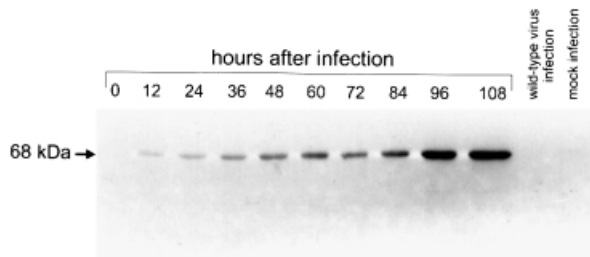


Fig. 3. Time course of expression of rAed a 1. Sf9 cells were infected with baculovirus containing Aed a 1 cDNA at an MOI of 10 and incubated at 27°C. At 12-h intervals after infection, culture media were taken and analyzed for the presence of rAed a 1 by immunoblot (see Methods). Cells infected with wild-type virus and uninfected cells were used as negative controls.

and that this recombinant protein has identical antigenicity as its native form. None of the negative controls (both cell lysates and culture media of wild-type baculovirus infected or mock infected Sf9 cell cultures) was found to have the 68-kDa protein (Fig. 2, lanes 4–8). Analysis of the time course of rAed a 1 expression showed that the maximum production of the recombinant protein occurred at 96 h post-infection (Fig. 3).

Purification and isoelectric point

rAed a 1 was purified from the expression medium supernatant by a combination of anion-exchange and gel-filtration chromatography. The first step was anion exchange using DEAE–Sephacel. rAed a 1 was not adsorbed to the matrix and was found in the effluent (Fig. 4A). Several other proteins including a protein of the same mol. wt as rAed a 1 were sequentially eluted by the Tris buffers (data not shown). After this step, the pooled fraction containing rAed a 1 was still contaminated with some other proteins, as detected by SDS–PAGE with silver stain. Therefore, the second step, gel filtration using Sephacryl S-100 HR, was employed to further purify this protein. As shown in Fig. 4(B), most contaminant proteins were excluded from the purified fractions. The purified rAed a 1, as indicated by the hatched lines, presented one band on the isoelectric focussing cell gel and its isoelectric point was 9.0.

ELISA and ELISA inhibition tests

As shown in Fig. 5, rAed a 1-specific IgE levels were significantly higher in the seven subjects with positive mosquito bite tests as compared to the subjects with negative mosquito bite tests. The difference was not statistically significant ($P = 0.14$) between the two groups due to the small number of subjects studied. Furthermore, in the ELISA inhibition test, the binding of serum rAed a 1-specific IgE was successfully inhibited by the addition of mosquito salivary gland extract in a dose-dependant manner (Fig. 6), indicating that the recombinant Aed a 1 allergen and the native Aed a 1 allergen in the salivary gland extract have identical antigenicity and that the rAed a 1 captured-ELISA was specific for the detection of Aed a 1-specific IgE in human serum.

Skin reactivity and clinical relevance

Although most people who are allergic to *Ae. aegypti* bites develop both immediate and delayed skin reactions to the

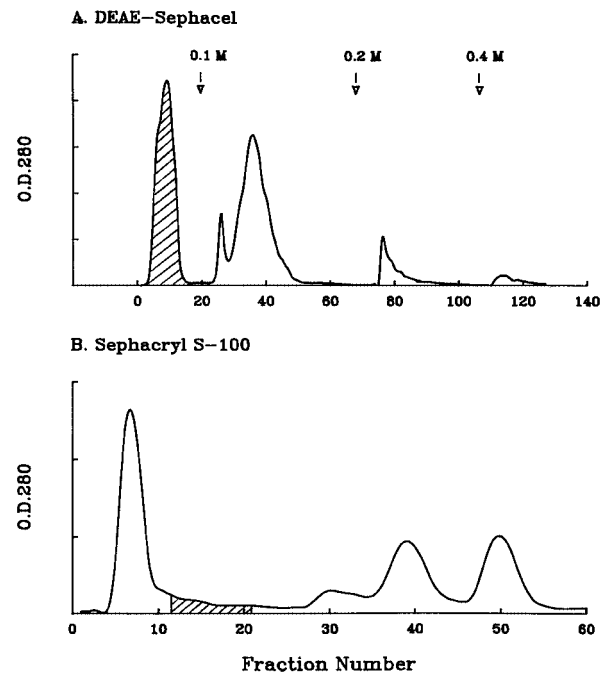


Fig. 4. Purification of the recombinant protein. rAed a 1 was purified from the culture medium by a combination of gel-filtration and ion-exchange chromatography. After concentration and dialyzation against Tris buffer the culture medium was loaded onto a DEAE–Sephacel column (A), and the column was then sequentially washed with Tris buffers containing 0.1, 0.2 and 0.4 M NaCl. Fractions containing rAed a 1 (indicated by the hatched lines) were pooled, concentrated and applied to a Sephacryl S100-HR column (B) as described in Methods.

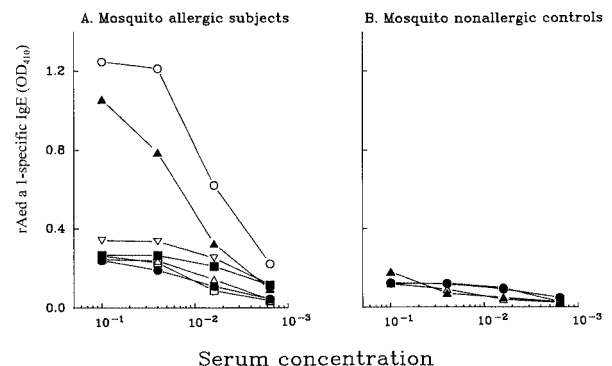


Fig. 5. rAed a 1-specific IgE measured by ELISA in seven mosquito bite test-positive subjects (A) and four mosquito bite test-negative subjects (B).

saliva, some only develop immediate reactions and others have only delayed reactions. Forty-eighty participants were divided into mosquito bite-positive and -negative groups according to the size of their immediate or delayed skin reactions in the bite tests. Thirty-one participants had positive immediate reactions to mosquito bites and seventeen participants had negative immediate reactions to mosquito bites. Thirty-three participants had positive delayed reactions to mosquito bites, while 15 had negative delayed reactions.

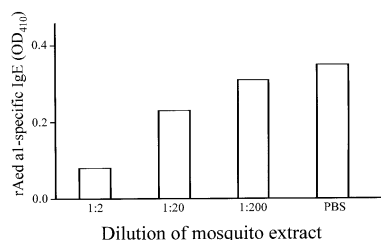


Fig. 6. ELISA inhibition tests. The mosquito salivary gland extract was 10-fold diluted and each dilution was mixed with mosquito-allergic serum. Serum mixed with PBS buffer extract served as a positive control. After incubation at 4°C overnight, the rAed a 1-specific IgE contained in the sera was measured by ELISA.

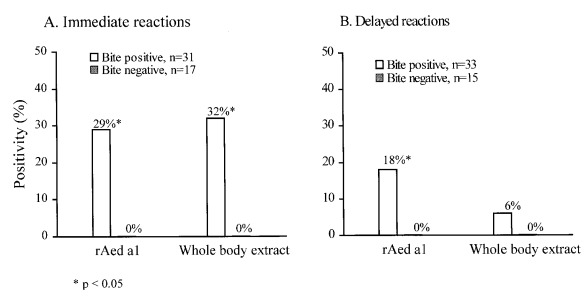


Fig. 7. Skin prick tests with rAed a 1. Forty-eight subjects were grouped into either bite test-positive or bite test-negative by their reactions to an *Ae. aegypti* bite as described in Methods. Epicutaneous tests were performed with purified rAed a 1 and a commercial mosquito *Ae. aegypti* whole-body extract. Immediate reactions were read at 20 min and delayed reactions were read 24 h after prick testing. Positive reactions are defined in Methods.

Epicutaneous tests with rAed a 1 and a commercial *Ae. aegypti* whole-body extract were performed in the mosquito bite test-positive and -negative groups. Nine of 31 (29%) of the subjects with positive immediate mosquito bite tests had a positive immediate skin reaction to rAed a 1 versus 10 of 31 (32%) for the commercial extract (Fig. 7A). Six of 33 (18%) of the participants with a positive delayed bite test had a positive delayed reaction in rAed a 1 tests versus two of 33 (6%) for the commercial extract (Fig. 7B). None of the participants with the negative immediate and/or delayed bite tests had a positive skin test to either rAed a 1 or the commercial extract, as measured at 20 min or at 24 h after the test. In the nine participants with positive rAed a 1 tests, rAed a 1-induced flare sizes significantly correlated with mosquito bite-induced flare sizes ($r = 0.88$, $P < 0.001$) (Fig. 8).

Discussion

Mosquito saliva contains up to 20 proteins (20), most of which are allergens (21). Due to the technical challenges in obtaining saliva and in isolating each allergen from the saliva, production of recombinant proteins is a promising approach for investigating the role of the individual allergens in the saliva and eventually leading to successful mosquito allergy diagnosis and immunotherapy. Aed a 1 is an important salivary allergen

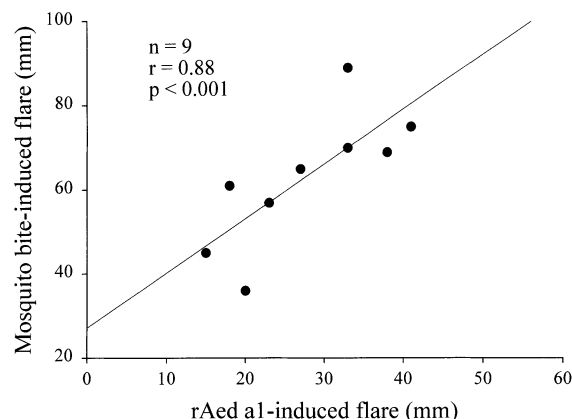


Fig. 8. Correlation of rAed a 1-induced flare sizes and mosquito bite-induced flare sizes.

A. aegypti and is composed of 562 amino acid residues, having a mol. wt of 68 kDa, based on observation by immunoblotting (23). Two segments in a cDNA library comprise the full-length Aed a 1 cDNA, the 5' segment (1–149 amino acid residues, λ SGG12-5) and the 3' significant portion (150–562 amino acid residues, λ SGG12) (23). In this study, we ligated the two cDNA segments to form the full-length Aed a 1 gene and expressed the intact protein using the baculovirus/insect cell system.

Baculovirus/insect cell systems have been used extensively for the production of large amounts of biologically active recombinant proteins (14,34,35). Their ability to perform many of the post-translational modifications found in eukaryotic cells is a major advantage over prokaryotic expression systems, but the efficiency of expression of baculovirus system varies by ~1000-fold from gene to gene (36). Most heterologous proteins are produced at amounts ranging from 1 to 100 mg/10⁹ cells (e.g. a 1-l culture) (36,37). The different expression vectors also may play a critical role in the expression levels of baculovirus system. In our study ~10 mg of recombinant protein was obtained from 1 l of culture medium by using small-scale batch fermentation in spinner cultures.

We used the baculovirus pBlueBacHis vector to express the C-end fragment of rAed a 1 (rAed a 1 3') and to purify this recombinant protein by means of immobilized metal-affinity chromatography (31). Because a stop codon is present just before the start codon of the full-length cDNA coding Aed a 1, the full-length rAed a 1 protein was expressed by the pBlueBacHis vector as a non-fusion protein and secreted into the serum-free culture medium. The full-length rAed a 1 was purified from the culture medium using a combination of ion-exchange and gel-filtration chromatography that resulted in the recovery of a highly purified rAed a 1 as shown in Fig. 4.

The purified full-length rAed a 1 was shown to bind to the serum IgE of mosquito allergic subjects as measured by ELISA (Fig. 5) and by immunoblot analyses (Fig. 2), and to the IgE on basophils of a mosquito allergic subject as detected by histamine release (unpublished data). The binding of rAed a 1 to the IgE of a mosquito-allergic serum could be inhibited by addition of natural Aed a 1 present in mosquito saliva

(Fig. 6). More importantly, rAed a 1 induced both skin immediate and delayed reactions in mosquito bite test-positive study participants (Fig. 7), and the sizes of skin test reactions to rAed a 1 significantly correlated with the sizes of *Ae. aegypti* bite test reactions (Fig. 8). All of the above evidence indicates that the rAed a 1 expressed using a baculovirus/insect cell system has identical antigenicity and biological activity to its natural form in the saliva. Proteins expressed using eukaryotic expression systems can be post-translationally modified, which is important for maintaining biological functions. These proteins can be used in both *in vitro* and *in vivo* tests (14,15). In contrast, proteins expressed by using prokaryotic expression systems, e.g. *Escherichia coli*, have no carbohydrates and are generally only used in *in vitro* assays (16,17).

Skin reactions to mosquito bites consisted of an immediate wheal and flare and delayed induration. To investigate the clinical relevance of rAed a 1, epicutaneous tests with rAed a 1 and a commercial *Ae. aegypti* whole-body extract were performed in *Ae. aegypti* bite-positive and -negative subjects. Although most people reacting to mosquito bites develop both immediate and delayed skin reactions, some develop only immediate reactions or only delayed reactions. Therefore, there were two bite-positive groups: one had a positive immediate bite test (31 subjects) and another had a positive delayed bite test (33 subjects). For immediate reactions, the positivity rate with this allergen (29%) was similar to that of the mosquito whole-body extract (32%) containing more than eight allergens. For delayed skin reactions, the single allergen, rAed a 1, induced an even higher positivity rate (18%) than the whole-body extract (6%), suggesting that one pure recombinant salivary allergen is more sensitive than many allergens (salivary and non-salivary) contained in the crude extract in the diagnosis of mosquito allergy.

The positivity rate of epicutaneous tests with rAed a 1 (29%) and the commercial whole-body extract (32%) are low in mosquito bite test-positive subjects. In mosquito bite tests, fresh salivary proteins are injected directly into the skin. In our previous experiments, each salivation of a female adult *Ae. aegypti* produces ~0.2 µg of saliva, while in epicutaneous tests, each prick introduces ~3 × 10⁻⁶ ml of the test solution into the epidermis (38), i.e. ~0.0030 µg of rAed a 1 or 0.0095 µg of the commercial extract was introduced in each epicutaneous test. The small amount of antigen extract introduced may count for the low positivity rate of both rAed a 1 and the commercial extract. The use of intradermal tests may increase the sensitivity of the skin tests.

Here, we report the first recombinant mosquito salivary allergen rAed a 1. In addition, two other salivary allergens of *Ae. aegypti*, rAed a 2 (a 37-kDa protein previously named D7) (26,40) and rAed a 3 (a 30-kDa protein) (27,40), have been also expressed and characterized. These recombinant salivary allergens will greatly facilitate the diagnosis and, in the future, the immunotherapy of mosquito allergy.

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