

Induction of IgE Responses Using a Recombinant Mosquito Salivary Allergen rAed a 2 without Adjuvant in Mice

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Key Words

Mosquito allergy · *Aedes aegypti* · IgE · IgG1 · IgG2a · rAed a 2

Abstract

Background: Reactions to mosquito bites are a global problem. Several salivary proteins from *Aedes (Ae.) aegypti*, the most common mosquito species, have been cloned and expressed. Plasmid DNA vaccination has been shown to be effective in the downregulation of IgE responses. To investigate the in vivo antigenicity of these recombinant proteins and to study the mechanisms underlying plasmid DNA vaccination, a mouse model sensitized with a recombinant antigen has been developed. **Methods:** BALB/c and C57BL/6 mice were injected intradermally with a 37-kD recombinant *Ae. aegypti* salivary allergen (rAed a 2) in the absence of adjuvant twice weekly for 8 weeks and then challenged twice with rAed a 2 at weeks 10 and 12. Serum rAed a 2-specific IgE, IgG1 and IgG2a were measured by ELISA. Intradermal tests were performed every 4 weeks. The binding capacity of rAed a 2-specific IgE to the native Aed a 2 was examined by immunoblotting. **Results:** In both strains, sensitization with rAed a 2 induced a significant increase in IgE and IgG1, but not IgG2a. In all sensitized mice, a positive immediate skin reaction was apparent, while delayed-type hypersensitivity reactions

were not observed. BALB/c mice produced significantly higher levels of IgE and IgG1 and larger wheals than C57BL/6 mice. The IgE antibodies elicited by rAed a 2 bound to not only rAed a 2 but also its natural form in mosquito saliva. **Conclusion:** (1) Repeated injections of rAed a 2 without adjuvant induce predominant Th2-type responses in mice. (2) BALB/c mice are better responders for IgE production than C57BL/6 mice. (3) rAed a 2 has identical allergenicity to its natural form.

Introduction

Cutaneous reactions to mosquito bites are common and consist of an IgE-mediated immediate wheal and flare reaction peaking at 20 min and a lymphocyte-mediated type IVa₂ delayed type hypersensitivity peaking at 24–48 h [1, 2]. Severe large local and systemic reactions, including urticaria and anaphylaxis, have been also reported [3, 4].

The proteins in the mosquito saliva are responsible for the induction of allergic responses [5]; as many as twenty proteins have been identified in the saliva of *Aedes aegypti*, the most common mosquito species in the world [6]. Among them, at least eight proteins are allergens which induce IgE responses in mosquito-allergic humans [5]. To date, 6 salivary proteins of *Ae. aegypti* have been cloned and sequenced [7–12]. Three of them, named rAed a 1

(apyrase), rAed a 2, and rAed a 3, respectively, have been shown to bind the IgE in mosquito-allergic human sera [5, 9, 13]. rAed a 2, previously named D7 [7], is a 37-kD salivary protein of *Ae. aegypti* with unknown function which has been found in the saliva of many other mosquito species with worldwide distribution [5].

During the past few years, numerous allergens have been cloned from plants, animals, and insects, facilitating an improvement in allergen-specific diagnoses [13]. Immunotherapy with recombinant allergens [14, 15] or plasmid DNA containing the cDNA sequence of the offending allergen [16, 17] has also been investigated. Nevertheless, knowledge of the in vivo immunological relevance of these recombinant allergens is very limited. Animal models are useful for the investigation of the antigenicity of recombinant allergens in vivo. IgE induction in mice is usually achieved by coadministration of protein antigens with adjuvant such as aluminum hydroxide [18], however, antigen/adjuvant-induced IgE does not reflect the induction of IgE responses under natural conditions [19].

Here, we describe IgE production in mice sensitized with a recombinant *Ae. aegypti* salivary protein, rAed a 2, in the absence of adjuvant. The production of antigen-specific IgE, IgG1, IgG2a and skin reactions to the antigen were analyzed in two mouse strains receiving different antigen doses.

Materials and Methods

Mice

Female, 8- to 10-week-old BALB/c and C57BL/6 mice were obtained from the Central Animal Care Services, University of Manitoba. All animals were kept under identical conditions in one room at the Service Facility. The experiments were approved by the University of Manitoba and the Animal Care and Use Committee, and the investigators adhered to Canadian Council on Animal Care guidelines for humane treatment of animals.

rAed a 2 and Mosquito Saliva

Recombinant Aed a 2 (rAed a 2) was expressed by a baculovirus/insect cell system using the methods previously described [20]. Briefly, *S. frugiperda* (Sf9) insect cells (Invitrogen, San Diego, Calif., USA) were cotransfected with the transfer vector pVL1392/Aed a 2 cDNA (provided by Dr. Anthony James, University of California, Irvine) [7] and baculoviruses by the cationic liposome method. The cotransfection media were then harvested for purification of the recombinant viruses. The virus stock was prepared for large scale infections using High-Five cells cultured in EX-CELL™ 405 media (JRH Biosciences, Lenexa, Kans., USA). Suspension cultures were harvested 4 days after infection and centrifuged. rAed a 2 was purified from the supernatant by anion exchange chromatography (DEAE Sephacel; Pharmacia Biotech, Uppsala, Sweden). The purified rAed a 2 which does not bind to

DEAE Sephacel beads) exhibited one band using SDS-PAGE and silver staining techniques (data not shown).

Mosquito saliva was collected from female *Ae. aegypti* in our laboratory using the methods reported previously [5].

Immunization

Four mice in each group were injected intradermally with 1 or 10 µg of purified rAed a 2 in the absence of adjuvant twice weekly. Sera, collected biweekly from the mice by tail bleeding, were stored at -30°C and initially screened by ELISA for rAed a 2-specific IgE levels at weeks 4 and 8 to define if there was a significant increase in rAed a 2-specific IgE. At week 8, rAed a 2-specific IgE levels were significantly increased in both sensitized groups, compared with the control mice. Intradermal injections with rAed a 2 were then stopped and the mice were challenged intradermally with 2 µg of rAed a 2 at weeks 9.5 and 11.5. Sera were collected 3 days after each challenge.

Intradermal Tests

Intradermal testing with rAed a 2 was performed every 4 weeks in rAed a 2-immunized mice and control mice. One microgram of rAed a 2 diluted in 10 µl of saline was injected intradermally into the shaved back of each mouse. Ten microliters of saline were injected as a negative control 2 cm away from the antigen injection site. The immediate reaction was read 20 min after the injection by measuring the largest and orthogonal diameters of the wheal using a micrometer (Mitutoyo, Tokyo, Japan). The delayed reaction was read 24 and 48 h later by measuring the induration. The visibility of the edge of the wheal or induration was enhanced by wrinkling the skin surrounding the injection site. After subtraction of the area of the saline-induced wheal or induration, the results were expressed using the average diameter of the wheal or the induration. A diameter ≥5 mm was considered to be a positive test.

Measurement of rAed a 2-Specific IgE, IgG1, and IgG2a by ELISA

Indirect enzyme-linked immunosorbent assays (ELISAs) were developed to measure rAed a 2-specific antibodies using techniques developed previously in our laboratory [21]. Optimal conditions were chosen by checkerboard titration with reference to the manufacturer's instructions. Each determination was performed in duplicate. The biotinylated rAed a 2 was prepared in our laboratory by labelling rAed a 2 with N-hydroxysuccinimidobiotin (Sigma, St. Louis, Mo., USA) according to the procedure described by Jansson et al. [22]. Monoclonal rat anti-mouse IgE antibodies and streptavidin-alkaline phosphatase conjugate were purchased from PharMingen (San Diego, Calif., USA). Alkaline phosphatase-conjugated monoclonal rat antibodies against mouse IgG1 and IgG2a were obtained from Jackson ImmunoResearch Laboratories (West Grove, Pa., USA).

For detection of serum antigen-specific IgE, polystyrene immunoplates (Nunc-Immuno Plate Maxisorp, Denmark) were coated with monoclonal rat anti-mouse IgE (0.2 µg/well) diluted in 0.05 M carbonate buffer (pH 9.6) and incubated overnight at 4°C. After rinsing the plates 3 times with washing buffer (0.05%) Tween 20 in 0.01 M PBS, pH 7.2) and blocking them with 2% bovine serum albumin (BSA) in washing buffer for 1 h at room temperature, 0.1 ml of the test sample or of the reference serum, diluted 1:10 in ELISA buffer (0.2% BSA in washing buffer), was added to each well. The plates were incubated overnight at 4°C. The wells were then washed, and 0.1 ml of biotinylated rAed a 2 (0.5 µg/ml) diluted in ELISA buffer were added and incubated overnight at 4°C. After washing, 0.1 ml of

enzyme-conjugated streptavidin (1:1,000) were added and incubated for 1 h at room temperature. After a final wash, 0.1 ml of the enzyme substrate (P-nitrophenylphosphate, 1 mg/ml in diethanolamine buffer, pH 9.8) were added and incubated overnight at 4°C. The reaction was stopped by addition of 0.1 ml of 1 N NaOH. Optical absorbance at 405 nm was read using the THERMOmax microplate reader (Molecular Devices, Calif., USA).

For measurement of serum-specific IgG1 and IgG2a to rAed a 2, the plates were coated with 0.25 and 1 µg/ml of rAed a 2, respectively. They were then incubated with serum samples or a reference serum (1:1,000 for IgG1; 1:50 for IgG2a), followed by incubation with alkaline phosphatase-conjugated monoclonal rat antibodies against mouse IgG1 (1:10,000) or IgG2a (1:1,000).

To avoid variations among microplates and among assays, the values of rAed-a-2-specific antibodies in test samples were expressed using normalized optical absorbance at 405 nm (OD₄₀₅). This was obtained after deduction of the OD₄₀₅ of PBS controls, and then normalized using the following formula:

$$\text{Normalized test sample OD} = \frac{\text{test sample OD} \times \text{standard reference serum OD}}{\text{test reference serum OD}}$$

Standard reference serum OD is the most suitable OD value of the reference serum in the assay. For example, if the standard reference serum OD is defined as 3.0, when the test reference serum OD is 2.8 and the test sample OD is 1.8, the normalized test sample OD should be 1.93.

Western Blotting

The mosquito saliva or rAed a 2 was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a discontinuous system according to the method previously described [5] using a BioRad Mini-PROTEAN II apparatus. Samples were boiled for 4 min in a reducing buffer and then loaded onto the wells (1–2 µg/well of rAed a 2 or mosquito saliva). Proteins in the sample were separated by 12% gel SDS-PAGE and then electronically transferred onto nitrocellulose membranes. Free binding sites on the membranes were blocked by incubation of the membranes with 3% BSA dissolved in phosphate-buffered saline containing 0.05% Tween 20 (PBS-T) for 2 h at room temperature. The membranes were incubated at 4°C overnight with a serum (diluted 1:20) pooled from the mice of the same group. After washing three times with PBS-T, the membranes were incubated with goat anti-mouse IgE (1:2,000; Immunological Laboratories, Tilburg, The Netherlands) followed by alkaline-phosphatase-conjugated rabbit anti-goat IgG (Jackson ImmunoResearch Laboratories) at room temperature for 1.5 h. The color was developed by incubation of the membranes with the alkaline phosphatase substrate (pNPP; Sigma). Prestained SDS-PAGE standards (Bio-Rad Laboratories, Hercules, Calif., USA) were used to determine the relative molecular weights of the electrophoresed components. In the negative strips, the membranes containing mosquito saliva proteins or rAed a 2 were incubated with the pooled sera from control mice or PBS-T. In the positive control strips, the membranes containing mosquito saliva proteins or rAed a 2 were incubated with mouse-anti saliva which was produced in our laboratory by immunizing mice with *Ae. aegypti* saliva proteins emulsified in complete Freund's adjuvant as previously described [23].

Table 1. Immediate skin reactions to rAed a 2 in mice

Mice	Immunization	Wheal diameter, mm (mean ± SEM)		
		week 4	week 8	week 12
BALB/c	1 µg	7.4±1.2	9.1±0.4	9.6±0.9
	10 µg	8.8±0.5	9.1±0.7	9.5±0.5
	saline	<3.0	<3.0	<3.0
C57BL/6	1 µg	6.9±0.5	7.5±0.3	8.2±0.5
	10 µg	8.9±0.6	6.3±0.4*	7.7±0.7
	saline	<3.0	<3.0	<3.0

*p<0.01 compared with week 4.

Statistical Analysis

The levels of rAed a 2-specific antibodies and the sizes of skin tests were expressed as means ± SEM of 4 mice per group. Statistical significances between groups were determined using Student's t test.

Results

rAed-a-2-Specific IgE, IgG1, and IgG2a Production

In BALB/c mice, immunization with either 1 or 10 µg of rAed a 2 induced a gradual significant increase in IgE production (p 's<0.001) which peaked around week 10 (fig. 1A, left panel). The overall rAed-a-2-specific IgE levels were slightly higher in the high-dose group than in the low-dose group. In contrast to the high-dose immunization, in C57BL/6 mice, immunization with a low dose of rAed a 2 appeared to induced more IgE peaking at week 10 (fig. 1A, right panel). In both mouse strains, the patterns of IgG1 production in the low- and high-dose-immunized groups (fig. 1B) were compatible with their IgE production. Interestingly, IgG1 appeared to be produced earlier than IgE in both strains (fig. 1A, 1B). The overall levels of IgE and IgG1 were significantly higher in BALB/c mice compared with C57BL/6 mice (p 's<0.05). The production of rAed a 2-specific IgG2a was slightly increased after week 4 in both strains.

Skin Tests

At week 4, both BALB/c and C57BL/6 mice immunized with either high or low doses of rAed a 2 developed a positive wheal reaction (6–12 mm in diameter) 20 min after skin testing with 1 µg of rAed a 2, whereas control mice developed a wheal reaction <3 mm in diameter (p 's<0.01; table 1). The mean sizes of the wheals increased gradually

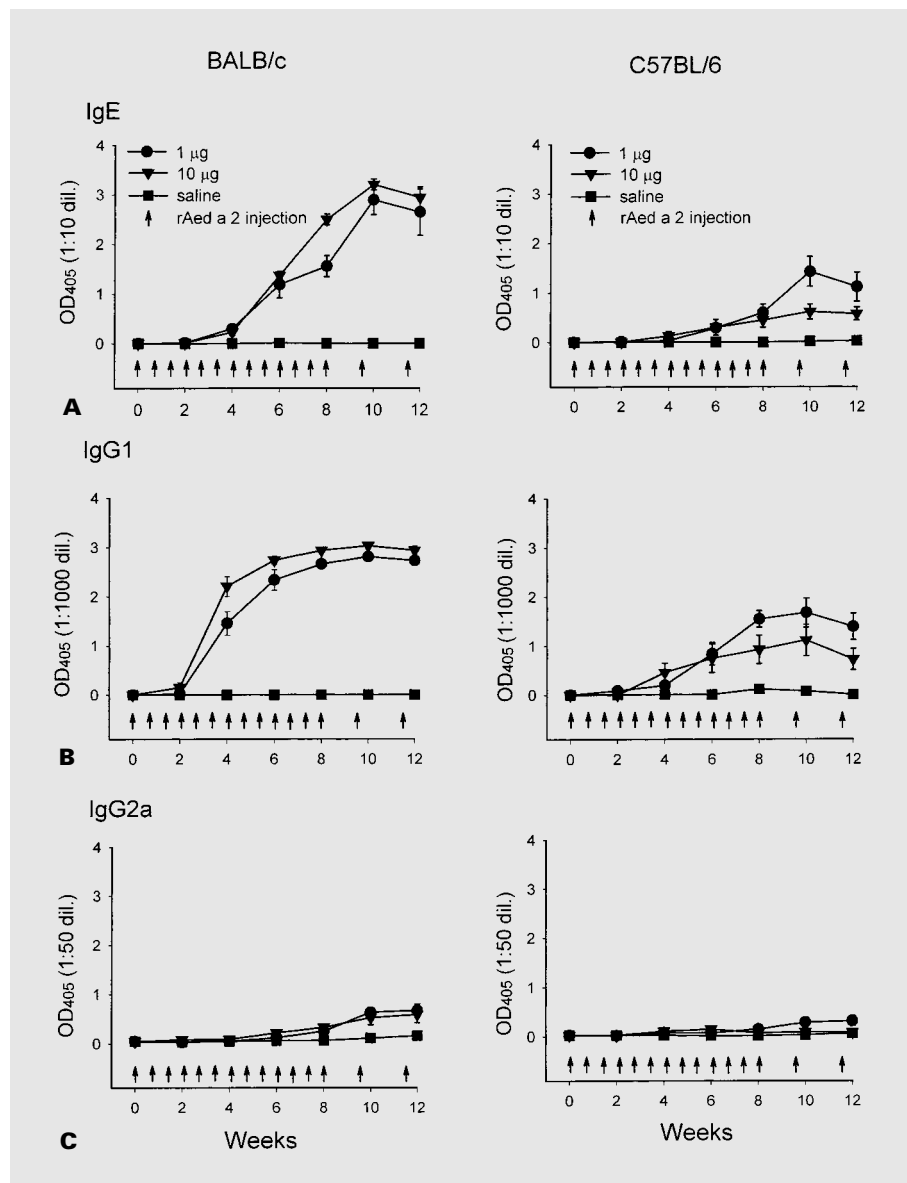


Fig. 1. Antibody responses to rAed a 2 in mice. BALB/c and C57BL/6 mice were injected with 1 or 10 µg rAed a 2 i.d. or saline twice weekly for 8 weeks. The mice were then challenged with 2 µg rAed a 2 i.d. at weeks 9.5 and 11.5. Serum rAed a 2-specific IgE (**A**), IgG1 (**B**) and IgG2a (**C**) were measured by ELISA.

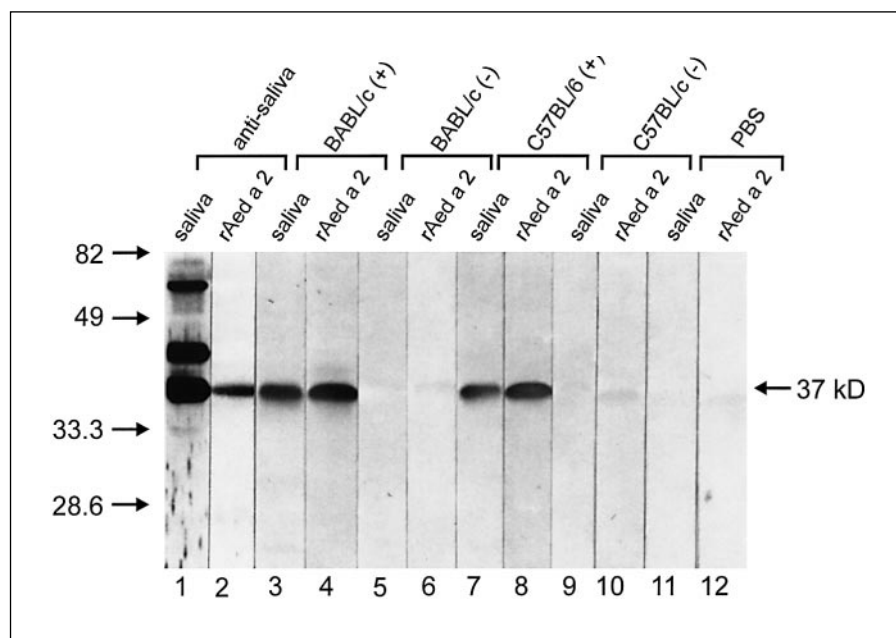
and significantly in BALB/c mice immunized with both high and low doses, and in C57BL/6 mice with low doses. However, in the high-dose-immunized C57BL/6 mice, the mean size of the immediate wheal reactions at week 8 was significantly reduced compared with that at week 4 ($p < 0.01$; table 1). Delayed skin reactions measured 24 and 48 h after testing were not observed in any mouse during the study.

Western Blot

To confirm the antigen-specific binding of IgE and to evaluate the IgE reactivity pattern to recombinant and native Aed a 2, Western blotting was performed with sera

from mice sensitized with rAed a 2. As shown in figure 2, the rAed-a-2-induced IgE in the sera from sensitized BALB/c (lanes 3 and 4) and C57BL/6 (lanes 5 and 6) mice reacted not only with the rAed a 2 (lanes 4 and 6) but also with its native counterpart in the saliva (lanes 3 and 5). The control sera (lanes 7–10) did not show any obvious IgE binding to either the recombinant or the natural antigen. Alternatively, the IgE induced by mosquito saliva bound to both natural (lane 1) and recombinant (lane 2) Aed a 2, suggesting that the rAed a 2 has identical antigenicity to its natural counterpart in the saliva.

Fig. 2. Western blot analysis of IgE binding to rAed a 2 and salivary proteins. *Ae. aegypti* saliva proteins (lanes 1, 3, 5, 7, 9 and 11) and rAed a 2 (lanes 2, 4, 6, 8, 10 and 12) were separated by 12% gels and then transferred onto nitrocellulose membranes. The membranes were incubated with (1) mouse anti-saliva (lanes 1 and 2), (2) rAed a 2-immunized BALB/c sera (lanes 3 and 4), (3) rAed-a-2-immunized C57BL/6 sera (lanes 5 and 6), (4) control BALB/c sera (lanes 7 and 8), (5) control C57BL/6 sera (lanes 9 and 10) or (6) PBS-T (lanes 11 and 12). This was followed by sequential incubations with goat anti-mouse IgE and rabbit anti-goat IgG conjugate as described in the Methods. The arrow indicates the 37-kD Aed a 2.



Discussion

Protein antigens are usually coadministered with adjuvants such as aluminum hydroxide, *Bordetella pertussis* or incomplete Freund's adjuvant to induce IgE responses [24]. At least two disadvantages are associated with the use of adjuvants. First, the adjuvants themselves may induce nonspecific T cell and inflammatory responses and affect several cellular functions [24]. Second, the immunization procedure usually consists of a primary and a secondary booster injection and the IgE titers are consistently higher after two or three injections of antigen in the presence of adjuvant than after a course of many injections without adjuvant [25]. There is a clear need to translate these findings into models which more closely approximate the triggering of IgE responses under natural conditions, that is, when the antigenic stimulus is not accompanied by an adjuvant, the portal of entry of the allergen is natural, and the exposure to the allergen is frequent. In this study, we report the successful establishment of an animal model in which mice are sensitized with a recombinant mosquito salivary protein in a manner similar to human natural sensitization to allergens.

It is well known that genetic background exerts striking selective pressures on the development of the phenotype of allergic responses. In general, BALB/c mice are good responders for IgE induction while C57BL/6 mice are more Th1 biased [26–28]. In this study, both BALB/c and C57BL/6 mice were used to examine whether genetic back-

ground could influence the outcome of IgE responses to rAed a 2. Our data showed that injection of as little as 1 µg of rAed a 2 induced a marked increase in rAed a 2-specific IgE and IgG1 in BALB/c mice. In contrast, the same dose used to immunize C57BL/6 mice induced significantly lower levels of IgE and IgG1, suggesting that genetic background does indeed influence IgE responses. IgG2a levels were low in both mouse strains.

The production of IgE and IgG1 is upregulated by Th2 cytokines, especially IL-4, which directly mediate IgE and IgG1 class switching [29, 30]. By contrast, IgG2a production is upregulated by Th1 cytokines [31, 32]. The significantly increased production of IgE and IgG1 and the low levels of IgG2a in rAed a 2-immunized mice suggest that a predominant Th2 response developed in both mouse strains. The development of immediate skin reactions, but not the delayed reactions, further confirms the nature of the predominant Th2-type response in the mouse model.

In summary, we have shown that intradermal administration of rAed a 2 without adjuvant can prime and sensitize both BALB/c and C57BL/6 mice to produce a dominant Th2-type immune response which is evidence by increased IgE and IgG1 levels and development of positive skin immediate reactions. Compared to BALB/c mice, C57BL/6 mice are poor IgE responders but may be susceptible to develop desensitization. This model provides a useful tool for future investigations of allergen and plasmid vaccination on prevention and treatment of IgE responses.

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