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Production and characterization of monoclonal antibodies to two new mosquito *Aedes aegypti* salivary proteins

Zhikang Peng ^{*}, Jian Yang, Hongsheng Wang, F. Estelle R. Simons

The Section of Allergy and Clinical Immunology, Department of Pediatrics and Child Health and Department of Immunology, Faculty of Medicine, University of Manitoba, Canada

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Abstract

Mosquito salivary proteins, which are fundamental to the process of blood feeding, also facilitate disease transmission and cause allergic reactions. The identification and characterisation of these proteins have been hampered by the difficulty of obtaining them in purified form. In this report, we describe the production of mouse monoclonal antibodies (mAbs) against mosquito salivary proteins. BALB/c mice were immunised with *Aedes aegypti* saliva proteins. Hybridomas were produced by fusion of spleen cells with a mouse myeloma cell line. Positive clones were selected using a saliva-capture ELISA and further identified using immunoblotting. Three mAbs reacted with a 44 kDa protein (Aed a X₁) in the saliva-immunoblotting, and did not react with 2 recombinant salivary proteins, rAed a 1 (apyrase) and rAed a 2 (D7), in both immunoblotting and ELISA. Two other mAbs reacted with a 37 kDa protein in saliva-immunoblotting, but failed to react with the 37 kDa rAed a 2 in either immunoblotting or ELISA, suggesting that there is a second 37 kDa protein (Aed a X₂) which is recognised by the two mAbs. The 44 kDa and 37 kDa proteins have not been previously identified.

These mAbs provide a means to purify proteins, to isolate new genes from the salivary gland cDNA library, and to standardise mosquito extracts, facilitating studies of disease transmission by mosquitoes and of mosquito allergy. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: *Aedes aegypti*; Mosquito; Salivary protein; Monoclonal antibody; Recombinant allergen/antigen; rAed a 1; rAed a 2

1. Introduction

Mosquitoes are not only the most important vector for the transmission of malaria, filariasis, and viral diseases (James, 1992), but also an important pest to humans, causing allergic responses which include local skin reactions and systemic reactions such as urticaria, angioedema, and, rarely, anaphylactic shock (Peng et al., 1996; Simons and Peng, 1999; McCormack et al., 1995). The transmission of pathogens occurs via salivation when the female mosquito takes a blood meal. Biochemically active protein molecules contained in the saliva counteract vertebrate hemostasis, allowing the mosquito to feed successfully (James, 1994; Ribeiro, 1995; Champagne and Valenzuela, 1996). They are also involved in the

enhancement of parasite infectivity (Titus and Ribeiro, 1990; Osorio et al., 1996; Edwards et al., 1998), and cause human IgE- and lymphocyte-mediated hypersensitivities (Peng et al., 1996; Peng et al., 1998a; Simons and Peng, 1999).

Protein visualisation techniques using gel electrophoresis and silver staining have revealed as many as 20 peptides in the saliva of the adult mosquito *Aedes aegypti* (Racioppi and Spielman, 1987), the world's most common vector for the transmission of yellow fever and dengue viruses (James, 1996). Some of these molecules such as the vasodilators sialokinin I (Ribeiro, 1992; Champagne and Ribeiro, 1994), anti-platelet aggregation factor (apyrase), and anticoagulant-factor Xa (Ribeiro et al., 1984; Champagne et al., 1995; Smartt et al., 1995; Stark and James, 1998) facilitate blood feeding. The biochemical functions of other molecules such as α -amylase (Grossman and James, 1993), esterase (Argentine and James, 1995), D7 (James et al., 1991), and Aed a 3 (Xu et al., 1998) remain unknown (Table 1). Immunological

^{*} Corresponding author. AE10-820 Sherbrook Street, Winnipeg, Manitoba, Canada R3A 1R9. Tel: +204-787-2537; fax: +204-787-5040.

E-mail address: zpeng@cc.umanitoba.ca (Z. Peng)

Table 1
Aedes aegypti salivary proteins

Proteins	Molecular weight	cDNA sequenced	Biological function and allergenicity	References
α -Amylase I	81.5 kDa	Yes	Unknown	Grossman and James, 1993 Grossman et al., 1997
Apyrase (Aed a 1)	68 kDa	Yes	Antiplatelet aggregation Allergen	Ribeiro et al., 1984 Smartt et al., 1995 Champagne et al., 1995 Peng et al., 1998b
α -Glucosidase(Maltase-like I)	67 kDa	Yes	Sugar digestion	James et al., 1989 Marinotti and James, 1990
Esterase	65 kDa	No	Unknown	Argentine and James, 1995 Kerlin and Hughes, 1992
Anticoagulant-factor Xa	54 kDa	Yes	Anticoagulant	Stark and James, 1998
Aed a X ₁	44 kDa	No	Unknown	present study
Aed a X ₂	37 kDa	No	Unknown	present study
Female-specific protein, D7 (Aed a 2)	37 kDa	Yes	Unknown Allergen	James et al., 1991 Peng et al., 1998b
Aed a 3	30 kDa	Yes	Unknown Allergen	Xu et al., 1998
Sialokinins	1.4 kDa	No	Vasodilator	Ribeiro, 1992 Champagne and Ribeiro, 1994
Anti-tumour necrosis factor	Unknown	No	Anti-tumour necrosis factor	Bissonnette et al., 1993
Bacteriolytic protein	Unknown	No	Bacteriolysis	Rossignol and Leuders, 1986

studies using SDS-PAGE and immuno-blotting techniques (Peng et al., 1998a) have revealed that at least 8 salivary proteins of *Aedes aegypti* are allergens which bind to the IgE of subjects with severe skin reactions to mosquito bites.

Isolation or purification of each of the salivary proteins is required in order to understand the mechanisms of blood feeding and disease transmission and for accurate diagnosis and immunotherapy of people who have severe reactions to mosquito bites. Obtaining a sufficient amount of highly purified saliva protein for research or clinic purposes is almost impossible, as collection of mosquito saliva is extremely time-consuming and labour-intensive. Although mosquito whole body extracts are available from different commercial sources for diagnosis or immunotherapy, these unstandardized products vary greatly in both quality and biological activity, with some entirely lacking in salivary proteins (Peng and Simons, 1996). Moreover, all whole body extracts contain many extraneous proteins that are not present in mosquito saliva (Peng and Simons, 1996) and potentially cause adverse effects in patients.

Molecular cloning of mosquito saliva proteins has provided a solution to this problem. To date, the cDNAs encoding for six salivary proteins of *Aedes aegypti* have been cloned and sequenced. These proteins are a 68 kDa apyrase (Champagne et al., 1995), a 37 kDa D7 (James et al., 1991), a 30 kDa Aed a 3 (Xu et al., 1998), a 67 kDa α -glucosidase (James et al., 1989), a 82 kDa α -amylase I and II (Grossman and James, 1993; Grossman et al., 1997), and a 54 kDa anticoagulant-factor Xa

(Stark and James, 1998). Among the six proteins, apyrase, D7, and Aed a 3 have been demonstrated to bind to the IgE in subjects with severe skin reactions to mosquito bites (Peng et al., 1998b; Xu et al., 1998). According to the allergen nomenclature (Larsen and Lowenstein, 1996) we have now named the apyrase Aed a 1 and the D7 Aed a 2 (Peng et al., 1998b). Due to the lack of specific tools such as DNA/mRNA probes or mAbs, progress in cloning and identification of other saliva allergens has been slow.

In order to facilitate studies of the role of mosquito salivary proteins in blood feeding, transmission of infectious diseases, and allergic reactions, we generated and characterized monoclonal antibodies to the salivary proteins of *Aedes aegypti*.

2. Materials and methods

2.1. Materials

Recombinant *Aedes aegypti* salivary gland proteins, rAed a 1 (r-apyrase) and rAed a 2 (rD7), were expressed by a baculovirus/insect system and purified by a combination of ion exchange and gel filtration in our laboratory (Xu et al. 1996, 1997). Rabbit antibodies to rAed a 1 and rAed a 2 were gifts from Dr. Anthony James, Department of Molecular Biology and Biochemistry, University of California, Irvine. Monoclonal antibody to human IgE (ascites, clone #7.12) came from Dr. Andrew Saxon, Department of Medicine, University of Califor-

nia. Alkaline phosphatase-conjugated goat-anti mouse IgG and alkaline phosphatase-conjugated goat anti-rabbit IgG were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Mouse anti-*Aedes aegypti* saliva was produced in our laboratory from saliva-immunised BALB/c mice as described below. The pooled human serum was from mosquito-allergic subjects with a large immediate skin reaction (wheal diameter ≥ 10 mm) in mosquito bite tests (Peng et al., 1996).

2.2. Mosquito saliva

The *Aedes aegypti* colony was obtained from the Department of Entomology at the University of Manitoba and maintained in our laboratory. Saliva was collected from 4–12 day old female adult *Aedes aegypti*. After being starved by feeding with water without sugar overnight, a mosquito was aspirated from the cage, placed in a test tube with water and shaken gently. The wet mosquito was then immobilized on the wall of a plastic box by putting Vaseline on its legs and wings, and its proboscis was inserted into a capillary tube filled with 20 μ l of water. Salivation was induced by applying a small amount of 0.5% malathion in acetone (v/v) to the thorax (Boorman, 1987). One hour later, the contents of capillary tubes were collected, pooled, and lyophilised. The saliva was reconstituted by dissolving the lyophilised proteins in PBS before use. The protein concentration of the saliva was 0.40 mg/ml as measured by a Bio-Rad Protein Assay kit (Bio-Rad Labs, Richmond, CA, USA).

2.3. Production of monoclonal antibodies

Six 6–8 week old female BALB/c mice were each immunised subcutaneously at two sites with 25 μ g of *Aedes aegypti* saliva proteins emulsified in complete Freund's adjuvant. This was repeated twice, at 3 week intervals, using incomplete Freund's adjuvant. Seven days after the last booster injection, the mice were bled and the sera were tested using an enzyme-linked immunosorbent assay (ELISA) in which mosquito saliva was used as capture antigens as described below. Two mice exhibiting the highest titres were selected and given a final intraperitoneal injection with 40 μ g of the saliva proteins. Three days later, the spleen cells were prepared, pooled, and fused with the myeloma cell line NS-1 using 50% polyethylene glycol 4000 (Sigma Chemical Co., St Louis, MO) according to the previously described procedure (Kohler and Milstein, 1975). The cells were cultured in 96-well plates in HAT medium (Sigma) with 20% FCS at 37°C with 5% CO₂. Fourteen days later, the culture supernatants from the growing hybridomas were screened by ELISA. Hybridoma cultures containing mAbs which reacted with the saliva in the ELISA were selected and subcloned by limiting

dilution in the presence of feeder cells. Finally, five clones were selected, expanded and their corresponding supernatants retested. At this stage the selected supernatants were further tested for their reactivity to the saliva proteins and two recombinant antigens, rAed a 1 and rAed a 2, using both ELISA and immunoblotting.

The isotypes of the five mAbs were determined by ELISA using a monoclonal antibody isotype kit according to the instructions (Amersham Life Science, Little Chalfont, Buckinghamshire, England). They all belonged to γ -1/kappa isotype of the immunoglobulins.

2.4. ELISA

An indirect enzyme-linked immunosorbent assay (ELISA) was used to screen or identify the hybridoma cultures. Polystyrene immunoplates (Nunc-Immuno Plate Maxisorp, Denmark) were coated with mosquito saliva proteins (0.05 μ g/well) or one of the two recombinant proteins (0.1 μ l/well) diluted in 0.05 M carbonate buffer (pH 9.6) and incubated overnight at 4°C. After rinsing the plates 3 times with PBS-T (0.05% Tween 20 in 0.01 M phosphate buffered saline, pH 7.2) and blocking with 2% bovine serum albumen (BSA) in PBS-T for 1 hour at room temperature, 0.1 ml of the test hybridoma supernatants was added and incubated at 37°C for 1.5 hours. The wells were washed, and 0.1 ml of enzyme-conjugated goat anti-mouse IgG (1:1000 diluted in PBS-T supplemented with 0.2% BSA) was added and incubated for 1.5 hours at 37°C. After a final wash, colour reaction was developed by adding 0.1 ml of the enzyme substrate (P-nitrophenylphosphate, 1 mg/ml in diethanolamine buffer, pH 9.8) and incubating at 4°C overnight. The optical absorbance at 410 nm was read, using the THERMOMax microplate reader (Molecular Devices, CA, USA). Negative controls included 0.2% BSA in PBS-T and the culture medium, and the positive control was saliva-immunised mouse serum (1:2000 dilution).

2.5. Western blot

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were performed to identify the mAbs. The saliva or the recombinant mosquito salivary proteins were separated by SDS-PAGE in a discontinuous system according to the method previously described (Peng et al., 1996) using a BioRad Mini-PROTEAN II apparatus. Samples were boiled 4 minutes in a reducing sample buffer, then loaded (1–2 μ g/well) and separated by 12% gel SDS-PAGE. The proteins were then electro-transferred onto nitro-cellulose membranes. Free binding sites on the membranes were blocked by incubation of the membranes with 3% BSA dissolved in PBS-T for 2 hours at room temperature. The immunoblot was completed by

incubation of the membranes with hybridoma culture supernatants at 4°C overnight. After washing three times with PBS-T, the membranes were incubated with enzyme-conjugated goat anti-mouse IgG at room temperature for 1.5 hours. The colour was developed by incubation of the membranes with the alkaline phosphatase substrate (pNPP) tablets (Sigma). Prestained SDS-PAGE standards (Bio-Rad) were used to determine the relative molecular weights of the electrophoresed components. In the control strips, the membranes containing mosquito saliva proteins were immunoblotted with one of the following: (1) mouse anti-saliva followed by incubation with goat anti-mouse IgG conjugate; (2) human mosquito-allergic serum followed by sequential incubations with mAb anti-human IgE and then goat anti-mouse IgG conjugate; (3) rabbit antibodies to rAed a 1 or to rAed a 2, followed by incubation with goat anti-rabbit conjugate. In the recombinant Aed a 1 and Aed a 2 control strips, the membranes containing the recombinant allergen were incubated with rabbit antibodies to rAed a 1 or to rAed a 2, followed by incubation with the relevant conjugate.

3. Results and discussion

As shown in Fig. 1, three mAbs (clone #1, #13 and #16) recognised a 44 kDa salivary protein (lanes 7–9). Two other mAbs (clone #12 and #15) reacted with a 37 kDa saliva protein (lanes 10 and 12) but not with the 37 kDa rAed a 2 (lanes 11 and 13). The failure of the mAbs to bind rAed a 2 suggests that in addition to Aed a 2 there is a second 37 kDa protein which is recognised by the two mAbs only. Studies using two-dimensional electrophoresis with mosquito saliva and immunoblot with the two mAbs will further elucidate this issue. The 44 kDa and the 37 kDa proteins recognised by the mAbs also bound to the IgE in mosquito-allergic human serum (lane 1) and in mouse anti-saliva serum (lane 2), indicating that both proteins are potential allergens. The other possibility is that due to incomplete glycosylation, the 37 kDa recombinant Aed a 2 has a distinct configuration from the native protein which is not recognised by the two mAbs. This is, however, unlikely because the recombinant Aed a 2 was expressed by a baculovirus/insect cell system which performs many post-translational modifications retaining all carbohydrate moieties of the molecule. No cross-reactivity to other salivary proteins was observed in the five mAbs, and none of them reacted with either rAed a 1 or rAed a 2 in both ELISA and Western blot, although they all reacted with saliva proteins. For convenience, here, we named the 44 kDa protein recognised by the 3 mAbs (#1,13,16) Aed a X₁ and the 37 kDa one reacted with the 2 mAbs (#12,#15) Aed a X₂.

To date, ten salivary proteins of *Aedes aegypti* have been

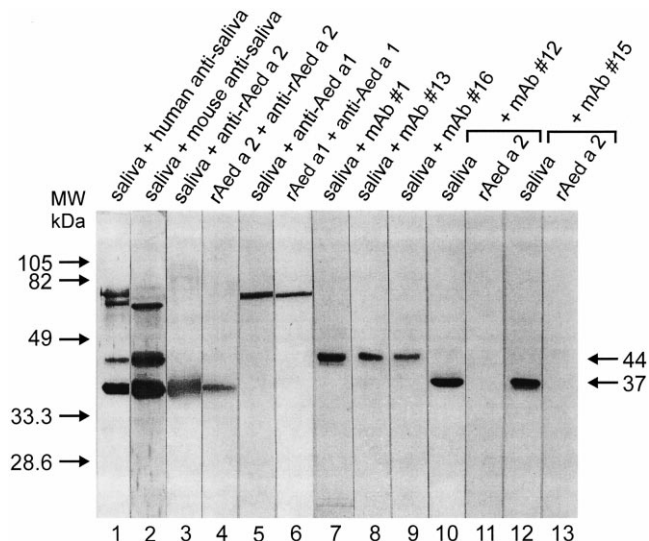


Fig. 1. Western blot analysis of monoclonal antibodies. Salivary proteins of *Aedes aegypti* (lanes 1–3, 5, 7–10, 12) and recombinant saliva proteins of *Aedes aegypti*, the 68 kDa rAed a 1 (r-apyrase) (lane 6) and the 37 kDa rAed a 2 (rD7) (lanes 4, 11, 13), were separated by SDS-PAGE under reducing conditions and then transferred to nitrocellulose membranes. After blocking with 3% BSA in PBS-T, the membranes were incubated with mAbs #1 (lane 7), #13 (lane 8), #16 (lane 9), #12 (lanes 10, 11), and #15 (lanes 12, 13) followed by incubation with goat anti-mouse IgG conjugate as described in the Materials and methods. Some membranes containing saliva proteins were incubated with sera from mosquito-allergic human subjects (lane 1), mouse anti-saliva antiserum (lane 2), rabbit antiserum against rAed a 2 (lane 3) or rAed a 1 (lane 5) as positive controls. Some membranes containing rAed a 2 were incubated with rabbit anti-rAed a 2 antiserum (lane 4) and those membranes containing rAed a 1 were incubated with anti-rAed a 1 antiserum (lane 6) as positive controls. Molecular mass markers were indicated on the left. The 44 kDa and 37 kDa proteins recognized by the mAbs were indicated on the right. A patent is pending on the monoclonal antibodies.

identified by their biochemical functions, allergenicity or molecular weights as illustrated in Table 1. Among these proteins, none of them has a 44 kDa molecular weight. The 37 kDa protein (Aed a X₂) recognised by the two mAbs has a distinct antigenicity from the 37 kDa Aed a 2 (formerly named D7). Therefore, the 44 kDa Aed a X₁ and the 37 kDa Aed a X₂ recognized by the mAbs represent two new salivary proteins of *Aedes aegypti* with potential allergenicity and unknown functions.

With the mAbs, Aed a X₁ and Aed a X₂ can be purified from mosquito whole body extracts or salivary gland extracts using affinity chromatography (Portnoy et al., 1990). These mAbs can be also used to screen *Aedes aegypti* salivary gland cDNA library for isolation of the genes encoding the two proteins, which allows for expression of pure molecules sufficient for characterisation of their biological functions and allergenicity.

Monoclonal antibodies to antigens have been extensively used to standardise, quantify and characterize antigen extracts (Chapman et al. 1987, 1988; Fahlbusch et al., 1994; Han et al., 1993; Portnoy et al., 1993), and

analyse antigen cross-reactivity (Ayuso et al., 1995). Our previous study showed that there are large variations in biological activity among commercially available mosquito whole body extracts used for diagnosis and immunotherapy of mosquito allergic subjects (Peng and Simons, 1996). However, to date mosquito extracts have not been standardised because of the lack of pure allergen components or antibodies to these pure components. With the mAbs we have produced it is now possible to standardise commercial mosquito extracts and to analyse mosquito salivary preparations. Furthermore, the monoclonal antibodies can be used to develop an mAb-capture ELISA (Peng et al., 1998c) to measure Aed a X₁- or Aed a X₂-specific antibodies using mosquito whole body extracts for study of mosquito allergy. Using the mAbs, cross-reactivity of either Aed a X₁ or Aed a X₂ with other mosquito species or insects can be examined.

Antibodies against mosquito salivary gland apyrase have been shown to inhibit enzyme activity (Mathews et al., 1996). Although mice produced these antibodies without apparent impediment to mosquito probing and feeding, the antibodies may have minimised the effect of mosquito saliva on the host animal's hemostasis. These mAbs might therefore be useful in elucidating the effect of antibodies on the enzyme activity and in designing of new strategies for prevention of disease transmission.

In summary, the 44 kDa and the 37 kDa proteins recognized by the mAbs represent two new salivary proteins of *Aedes aegypti* which have not been previously identified. Their biological functions and allergenicity remain to be investigated. For the two salivary proteins, the monoclonal antibodies provide a means to identify, purify, and measure specific antibodies in host sera, and to isolate genes from the salivary gland cDNA library. The monoclonal antibodies can be also used to standardise mosquito extracts, thus facilitating studies of disease transmission and allergic reactions to mosquito saliva.

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