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Immunization of Rabbits with Mosquito Bites: Immunoblot Analysis of IgG Antimosquito Antibodies in Rabbit and Man

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Abstract. Rabbits immunized with 50–60 *Aedes communis* mosquito bites developed IgG antibodies recognizing a 21.5-kD mosquito antigen detected by the immunoblot technique. This protein seems to be a major immunogen in the saliva of *A. communis* mosquitoes. Studies on 60 human sera revealed that IgG antibodies recognizing the 21.5-kD antigen occured in about 50% of young children and adults but were not found in the sera of 20 unexposed infants. The present immunoblot method allows further characterization of antigens and antibodies specific to mosquito saliva and contributes to understanding their role in mosquito bite reactions.

Introduction

Mosquito bites cause great discomfort to many people, especially in northern Europe, Asia and North America, where mosquitoes constitute a common problem. Cutaneous reactions vary from mild pruritic wheals and papules to long-lasting Arthus-type lesions with systemic symptoms [1]. Mechanisms causing these reactions are generally considered immunologic in nature, but knowledge about mosquito antigens and antibodies is scant. There is direct evidence that the salivary secretion in the mosquito bite is responsible for the reactions [2–4]. Attempts have been made to define the causative proteins chemically [3, 5] and immunologically [6–12]. Although many of these studies have added to our information on the different components of the whole-body and the salivary gland extracts of different mosquito species, they do not necessarily point out which components are immunogenic.

In a previous report from our laboratory, precipitating and ELISA-reactive IgG antimosquito antibodies were shown to occur frequently among the adult Finnish population [13]. In the present study we have examined, by immunoblotting, the development of mosquito-reactive IgG antibodies in rabbits during controlled feeding experiments using *Aedes communis* mosquitoes, the most frequent man-biting mosquito species in Finland [14]. We also investigated selected human serum samples in order to study the occurrence of antimosquito antibodies in man. A preliminary report dealing with some of these results was published earlier in abstract form [15].

Materials and Methods

Mosquitoes and the Immunization of Rabbits

Larval stage mosquitoes were collected from a single pond where *A. communis* mosquitoes are dominant (over 99.5%) [16]. The larvae were reared in vessels containing 20–30 larvae in 1 liter of pond water. After the adults had emerged, females were collected and sustained on a sugar diet until they were allowed to feed on the rabbits.

Two rabbits were immunized with mosquito saliva by allowing an average of 2–6 mosquitoes to eat to repletion 5–7 times a week, for 6 weeks of the year over a 2-year period. This was done by allowing the mosquitoes to feed through a cage (10 × 2.5 × 2.5 cm) placed on a shaved area of the rabbits' skin. Only bites where it could be clearly seen that the mosquito had succeeded in drawing blood were counted. A third rabbit was kept as a control. Blood samples were taken once a week during the immunization periods and 3 times between these periods.

Mosquito Antigens

Some of the mosquitoes were killed at −20 °C within 4 days of emerging. Four different *A. communis* mosquito extracts were used as antigens: (1) whole-body, (2) head and thorax and (3) abdomen extracts from female mosquitoes and (4) a whole-body extract from male mosquitoes.

Whole-body extracts were made by grinding 300 mg of mosquitoes in 3 ml of phosphate-buffered saline (PBS) on ice. The extract

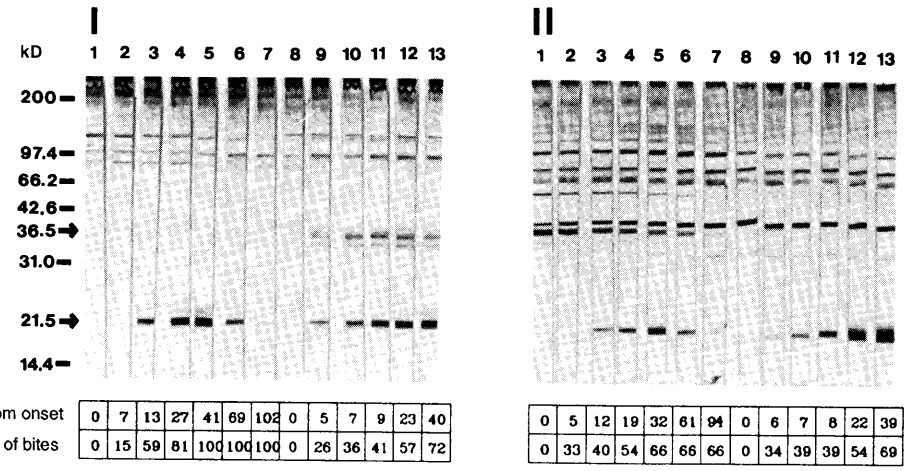

Fig. 1. Immunoblot analysis of the development of IgG antimosquito antibodies in rabbit sera following *A. communis* mosquito bites. Serial bleedings (1-13) of 2 rabbits (I, II) were tested against whole-body extracts of female *A. communis* mosquitoes. The cumulative number of bites in relation to days from the onset of the experiment is given in the lowest section of the figure. Response to 21.5-kD protein is seen in both rabbits and a weak response to 36.5-kD protein in rabbit I.

was sonicated at 20 W for 4 × 15 s (Vibra cell; Sonics and Materials, Danbury, Conn., USA) on ice and centrifuged at 12,000 *g* for 30 min at +4°C. The supernatant was filtered through a 0.45-μm filter (Miller; Millipore, Molsheim, France), and the filtrate was divided into small aliquots and stored at −70°C. Head-thorax extracts and abdomen extracts were prepared in the same way except that 300 mg of mosquitoes were first divided into head-thorax and into abdomen parts by cutting the mosquitoes, after which 3 ml of PBS was added to each portion.

Immunoblotting

For electrophoresis the samples were diluted 1:3 into electrophoresis sample buffer [17] containing 2% sodium dodecyl sulfate and 5% 2β-mercaptoethanol and kept in boiling water for 2 min. The dissolved samples, 30 μl/cm of gel, were loaded on 12% acrylamide gels (0.75 mm) containing 0.1% sodium dodecyl sulfate with a 4% stacking gel [18]. Molecular weight protein markers (14.3-200 kD) were included in all gels (Amersham, UK). The molecular weight values for mosquito proteins were the means of five different electrophoresis runs.

Electrophoresis was carried out for 45 min at a constant voltage of 200 V (Mini Protean II Cell; Bio-Rad, Richmond, Calif., USA). After electrophoresis, the antigens were transferred from the gel to 0.2-μm pore size nitrocellulose filters (Trans-Blot, Bio-Rad), in an electroblotting buffer containing 25 mmol of Tris-HCl, 150 mmol of glycine and 20% methanol, pH 8.3, for 1 h at 40 V and 15°C and for 1 h at 100 V in melting ice (Mini Trans-blot cell, Bio-Rad).

After blotting the membrane was stained with Ponceau-S red to demonstrate successful transfer of proteins to the nitrocellulose membrane.

The filter was cut into 3-mm-wide strips, washed 3 times for 10 min in 0.02% Tween-PBS and incubated for 20 min in a blocking buffer containing 5% nonfat milk powder (Valio, Finland) in PBS. Serum samples were added (human sera 1:50 and rabbit sera 1:100 as final dilution), and the strips were incubated overnight at room temperature with continuous shaking. As negative controls, strips to which no serum samples had been added were also incubated. The strips were washed 3 times for 15 min in 0.02% Tween-PBS, and horseradish-peroxidase-conjugated goat antihuman (1:500) and goat antirabbit (1:2000) IgG antibodies (Bio-Rad) were added in 5% milk in PBS. The incubation was for 90 min at room temperature, with shaking. The strips were then washed 3 times for 10 min in 0.02% Tween-PBS, once for 5 min in PBS and placed in a color-developing solution (15 mg 4-chloro-I-naphitol dissolved in 5 ml methanol, 15 μl 30% hydrogen peroxidase and 25 ml PBS). The reaction was stopped after 15 min by rinsing the blots in tap water.

Human Sera

Sera from 20 infants aged 6-8 months, 20 children aged 2-3 years and 20 adults aged 20-40 years were studied. Sera from the older children and adults were collected in July-August. All the infants were born in the fall, and the serum samples were collected in early spring, thus ensuring no contact with mosquitoes. All the human sera were collected from the Helsinki city area, i.e. the southern part of Finland, and were stored at −20°C before being examined.

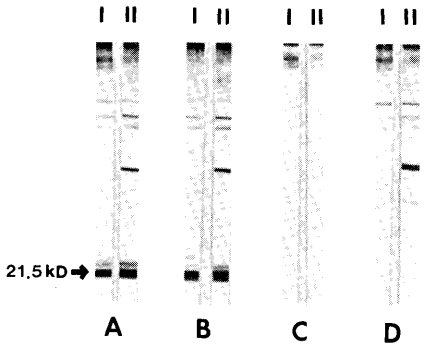

Fig. 2. Identification of 21.5-kD antigen by immunoblot analysis in various mosquito extracts, by using IgG antibodies from 2 immunized rabbits (I, II). A = Female *A. communis* mosquito whole-body extract; B = female *A. communis* head-thorax extract; C = female *A. communis* abdomen extract; D = male *A. communis* whole-body extract.

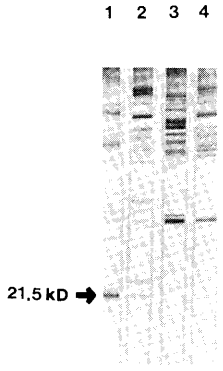

Fig. 3. IgG class antibody binding to whole-body extract of female *A. communis* mosquitoes from 4 normal human sera. Individuals 1 and 2 show positive reactions with 21.5-kD antigen.

Results

Antimosquito Antibodies in Rabbit Sera

Figure 1 shows the results of immunoblot studies. The preimmune sera (lane 1) of both rabbits (I, II), revealed antibodies binding to various antigens of the whole-body extract of *A. communis* mosquitoes. The molecular weight of the bands ranged from 26 to over 200 kD.

Macular and papular skin reactions developing within 1 h and persisting for several hours were observed in the rabbits after they had received 40–60 mosquito bites. At the same time, IgG class antibodies recognizing a major antigenic component (21.5 kD) in whole-body extract of female mosquitoes developed in both exposed rabbits (I, II; lane 3) but not in the control rabbit. When the rabbits were not exposed to mosquitoes, these antibodies disappeared (I, II; lane 8). On re-exposure, the following year, the antibodies reappeared in both rabbits after 20–30 bites (I, II; lane 9). After a total of some 120 bites the serum from rabbit I (lane 9) reacted also weakly against a second protein (36.5 kD).

When using extracts of the thorax-head region of female *A. communis*, containing the salivary glands, antisera from both immunized rabbits showed strong reaction to the 21.5-kD protein (fig. 2; B: I, II). Extracts made from the abdomen of female *A. communis* did not contain the 21.5-kD protein in immunoreactive amounts (fig. 2; C: I, II), and only minute amounts of the 21.5-kD protein were found in the whole-body extracts of male *A. communis* mosquitoes (fig. 2; D: I, II).

Antimosquito Antibodies in Human Sera

None of the 20 infants revealed antibodies against the 21.5-kD protein, while 9/20 (45%) of the older children had these antibodies. The reaction pattern in young adults was essentially similar: 10/20 (50%) had antibodies against the 21.5-kD protein. All human sera tested showed IgG class antibodies binding to several other components besides the 21.5-kD protein of whole-body extracts to female *A. communis* mosquitoes. The molecular weight of these bands ranged from 14 to over 200 kD. Typical immunoblot results from 4 adult sera are shown in figure 3.

Discussion

By immunizing rabbits using *A. communis* mosquito bites, we were able to demonstrate, by immunoblotting, the development of IgG antibodies recognizing a 21.5-kD protein component present in whole-body and head-thorax extracts of female mosquitoes. Initial immunization required 40–60 mosquito bites.

After exposure to mosquitoes had ceased, these anti-mosquito antibodies gradually disappeared but after re-exposure to 20–30 bites they reappeared in the rabbits serum. These findings suggest that the 21.5-kD protein is a major immunogen in the saliva of *A. communis* mosquitoes.

Hudson et al. [4] showed that less than 12 bites during a period of 12 days were sufficient to cause immediate skin reactions in rabbits. These authors were unable to demonstrate antimosquito antibodies with Ouchterlony immunodiffusion in bite-sensitive rabbit sera even after 60 bites. McKiel [9] demonstrated that experimental animals can only be sensitized to mosquito bites with extracts from the head-thorax part of female mosquitoes containing the salivary glands. According to McKiel [9], sensitization can also be achieved using whole-body extracts made from male mosquitoes, though the males do not suck blood and their salivary glands do not differentiate in the same way as the glands of female mosquitoes [19, 20]. Our findings are consistent with this, since large amounts of the 21.5-kD antigen were found in the head-thorax region of female mosquitoes compared to very small amounts in whole-body extracts from male mosquitoes. Our preliminary results have now shown that the saliva of *A. communis* contains three major antigens, one of which is the 21.5-kD protein and the second a 36.5-kD protein [H. B.-K., unpubl. data].

Many authors have tried to isolate and characterize substances in the mosquito saliva [3, 5, 10, 11], but no previous studies have been performed using *A. communis*. Newsome et al. [3] isolated a glycoprotein with a minimum molecular weight of 33.4 kD from the saliva of *Aedes aegypti*. This glycoprotein elicited skin reactions in sensitized human volunteers. Racioppi and Spielman [11], by means of silver staining, demonstrated 20 polypeptides in the saliva of *A. aegypti* mosquitoes. Twelve of these peptides were secreted during feeding and were immunogenic in guinea pigs.

Immunoblotting has been employed by Penneys et al. [10] in studies of human sera against mosquito salivary gland extracts from five different mosquito species: *Culex nigripalpus, Culex quinquefasciatus, Aedes taeniorhynchus, A. aegypti* and *Anopheles quadrimaculatus*. Several antigenic components sized 14–126 kD were found. However, none of them was the 21.5-kD protein typical of *A. communis* mosquitoes, and only 62-kD and 67.5-kD components seemed to be common to all mosquito species examined. Skin reactivity to the bites of mosquitoes may be species specific [21], and there is evidence that the salivary antigens of different mosquito species may vary [10, 19, 22]. In agreement with this we could not find by immunoblotting the 21.5-kD and 36.5-kD proteins characteristic of *A. communis* in whole-body extracts of Culex and Culiceta mosquitoes [unpubl. data].

The 5 human sera studied by Penneys et al. [10] revealed very complex patterns in immunoblot when mosquito salivary extracts were used as antigens. In a recent study, Shen et al. [12] used immunoblotting to analyze IgG antibodies to proteins in whole-body extracts from five mosquito species: *Aedes albopictus, A. egypti, Aedes togoi, Armigeres subalbatus* and *C. quinquefasciatus*. Complex binding patterns resembling those reported by Penneys et al. [10] and by us, showing much variation between the individual sera, were found. Some of the antigens found by Shen et al. [12] were within the range of 21–23 and 36 kD, and could thus be similar to the 21.5-kD and 36.5-kD antigens found in the present study in *A. communis* mosquitoes. The fact that we could not find antibodies binding to the 21.5-kD protein, either in non-exposed infants or in the rabbits, but observed a variety of antibodies binding to other whole-body extract components, suggests that these antibodies are not derived from mosquito bites. These antibodies could be cross-reacting antibodies originating from immune response against various insect antigens derived via inhalant or alimentary routes. Supporting this assumption, Baldo and Panzani [23] have recently shown IgE cross-reactivity between different airborne insect allergens in humans.

In conclusion, we have demonstrated by immunoblotting the development of IgG antibodies in rabbits after receiving 40–60 *A. communis* mosquito bites. These antibodies recognize a 21.5-kD antigen in whole-body and head-thorax extracts but not in abdominal extracts from female *A. communis* mosquitoes. Similar IgG antibodies were frequently encountered in human sera, but the determination of their role in mosquito bite reactions requires further study.

References

1 Frazier CA: Diptera - Mosquitoes and flies; in Frazier CA (ed): Insect Allergy. St. Louis, Green, 1987, pp 136–200.
2 Hudson A, Bowman L, Orr CWM: Effects of absence of saliva on blood feeding by mosquitoes. Science 1960;131:1730–1731.
3 Newsome WH, Jones JKN, French FE, West AS: The isolation

and properties of the skin-reactive substance in *Aedes aegypti* oral secretion. Can J Biochem 1969;47:1129–1136.
4 Hudson A, McKiel JA, West AS, Bourns TKR: Reactions to mosquito bites. Mosquito News 1958;18:249–252.
5 Poehling HM, Meyer W: Esterases and glycoproteins in the salivary glands of *Anopheles stephensi*. Insect Biochem 1980;10: 189–198.
6 McKiel JA, West AS: Nature and causation of insect bite reactions. Pediatr Clin North Am 1961;8:795–816.
7 Dublin IN, Reese JD, Seamans LA: Attempt to produce protection against mosquitoes by active immunization. J Immunol 1948;58:293.
8 McKiel JA: Reactions to Mosquito Bites. Studies of Causation and Remedial Measures; PhD thesis, Queen's University, Kingston (Ontario), 1955.
9 McKiel JA: Sensitization to mosquito bites. Can J Zool 1959; 37:341–351.
10 Penneys NS, Nayar JK, Bernstein H, Knight JW, Leonardi C: Mosquito salivary gland antigens identified by circulating human antibodies. Arch Dermatol 1989;125:219–222.
11 Racioppi JV, Spielman A: Secretory proteins from the salivary glands of adult *Aedes aegypti* mosquitoes. Insect Biochem 1987;17:503–511.
12 Shen HD, Chen CC, Chang HN, Chang LY, Tu WC, Han SH: Human IgE and IgG antibodies to mosquito proteins detected by the immunoblot technique. Ann Allergy 1989;63:143–146.
13 Ailus K, Palosuo T, Brummer-Korvenkontio M, Rantanen T, Reunala T: Demonstration of antibodies to mosquito antigens in man by immunodiffusion and ELISA. Int Arch Allergy Appl Immunol 1985;78:375–379.
14 Utrio P: Distribution of mosquitoes (Diptera Culicidae) attracted by man in Finland in early July. Notulae Entomol 1978;58:107–114.
15 Brummer-Korvenkontio H, Palosuo T, Reunala T: IgG antibodies in rabbit and man to mosquito antigens detected by immunoblot technique. Abstracts of SSI 20th AM, Copenhagen. Scand J Immunol 1989;30:633.
16 Brummer-Korvenkontio M, Korhonen P, Hämeen-Anttila R: Ecology and phenology of mosquitoes (Dipt., Culicidae) inhabiting small pools in Finland. Acta Entomol Fenn 1971;28:51–73.
17 Mini-Protean II Dual Slab Cell Instruction Manual. Richmond, Bio-Rad Laboratories, 1988.
18 Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–685.
19 Wright KA: The anatomy of salivary glands of *Anopheles stephensi* (Liston). Can J Zool 1969;47:579–587.
20 Barrow PM, McIver SB, Wright KA: Salivary glands of female *Culex pipiens:* Morphological changes associated with maturation and blood-feeding. Can Entomol 1975;107:1153–1160.
21 Rockwell EM, Jonson P: The insect bite reaction. II. Evaluation of the allergic reaction. J Invest Dermatol 1952;19:137–155.
22 Poehling HM: Distribution of specific proteins in the salivary gland lobes of culicidae and their relation to age and blood sucking. J Insect Physiol 1979;25:3–8.
23 Baldo BA, Panzani RC: Detection of IgE antibodies to a wide range of insect species in subjects with suspected inhalant allergies to insects. Int Arch Allergy Appl Immunol 1988;85: 278–287.

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