Differential Recognition of Saliva Antigens from the Ixodid Tick *Amblyomma hebraeum* (Acari: Ixodidae) by Sera from Infested and Immunized Rabbits

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ABSTRACT We determined the protein composition and antigenic content of saliva from *Amblyomma hebraeum* female ticks of different weight classes. The mean protein concentration of saliva of small partially fed ticks (<100 mg) was 333 ± 83 μg/ml and that of large partially fed ticks (150–420 mg) was 59 ± 14 μg/ml. The reduction in concentration mostly was caused by the significantly higher fluid volume per minute secreted by large ticks. Polypeptide analysis of saliva indicated the presence of a protein (14 kilodaltons [kD]) only in ticks weighing <60 mg. Other saliva proteins of 21 and 26 kD were present only in ticks weighing <150 mg, whereas 68-kD protein was absent or very faint in ticks >100 mg. Immunoblot analysis indicated that sera from rabbits infested with ticks recognized 13 saliva antigens ranging in size from 23 to 200 kD. The antigens were present in detectable quantities in the saliva of small ticks only. In contrast, the sera from rabbits immunized with tick saliva recognized only four antigens from 63 to 200 kD. The 63-kD antigen was not present in the saliva of large ticks. These data indicate that the saliva of *A. hebraeum* from small ticks is antigenically more complex than that of large ticks and that the route of immunization influences the humoral immune response of the host to the saliva antigens.

KEY WORDS *Amblyomma hebraeum*, saliva antigens, rabbits

BECAUSE OF THE numerous functions performed by the salivary glands of ticks (Kaufman 1989), the saliva composition is likely to change considerably throughout the feeding period. Although proteins have already been detected in saliva (e.g., Tatchell 1971, McSwain et al. 1992), a comprehensive study of saliva composition throughout the feeding cycle has not been done. In most cases, characterization of the protein has not gone beyond an estimate of molecular size, and only rarely has a specific peptide been associated with a biological function. For example, Geczy et al. (1971) identified a 30-kilodalton (kD) macromolecule, which they suggested was an esterase. The function of this esterase may be to hydrolyze cholesterol esters in the plasma membrane of certain cells (e.g., mast cells), thereby releasing vasoactive mediators such as histamine, 5-hydroxytryptamine and other substances (Ribeiro et al. 1985, Ribeiro 1989). Ribeiro (1987) associated anticomplement activity of *Ixodes dammini* with a 49-kD polypeptide. More recently, Limo et al. (1991) purified a 65-kD protein from the salivary glands of *Rhipicephalus appendiculatus*, which possessed anticoagulant activity.

The host mounts an immune response to many of the proteins that have been detected in tick saliva or in salivary gland extracts (SGE) (reviewed by Kaufman 1989). The salivary gland antigens (SGA) have been characterized by immunoblot analysis following sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) (Brown 1985, 1988; Gill et al. 1986; Shapiro et al. 1986, 1987, 1989; Gordon & Allen 1987), and, in some cases, a limited degree of passive resistance can be induced in a host by immunizing with a mixture of SGA. In all cases, however, this passive immunity is inferior to that attained by simply feeding ticks on the host. Thus, the development of host resistance to tick infestation is subtler than merely producing antibodies to a wide variety of SGA. Nevertheless, there remains a considerable incentive to reduce dependence on acaricides as a means for controlling ticks, and much attention is being focused on potential protection by vaccination. We demonstrate that the number of saliva antigens of the ixodid tick, *Amblyomma hebraeum* Koch, recognized by the host (rabbit), is greater following natural feeding than following inoculation with SGA, and this may explain why inoculation affords only minimal protection.

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Materials and M

Ticks and Rabbits. Exper
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Materials and Methods

Ticks and Rabbits. Experimental ticks were taken from a colony maintained in our laboratory at 26°C in darkness and at >95% RH. One day after applying 20 males, 30 unfed females (28–33 mg) were placed on the shaved backs of rabbits (a cross between the Flemish giant and the French lop-eared breeds) to feed as outlined by Kaufman & Phillips (1973). Ticks were removed from the rabbit once they attained the desired size, rinsed in water, and weighed.

Collection of Saliva. Salivation was induced within 3 h of removal from the rabbit by injecting 10 μl per 100 mg body weight of 5 m*M* dopamine made up in 1.2% NaCl. The drug was injected into the haemocoel through the camerastomal fold with an Agla micrometer syringe (Wellcome Reagents) attached to a 30-gauge needle. A capillary tube (20 or 50 μl) was placed over the chelicerae and hypostome to collect the saliva as described by Kaufman (1978). Duration of salivation varied from 10 min for the small ticks to 60 min for the large ticks. Following collection of saliva, the capillary tubes were plugged with Sealease (Clay Adams, Parsippany, NJ) and stored at −35°C. For SDS–PAGE and immunoblot analysis, saliva samples were pooled from ticks in five weight ranges: (1) 30–59 mg; (2) 60–99 mg; (3) 100–151 mg; (4) 152–202 mg; and (5) 203–420 mg. Saliva samples were lyophilized and reconstituted in phosphate-buffered saline (PBS) before use in the SDS–PAGE and immunoblot analysis.

Protein Assay. The Bio-rad Protein Assay Kit (Bio-rad, Richmond, CA) was used to determine the protein concentration of saliva samples; bovine serum albumin (Sigma Chemical, St. Louis, MO) was used as the protein standard.

Preparation and Collection of Sera. Two sera were used for the immunoblot analysis. Rabbit antifeeding tick (RAFT) serum was obtained from one rabbit exposed to *Amblyomma hebraeum* as follows: 20 female ticks plus males were allowed to feed to repletion on a rabbit. Three weeks after initial exposure, the rabbit was reexposed to 20 females. Two weeks after the second exposure, blood was collected by heart puncture. Rabbit antisaliva (RAS) serum was obtained from two rabbits immunized with tick saliva in Freund's complete adjuvant (FCA) and Freund's incomplete adjuvant (FIA) as follows: Pooled saliva obtained from partially fed females of various sizes containing 500 μg of protein was mixed with equal volumes of Dulbecco's PBS (Gibco) and FCA (Gibco) and administered by subscapular and intramuscular injection. Rabbits were boosted 3 wk later with saliva containing 200 μg of protein mixed in equal volumes of Dulbecco's PBS and FIA. At 1–2 wk after the boost, the immunized rabbits were bled, the blood was allowed to cool to room temperature for 30 min, and was refrigerated for 6 h. The clotted blood was centrifuged at 800 × *g* for 20 min. The serum was removed and stored at −80°C before use in the assays. The antibody titers of RAFT and RAS used in the immunoblot analysis was determined using enzyme-linked immunosorbent assay (ELISA), and were 1:8,000 and 1:10,000, respectively. The sera from two RAS rabbits recognized identical profiles of antigens as tested by immunoblot. Consequently, all immunoblot analyses were done using serum from a single RAS rabbit. In addition, preimmune serum from RAFT and RAS rabbits was tested using ELISA and immunoblot analysis and did not recognize pooled tick saliva antigens. All rabbits were cared for and bled according to the guidelines of the Canadian Council of Animal Care.

Gel Electrophoresis. Saliva proteins were separated by SDS–PAGE. Samples of the same protein concentration were prepared as 1 part saliva to 3 parts sample buffer (0.5 *M* Tris, pH 6.8, 12.5%; deionized water, 50%; 20% SDS [10% stock solution]; glycerol, 10%; 2-β-mercaptoethanol, 5%; and 2.5% bromophenol blue [0.05% stock solution]). Samples were boiled for 4 min, then were loaded into wells of a 1-mm thick 5% stacking gel (upper 5 mm) on a 12% resolving gel (Laemmli 1970). The gels were run at 100 V until the dye front cleared the stacking gel, then at 200 V for the rest of the electrophoretic run. After electrophoresis, gels were electrophoretically transferred to nitrocellulose sheets or stained with Coomassie blue followed by silver stain (Bio-rad Silver Stain Kit). The electrophoretic transfer (Bio-rad Blot Kit) was done at 40 V, overnight at 4°C in transfer buffer (25 m*M* Tris base, 192 mM Glycine, 20% vol/vol methanol, pH 8.3). After transfer, nitrocellulose blots were blocked for 6 h in tween-Tris buffered saline (TTBS) containing 2.5% skim milk powder. After blocking, filters were washed 10 min in TTBS and incubated with antisera (either RAFT or RAS) for 18 h. The antisera were diluted to 1:500 in antibody buffer (2.5% milk powder and 1 mM NaN_3 in TTBS; pH 7.5). After the first incubation, the filters were rinsed three times in TTBS and incubated for 6 h with goat–antirabbit IgG alkaline phosphatase conjugate (Bio-rad) diluted to 1:3000 in antibody buffer. Avidin-phosphatase conjugate (also at a dilution of 1:3000) was used to develop the biotinylated high and low molecular weight standards (Bio-rad). Filters were washed three times in TTBS for 5 min, in Tris-buffered saline (TBS) twice for 5 min, and developed using Bio-rad developing solution.

Statistical Analysis. Protein concentration of saliva obtained from the five groups of ticks were compared using one-way analysis of variance (ANOVA) from Statview SE + Graphics software (Abacus Concepts) on a Macintosh computer. Values are reported as mean ± SEM.

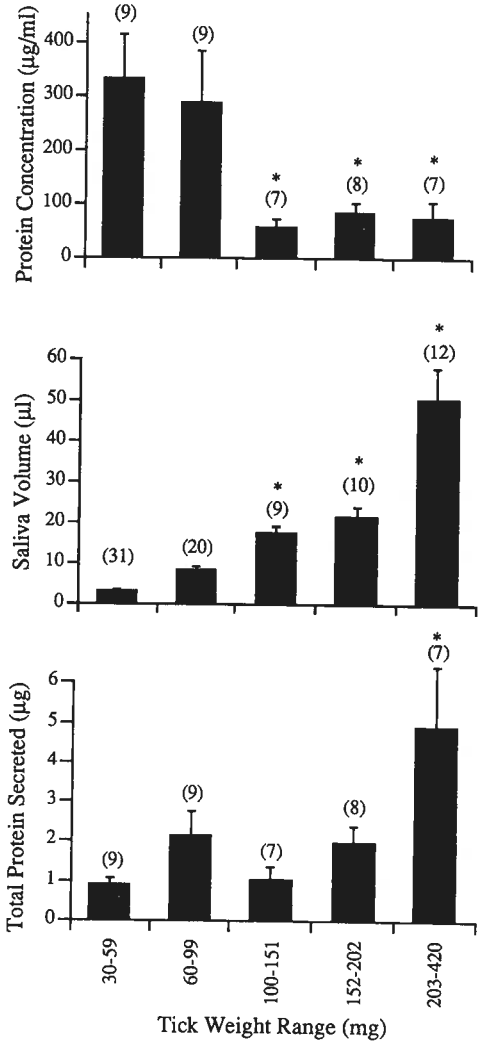

Fig. 1. (A) Protein concentration of dopamine-stimulated saliva from partially fed females as a function of tick weight. (B) Total volume (in microliters) of fluid collected during salivation from the ticks shown in A. (C) Total protein (in micrograms) secreted by the above ticks (calculated as concentration × volume). Mean, SEM, and *n* are indicated for each group. Statistical significance is compared to the 30–59 mg weight range. *, $0.01 < P < 0.05$; **, $P < 0.01$.

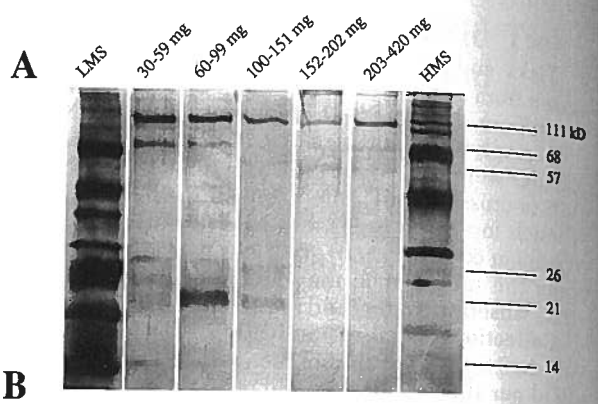

Fig. 2. SDS–PAGE of saliva polypeptides stained with Coomassie brilliant blue followed by silver staining of partially fed females. LMS, Low molecular-weight standards; HMS, high molecular-weight standards. Tick weight range (in milligrams) is indicated above each lane. The lanes were loaded with the same volume of saliva (25 μl) containing equal protein concentrations.

Results

Protein Content of Saliva. Protein concentration in the saliva of small ticks (<100 mg) was 333 ± 83 μg/ml but it fell to 59 ± 14 μg/ml in ticks weighing >100 mg ($P < 0.05$) (Fig. 1A). Fig. 1B demonstrates that the total volume of saliva secreted increased from 3.17 ± 0.29 to 50.5 ± 7.5 μl as the tick engorged. Similarly, when the data of the volume of saliva secreted per unit time (μl/min) were analyzed, the volume of saliva increased from 0.16 ± 0.3 to 1.34 ± 0.13 μl/min as the tick engorged ($P < 0.001$). This seems to account largely for the observed fall in protein concentration, because the total protein secreted did not vary significantly until the tick reached ≈200 mg, and only then appeared to rise ($P < 0.05$) (Fig. 1C).

Polypeptide Analysis of Tick Saliva. Silver stained polyacrylamide gels revealed major protein bands of ≈111, 68, and 21 kD (Fig. 2). Other minor bands were observed at 57, 26, and 14 kD. The 68-kD band was notably absent or faint in ticks weighing >100 mg. The 14-kD protein was detected only in ticks weighing <60 mg. It may be a component of tick attachment cement, because it is secreted in appreciable amounts only by small ticks. The 26- and 21-kD proteins were absent from saliva of ticks weighing >150 mg. These data indicate generally that the number of saliva proteins secreted diminishes as the tick engorges.

Immunoblot Analysis. RAS antiserum recognized one major (108 kD) and three minor (200, 160, and 63 kD) antigens (Fig. 3A). The 63-kD antigen was undetected in saliva of ticks weighing >100 mg, and the concentrations of the other antigens appeared to be lower in the saliva of large ticks.

In contrast to RAS, RAFT antiserum recognized as many as 13 antigens ranging in molecular weight from 23 kD to 200 kD. Major antigens included 77, 66, 59, 41, 39, 31, and 28 kD. Minor antigens included a 200, 35, 34, 26, 25, and 23 kD (Fig. 3B). All antigens were obvious only in ticks weighing <100 mg.

Discussion

The main objective of this study was to identify major saliva proteins and saliva antigens of

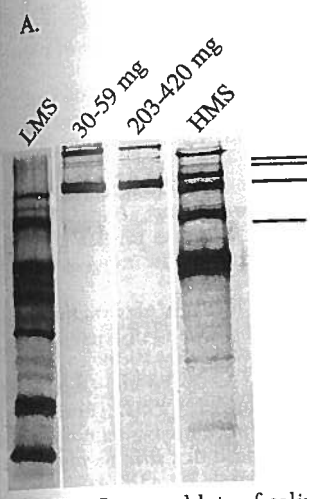

Fig. 3. Immunoblots of sali saliva from a small and a large standards; HMS, high molecula range probed with RAFT antise protein concentrations.

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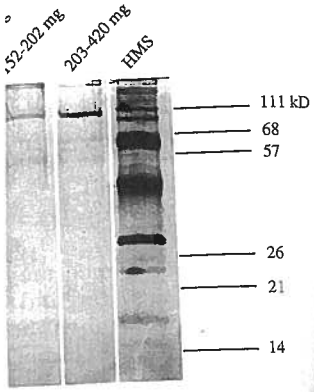
We compared the saliva RAS and RAFT antisera to c of immunization affected th recognize saliva antigens were recognized by RAFT four by RAS antiserum. T that ticks feeding on the h genic substances than un lated to secrete artificially however, that RAFT an against both feeding males RAS antisera were obtaine rabbits with female saliva determined whether some nized by RAFT are presen

The recognition of mo RAFT antiserum may also ferential ability of the host response to immunogens. that the induction of an dependent on the kind an immunogen (Karush 1963) that the route of immuniz moral immune responses 1983). During feeding, the female secrete excess flui back into the host's circul ulate its haemolymph vol sition (Kaufman 1989). Th tial that a considerable



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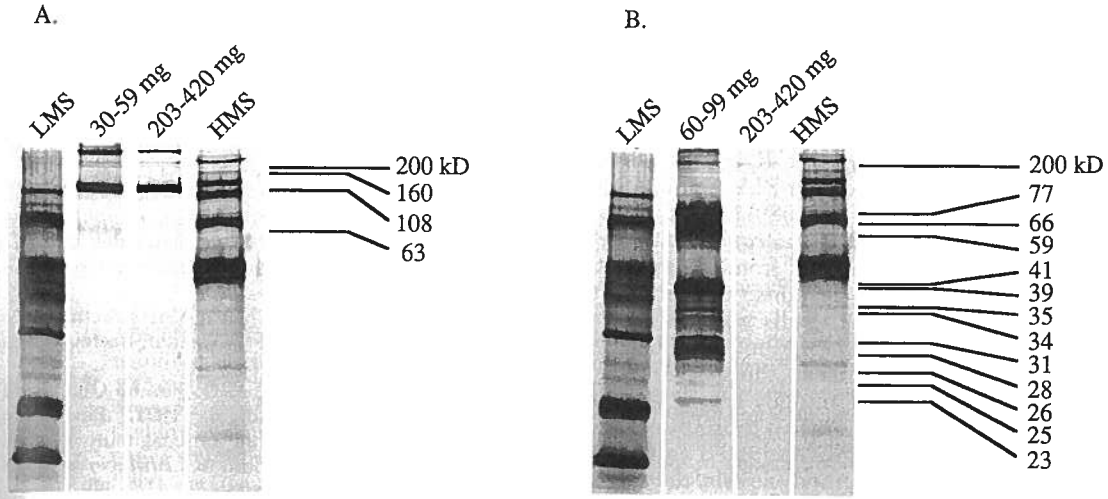

Fig. 3. Immunoblots of saliva from partially fed females using RAS and RAFT antisera. A, Immunoblot of saliva from a small and a large tick weight range probed with RAS antiserum. LMS, Low molecular-weight standards; HMS, high molecular-weight standards. B, Immunoblot of saliva from a small and a large tick weight range probed with RAFT antiserum. Lanes were loaded with the same volume of saliva (25 μl) containing equal protein concentrations.

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Discussion

ective of this study was to iden-
a proteins and saliva antigens of *A. hebraeum*. We also determined the protein concentration of saliva obtained from feeding females of the five weight ranges. As the ticks fed, the protein concentration of their saliva decreased. Lower protein concentration of saliva from large ticks (Fig. 1A) is explained (Fig. 1C) largely by the increased fluid volumes (Fig. 1B).

We compared the saliva antigens detected by RAS and RAFT antisera to determine if the route of immunization affected the ability of the host to recognize saliva antigens. Thirteen antigens were recognized by RAFT antiserum, and only four by RAS antiserum. These results suggest that ticks feeding on the host secrete more antigenic substances than unattached ticks stimulated to secrete artificially. It should be noted, however, that RAFT antiserum was raised against both feeding males and females, whereas RAS antisera were obtained by immunization of rabbits with female saliva only. It remains to be determined whether some of the antigens recognized by RAFT are present in male saliva only.

The recognition of more saliva antigens by RAFT antiserum may also be related to the differential ability of the host to mount an antibody response to immunogens. It is well established that the induction of an antibody response is dependent on the kind and concentration of the immunogen (Karush 1963). It is also well known that the route of immunization affects host's humoral immune responses (Davies & Metzger 1983). During feeding, the salivary glands of the female secrete excess fluid from the blood meal back into the host's circulation, and thereby regulate its haemolymph volume and ionic composition (Kaufman 1989). Thus, there is the potential that a considerable amount of antigens is secreted into the host throughout feeding. Over time, the concentration of these antigens may reach levels in the host's circulation that would trigger an antibody response. Immunization by injection of tick saliva, in contrast, might not contain critical levels of various saliva antigens for the host to mount as complete an antibody response.

Our observations on the recognition of saliva antigens of small ticks by RAFT are surprising, because one would expect that small ticks would have evolved mechanisms to mask their antigens from the host's antibody mediated defenses, particularly during the critical phase of establishment on the host. It is possible that small ticks use the alternate strategy of overburdening the immune system of the host by secreting many substances that are not essential for their survival. The inability of RAFT antiserum to recognize proteins from the saliva of large ticks (Fig. 3B), on the other hand, suggests that this stage of the parasite has evolved the ability to decrease the antigenicity of its saliva. Although the precise mechanism of this disguise remains to be determined, the obvious advantage of this adaptation is prolonged survival of ticks on the host.

Two of the three dominant salivary antigens of *A. americanum* recognized by rabbits (39, 40, and 41 kD) (Brown 1988) were also recognized as major antigens by RAFT in this study (39 and 41 kD). The 65-kD molecule with anticoagulant activity reported in salivary glands of *R. appendiculatus* by Limo et al. (1991) might be the 66-kD molecule recognized by our RAFT antiserum.

The control of ticks by immunological means has met with some success recently. Shapiro et al. (1989) induced resistance to *R. appendiculatus* by inoculating rabbits with a purified 94 kD attachment-cement antigen and Nyindo et al. (1989) immunized rabbits to *R. appendiculatus* by using salivary antigens in FIA. Opdebeeck et al. (1988) and Kemp et al. (1989) immunized and protected cattle against *B. microplus* with membrane components extracted from the midgut of the tick. The immune response of the host resulted in lysis of the gut cells with subsequent severe physiological disruption. Opdebeeck & Daly (1990) reported that the antibody levels of cattle vaccinated with midgut extract antigens were significantly higher than those of naturally infested cattle. Although vaccinated animals appear to have higher antibody levels, they do not necessarily have to recognize as many antigens as infested animals, because antibody diversity does not parallel protection afforded against a parasite. Here, we identified several antigens in the saliva of feeding and partially fed detached *A. hebraeum*, but it remains to be determined whether any of the identified antigens are protective in vivo.

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