NEURAL INVOLVEMENT IN THE CONTROL OF SALIVARY GLAND DEGENERATION IN THE IXODID TICK AMBLYOMMA HEBRAEUM

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SUMMARY

1. We have developed a simple, quantitative assay to monitor salivary gland function in ixodid ticks.
2. Salivary glands of engorged (1·0–3·0 g), or partially engorged (0·40–1·0 g), Amblyomma hebraeum Koch lose virtually all secretory function within 4 days after engorgement.
3. Salivary glands from partially-fed ticks (0·20–0·30 g) lose 75% of their secretory ability by 4 days post-removal, but retain this level of function for at least another 11 days.
4. Partially-fed ticks (0·20–0·30 g) removed from the host for 4 days and then allowed to re-attach and resume feeding for a further 2 days, recover much of their lost function.
5. Cutting the opisthosomal nerves of partially-fed ticks (0·40–1·0 g) inhibits salivary gland degeneration.
6. Excising the seminal receptacle from partially-fed ticks (0·40–1·0 g) inhibits salivary gland degeneration. Replacement of the seminal receptacle permits salivary gland degeneration to proceed normally. The factor from the seminal receptacle appears to be distinct from tick salivary gland degeneration factor (TSGDF; Harris & Kaufman, 1981).
7. Injecting a crude extract of male genital tracts into large, partially-fed ticks (0·40–1·0 g), which had had their seminal receptacles removed, caused virtually complete salivary gland degeneration. Such ticks, when injected with an extract of male salivary glands, showed no such degeneration. This suggests that the factor associated with mating originates in the male and is transferred to the female during copulation.

INTRODUCTION

Female ixodid ticks (Acari: Ixodidea) feed only once as adults, increasing in weight by up to 100-fold. During attachment (7–10 days), periods of blood intake alternate with periods of rest and with periods of fluid excretion. The salivary glands are the organs which secrete the excess fluid back into the host (Tatchell, 1967; Kaufman & Phillips, 1973). Males imbibe much less blood and can feed several times; their

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salivary glands appear not to play a significant role in fluid balance (Kaufman, 1976). Within a few days of engorgement, the salivary glands of females are resorbed (Till, 1961). Autolysis of the salivary gland is controlled by tick salivary gland degeneration factor (TSGDF; Harris & Kaufman, 1981). In this paper we describe a physiological assay for monitoring salivary gland degeneration, we demonstrate the time course for this degeneration, and we illustrate some neural and blood-borne components of the system controlling salivary gland degeneration in *Amblyomma hebraeum*.

**MATERIALS AND METHODS**

**Animals**

Adult *Amblyomma hebraeum* were obtained from a laboratory colony maintained in darkness, at 26 °C and at a relative humidity of 95%. Adult ticks were confined to the backs of rabbits as described by Kaufman & Phillips (1973). Since mating (which takes place on the host) is necessary for complete engorgement, approximately equal numbers of males and females were confined together.

**Assay for secretory competence**

In order to collect salivary glands, each tick was first glued (with a cyanoacrylate compound; alpha aron, Toagosei Chemical Co., Japan) to a strip of adhesive tape which was stuck to the bottom of a small Petri dish. The tick was flooded with tissue culture medium (TCM 199; Gibco) without sodium bicarbonate, buffered (pH 7.2) with 10 mmol L⁻¹ morpholinopropanesulphonic acid (MOPS; Sigma), and adjusted to 360 mosmol L⁻¹ with 2.1 g L⁻¹ NaCl. The dorsum was removed with the aid of a fine razorblade scalpel and the main salivary ducts ligated with 8-0 silk (Davis & Geck). The ducts were severed distal to the ligatures and the glands transferred to fresh medium where they remained for approximately 15 min before further treatment.

Wet weights were measured on a Sartorius 2474 microbalance 60 s after the glands were blotted according to a standard procedure. The glands were then incubated in constantly stirred TCM 199 containing 10 μmol L⁻¹ dopamine (Sigma). This concentration elicits a maximal rate of fluid transport (Kaufman, 1976). Fluid transport, as measured by net increase in wet weight, was taken as an index of secretory competence.

**Nerve tracing**

Ticks were immobilized and eviscerated as described above and bathed in a solution of Janus Green B (0.001% dye in 1.2% NaCl) for approximately 5 min. At this concentration, the stain was reasonably specific for neural tissue in the tick.

**Surgical procedure**

Ticks were suspended in a hypobaric chamber designed so that the operative site remained open to atmospheric pressure (Fig. 1; see also Harris & Kaufman, 1981), thus preventing escape of the gut diverticula.

Large partially-fed ticks (>0.40 g) were fixed in the hypobaric chamber so that the area between the mouthparts and the genital pore was exposed as the operative sit
a semicircular incision was made as shown in Fig. 1. Retraction of the gut diverticula exposed both the synganglion (=CNS), and the seminal receptacle. Either distinct groups of nerves were severed at their juncture with the synganglion or the seminal receptacle was excised after ligating it near the vagina. In some cases the seminal receptacle was replaced within the haemocoel immediately after removal. After surgery, the incision was sealed with glue. Salivary glands were extirpated 4 days later, and tested for secretory competence. Sham-operated controls were surgically opened and closed in the same manner as the experimental ticks.

**Injection of male tissue extracts**

Fed male ticks were immobilized as previously described and flooded with ice-cold 1.2% NaCl. Tissues were removed and homogenized in ice cold 1.2% NaCl (20 μl/male). Homogenates were then centrifuged at 12,000 g for 5 min. The supernatant was removed and diluted to 25 μl per male equivalent. One male equivalent of crude extract was then injected into each subject female which had had its seminal receptacle removed as previously described. Injections were made using 250 μl syringes (Hamilton) fitted with 30 gauge needles, inserted into the haemocoel through the articulation between the capitulum and the scutum. The ticks were left on the needle for 2 min to prevent the loss of extract through the wound upon removal of the needle.

**Electron microscopy**

Salivary glands were dissected out under TCM 199, and the large tracheae
removed. The glands were then fixed for 12 h in 2.5 % glutaraldehyde in 0.05 mol l\(^{-1}\) cacodylate buffer made up in 2.5 % sucrose. After washing in cacodylate buffer, the glands were then post-fixed in 4 % OsO\(_4\)-0.05 mol l\(^{-1}\) cacodylate buffer for 1 h. After washing in buffer, the tissue was stained in aqueous uranyl acetate (2 %) for 20 min. The tissue was then passed through an ascending series of ethanol concentrations followed by two changes of propylene oxide (100 %). Tissues were infiltrated overnight in Araldite 502-propylene oxide (1:1, v/v) and then washed twice with pure Araldite 502. The tissue was then transferred to plastic moulds, covered with Araldite 502 and polymerized at 60 °C for 48 h. Thin sections were taken on a Porter-Blum Ultramicrotome and mounted on copper grids (150 mesh). Grids were stained in 5 % uranyl acetate (in methanol) for 25 min and counter-stained in lead citrate (Reynolds, 1963) for 2 min. The grids were then examined on a Philips 200 transmission electron microscope.

All data are reported as mean ± s.e.m. (N).

RESULTS

In preliminary experiments we established that (1) salivary glands incubated in dopamine-free medium did not gain weight significantly [0.10 mg/15 min ± 0.09 (11)] and (2) the rate of fluid uptake in glands incubated with 10 \(\mu\)mol l\(^{-1}\) dopamine was constant for at least 20 min. Therefore we routinely adopted an incubation time of 15 min so as to remain in the linear portion of the transport curve.

In the first experiment we monitored the loss of secretory competence of salivary glands as a function of time post-repletion (Fig. 2). Partially-fed ticks, weighing 0.20–0.30 g, also lost secretory function over the first 4 days post-removal; however,
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This decline was neither as rapid nor as extensive as that seen in the engorged ticks. Indeed, secretory rates levelled off at a plateau significantly higher than that for engorged ticks, and maintained this level for at least 15 days (Fig. 2). Some partially-fed ticks (0.20–0.30 g) were removed from the host for a sufficient period (4 days) to attain the plateau level of secretory function. When allowed to re-attach to the host and feed for 2 days they regained much of their lost secretory function (Fig. 2).

We next tested salivary glands from ticks of intermediate weights at 4 days post-removal. As shown in Fig. 2, glands from ticks between 0.20 and 0.30 g transported 3.10 mg fluid gland⁻¹ 15 min⁻¹ ± 0.15 (13). Glands from ticks between 0.40 and 1.0 g transported 0.44 mg fluid gland⁻¹ 15 min⁻¹ ± 0.15 (12), a figure which was not significantly different from the secretory rate of glands from engorged ticks [0.30 ± 0.10 (15); P > 0.05]. Glands from females weighing 0.30–0.40 g transported 0.97 ± 0.27 mg fluid gland⁻¹ 15 min⁻¹ (15), significantly less than glands from 0.20–0.30 g partially-fed females (P < 0.05).

We next attempted to inhibit salivary gland degeneration surgically. Since most ticks engorge during the night (Rechav, 1978) there can be a delay of over 12 h between detachment and surgery. Consequently, for these experiments, we used large ticks (>0.40 g) which were still attached to the host. Control glands were taken from partially-fed ticks weighing 0.20–0.30 g. We did not use ticks weighing less than 0.20 g because their salivary glands would not yet have developed maximal secretory ability (Kaufman, 1976; Fawcett, Doxsey & Buscher, 1981).

To determine which, if any, neural components were involved in the release of TSGDF, we severed nerves at their juncture with the synganglion. The structure of the synganglion is given in Fig. 3. Nerves were severed in defined groups (Table 1). The extent of surgery in all experimental cases was confirmed by subsequent dissection.

Severing all the opisthosomal (Groups C and D) nerves resulted in a significant inhibition of salivary gland degeneration (P < 0.05), whereas severing all the cephalic nerves or the pedal nerves did not (P > 0.05; Fig. 4).

In a few ticks, the surgery resulted in bilateral section of the medial group of opisthosomal nerves, but only unilateral section of the lateral group of opisthosomal

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**Table 1. Groups of nerves which were severed in the denervation experiment**

<table>
<thead>
<tr>
<th>Group</th>
<th>Nerves</th>
<th>Organs innervated</th>
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<tbody>
<tr>
<td>A</td>
<td>Cephalic</td>
<td>Palps</td>
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<td></td>
<td></td>
<td>Eyes</td>
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<td></td>
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<td>Chelicerae</td>
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<td></td>
<td></td>
<td>Gené’s organ</td>
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<td></td>
<td></td>
<td>Integument</td>
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<tr>
<td>B</td>
<td>Pedal + haemal</td>
<td>Legs</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Coxal muscles</td>
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<td>Salivary glands</td>
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<tr>
<td></td>
<td></td>
<td>Integument</td>
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<tr>
<td>C</td>
<td>Lateral-opisthosomal</td>
<td>Seminal receptacle</td>
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<td></td>
<td></td>
<td>Genital pore</td>
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<td>Spiracle</td>
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<td></td>
<td>Integument</td>
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<td>D</td>
<td>Medial-opisthosomal</td>
<td>Lateral and medial dorsoventral</td>
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<td>muscles</td>
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"EXB 109"
Fig. 3. Structure of synganglion in *Amblyomma hebraeum*. Major nerve trunks were traced under a dissecting microscope. Terminology follows that of Binnington (1981). Nerves are identified as follows: Pedal nerves (PN), haemal nerves (HN), lateral segmental organs (LSO) and the salivary gland nerve (SGN). The opisthosomal nerves have been divided into two groups: the lateral opisthosomal nerves comprise a nerve to the genital pore (GP), a nerve to the seminal receptacle (SR) and the paraspiracular nerve (PS). The medial opisthosomal nerves innervate the medial dorsoventral muscles (MDVM) and the lateral dorsoventral muscles (LDVM). The cephalic group of nerves serve the palps, eyes, chelicerae, salivary glands and Gené's organ (GO). The synganglion is surrounded by a neurolemma (NL).

nerves. This treatment did not inhibit salivary gland degeneration \[0.52 \pm 0.20 \text{ mg gland}^{-1} 15 \text{ min}^{-1} (8); P > 0.05\] as compared to sham-operated ticks over 0.40 g \[0.44 \pm 0.15 \text{ mg gland}^{-1} 15 \text{ min}^{-1} (12)\]. Bilateral section of either the lateral opisthosomal nerves (group C), or the medial opisthosomal nerves (Group D) alone did not result in an inhibition of salivary gland degeneration (see Fig. 4).

Since copulation is necessary for ticks to feed to repletion (Snow, 1969; Pappas & Oliver, 1971) mating could directly or indirectly influence salivary gland degeneration. Severing the nerves to the seminal receptacle did not inhibit salivary gland degeneration (Fig. 4). We then removed seminal receptacles from partially-fed ticks, 0.40–1.0 g, and either replaced the seminal receptacles of such ticks, or injected crude homogenates of male salivary glands or genital tracts. Salivary glands of those tick
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Fig. 4. Fluid secretory ability of salivary glands extirpated 4 days after various groups of nerves were severed. For a definition of experimental groups see Table 1. It is only by cutting all the opisthosomal nerves (Groups C, D), that salivary gland degeneration could be attenuated. Mean, s.e. and N are indicated for each treatment. Bars under a common solid line are not significantly different. *P < 0.05 (Student's t-test).

Fig. 5. Influence of the seminal receptacle on salivary fluid secretion. Seminal receptacles were excised from mated, large, partially-fed ticks (0.40–1.0 g). In one experiment, the excised seminal receptacles were replaced in the haemocoel. Four days later, salivary gland function was monitored. Complete removal of the seminal receptacle was necessary for attenuation of salivary gland degeneration. In other experiments such females were injected with homogenates of male genital tracts or salivary glands. Only the injection of male genital tract extract resulted in significant salivary gland degeneration. Statistical symbols as in Fig. 4.
which had their seminal receptacles replaced after extirpation transported significantly less fluid than glands from ticks without their seminal receptacles (Fig. 5).

Injection of a crude extract of male salivary glands did not cause a significant loss of function \( [2.2 \pm 0.5 \text{ mg gland}^{-1} \text{ 15 min}^{-1} (10); P > 0.05] \) in large ticks which had had their seminal receptacles removed. However, injection of male genital tract extract into such ticks resulted in a significant loss of function \( [0.80 \pm 0.25 \text{ mg gland}^{-1} \text{ 15 min}^{-1} (13); P < 0.05] \). This level is not significantly different \( (P > 0.05) \) from large \((0.40-1.0 \text{ g})\) unoperated partially-fed ticks (Fig. 4). All ticks were tested 4 days after removal from the host.

**DISCUSSION**

The loss of salivary gland function observed in partially-fed ticks \((0.20-0.30 \text{ g})\) is not due to the same process which causes degeneration of glands in replete ticks, for at least two reasons. First, the characteristic ultrastructural changes associated with degeneration in replete ticks do not manifest themselves in partially-fed ticks \((0.20-0.30 \text{ g})\) for at least 5 days post removal (Fig. 6), even though it is during this period that glands from such ticks lose much secretory function (Fig. 2). Secondly, the cytological disruption which occurs in engorged ticks by 4 days post-repletion would seem to be irreversible. Unfortunately, this conjecture cannot be tested experimentally because ticks over \(0.40 \text{ g}\) will not re-attach and feed once they have been removed from the host. In contrast, \(0.20-0.30 \text{ g}\) ticks removed from the host for a sufficient period of time for their salivary glands to lose secretory function, quickly regain the lost function when given the opportunity to resume feeding (Fig. 2). The cause of this reversible loss of secretory competence in partially-fed ticks remains to be investigated.

From the denervation experiments, we have concluded that a neural component for the release of TSGDF exists in the opisthosomal nerves. These experiments do not reveal whether information carried by the nerves is afferent, efferent, or both. All the opisthosomal nerves, with the possible exception of the nerve supplying the genital tract, appear to terminate in somatic muscle bundles. Unless these muscles also function as release sites for neurosecretory products, it is attractive to suggest that they contain stretch receptors, the afferent signals of which trigger the release of TSGDF. At least one group of stretch receptors has already been tentatively proposed in *A. hebraeum*: stretch receptors which trigger salivary fluid secretion when fluid is imbibed (Kaufman, Aeschlimann & Diehl, 1980; Kaufman & Harris, 1983).

The initiation of salivary gland degeneration is also delayed by extirpation of the seminal receptacle. If the seminal receptacle is replaced, degeneration proceeds normally. Thus a 'mating factor' (MF) from the seminal receptacle appears to be involved.

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Fig. 6. Ultrastructural appearance of the secretory labyrinth in a Type III acinus. (A) Typical appearance of the secretory labyrinth from partially-fed ticks \((0.20-0.30 \text{ g})\) 5 days post-removal. The clear vacuoles \(v\) noticed in these specimens have not yet been described in ticks freshly removed from the host. The function of these vacuoles is not known. (Scale bar, 1 \(\mu\)m; \(\times 35\ 000\)). (B) Secretory labyrinth from salivary gland of an engorged tick \((2.34 \text{ g})\), 2 days post-removal. Arrows denote autophagic vacuoles which are absent from partially-fed ticks \((0.20-0.30 \text{ g})\). Photomicrograph taken from Harris & Kaufman (1981). (Scale bar, 1 \(\mu\)m; \(\times 19\ 000\)).
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In salivary gland degeneration, MF appears not to be TSGDF itself since, (a) the salivary glands of 0·20–0·30 g ticks did not degenerate (Fig. 2) even though these ticks were all mated and, (b) salivary gland degeneration in large ticks (>0·40 g) can be blocked by procedures (e.g. the severing of opisthosomal nerves) which do not remove the seminal receptacle. Salivary gland degeneration still proceeds when nerves to the seminal receptacle have been cut (Group C), indicating that innervation of the seminal receptacle does not mediate the release of MF. The salivary glands of large, partially-fed, virgin ticks (>0·40 g) do not lose secretory function beyond the level characteristic of small (0·20–0·30 g) partially-fed, mated ticks (Kaufman, 1983). Salivary gland degeneration can be initiated in large partially-fed ticks (>40 g), which have had their seminal receptacles removed, by injection of extracts from male genital tracts. The latter indicates that MF originates in the male and is transferred during copulation. This appears not to be a non-specific response to extracts of male tissues, since extracts of male salivary glands did not elicit a response (Fig. 5).

It is not yet understood how MF and the neural information carried via the opisthosomal nerves interact. However, it appears that both the neural information and MF are necessary for salivary gland degeneration. For example, the salivary glands of large, partially-fed, virgin ticks (>0·40 g) do not lose secretory function beyond the level characteristic of small, partially-fed, mated ticks (Kaufman, 1983). We are currently investigating the origin of MF, as well as the nature of the signals carried via the opisthosomal nerves.

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