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Rhipicephalus appendiculatus (Acari: Ixodidae): dynamics of Thogoto virus infection in female ticks during feeding on guinea pigs

W. Reuben Kaufman* and Patricia A. Nuttall

NERC Center for Ecology and Hydrology, Mansfield Road, Oxford OX1 3SR, UK

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Abstract

Engorged nymphs (*Rhipicephalus appendiculatus*) were inoculated parenterally with Thogoto (THO) virus (~1 μl per nymph; 10^6–10^7 PFU/ml). The adult females which resulted were used as the source of infected ticks for this study. Hemolymph, salivary glands, synganglion, gut, ovary, and Malpighian tubule were collected on each day of the blood meal and titrated for THO virus by plaque assay. The percent of tissues infected with virus was 16% or less on the day of attachment. Percent infection rose for all tissues throughout 6–7 days of feeding, reaching 40–100% infection during the rapid phase of engorgement. For the first 4 days of feeding, virus titer in the synganglion was higher than in salivary glands (means of 6.4–34.7 PFU/synganglion and 1.6–8.8 PFU/salivary gland pair). From days 5–7, virus titer was generally higher in the salivary gland than the synganglion (means of 422, 408, and 817 PFU/gland pair and means of 62, 811, and 9 PFU/synganglion). However, because a salivary gland pair is much heavier than a synganglion, the virus concentration in the synganglion was much higher than in the salivary gland during the slow phase of feeding. During the rapid phase of feeding, the difference in virus titer between the synganglion and salivary gland reduced. This difference between the early and late stages of feeding may explain why a previous study [J. Gen. Virol. 70 (1989) 1093], using immunofluorescence and immuno-gold labelling, failed to detect virus in the salivary gland early in feeding. These data provide evidence to explain that *R. appendiculatus* can transmit THO virus within 24 h of attachment, an important epidemiological finding.

Index Descriptors and Abbreviations: Arboviruses, arthropod-borne viruses; PFU, plaque-forming units; *Rhipicephalus appendiculatus* (Acari: Ixodidae); THO virus, Thogoto virus; ticks.

1. Introduction

Successful transmission of arboviruses from vector to host requires that imbibed virus survives in the vector's gut for a period of time, enters the hemolymph through the gut wall and, during a subsequent meal, enters the saliva through the salivary gland epithelium. For certain vectors (most tick species) a molting period, involving extensive tissue histolysis and renewal, frequently intervenes during this whole process. Little is known about the specific mechanisms that permit survival of the pathogen in a vector undergoing molting, and about the vector's cellular mechanisms that influence transmission to the host. These factors probably vary significantly from one vector–pathogen system to another (Nuttall et al., 1994; Schwan, 1996).

In an earlier study, THO virus was not detected in the salivary glands of trans-stadially infected *Rhipicephalus appendiculatus* until the ticks had fed on a host for about 7 days (Booth et al., 1989). Moreover, the latter study suggested that virus resides primarily in the synganglion by the time the adult molt is complete. This observation was in harmony with an assumption that the synganglion probably does not suffer histolysis during the molting period (Till, 1961). Nevertheless, such infected ticks apparently are able to transmit virus to a susceptible host (Syrian hamster) as early as 24 h following attachment (Davies, 1988), and accelerated transmission of THO virus was demonstrated when the feeding of infected ticks was interrupted for a period of up to 28

* Corresponding author. Permanent address: Department of Biological Sciences, University of Alberta, Bio Sci Z606, Edmonton, Alta., Canada, T6G 2E9. Fax: +780-492-9234.

E-mail address: reuben.kaufman@ualberta.ca (W. Reuben Kaufman).

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days (Wang and Nuttall, 2001). How ticks can transmit virus via the salivary secretions during a time that no virus can be detected in the salivary glands was an enigma for some years. Then we observed that virus can pass directly from the hemolymph to the saliva through the salivary gland epithelium independently of viral replication within the tick (Kaufman and Nuttall, 1996); the mechanism of this passage of virus through the salivary gland epithelium remains unknown.

It seems surprising that virus would persist for several weeks in a molting tick and be excluded from the very tissue (salivary gland) that will ultimately be responsible for viral transmission. One possibility is that virus is present in the salivary glands, but the detection system used by Booth et al. (1989; immunofluorescence by light-microscopy and immuno-gold labelling by electron microscopy), although eminently suitable for determining the intracellular localization of virus, may not have been sensitive enough to detect low virus titers in the tissue. Thus we re-examine here the question of transstadial virus persistence, and virus titers in various organs throughout a 7-day feeding cycle on guinea pigs, using a plaque assay to quantify infectious virus.

2. Materials and methods

2.1. Source of virus and infected ticks

Rhipicephalus appendiculatus nymphs were taken from a long-established colony maintained at CEH-Oxford as described by Jones et al. (1988). The Sicilian isolate of THO virus (10^6–10^7 PFU/ml Eagle's minimal essential medium supplemented with 6–10% fetal calf serum) was prepared as described by Davies et al. (1986). Nymphs were fed on guinea pigs (Dunkin Hartley strain) and engorged ticks were inoculated parenterally with virus as described by Davies et al. (1986). When such inoculated ticks molt to adults, almost all of them are infected (unpublished observations).

2.2. Plaque assays

Titration of tissue samples for THO virus was performed by plaque assay in Vero cells at 37 °C for 4 days according to the method of Davies et al. (1986), as modified slightly by Kaufman and Nuttall (2000).

2.3. Feeding of adult ticks

Twenty pairs of THO-virus-infected male and female ticks (up to 1 year post-molt) were confined to the shaved back of each guinea pig by means of a gauze-covered neoprene capsule glued to the back (Jones et al., 1988). Although virus titers were not determined in male tissues, females will not feed to engorgement unless they mate (Kaufman and Lomas, 1996). We studied unfed ticks, as well as ticks that had been allowed to feed for 1–7 days; ticks normally engorged on day 6–7. Ticks were rinsed in tap water before weighing them on a microbalance and attaching them to a disposable petri dish with a cyanoacrylate glue (Locktite) for dissection.

2.4. Media

2.4.1. Modified TC mediuim 199

Commercial formula from Gibco (Long Island New York) containing Hank's salts, plus 2.1 g/L extra NaCl to make it isosmotic to tick hemolymph and buffered to pH 7.0–7.3 with 2.09 g/L MOPS buffer; sterilized by membrane filtration. *Hank's saline* (composition in g/L: NaCl 11.5; glucose 1.6; KCl 0.4; $CaCl_2$ 0.14; $MgSO_4$ 0.1; KH_2PO_4 0.06; Na_2HPO_4 0.06; phenol red 0.01; pH adjusted to 7.1–7.3 with 1 N NaOH). *Virus storage medium*: Eagle's minimal essential medium, supplemented with 10% fetal calf serum, 2% penicillin/streptomycin, and 1% fungizone (all from Gibco).

2.5. Harvesting of tissue

Salivary glands, synganglion, and pieces of gut were harvested from most females on each day of the feeding period, and later in the study we made more limited collections of ovary and Malpighian tubules. The following protocol was used to collect and store the tissue. Females (usually 3 or 4 at a time) were glued to a disposable petri dish and a superficial cut was made in the dorsal cuticle in order to collect some hemolymph in a calibrated glass capillary tube. As the gut ruptures frequently during this process we discarded all hemolymph samples that were contaminated with gut contents. The ticks were then flooded with modified TC medium 199, and the dorsum removed using a microscalpel. As each tissue sample was collected, it was transferred to a petri dish containing fresh TC medium 199 in order to dilute away virus loosely associated at the surface of the tissue. Following the removal of extraneous tissue (usually larger tracheae, fragments of fat body, etc.), tissue samples were transferred once again to a petri dish containing fresh TC medium 199 and gently swirled. A 25–75 μl sample of medium ('wash medium control') was transferred to 160 μl of virus storage medium (kept on ice) in order to assess the amount of virus still loosely associated with the tissue sample. Each tissue sample was then removed from the medium and transferred to ice-cold 160 μl virus storage medium. Immediately following the collection of tissue from each group of 3–4 ticks, the samples were stored at −70 °C. Samples of salivary gland and Malpighian tubule consisted of paired organs. Samples of synganglion contained the single organ of one tick. For very small ticks (fed for

only a few days), most of the gut was taken for each sample. In larger ticks, the gut sample consisted of only a single lobe or two; thus, unlike the case for the other tissues, the virus titers recorded for gut were not normalized in a consistent manner.

2.6. Exposing non-infected tissue to virus ('exogenous virus control')

As a second control to determine the amount of virus only loosely associated with the tissue, the dorsum of a number of uninfected ticks was removed as described above, and the bathing TC medium 199 poured off. About 100 μl of stock virus (~30,000 PFU/ml; stored on ice) were then squirted into the open hemocoel of each tick, such that all the internal tissues made contact with virus. Following 2 min of exposure, the dish was flooded with virus-free TC medium 199, and the dissecting out of tissues was continued as usual. Tissue samples from these ticks were processed and assayed for virus content in the normal way.

2.7. Statistics

Results are reported as means ± SEM (n) unless otherwise reported. Patterns of infection rates in salivary gland, synganglion, and gut were analysed by logistic regression (PROC LOGISTIC, SAS Institute Inc., 1990).

3. Results

3.1. Controls for loosely associated virus

'Wash medium control' was titrated for THO virus in 23 samples and 'exogenous virus control' was titrated for virus in six samples. None of these controls contained a detectable titer of virus. On the basis of these observations, we believe that the virus titers reported in this study represent virus endogenous to the tissue.

3.2. Analysis of percent infection of tissues

The percent of tissues infected with virus from days 0 to 7 of feeding varied among tissue types (Table 1). On day 0, about 14% of the salivary gland pairs were infected. Percent infection rose to the 30–50% range between days 2 and 5 (slow phase of engorgement), and then rose further to 86–100% on days 6–7 (rapid phase of engorgement).

On day 0, about 15% of the synganglia were infected. Percent infection rose to 43% on day 1, and then was maintained at the 33–67% range for the remainder of the feeding period (Table 1).

Only about 7% of gut samples were infected on day 0, but the percent infection rose to the range of 21–35% during the slow phase of engorgement and 74–100% during the last 2 days of feeding (Table 1).

Logistic regression analysis of the data in Table 1 indicated that the changes in percent infection as a function of feeding differed among salivary gland, synganglion, and gut. The interaction term (day * tissue) in the regression model was significant (Wald $\chi^2 = 12.41$, df = 2, $p = 0.002$), indicating differences in infection rates among tissue types. However, most of this effect was due to differences between infection rates in salivary gland and synganglion. When the logistic models were re-run for each combination of two tissues, the day * tissue term remained significant for the salivary gland/synganglion model only (Wald $\chi^2 = 12.36$, df = 1, $p = 0.0004$). The interaction terms in the other 'two-tissue' models were marginally not significant (salivary gland/gut: Wald $\chi^2 = 2.64$, df = 1, $p = 0.104$; synganglion/gut: Wald $\chi^2 = 2.92$, df = 1, $p = 0.087$). The model, using frequency data from all three tissue types, fitted the probability of infection (P_i) to the function defined by:

$$P_i = \frac{[e^{(b_0 + b_1 * \text{day})}]}{[1 + e^{(b_0 + b_1 * \text{day})}]},$$

where $b_0 = -2.43$ (salivary gland); -0.88 (synganglion); -1.93 (gut), and $b_1 = 0.67$ (SG); 0.19 (synganglion); 0.43 (gut).

Table 1
Frequency of THO-virus infection in various tissues as a function of feeding

Day of feeding	Percent of samples infected (n)[a]				
	Salivary gland	Synganglion	Gut	Ovary	Malpighian tubule
0	14.3 (14)	15.4 (13)	7.1 (14)	—	—
1	14.3 (14)	42.9 (14)	21.4 (14)	0.0 (3)	0.0 (4)
2	41.2 (17)	35.3 (17)	29.4 (17)	—	0.0 (1)
3	28.6 (21)	33.3 (21)	45.4 (22)	0.0 (11)	0.0 (7)
4	46.2 (13)	50.0 (14)	33.3 (15)	0.0 (4)	—
5	46.7 (15)	43.8 (16)	35.3 (17)	66.6 (9)	—
6	86.1 (36)	57.1 (21)	73.9 (23)	50.0 (20)	44.4 (9)
7	100 (28)	66.6 (6)	100 (2)	50.0 (4)	40.0 (5)
8 and 9	100 (5)	—	—	—	—

[a] See text for logistic regression analysis on percent infection. Missing data are indicated by a dash.

We had insufficient data to include ovary and Malpighian tubule in the logistic regression analysis, but by visual inspection, the pattern of infection in these tissues seemed substantially different from those of the salivary gland, synganglion, and gut. Ovary and Malpighian tubule remained apparently free of virus throughout the slow phase of engorgement, although the sample sizes were small. Then, percent infection of ovary rose to 50–67% (days 5–7) and Malpighian tubule to 40–44% (days 6–7; Table 1).

3.3. Virus titer of tissue as a function of feeding

Mean virus titer throughout the feeding cycle for all tissues is displayed in Fig. 1. Samples which contained no detectable virus were excluded from the data contributing to this figure. In *R. appendiculatus* it was difficult to collect a sufficient volume of uncontaminated hemolymph from ticks in the slow phase of feeding. In any case, the hemolymph titers of THO virus were so highly variable, that it was uninformative to report them as a function of feeding duration. The mean hemolymph virus titer during the rapid phase of engorgement (data pooled for days 6–9) was 256 PFU/μl ± 101 (SEM); $n = 34$.

3.4. Salivary gland and synganglion

There was a progressive increase in virus titer in both synganglion and salivary gland over the first 4 days of feeding (Fig. 1a). The synganglion rose from about 6.4 ± 0.0 PFU (2) on day 0 to 35 ± 5 PFU (7) on day 4. The salivary gland rose from 1.6 ± 0.0 PFU (2) on day 0 to 6.9 ± 0.9 PFU (6) on day 4. Virus titer of the salivary gland increased by an astounding degree thereafter, attaining 422 ± 233 PFU (7), 408 ± 162 PFU (31), and 817 ± 227 PFU (28) per gland pair on days 5, 6, and 7 respectively. Virus titer continued to rise in the synganglion as well toward the end of feeding, attaining 62 ± 17 PFU (7), and 811 ± 560 PFU (12) on days 5 and 6, respectively. There seemed to be a precipitous drop to 8.8 ± 3.1 PFU (4) on day 7, but this may be an anomaly arising from the small sample size.

3.5. Ovary and Malpighian tubule

Virus was not detected through the slow phase of feeding in ovary and Malpighian tubule (Table 1), although once again the sample sizes were small. Virus was detected in both tissues toward the end of feeding, but titers were significantly lower than in salivary gland, synganglion and gut (Fig. 1a). Thus, on day 6, virus titer for ovary and Malpighian tubule were 46 ± 14 PFU (10) and 8.8 ± 4.2 PFU (4), respectively. The corresponding day 6 values for salivary gland, synganglion, and gut, in contrast, were 408 ± 162 PFU (31), 811 ± 560 PFU (12), and 233 ± 91 PFU (17), respectively.

3.6. Gut

The gut showed a similar pattern to that of the salivary gland and synganglion (Fig. 1b), beginning at 6.4 PFU (1) per sample initially, rising to 9.4 ± 3.3 PFU (10) on day 3, and then peaking at about 200–230 PFU per sample on days 6–7. Note, however, our caution in Section 2 regarding the semiquantitative nature of the data for gut samples. But because only a portion of the gut was taken from ticks during the rapid phase of engorgement, the amount of virus reported here is bound to significantly underestimate the true peak values.

4. Discussion

The time at which an arbovirus appears in the salivary gland of its vector is critically important epidemi-

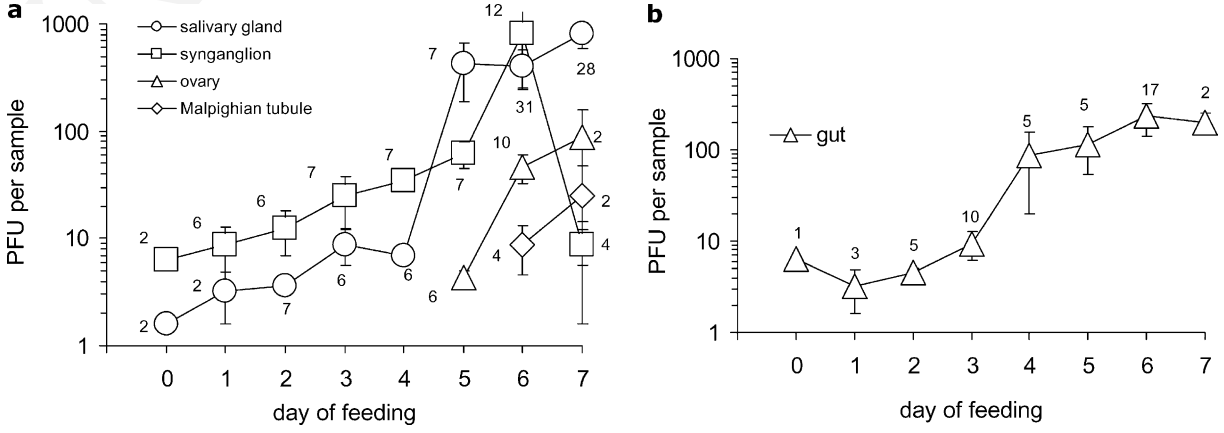

Fig. 1. Amount of virus (PFU) per sample as a function of feeding duration for samples of: (a) salivary gland, synganglion, ovary, and Malpighian tubule as indicated and (b) gut. Means ± SEM and *n* are indicated for each sample.

ologically. This period determines the safety margin during which an infected vector can be detached from a host without the host becoming infected. Booth et al. (1989) suggested that THO virus could not be detected in the salivary gland of unfed, infected female *R. appendiculatus*. Instead, they detected virus primarily in the synganglion prior to feeding. Only towards the very end of the feeding period (day 7) were the salivary glands recorded as virus-positive. Consistent with the observations of Booth et al. (1989), we detected no virus in most of the salivary glands of unfed ticks. Unlike them, however, we found a very similar low percentage of virus-negative synganglia in unfed ticks. Although the percentages were similar, the virus-positive salivary glands were not inevitably found in the same ticks as the virus-positive synganglia; thus, 3 of the 4 infected salivary glands on days 0–1 corresponded to 3 of the 8 infected synganglia on those two days.

As feeding progressed, the frequency of ticks possessing infected salivary glands rose substantially faster than suggested by Booth et al. (1989). Whereas they reported that virus did not appear in the salivary glands until 7 days of feeding, we find here a small but detectable frequency on day 0 (~14%) and a substantial percentage of infected glands (41%) by day 2 (Table 1). The logistic regression analysis demonstrated a highly significant difference between salivary gland and synganglion in the rate of change of virus titer as a function of feeding duration.

The different conclusions between the older study and this one may have resulted from the difference in technique used to detect virus. During the slow phase of feeding, we found 3–5 times more virus in the synganglion compared to the salivary glands (Fig. 1a). These numbers, however, grossly underestimate the relative concentration of virus in the two organs. In *Amblyomma hebraeum*, the pair of salivary glands weighs ~50 times more than the synganglion (Kaufman, unpublished). If a similar weight ratio should pertain to *R. appendiculatus*, and ignoring the possibility that virus may be unevenly distributed within the tissue, the concentration of THO virus in a synganglion was likely to be greater than 100-fold higher than in a pair of salivary glands. As the sensitivity of an immunofluorescence assay is expected to vary with the concentration of antigen, this might explain why Booth et al. (1989) detected virus in the synganglion much earlier than in the salivary glands. The sensitivity of a plaque assay, by comparison, is greater because the amount of infectious virus present is amplified during the assay to enable detection. Toward the end of feeding, virus titer in the salivary gland rose at least 50-fold, and that in the synganglion a more modest 10-fold (Fig. 1a). As a consequence, the difference in virus concentration between the two organs would fall significantly toward the end of feeding, and the likelihood of detection by both assays perhaps would be similar. Also, the sample sizes reported by Booth et al. (1989) were much smaller than those reported here. This factor, coupled with the low frequency of infection during the slow phase of engorgement, may also have contributed to the failure of Booth et al. (1989) to detect virus in the salivary glands early in feeding.

The ovary and Malpighian tubules showed a pattern of virus development that was qualitatively and quantitatively different from that of the salivary gland and synganglion, differences that cannot be explained in terms of differences in tissue weight. Both organs in *A. hebraeum* are considerably heavier than the synganglion, and somewhat lighter than the salivary glands (Kaufman, unpublished). Without knowing which specific factors allow virus to accumulate in the synganglion and salivary gland, it is not possible to speculate on whether these factors are absent in the ovary and Malpighian tubule. However, the low titers in the ovary are consistent with the absence of transovarial transmission of THO in *R. appendiculatus* (Davies et al., 1986).

Although the above-mentioned tissues would all normally become infected via the hemolymph (i.e., baso-lateral cell surface), the gut would normally become infected via the mucosal (apical) surface following feeding. The ticks used in this study were infected by injecting virus into the hemocoel of fed nymphs (Section 2); thus, virus appearing in the gut was presumably through invasion from the serosal (baso-lateral) surface. Although these data clearly indicate that virus can penetrate the gut wall from the serosa, this would not be the normal route.

In conclusion, our data support previous observations that the synganglion is a significant focus of THO virus infection in its tick vector. However, the model presented by Booth et al. (1989) for the dissemination of virus has to be modified in light of the present findings. They hypothesized that during the early phase of feeding, virus gains access to the salivary glands via the nerves, and a focus of infection is established in the salivary glands only toward the end of feeding. This hypothesis failed to explain how a sensitive host (Syrian hamster) could become infected with THO virus within only 24 h of feeding (Davies, 1988). We subsequently demonstrated that THO virus can be transported directly from hemolymph to saliva without need for replication within the salivary gland (Kaufman and Nuttall, 1996), and virus titers in the hemolymph are high (Kaufman and Nuttall, 1996, and present results). From the present results (Table 1) it appears that in many individuals, replication within the salivary gland also represents an important source of virus early in feeding. In many ticks, the salivary glands gain maximal fluid secretory competence during the rapid phase of engorgement (e.g., Kaufman, 1976; Tatchell, 1967), so this period is also likely to be when most virus is transmitted to the host, although data supporting this conjecture are still lacking.

Ticks infected with THO virus secrete ~25% less saliva than uninfected controls (Kaufman and Nuttall, 1996), a reduction attributed to unknown local effects by the virus (Kaufman et al., 2002). The very high concentration of virus in the synganglion raises the interesting, though still untested, possibility that virus might exert significant behavioural effects from this locus as well.

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