Experimental & Applied Acarology, 9 (1990) 79–86
Elsevier Science Publishers B.V., Amsterdam

Organ culture of ixodid-tick salivary glands

W. Reuben Kaufman
Department of Zoology, University of Alberta, Edmonton, Alberta T6G 2E9, Canada

(Accepted 10 April 1989)

ABSTRACT

Kaufman, W.R., 1990. Organ culture of ixodid-tick salivary glands. *Exp. Appl. Acarol.*, 9: 79–86.

This work describes an organ-culture method for isolated salivary glands of the ixodid tick *Amblyomma hebraeum* Koch. The support medium is a TC 199-1% agarose gel with no hormonal or undefined supplements. I used a fluid-transport assay for measuring viability of salivary glands. When cultured at 26°C, female glands secreted fluid at 65% on day 2, 59% on day 4 and 42% on day 6 post-culture compared to glands tested on day 0. Fluid secretory rates in culture were higher than rates of glands from partially fed ticks (about 180–250 mg) on days 2, 4 or 6 post-removal from the host. Salivary glands from fed males cultured for 4 days lost only 5% of their fluid secretory competence, whereas glands dissected from males 4 days post-removal lost 63% of their fluid secretory competence.

INTRODUCTION

For many years, the salivary glands of ixodid ticks have captured the interest of several disciplines in tick biology. Epidemiologists recognize that pathogenic organisms take up residence, and in many cases undergo some development, in the salivary glands (Fawcett et al., 1982). Immunologists recognize the salivary gland as a source of antigens that provoke the immune response against subsequent tick infestation (Brown and Askenase, 1986); moreover, in recent years, they have attempted to develop vaccines against ticks by using immunogens derived from the salivary glands (Shapiro et al., 1987). Physiologists recognize the salivary gland as a vital organ system for osmoregulation and some other functions. For example, in females, the glands secrete excess fluid imbibed with the blood meal (Kaufman, 1983); in the male, saliva is used to lubricate the spermatophore during its transfer through the female genital orifice (Feldman-Muhsam et al., 1970). In both sexes, as well as in the larvae and nymphs, the glands participate in the water-vapour-uptake mechanism in unfed specimens (Knülle and Rudolph, 1982). Finally, cell biologists have been intrigued by the astounding transformations that take place in the female salivary gland during feeding (Fawcett et al., 1981) and

0168-8162/90/$03.50

then after the meal when the glands undergo autophagocytosis (Harris and Kaufman, 1981).

All of these disciplines would benefit from the ability to maintain the salivary glands alive and functional in organ culture. To date, several culture systems have been described. Hoffmann et al. (1970) and Hoffmann (1972) cultured several explanted organs of ticks, and tested their viability by histological criteria. Success in culture depended on degree of engorgement and on whether the tissue was infected with *Babesia bigemina*. Kaufman and Barnett (1977) introduced a method in which the glands were completely isolated. Viability was assessed by measuring fluid secretory competence in vitro. Compared to fluid secretory rate on day 0, the glands secreted at 73% on day 3, 25% on day 7 and 8% on day 14 post-culture.

Bell (1980) introduced a 'backless tick explant' method which proved successful in studying *Theileria* development in *Rhipicephalus appendiculatus* and which was soon adopted by my own laboratory for studying the effects of steroids on salivary-gland function (Harris and Kaufman, 1985; Lindsay and Kaufman, 1988). However, with this technique the glands are not completely isolated from other tissues. Thus, some experimental treatments may exert effects on salivary-gland function only indirectly. Also, Bell's technique requires up to about 10 ml of culture medium to submerge each explant of the large tick *A. hebraeum*. Some of the steroids we use are in short supply, prompting us to look for ways to reduce incubation volume.

For the aforementioned reasons, I attempted to develop a new culture method, the results of which are presented here. The principle was similar to the method of Kaufman and Barnett (1977), but there was significant improvement in viability by changing the substrate supporting the tissue. Whereas before we supported the tissue on stainless-steel wire mesh, in the method described here I use instead an agarose-gel support medium. Also, the hormonal supplements found beneficial in the earlier method seemed not to be necessary in this one, and the total volume of culture medium plus gel is less than 2 ml.

MATERIALS AND METHODS

Preparation of TC 199/agarose-gel culture medium

Purified, low-gelling-temperature (LGT) agarose (Miles Laboratories, Elkhart, Indiana, U.S.A.) was dissolved in hot distilled water to a concentration of 2% (*w/v*), dispensed into 10-ml aliquots and autoclaved. Sterilized gels were stored at room temperature until used.

Double-strength tissue-culture medium 199 (Hank's salts, without $NaHCO_3$; Gibco) was supplemented with 4.18 g/l MOPS buffer plus 4.20 g/l NaCl, and the pH adjusted to 7.1–7.3 with NaOH. All media, when diluted

to final concentration, contained 50 μg/ml gentamicin (Sigma, St. Louis, Missouri) in order to inhibit bacterial growth. When diluted to working strength with the agarose or distilled water, this medium is approximately isosmotic (360 mosmolar) to tick haemolymph. Double-strength TC 199 was sterilized by Millipore filtration. Any heat-labile test substances, such as insulin (Insulin-Toronto; beef and pork; injectable; Connaught-Novo) to be included in a given culture protocol were dissolved (double strength) in the double-strength TC 199 prior to sterile-filtration.

Within a day of setting up cultures, the gelled agarose was liquified in hot water, allowed to cool to about body temperature and mixed 50/50, under aseptic conditions, with warm, double-strength TC 199 containing the desired heat-labile supplements. Steroid or ecdysteroid supplements were added only at this time; they were first prepared in 1000× concentrated stock solutions in 70% ethanol (ETOH) and then diluted to working concentration in the TC 199/agarose-gel.

The warm, liquid TC 199/agarose was taken up in a sterile syringe and dispensed into small, disposable culture dishes (approximately 0.5–1 ml) and allowed to set at room temperature. Salivary-gland explants (see below) were laid upon the gelled medium and a small volume of single-strength TC 199, containing the appropriate supplements, was added so as to just cover the explants, yet allow the top surface of the glands to remain at the gas/liquid interface. All cultures were incubated at 26°C for the time indicated, before testing the glands for fluid secretory competence.

Preparation of salivary glands for organ culture

Salivary glands were dissected out under aseptic conditions as follows: Just prior to dissection, ticks were surface-sterilized by submersion for 1 min, each in 70% ETOH and 0.1% thimerosal (Sigma) and blotted dry. They were then attached, ventral side down, to sterile disposable petri dishes by a drop of cyanoacrylate glue (Alpha Aron; Tagosei Chemical Co., Japan). Ticks were transferred to a sterile air cabinet (horizontal laminar flow) and covered with modified Hank's medium (composition in g/l: NaCl 11.5; D-glucose 1.6; KCl 0.4; $CaCl_2$ 0.14; $MgSO_4$ 0.1; KH_2PO_4 0.06; Na_2HPO_4 0.05; phenol red 0.01; 360 mosmolar; pH 7.1–7.3). The dorsal integument was removed with a razor-blade scalpel and the salivary glands dissected out so as to retain a fair length of the main salivary duct. The glands were washed at least three times with TC medium 199 before placing them into culture.

Assay for salivary-gland viability

Glands from partially fed females are very adept at transporting copious volumes of fluid in vitro, and so I used fluid secretory competence in vitro as

an index of viability. Salivary-fluid secretory competence was measured as described by Harris and Kaufman (1984). In brief, the glands were excised from partially fed females (about 200–400 mg), the main duct ligated, and the net weight increase per gland was recorded following incubation in TC medium 199 containing 10 μM dopamine (a salivary-fluid secretory agonist). There is good reason for not normalizing net weight increase in the assay to initial salivary-gland weight or to protein content. The water content of salivary glands in feeding ticks can exceed 90% (Kaufman, 1976) because of the large extracellular fluid spaces (acinar lumina, canaliculi of the secretory labyrinth, etc.). It thus appears that the wet weight of the tissue is more a reflection of how expanded the extracellular space is at any given moment rather than how much metabolic tissue there is. For example, linear-regression analysis on data from Table 3 (0 days in organ culture, using 32 of the 33 observations) indicates only a weak correlation between rate of fluid uptake and initial wet weight ($r^2=0.138$). Likewise, protein content of this tissue increases dramatically throughout feeding (Shelby et al., 1987); much of this protein is destined for secretion and so presumably is not a direct reflection of the amount of cytoplasm. In such a situation it is also unhelpful to normalize transport rate to a constantly fluctuating protein content. Although one would expect there to be a correlation between transport rate and dry-weight of tissue, in fact the dry-weight of the glands changes rather little in ticks between 180 and 500 mg (Kaufman, 1976). But in any case, interpretation of the data makes most sense physiologically when transport rate is expressed simply on a per-gland basis. For any individual treated with ecdysteroids, the important factor is the quantity of fluid that can be secreted by its pair of glands, and not the secretory ability of any tissue which has not yet succumbed to the hormone.

Glands from fed males secrete at much lower rates than female glands (Kaufman, 1976), yet the assay is sensitive enough to measure transport rate in males, provided one increases the incubation time to 20 or 30 min. Fed males (i.e. those allowed to feed for 4 days or more) were removed from the host and subjected to one of four treatments: (A) glands dissected out and tested on the day of tick removal; (B) males held for 4 days at 26°C before dissecting out and testing the glands; (C) glands cultured for 4 days at 26°C submerged in liquid TC 199; and (D) glands cultured for 4 days on an agarose-gel medium supplemented with insulin plus cortisol. (In this experiment, the medium contained no amphotericin B, the antibiotics were 200 units/ml Penicillin G and 100 μg/ml streptomycin sulphate, and fluid transport was stimulated by 1 μM dopamine.)

RESULTS

Kaufman and Barnett (1977) suggested that supplementing TC 199 with cortisol and insulin was beneficial to culture. Table 1 shows that these supple-

TABLE 1

Effect[1] of hormone supplements to TC 199/agarose-gels on female salivary-gland function following 4 days of organ culture

Treatment	Gland wt. (mg)	Rate of transport (mg/gland/15 min)
No hormonal supplement	5.62±0.20 (16)	8.44±0.56 (16)
0.125 μg cortisol/ml	6.96±0.28 (8) (**)	6.22±0.91 (8) (*)
1.0 μg cortisol/ml	7.22±0.30 (7) (**)	4.98±0.27 (7) (**)
3.0 μg cortisol/ml	7.42±0.29 (6) (**)	5.40±0.90 (6) (**)
100 μ Units insulin plus 0.125 μg cortisol/ml)	4.90±0.25 (25) (*)	5.25±0.46 (25) (**)

[1] *n.s.*, $P > 0.05$; (*), $0.01 < P < 0.05$; (**), $P < 0.01$; compared to no hormonal supplement, by one-way ANOVA. Mean ± standard error and *n* (in parentheses) are reported.

TABLE 2

Effect[1] of hormone supplements to TC-199/agarose-gels on female salivary-gland function following 2 or 6 days of organ culture

Treatment	Gland wt. (mg)	Rate of transport (mg/gland/15 min)
2 days in culture		
No supplement	8.16±0.60 (15)	9.35±0.80 (15)
100 μ Units insulin/ml	9.39±1.75 (6) (n.s.)	11.85±2.27 (6) (n.s.)
100 μ Units insulin plus 0.125 μg cortisol/ml	7.96±0.36 (5) (n.s.)	10.63±0.56 (5) (n.s.)
6 days in culture		
No supplement	7.22±0.61 (13)	6.08±0.69 (13)
100 μ Units insulin/ml	7.57±0.16 (6) (n.s.)	4.02±0.64 (6) (n.s.)
100 μ Units insulin plus 0.125 μg cortisol/ml	7.43±0.24 (5) (n.s.)	5.17±0.64 (5) (n.s.)

[1] n.s., $P > 0.05$; (*), $0.01 < P < 0.05$; (**), compared to no hormonal supplement, by one-way ANOVA. Mean ± standard error and *n* (in parentheses) are reported.

ments did not improve fluid secretory competence after 4 days in culture. On the contrary, cortisol appeared to be somewhat deleterious under these conditions. The results with insulin plus cortisol were similar. The apparent inhibition by insulin plus cortisol on day 4 was not observed for day 2 or day 6 (Table 2). A few cultures were supplemented with insulin alone on days 2 and 6, and these also showed no improvement over unsupplemented medium (Table 2). Salivary-gland function after 0–6 days of organ culture in unsup-

plemented gelled medium is displayed in Table 3. There was no consistent change in gland weight over 6 days in culture. On days 2, 4 and 6, glands secreted 65%, 59% and 42% the rate of day-0 glands.

Results for male glands are presented in Table 4. Day-0 glands secreted 0.57 mg/gland/20 min. After 4 days off the host, male glands lost 63% of their fluid secretory competence. Cultured glands submerged in liquid medium for 4 days lost 77% of their fluid secretory competence, but glands cultured on gelled medium lost only 5% of their fluid secretory competence after 4 days.

TABLE 3

Organ culture of female salivary glands on an unsupplemented TC 199/agarose-gel medium at 26°C for up to 6 days[1]

Days in culture	Gland wt. (mg)	Rate of transport (mg/gland/15 min)
0	5.94±0.25 (33)	14.40±0.80 (33)
2	8.16±0.60 (15) (**)	9.35±0.80 (15) (**)
4	5.62±0.20 (16) (n.s.)	8.44±0.56 (16) (**)
6	7.22±0.61 (13) (*)	6.08±0.69 (13) (**)

[1]n.s., $P>0.05$; (*), $0.01<P<0.05$; (**), $P<0.01$; compared to day 0, by one-way ANOVA. Mean ± standard error and n (in parentheses) ar reported.

TABLE 4

Male salivary-gland function[1] following a variety of treatments

	Treatment	Gland wt. (mg)	Rate of transport (mg/gland/20 min)
(A)	Glands tested on same day as tick removal from host	0.69±0.03 (11)	0.57±0.12 (11)
(B)	Glands tested 4 days following tick removal from host	0.61±0.04 (15) (n.s.)	0.21±0.03 (15) (*)
(C)	Glands cultured for 4 days submerged in liquid TC 199	0.95±0.11 (13) (*)	0.13±0.04 (13) (**)
(D)	Glands cultured for 4 days on TC-199/agarose-gels	0.78±0.07 (14) (n.s.)	0.54±0.14 (14) (n.s.)

[1]n.s., $P>0.05$; (*), $0.01<P<0.05$; (**), $P<0.01$; compared to treatment A, by one-way ANOVA. mean ± standard error and n (in parentheses) are reported.

DISCUSSION

The method described in this study is suitable for studying physiological and developmental events in completely isolated tick salivary glands. When

small partially fed ticks are removed from a host and held at 26 °C for 4 days, they lose approximately 25% of their fluid secretory competence by day 2 and 75% by day 4 post-removal; they then plateau at this level for at least another 10 days (Harris and Kaufman, 1984). This loss of function is reversed on allowing the tick to resume feeding on a host (Harris and Kaufman, 1984), and a possible reason for this decline in function is discussed by Kaufman (1986). The glands cultured in this study fared somewhat better than glands remaining in the tick, losing only 41% of function by day 4 and 58% by day 6 in culture. Although I assume that viability would continue to fall beyond 6 days, certainly over the short term the glands function better after being held in culture compared to remaining in situ.

Cortisol, with or without insulin, did not improve the function of female salivary glands in this study. This result is difficult to explain in light of earlier work demonstrating that several vertebrate steroids improve the viability of glands cultured as 'backless tick explants' (lindsay and Kaufman, 1988). This might lead one to suggest that the site of action of vertebrate steroids is not directly on the salivary gland epithelium. But if that were so, it would then be difficult to explain why cortisol and insulin were found to be beneficial in Kaufman and Barnett's (1977) study, since they also used completely isolated tissue.

The benefit of this culture method to male salivary glands was quite dramatic. They lost almost 63% of their function after 4 days in the intact male detached from its host, but only 5% after 4 days on agarose gels. Thus, the unknown factor which normally causes the decline in male salivary gland function is absent in culture.

ACKNOWLEDGEMENTS

I am grateful to Mr. Stephen Cozens and Mr. Gordon Pellerin for most capable technical assistance. Research in my laboratory is generously supported by an operating grant from the Natural Sciences and Engineering Research Council (NSERC) of Canada.

REFERENCES

Bell, L.J., 1980. Organ culture of *Rhipicephalus appendiculatus* with maturation of *Theileria parva* in tick salivary glands in vitro. Acta Trop., 37: 319–325.

Brown, S.J. and Askenase, P.W., 1986. Characterization of *Amblyomma americanum* - derived salivary gland proteins responsible for the elicitation of host immunity. In: J.R. Sauer and J.A. Hair (Editors), Morphology, Physiology and Behavioural Biology of Ticks. Ellis Horwood, Chichester, pp. 300–328.

Fawcett, D.W., Doxsey, S. and Büscher, G., 1981. Salivary gland of the tick vector (*R. appen-*

diculatus) of East Coast Fever. II. Cellular basis for fluid secretion in the type III acinus. Tissue Cell, 13: 231–253.

Fawcett, D.W., Büscher, G. and Doxsey, S., 1982. Salivary gland of the tick vector (*R. appendiculatus*) of East Coast Fever. III. The ultrastructure of sporogony in *Theileria parva*. Tissue Cell, 14: 183–206.

Feldman-Muhsam, B., Borut, S. and Saliternike-Givant, S., 1970. Salivary secretion of the male tick during copulation. J. Insect Physiol., 16: 1945–1949.

Harris, R.A. and Kaufman, W.R., 1981. Hormonal control of salivary gland degeneration in the ixodid tick *Amblyomma hebraeum*. J. Insect Physiol., 27: 241–248.

Harris, R.A. and Kaufman, W.R., 1984. Neural involvement in the control of salivary gland degeneration in the ixodid tick *Amblyomma hebraeum*. J. Exp. Biol., 109: 281–290.

Harris, R.A. and Kaufman, W.R., 1985. Ecdysteroids: possible candidates for the hormone which triggers salivary gland degeneration in the ixodid tick *Amblyomma hebraeum*. Experientia, 41: 740–742.

Hoffmann, G., 1972. Haltung babesieninfizierter Zeckengewebe in künstlichern Nährmedium. Z. Angew. Entomol., 71: 26–34.

Hoffmann, G., Schein, E. and Jagow, M., 1970. Untersuchungen an extirpierten und in der Kultur gehaltenen Zeckengeweben. Tropenmed. Parasitol., 21: 46–61.

Kaufman, W., 1976. The influence of various factors on fluid secretion by in vitro salivary glands of ixodid ticks. J. Exp. Biol., 64: 727–742.

Kaufman, W.R., 1983. The function of tick salivary glands. In: K.F. Harris (Editor), Current Topics in Vector Research, Vol. 1. Praeger Scientific, New York, pp. 215–247.

Kaufman, W.R., 1986. Salivary gland degeneration in the female tick, *Amblyomma hebraeum* Koch (Acari: Ixodidae). In: J.R. Sauer and J.A. Hair (Editors), Morphology Physiology and Behavioural Biology of Ticks. Ellis Horwood, Chichester, pp. 46–54.

Kaufman, W.R. and Barnett, S.F., 1977. *Dermacentor andersoni* Stiles (Acari: Ixodidae): Organ culture of whole salivary glands in artificial media. Exp. Parasitol., 42: 106–114.

Knülle, W. and Rudolph, D., 1982. Humidity relationships and water balance of ticks. In: F.D. Obenchain and R. Galun (Editors), The Physiology of Ticks. Pergamon, Oxford, pp. 43–70.

Lindsay, P.J. and Kaufman, W.R., 1988. Action of some steroids on salivary gland degeneration in the ixodid tick, *Amblyomma americanum* L. J. Insect Physiol., 34: 351–359.

Shapiro, S.Z., Büscher. G. and Dobbelaere, D.A.E., 1987. Acquired resistance to *Rhipicephalus appendiculatus* (Acari: Ixodidae): Identification of an antigen eliciting resistance in rabbits. J. Med. Entomol., 24: 147–154.

Shelby, K.S., Bantle, J.A. and Sauer, J.R., 1987. Biochemical differentiation of lone star tick, *Amblyomma americanum* (L), salivary glands: Effects of attachment, feeding and mating. Insect Biochem., 17: 883–890.