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Organ culture of *Rhipicephalus appendiculatus* with maturation of *Theileria parva* in tick salivary glands in vitro

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Summary

A technique is described for the organ culture of *Rhipicephalus appendiculatus* ticks. Whole, unfed adult ticks with the dorsal integument removed, known as backless tick explants, were cultured in enriched Leibovitz' L-15 medium in which they remained active for at least 32 days at 28° C and 9 days at 36° C. Development of *Theileria parva*, as demonstrated by methyl green-pyronin staining, occurred in the salivary glands of infected backless tick explants held for 8 days at 28° C or 3 days at 36° C. Maturation in vitro of *T. parva* in backless tick explants was compared with that in cultured excised salivary glands. After 3–7 days at 36° C glands from backless tick explants and excised salivary glands showed similar numbers of infected acini per infected tick. However, after 12 days at 28° C backless tick explants showed 20–30 times as many infected acini per infected tick as excised salivary glands, in two experiments. No assessment was made of degree of parasite maturity or infectivity. It was concluded that both organ culture techniques supported development in vitro of the salivary gland stages of *T. parva*, but the backless tick explant technique was simpler and gave generally better results than culture of excised salivary glands.

Key words: organ culture; *Rhipicephalus appendiculatus; Theileria parva;* salivary gland.

Introduction

Ixodid ticks are vectors of many economically important disease-causing viruses, rickettsiae and protozoa, affecting man and domestic animals. Primary tick cell cultures and established cell lines are used routinely in research on tick-

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borne viruses and rickettsiae (Řeháček, 1972, 1976); cell culture techniques are reviewed by Kurtti and Büscher (1979). Tick organ culture techniques also have potential importance in the study of protozoan haemoparasites, especially *Theileria* and *Babesia* which reach their infective stages in the tick salivary glands. To date, tick organ culture techniques have been mainly concerned with maintenance in vitro of excised salivary glands for physiological studies (Needham and Sauer, 1975; Kaufman, 1976). Salivary glands and other organs of various ixodid ticks have been cultured with the aim of maintaining babesial parasites in vitro (Hoffmann et al., 1970; Hoffmann, 1972), and excised salivary glands of *Hyalomma anatolicum excavatum* have been used in studies of the development in vitro of *Nuttallia danii* (Hadani et al., 1978).

Theileria parva, the causative organism of East Coast fever (ECF) in cattle, also undergoes part of the cycle of development in the salivary glands of the tick *Rhipicephalus appendiculatus*. Tissues from developing adult ticks of this species were first cultured by Martin and Vidler (1962); later Varma et al. (1975) established cell lines from nymphal *R. appendiculatus*. Little has been achieved in the cultivation in vitro of tick stages of *T. parva;* McCall (1977) investigated the effect of salivary stimulants on *T. parva* in excised *R. appendiculatus* salivary glands cultivated for short periods. The salivary gland stages of *T. parva* are of growing interest for the efficient production of sporozoites infective for cattle, which have potential value in control measures against ECF (Cunningham et al., 1973).

This paper describes a new tick organ culture system using explants of whole, unfed adult *R. appendiculatus* ticks with the dorsal integument removed, known as "backless tick explants", which can be used to study the relationship of the developing parasite *T. parva* to its tick host. The system permits the maintenance in vitro of the parasite under conditions closely related to those experienced in naturally feeding ticks. The degree of *T. parva* maturation attained in vitro in backless tick explant cultures has also been compared with that in more conventional excised salivary gland cultures.

Materials and methods

Ticks

R. appendiculatus nymphs, infected by engorging on a calf with a *T. parva* (Muguga) rising parasitaemia of 18–40%, were held at 28° C, 85% relative humidity (r.h.) for 18 days while they moulted to adults, and for a further 10 days to allow them to harden. The ticks were then stored for 1–2½ months at 18° C, 85% r.h. The tick strain was originally established at Muguga, Kenya, but had been selectively bred in Berlin for susceptibility to *T. parva* infection (Schein et al., 1977). An infection rate of 73%, with a mean number of 48.6 infected acini per infected tick, was found by examination of methyl green-pyronin stained salivary glands from 53 ticks of the batch after feeding for 4 days on a rabbit (Walker et al., 1979).

Backless tick explant cultures

Unfed adult ticks harboured moulds in their mouthparts which were not killed by standard surface sterilization procedures. To minimise contamination from this source, ticks were shaken for 2 min in a 0.1% (w/v) aqueous solution of thiomersal (B.D.H. Chemicals, Poole, England), and allowed to dry without rinsing for 24–48 h before use. When required for culture, ticks were washed for 2 min in tap water and transferred to a laminar flow hood (Microflow, Fleet, England) where the remaining procedures were carried out under aseptic conditions. Surface sterilization was achieved by two 1 min rinses in 1% benzalkonium chloride ("Roccal", Winthrop Laboratories, Surbiton-upon-Thames, England), one 30 sec immersion in 70% ethanol, followed by two rinses in sterile distilled water. The ticks were allowed to dry for at least 10 min on sterile filter paper. In a 100 mm glass Petri dish containing histological wax (melting point 57° C) previously sterilized by heating, ticks were embedded dorsal side uppermost, and covered with 30 ml of insect balanced salt solution (Jones and Cunningham, 1961). With the aid of a stereoscopic dissecting microscope ($\times 20$ magnification), a scalpel (Swann Morton Ltd., Sheffield, England, No. 11 blade) and two pairs of watchmakers forceps (Nos. 4 and 5 A), the dorsal integument was removed. The legs and mouthparts were freed from the surrounding wax, any wax adhering to or between the mouthparts was carefully removed, and the now backless tick was transferred to a 35 mm plastic Petri dish (Falcon Plastics, Oxnard, California, USA) containing 1 ml of culture medium. When ten ticks had been dissected they were placed dorsal side uppermost in a 25 cm^2 culture flask (Falcon) containing 1 ml of medium. The tick explants were partly submerged, with their mouthparts in contact with the medium and their exposed viscera kept moist; this allowed free diffusion of oxygen to most tissues through the dorsal surface. Medium was changed weekly in long-term cultures. Backless tick explants were observed either by inverted microscope or, more conveniently, by stereoscopic dissecting microscope ($\times 10$ or $\times 50$ magnification) from above.

Excised salivary gland cultures

Surface sterilization and embedding of unfed, adult ticks were carried out as above. The dorsal integument was removed and the salivary glands dissected out by a modification of the technique of Purnell and Joyner (1968). After excision, the salivary glands were rinsed once in culture medium and placed singly or in pairs in approximately 0.1 ml of medium in wells of a 96-well tissue culture plate (Nunc, Gibco-Europe, Paisley, Scotland). Humidity levels were maintained during incubation by enclosing the culture plate in a sealed polythene bag containing moist tissue paper.

Culture medium and incubation temperatures

A modification of the medium used by Pudney et al. (1973), Leibovitz' L-15 medium supplemented with 20% foetal bovine serum and 10% tryptose phosphate broth (Gibco-Europe), was used in all experiments. Penicillin and streptomycin (Glaxo, Greenford, England) were added to give a final concentration of 100 units and 100 μg/ml, respectively. Cultures were incubated at 28° C or 36° C.

Assessment of T. parva infection rate

Salivary glands were dissected out of cultured backless tick explants, or taken directly from excised salivary gland cultures and stained whole with methyl green-pyronin by the technique of Walker et al. (1979). Mounted preparations were examined for the presence of *T. parva* inclusions using a stereoscopic dissecting microscope ($\times 50$ or $\times 100$ magnification); infected acini were counted.

Results

Characteristics of organ culture systems

The majority of backless tick explants showed resumption of activity 5–30 min after culture initiation. Peristaltic contractions of gut caeca, Malpighian tubules and rectal sac were consistently seen in explants which survived the initial trauma of dissection, and were often accompanied by contractions of the heart and movements of legs and mouthparts. Viability was undiminished for at least 32 days at 28° C; tick survival was affected at 36° C, with death of some individuals and deterioration or "browning" of salivary acini in others after 3–9 days. Guanine deposits in the Malpighian tubules and rectal sac built up to levels greater than those observed in vivo; this was apparently harmless, and supplied evidence of continued metabolic activity in healthy ticks. Some of the explants were seen to imbibe the culture medium, as revealed by microscopic observation of suspended particles being sucked into the submerged mouthparts, and by distension of the gut caeca and rectal sac with a clear brownish fluid. Imbibing commenced between 8 and 48 h after culture initiation and continued for varying lengths of time up to culture termination. Prewashing with thiomersal minimised the problem of fungal contamination; when a mould developed on an explant it only caused deterioration when at an advanced stage, and had no apparent effect on other explants in the same flask.

Excised salivary glands in vitro remained healthy for at least 11 days at 28° C, and for 7 days at 36° C. Contractions of salivary ducts were observed during the first 2–5 days. Salivary glands stained with methyl green-pyronin after 6 days in culture at 36° C appeared similar, both in morphology and in distribution of nucleic acids, to freshly excised glands from unfed ticks.

Maturation of T. parva in tick organ cultures

A control group of 20 unfed infected ticks were examined straight from storage at 18° C; no *T. parva* inclusions were found in their salivary glands when stained with methyl green-pyronin. The presence of stained parasitic inclusions in cultured salivary glands therefore indicated that a degree of parasite maturation had occurred in vitro.

Preliminary experiments with groups of 10 or 20 backless tick explants were carried out to establish the earliest time of appearance of *T. parva* in the salivary glands. After 8 days in culture at 28° C, 28% of ticks showed small theilerial inclusions in the salivary glands; by day 12 the parasite masses occupied more than half of each infected acinus and were more numerous, with 32% of ticks infected. At 36° C, large *T. parva* inclusions were visible in the salivary glands of 30% of ticks on day 3.

An experiment was then carried out to compare *T. parva* maturation in backless tick explant and excised salivary gland culture systems (Table 1). Sample batches of 10 ticks were set up in culture by one of the two techniques;

Table 1. Comparison of maturation of *T. parva* in backless tick explant and excised salivary gland cultures

Type of culture	Incubation temperature °C	Number of days in culture	Number of ticks infected in sample of 10	Mean number of infected acini/ infected tick
Backless tick explants	28	12	9	28.3
Excised salivary glands	28	12	2	1.0
Backless tick explants	28	12	10	51.6
Excised salivary glands	28	12	6	2.5
Backless tick explants	36	3	6	126.0
Excised salivary glands	36	3	5	128.8
Backless tick explants	36	5	7	52.6
Excised salivary glands	36	5	7	94.2
Backless tick explants	36	6	5	52.4
Excised salivary glands	36	6	5	24.4
Backless tick explants	36	7	5	30.0
Excised salivary glands	36	7	5	60.0
Backless tick explants	36	9	4	23.0
Excised salivary glands	36	9	6	7.6

the cultures were incubated at 28° C for 12 days, or at 36° C for 3, 5, 6, 7 or 9 days. The salivary glands were then stained and examined for *T. parva* inclusions and the infection levels recorded as the mean number of infected acini per infected tick (Table 1). At 28° C the backless tick explant technique showed a 20–30-fold greater infection rate than that demonstrated in the excised salivary gland cultures. At 36° C the two methods gave broadly similar results, although some variation was evident between preparations compared after similar periods in culture.

Discussion

The backless tick explant technique for organ culture of *R. appendiculatus* adults has been developed with the primary aim of stimulating and supporting maturation in vitro of *T. parva* in the salivary glands. Culture initiation and maintenance are simple; with weekly medium changes explants remain healthy and active for at least 32 days at 28° C. Microscopic examination from above allows observation of tick behaviour, visceral activity in situ and evidence of continued metabolism over a time period. A culture system using the entire body contents of the adult tick, allowing interaction of the various organs, has potential application in a wider field. In vitro studies of salivation in ixodid ticks have used isolated salivary glands maintained in an artificial medium (Kaufman, 1976; Kaufman and Barnett, 1977); in a backless tick explant the salivary glands are contained within their natural environment. Substances such as

hormones or salivary stimulants could be added to the culture medium and the effect on the entire viscera observed, extending the work of McCall (1977) who has previously used excised salivary gland cultures of unfed adult *R. appendiculatus* infected with *T. parva.* In an attempt to simulate conditions experienced during tick feeding, McCall applied various chemical salivary stimulants to infected glands maintained in an artificial medium for 96 h at 28° C. The presence of 0.05 mM ATP in the culture medium resulted in changes in *T. parva* morphology within infected acini similar to those observed in ticks feeding for 20 h.

Development of *T. parva* to the infective stage normally occurs after 3–5 days feeding on a host (Purnell and Joyner, 1968), though occasionally after as little as 24 h (Martin et al., 1964). Recently, however, it has been shown that mature infective parasites develop in *R. appendiculatus* ticks held without feeding at 37° C for 6–10 days (Young et al., 1979). Using the organ culture systems described here in vitro development of *T. parva* in unfed ticks was evident after 3 days at 36° C, and after 8 days at 28° C. Hadani et al. (1978) reported development of *N. danii* in cultured salivary glands of *H. a. excavatum* nymphs only after the ticks had fed for 2 days on a host.

Of the two culture systems examined, the backless tick explant technique was simpler in terms of initiation and maintenance, and resulted in generally better survival of salivary glands. At 28° C, maturation of *T. parva* in a limited number of backless tick explants was superior to that in excised salivary gland cultures, both in numbers of infected ticks and in numbers of infected acini demonstrated. In preliminary experiments with backless tick explants a total of 190 ticks were cultured for between 5 and 14 days at 28° C; 30% of these ticks showed development of *T. parva* in vitro. At 36° C parasite development, as shown by the staining technique used, was apparently similar in the two culture systems; however, no assessment was made of the infectivity of parasites thus produced. Both culture techniques are currently being used in studies on the maturation of *T. annulata* in *H. a. anatolicum* ticks in vitro and tests are being carried out on the infectivity of *T. parva* and *T. annulata* parasites produced in such systems.

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