Primary Culture of Developing Adult Tissue in a Nymphal Tick, *Haemaphysalis longicornis* (Acarina: Ixodidae)

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Primary cultures of the developing adult tissues in nymphs of *Haemaphysalis longicornis* were described. All cultures were incubated at 25°C in Leibovitz L-15 medium, pH 7.0, supplemented with 10% tryptose phosphate broth and 10% fetal bovine serum. The migrated cells from explants were of mostly fibroblast-like, epithelial-like or freely suspended type. They started to migrate within 24 hr after the culture was set up and formed cell networks by 30 days later. The cultures could be maintained for about 100 days. Sub-culturings of migrated cells, however, were unsuccessful and the cells began to degenerate resulting in remarkable reduction of their numbers after about 1 month.

INTRODUCTION

The developments in tick cell cultures have been reviewed by KURTTI and BÜSCHER (1979). The application of tick cell cultures to tick borne diseases has so far been restricted to studies of the arboviruses (ŘEHÁČEK, 1976; PUDNEY et al., 1978) or to protozoa and rickettsia. However, BELL (1983) showed recently that tick cell culture is an effective tool with which to study the development of theilerian protozoa. There are no records of culturing cells or tissues of *Haemaphysalis longicornis*, although this species is the most important vector of bovine theileriosis and babesiosis in Japan (ISHIHARA, 1968). The present study was undertaken to establish a good *in vitro* system to investigate these diseases. This report describes primary cultures of developing adult tissues from nymphal *H. longicornis*.

MATERIALS AND METHODS

Rabbits. Immature commercial domestic rabbits were used as hosts for infestation.

Ticks. The colony of *H. longicornis* used in the present study was a parthenogenetic race which had been collected in Okayama Prefecture, Japan, reared and maintained by feeding on rabbits in this laboratory since 1961. Nymphal ticks were kept at 25°C under 100% RH by feeding on rabbits until dissection for cell cultures. Nymphs 4 to 11 days after engorgement were used as the source of culture explants.

Medium. According to VARMA et al. (1975), Leibovitz L-15 medium, supplemented with 10% tryptose phosphate broth and 10% fetal bovine serum (L-15) was used for

culturing cells. One ml of an antibiotics mixture (1 g of dihydrostreptomycin, 100,000 IU of penicillin G potassium salt, 1 g of kanamycin sulfate and 0.1 g of Novobiocin in 100 ml of distilled water) was added to 100 ml of the medium and the pH was adjusted to 7.0. The medium was sterilized by filtration.

Dissection. The surface of ticks was sterilized by rinsing in 70% ethyl alcohol for 3 min and by double washing in distilled water. Individual ticks were fixed with a fine entomological needle to a small piece of cork which had been embedded in paraffin wax and then sterilized with 70% ethyl alcohol. The tick on the cork strip was covered with sterilized Carlson solution (SCS) (CARLSON, 1946) and the dorsal integument was removed by cutting the mid-line between the tick's dorsal and ventral parts. The alimentary canal, Malpighian tube, and trachea were also removed using needles and forceps. The remaining tissues in the ventral part of the tick consisted of the salivary glands, muscles, central ganglion and the hypoderm elements of a developing adult (ŘEHÁČEK, 1965; MOULTON, 1978). These tissues from individual ticks were dissected and pooled in SCS. They were cut into small pieces with needles in a few drops of L-15 after thorough washing in SCS. The tissue fragments were transferred to culture tubes (15×35×15 mm) and then 1 ml of L-15 was added to these tubes. Tissues from 10 to 20 nymphs were usually used to set up one culture. The cultures were maintained at a stationary state and 25°C. Change of the medium, that is, replacement of a half volume of used medium with fresh medium, was made at weekly intervals.

RESULTS

Results of observation are summarized in Table 1. In all cultures, explanted tissue fragments attached to the glass surface of culture tubes within 24 hr. The migration of numerous polymorphic cells containing many granules was observed around explants within 24 hr.

In cultures of tissues from nymphs 4 days after engorgement, many fibroblast-like cells began to migrate around the 4th day after incubation, and numbers of freely suspended cells (30 to 50 μ m in diameter) appeared from explants after about 14 days (Fig. 1). Vigorous growth of these cells continued for 2 to 3 weeks after their appearance. The number of cells increased and some of them aggregated (Fig. 2); subsequently characteristic cell networks were formed in some cultures.

In cultures of nymphs 5 days after engorgement, slender elongated fibroblast-like cells (about 200 μ m in length) started to grow after about 6 days. This type of cell increased gradually in number and survived for about 40 days (Fig. 3). The freely suspended spherical cells began to migrate about 14 days after inoculation. A coarse

Table 1. Summarized results of primary cultures of *Haemaphysalis longicornis*

Explants, days after engorgement	Number of cultures	Days of most active proliferation after incubation		
		Fibroblast- like cells	Epithelial- like cells	Cell networks
4	5	4-5	14	28-35
5	8	6	14	30
6	5	2-7	14	50-60
7-11	8	No active proliferation	No active proliferation	No active proliferation

Figs. 1–4. Phase contrast photography of primary cultured cells from *Haemaphysalis longicornis*.

Fig. 1. Numerous number of freely suspended cells migrated and proliferated from explants: 14 days after incubation.

Fig. 2. A vigorous growth of small epithelial-like cells: 26 days after incubation.

Fig. 3. Slender elongated fibroblast-like cells: 34 days after incubation.

Fig. 4. A cell network: 29 days after incubation.

cell network was formed around the 30th day.

In cultures from nymphs 6 days after engorgement, fibroblast-like cells with a granulated cytoplasm were observed from the 2nd to 7th day. Slender fibroblast-like cells with a clear cytoplasm appeared by the 14th day and freely suspended small spherical cells began to increase in number at about the same time. Many epithelial-like cells containing granules attached to each other and/or to other types of cells to form a characteristic cell network from the 50th to 60th day after incubation (Fig. 4).

In cultures of nymphs 7 to 11 days after engorgement, a few small fibroblast-like cells with granules and small polymorphic epithelial-like cells started to migrate on the 2nd day. Their growth, however, ceased after a few days.

All cell networks which appeared in cultures of nymphs 4, 5 and 6 days after engorgement showed degenerative changes after 60 days cultivation. When cell networks were formed and the number of the cells had sufficiently increased, part of these cells were transferred to a new culture tube as an attempt to subculture them. The active outgrowth of transplanted cells was observed temporarily but such cell proliferation was not continuous. After a few days these cells began to show degenerative changes such as heavy granulation.

DISCUSSION

Although subcultures were not successful, the primary cultures of developing adult tissues could be maintained for about 100 days in this study. MARTIN and VIDLER (1962) described that nymphs 4 to 7 days after engorgement seemed to be most suitable for the preparation of tissue culture of *Rhipicephalus appendiculatus*. Results of the present study on *H. longicornis* were in good agreement with their report. Since the cultures of tissues from nymphs 4 to 6 days after engorgement showed a good cell proliferation, it was considered that the developing adult tissues around this period might be able to metabolize the nutrients in the culture medium most actively. The very thin veil-like adult cuticle which enclosed the whole body of developing adult was sometimes observed at the dissection, but the cellular outgrowth from such tissues *in vitro* was poor and not continuous.

It was difficult to determine the exact origin of the migrated cells showing various sizes and shapes. Predominant cells, however, were classified as the following three types; spindle shaped fibroblast-like cells, round or irregular shaped epithelial-like cells and freely suspended cells. The former two types of cells were major components of cell networks formed in cultures.

Almost all the established cell lines of ticks were derived from the embryonic cells (PUDNEY et al., 1973; HOLMAN and RONALD, 1980; YUNKER et al., 1981) and exceptionally VARMA et al. (1975) succeeded in using the developing adult tissues in engorged nymphs. They employed Leibovitz L-15 medium (LEIBOVITZ, 1963) for their cultivation of *R. appendiculatus*. Therefore, this medium was used as a basal medium in this study. The fetal bovine serum and tryptose phosphate broth were supplemented to this medium since KURTTI et al. (1982) demonstrated their growth promotion effects on tick cells. Subcultures were unsuccessful in this study, and it might be necessary to improve culture media and a technique of subculture for this purpose. However, survival of *H. longicornis* cells for more than 3 months *in vitro* will permit the *in vitro* study of sexual stages of theilerian parasites transmitted by this tick species.

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