Hemocyte Types and Their Primary Cultures in the Argasid Tick, *Ornithodoros moubata* MURRAY (Ixodoidea)

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The hemolymph of adult *Ornithodoros moubata* was found to contain 5 types of hemocytes, namely, prohemocytes, early plasmatocytes, advanced plasmatocytes, early spherule cells and advanced spherule cells.

Primary cultures of hemocytes were successfully maintained in MITSUHASHI and MARAMOROSCH's and ŘEHÁČEK and BRZOSTOWSKI's culture media for up to 8 months by replenishing these media at 10 day intervals. The hemocytes floated freely in the media a few days after initiation of the cultures. Although no apparent cell proliferation occurred, the composition of the cell population changed considerably during cultivation; the ratio of prohemocytes increased gradually in both media and that of spherule cells decreased significantly.

INTRODUCTION

Unlike insects, continuously propagated lines of cells originating from ticks have not yet been established. Ever since ŘEHÁČEK (1958) reported on the culture of tissues from developing adults of *Dermacentor marginatus*, many investigators (ŘEHÁČEK, 1962, 1965; ŘEHÁČEK and HANA, 1961; MARTIN and VIDLER, 1962; VARMA and WALLERS, 1965; YUNKER and CORY, 1965, 1967; ŘEHÁČEK and BRZOSTOWSKI, 1969; HOFFMANN et al., 1970; CORY and YUNKER, 1971; MEDVEDEVA et al., 1972; PUDNEY et al., 1973) have attempted to initiate primary cultures of various tick cells. The potential application of these tick cell cultures in the cultivation of viruses and rickettsiae has been reviewed by ŘEHÁČEK (1971, 1972).

Until recently, there has been only one published record of an attempt to culture tick hemocytes (CORY and YUNKER, 1971) although several stable cell lines of hemocyte origin have been established in insects, e.g. *Chilo suppressalis* (MITSUHASHI, 1967), *Samia cynthia* (CHAO and BALL, 1971) and *Malacosoma disstria* (SOHI, 1971). There are considerable advantages in hemocyte culture as there is no need for skillful microdissection and it is easy to obtain sterile material. This report describes primary cultures of hemocytes of the argasid tick, *Ornithodoros moubata* MURRAY, and the changes in hemocyte cell types in the course of culture.

MATERIALS AND METHODS

Ticks. Adult *Ornithodoros moubata* used in the present study were from a colony that had been maintained in our laboratory since 1970. It originated from Africa

but has been maintained in the Institute of Medical Science, University of Tokyo.

Body weight, hematocrit value, differential hemocyte counts, volume of collectable hemolymph and the degree of facility in sampling hemolymph from the following tick physiological stages were examined;

“Unfed males or females” which had not been exposed to a host after moulting from nymphs.

“Engorged+1 day males or females” which had been held for one day after the first feeding at a temperature of 25°C and relative humidity of 50 to 60%.

“Engorged+3 day males or females” and “engorged+7 day males or females” which had been held as above for 3 and 7 days after the first feeding.

On the basis of the results of the preliminary examinations “engorged+1 day females” were selected as a suitable hemolymph source for cultivation in the present study.

Rabbits were used as hosts, and ticks were allowed to engorge by the feeding method previously reported (KITAOKA, 1971).

Hemolymph. The surface of ticks were sterilized by immersing and washing in 70% ethyl alcohol for 5 min followed by brisk rubbing between sterile gauze pads. Ticks were bled by amputating the tibial-tarsal segments of one or more legs with forceps under a dissecting microscope. Five to 20 μ l of exuded hemolymph was collected in a sterile micropipette (Microcaps®, DRUMMOND Co. U.S.A.). Two to 3 μ l were immediately transferred to a glass slide and a thin film was spread with a cover slip, air dried, fixed in acetone for 3 min, stained with Giemsa solution for 30 min and rinsed in running water. A hundred to 400 differential hemocyte counts were made according to DOLP's classification (DOLP, 1970).

For determination of hematocrit values, hemolymph was collected in a 20 μ l micropipette, and the opposite end was heat-sealed. Samples were centrifuged at $13,000 \times g$ for 5 min. Measurement was made by placement of the sample on a glass microscale and observation under a stereoscopic microscope. The hematocrit value was expressed as the percentage of packed cells in a given volume of hemolymph.

Culture medium. MITSUHASHI and MARAMOROSCH's (M.M.) culture medium (MITSUHASHI and MARAMOROSCH, 1964) and a modification of ŘEHÁČEK and BRZOSTOWSKI's (R. B.) culture medium (ŘEHÁČEK and BRZOSTOWSKI, 1969) using fetal bovine serum were employed in the present study, rather than hemolymph from *Antheraea pernyi*.

Preparation for culture. Culture vessels consisted of two glass slides (30 $\times$ 30 mm) and one glass ring (25 mm in diameter; 5 mm in height); these were partially assembled just before use according to the method of MITSUHASHI and MARAMOROSCH (1964). Approximately 0.5 ml of the culture medium was pipetted into an open vessel, followed by the addition of about 20 μ l of aseptically collected hemolymph. The upper edge of the glass ring was sealed with silicone grease, and a pre-heated glass slide positioned upon it to serve as a cover. Completely closed culture systems were thus prepared and the vessels were incubated at a temperature of 25 to 28°C. About 0.2 ml of the culture medium was replenished once every ten days.

Observations. During cultivation living hemocytes were observed daily under a phase-contrast microscope; fixed and stained (Giemsa) hemocytes were observed with an ordinary light microscope. In order to evaluate hemocyte growth the following procedure was employed from the time cultures were initiated until the 11th

day: equal volumes of an aqueous 0.02% crystal violet and 1 M citric acid solutions were added to the culture medium, and after mixing thoroughly with an injector a sample was introduced into a Neubauer hemocytometer and the number of hemocytes per ml of medium was counted.

RESULTS

Examination of ticks as the suitable hemolymph source

Male ticks. Fig. 1 shows changes in body weight, collectable volume of hemolymph, hematocrit value and the percentage of differential hemocyte counts of both unfed and post-engorged male ticks. The body weights of male ticks increased to 3 times after feeding and gradually decreased with time. The collectable hemolymph volume of unfed ticks was greater than that of post-engorged ticks. Hematocrit values for the unfed state were 2 to 7 times higher than those for the post-engorged condition.

The hemolymph of *O. moubata* males contained the following 3 basic types of hemocytes as classified according to their general structure and staining properties; prohemocytes, plasmatocytes and spherule cells. The latter two were subdivided into two variants (Figs. 2–6).

Prohemocytes were relatively small, round to slightly ovoid and distinguished by

Fig. 1. Changes in body weight (○), collectable volume of hemolymph (◐), hematocrit value (●) and percentage of differential hemocyte counts of male *O. moubata* in various physiological states. Each circle and vertical line indicate mean and standard deviation, respectively.

Fig. 2~6. Five types of hemocytes of *O. moubata*. Giemsa, 2,000 $\times$.
 2. Prohemocyte; 3. Early plasmatocyte; 4. Advanced plasmatocyte;
 5. Early spherule cell; 6. Advanced spherule

a high nuclear-cytoplasmic ratio. The average cell size measured 4.9 ± 0.9 by 5.7 ± 0.9 μm with average nuclear dimensions of 3.7 ± 0.4 by 4.0 ± 0.5 μm .

Early plasmatocytes contained from 1 to 3 cytoplasmic vacuoles of various sizes. The average cell size was 6.2 ± 1.3 by 7.5 ± 1.3 μm with a nucleus having average dimension of 4.4 ± 0.7 by 5.0 ± 1.2 μm .

Advanced plasmatocytes were usually larger and possessed a lower nuclear-cytoplasmic ratio than early plasmatocytes; furthermore, they were seen to contain 4 or more vacuoles. Cells averaged 9.3 ± 1.5 by 12.4 ± 2.9 μm in sized with average nuclear dimensions of 5.9 ± 0.9 by 6.4 ± 0.9 μm .

Early spherule cells were characterized by the presence of a few distinct, purple staining spherules in the cytoplasm. The average cell size was 7.8 ± 1.8 by 14.7 ± 4.1 μm with a nucleus averaging 3.5 ± 0.5 by 4.1 ± 0.4 μm .

Advanced spherule cells contained extremely dense spherules to the extent that the cytoplasm and/or nucleus were often obscure. These cells had average dimensions of 9.1 ± 1.7 by 14.4 ± 3.6 μm while nuclei could not be measured due to their obscurity.

Regarding the relative percentage of differential hemocyte counts, plasmatocytes usually occupied a larger portion of the total cell population (40 to 80%) than did the other cell types for all physiological states. The percentage of early plasmatocytes was high in the unfed state while that of advanced plasmatocytes displayed on increased after engorgement. Prohemocytes and spherule cells each comprized about 10% of the total in the unfed state, but the ratio increased after engorgement (Fig. 1).

It was relatively difficult to collect hemolymph from males under the dissecting microscope as male ticks in all physiological states showed active movement.

Female ticks. Fig. 7 shows changes in body weight, collectable volume of hemolymph, hematocrit value, and percentage of differential hemocyte counts of both unfed and post-engorged female ticks. Female body weight increased to about 5 times after feeding and showed a gradual decrease similar to that observed for males. The average collectable hemolymph volume of unfed females was about 6.5 μl and that of the "engorged+1 day females" was about 15.5 μl , which was twice that of the unfed ones. This volume decreased gradually after engorgement. The average hematocrit value in the unfed state was nearly 9 times that of the post-engorgement states.

Fig. 7. Changes in the body weight (○), collectable volume of hemolymph (◐), hematocrit value (●) and percentage of differential hemocyte counts of *O. moubata* females in various physiological states, respectively. Each circle and vertical line indicate mean and standard deviation, respectively.

The hemolymph of females also contained the 5 types of hemocytes as described for males. The percentage of plasmatocytes was always the highest among the 3 basic cell types, and early plasmatocytes comprised a maximum of about 72.6% in the unfed state. The ratio of prohemocytes and spherule cells also increased after engorgement. Following engorgement females were not very active, and consequently hemolymph sampling from these ticks was rather easily accomplished.

Cultivation of hemocytes.

Cell growth. Fig. 8 shows changes in the density of hemocytes after inoculation of 5 μ l of hemolymph from "engorged+1 day females" into 0.5 ml of both the M.M. and R. B. culture media over a period of 11 days after commencement of the culture.

The hemocyte number in the M.M. medium did not appreciably increase during the first 24 hours following inoculation, but multiplied about twice by the 3rd day. Thereafter, a gradual decrease was observed and by the 11th day the density was about the same as that of the initial culture. The hemocyte number in the R.B. medium maintained a nearly constant level of about 2.0×10^5 per ml of medium throughout

Fig. 8. Changes in the hemocyte number per ml of medium, when 5 μ l of hemolymph from *O. moubata* females were cultivated in 0.5 ml of MITSUBASHI and MARAMOROSCH's (○) or ŘEHÁČEK and BRZOSTOWSKI's (●) culture medium for 11 days. The females used were at a temperature of 25 °C and 50 to 60% relative humidity for one day after the first feeding. Each circle and vertical line indicate mean and standard deviation, respectively.

the 11-day observation period.

Percentage of differential hemocyte counts. Fig. 9 shows changes in the percentage of differential hemocyte counts during the culture/experimental period (11 days or 8 months), when 20 μ l of hemolymph from the "engorged+1 day females" were inoculated in either 0.5 ml of the M.M. or R.B culture medium. The percentage of prohemocytes in both media increased gradually. In the M.M. medium the ratio of prohemocytes increased significantly about 3 months after inoculation (Fig. 10), and some cultivation vessels were observed to contain almost exclusively this kind of cell after 5 months (Fig. 11). In general, plasmatocytes and prohemocytes were both observed during the 8 month period in both media (Fig. 12).

Spherule cells were numerous even after a few days following inoculation in the M.M. medium (Fig. 13), but existed only in low numbers even 5 minutes after inoculation in the R.B. medium.

A characteristic cell network consisting mainly of plasmatocytes and some spherule cells was observed in the R.B. medium about 7 days after inoculation (Fig. 14). This network, however, was not apparent in the M.M. medium; even in those few cases where a small network was formed, it was observed to disappear 3 days after inoculation.

Almost all hemocyte began to float 5 days after inoculation in the M.M. medium (Fig. 15) and settled to the bottom of the culture vessels thereafter. These hemocytes were cultivated up to 8 months. Similarly, a floating state of cells in the R.B. medium was initially observed 10 to 20 days after inoculation and continued during the 8 month experimental period.

Additionally, subcultures in both media were carried out 2 and 4 months after

Fig. 9. Changes in the percentages of differential hemocyte counts during cultivation, when 5 to 20 μ l of hemolymph from *O. moubata* females in 0.5 ml of ŘEHÁČEK and BRZOSTOWSKI's (A) or MITSUHASHI and MARAMOROSCH's (B) culture medium. Although hemocytes were cultivated in ŘEHÁČEK and BRZOSTOWSKI's medium for 8 months, cell classification was not carried out after the 11th day of cultivation.

inoculation, but these attempts failed because of bacterial or fungal contamination of the media.

DISCUSSION

Hemocytes of argasid ticks have been studied for *Argas persicus*, *A. arboreus* and *Ornithodoros papillipes* (BALASHOV, 1968; DOLP, 1970), and cells of each species have been given different classifications by both investigators. Despite minor discrepancies in the systematic order, there is an agreement in that all investigators concur that hemocytes should be classified into 3 major categories. In the present study, hemocytes of adult *O. moubata* were classified into 3 major groups, i.e., prohemocytes, plasmotocytes and spherule cells, based on Giemsa-staining properties. Furthermore, each of the latter two groups were subdivided into early and advanced forms. Recently, spherule cells of *Dermacentor andersoni* were subdivided into 4 forms according to electron microscopic observation (BRINTON and BURGDORFER, 1971). Therefore, the present classification is likely to undergo revision in the future. DOLP (1970) has made quantitative studies on hemocytes of 4 species of ticks and concluded that the value

Fig. 10. *O. moubata* hemocytes in MITSUHASHI and MARAMOROSCH's (M.M.) culture medium, 3 months after inoculation. Bright phase contrast, $140\times$. (A): prohemocyte, (B): plasmatocyte.

Fig. 11. *O. moubata* hemocytes in M.M. medium, 5 months after inoculation. Bright phase contrast, $50\times$.

Fig. 12. *O. moubata* hemocytes in M.M. medium, 8 months after inoculation. Bright phase contrast, $100\times$. (A): prohemocyte, (B): plasmatocyte.

Fig. 13. *O. moubata* hemocytes in M.M. medium, one day after inoculation. Bright phase contrast, $100\times$.

Fig. 14. *O. moubata* hemocytes in ŘEHÁČEK and BRZOSTOWSKI's culture medium, 7 days after inoculation. Bright phase contrast, $50\times$.

Fig. 15. *O. moubata* hemocytes in M.M. medium, 5 days after inoculation. Bright phase contrast, $140\times$.

of differential hemocyte counts might be influenced greatly by the nutritional state. Results of present study are in good agreement with his findings, namely the number of advanced forms and spherule cells of *O. moubata* changed considerably in the different nutritional states, i.e., the hunger, engorgement and pre-oviposition state.

The only report on the primary cultures of tick hemocytes is that of CORY and YUNKER (1971). Their primary cultures of hemocytes of *Dermacentor andersoni* were maintained in variable condition for 72 to 74 days as adjusted by their ability to support the growth of Colorado tick fever virus. The principal object of their report, however,

was to sustain growth of arboviruses in a new culture system and description of hemocytes morphology was rather limited.

In the present study the initial number of homocytes was about 3×10^5 per ml of medium, such that the number per μl of hemolymph for "engorged+1 day females" of *O. moubata* was estimated to about 6×10^4 . This figure is higher than that of *Argas persicus* or *A. arboreus* (DOLP, 1970) (i.e., ca. 2×10^4 per μl of hemolymph).

The collectable volume of hemolymph from "engorged+1 day female" was about 15 μl , which was approximately 10% of the body weight. HEFNAWY (1972) reported that the absolute hemolymph volume of "engorged+1 day females" of *Argas arboreus* and *A. persicus* as determined by dye and ^{14}C -dilution methods was about 8 μl or 15 to 30% of the body weight. Their results may suggest that nearly 50% of the absolute hemolymph volume of *O. moubata* can probably be collected for culture.

An apparent increase in hemocyte number was not seen in both media, so it might be necessary to carry out further trials to examine improvements in the medium, the method of medium change and the number of hemocytes which should be used for inoculation in order to successfully establish cell lines.

The composition of hemocytes in the two media underwent distinct changes during the cultivation period; the percentage of prohemocytes increased during this time. Difficulty was encountered in making stained preparations of hemocytes since almost all cells were in a floating condition. In order to surmount this problem, observations on cell morphology were carried out mainly by phase contrast microscopy. The subgrouping of plasmatocytes or spherule cells for the two variants is impossible until more knowledge is acquired concerning the cell structure of hemocytes.

MITSUHASHI (1966) suggested that prohemocytes possibly undergo change and become plasmatocytes in the cultivation of *Chilo suppressalis* hemocytes. Concerning the observed changes in the composition of hemocytes in the present study, a similar change of one hemocyte type into another is a very likely possibility.

M.M. and R.B. media were used for the cultivation of hemocytes in the present study. MEDVEDEVA *et al.* (1972) succeeded in subculturing embryonic cells of *Hyalomma asiaticum* using M.M. medium, ŘEHÁČEK and BRZOSTOWSKI (1969) cultured cell of *Rhipicephalus sanguineus* up to 9 months in a medium which they formulated. The results of this study suggest that both media are relatively suitable for tick tissue culture.

The formation of cell networks and the ratio of spherule cells were considerably different according to the medium employed. The mode of proliferation of hemocytes derived from the same origin differed considerably according to the media used. This fact is noticeable in application of these media for arthropod tissue culture. Although primary cultures of hemocytes were maintained by the use of these media up to 8 months, successful subcultures were not obtained and therefore, it might be necessary to find more suitable media for this purpose.

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