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Long-Term Primary Culture of Cells of the Mosquito *Anopheles stephensi*[1,2]

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ABSTRACT

Long-term primary cultures of cells in which fragments of newly hatched larvae of *Anopheles stephensi* Liston (Diptera: Culicidae) serve as sources have been initiated. The various outgrowths which appear at different times during the cultivation period can be distinguished morphologically as large and small epithelial cells, fibroblastlike cells, neuronlike cells, polygonal cells, 2 types of myoblastlike cells, and noncellular and cellular vesicles. Viability of the various outgrowths for a period of about 60 days without change of nutrient medium has been observed. Efforts are being directed toward developing the primary cell culture into a receptive system for the in vitro cultivation of the sporogonic form of the malarial parasite *Plasmodium berghei*.

A review of the efforts of several workers in their aim to cultivate in vitro the sporogonic forms of the malarial parasite indicates the need for an insect-oriented environment that will support the growth and development of the parasite (Ball and Chao 1957; Chao and Ball 1964; Ragab 1947; Schneider 1968a, b; Walliker and Robertson 1970). Partial success has been reported using cell-cultured substrate derived from tissues of the mosquito *Aedes aegypti* L. (Schneider 1968a, b).

This paper describes the establishment of a long-term primary culture of mosquito cells derived from newly hatched *Anopheles stephensi* Liston and the possible utilization of the culture system in the in vitro cultivation of the mosquito-borne phase of the rodent malarial parasite *Plasmodium berghei*.

MATERIALS AND METHODS

Newly deposited eggs were obtained from laboratory-bred *A. stephensi* maintained under standard conditions in our laboratory (Alger and Undeen 1970). Sterilization procedures were essentially the same as those described by Schneider (1969). Eggs were incubated at 27°C in the dark. Embryogeny was completed in ca. 58 hr. Hatching took place at room temperature in trypsinized Rinaldini's salt solution.

About 150 newly hatched larvae were used to inoculate each glass V-bottom Bellco culture flask. Dissections of the larvae were done in a depression slide containing trypsinized Rinaldini's salt solution, under sterile conditions using surface-sterilized ultramicroprecision instruments (Trident).

Techniques used to expose and to draw out the entire digestive system intact were essentially the same as those employed by Ronquillo (1968)[3] and Ronquillo and Horsfall (1969). The intact gut and remaining bulk of the larval matrix were then cut into 1–3 mm³ fragments as suggested by Schneider (1967). These explants were pipetted into 2 ml of fresh trypsinized Rinaldini's salt solution, and incubated for 45 min at 27°C. One ml inactivated foetal bovine serum (FBS) was added to the trypsinized explants which were then allowed to stand for 5 min at room temperature. The trypsinized FBS-treated explants were centrifuged at 500 rpm (RCF = 36.3) for 6 min, resuspended in 1.5 ml of the culture medium, and pipetted into a culture flask. All cultures were kept at either 21±0.1 or 27±0.1°C in the dark.

The protocol for preparing the culture medium was outlined by Schneider (1969). However, for our purposes, the *p*H of the medium was adjusted to either 6.8, 7.0, 8.0, or 8.5. Culture media either with or without antibiotics were employed.

This report is based on examination of 135 primary cultures which were initiated during the period July 1970 to August 1971. The cultures were examined under a Zeiss UPL inverted microscope at 2-day intervals after the initial seeding. All photomicrographs (Fig. 1–24) were taken with a Zeiss C-35 Camera. Critical appraisal of the pattern of cellular outgrowths in the primary cultures has been made

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[3] M. C. Ronquillo. 1968. Organogenesis of the female reproductive system of an aedine mosquito. Doctoral dissertation, University of Illinois, Urbana. 115 p.

possible with phase-contrast time-lapse cinephotomicrography.[4]

RESULTS

Monolayer Cell Outgrowths.—Large Epithelial Cells (LE).—These cells varied from round, ovoid forms to starlike configurations (Fig. 1–3). Each cell had a large nucleus which was spherical or irregular in shape, a granular cytoplasm, and a thin membrane (M) at the periphery. This membrane which was either slightly curled up or folded, was hardly visible in the more compact, subspherical cells but distinct in flat cells (Fig. 1, 11). These unusually large cells became evident 4 days after the initial seeding and were generally attached to the glass. Each cell attained its maximum size at ca. 20 days in culture and was ca. 120 μ diam. As the culture progressed, small ovoid and spindle-shaped epithelial cells (Fig. 6, SE) began to appear in the vicinity of the large epithelial cells (Fig. 6, LE).

Each cell exhibited vigorous nonoriented gliding movement 3 days after its appearance. This activity was accompanied by contraction and relaxation and a continual wavy motion of the thin peripheral membrane. The extent and character of this type of motility was best seen in phase-contrast time-lapse cinephotomicrography.

Cellular morphological changes and gradual decline of motility were evident as the culture aged. The cells assumed rounder, denser forms by shrinking back from their expanded state. Some of these cells became increasingly filled with highly refractile granules (Fig. 3, G) and minute vacuoles (V) appearing first around the nucleus and finally filling the entire cytoplasmic area. With passage of time, the large epithelial cells disintegrated, and the cell membrane ruptured, resulting in the extrusion of the contents into the nutrient medium. In some individually scattered aging cells, the cytoplasm was transformed into a network of fine fibrils and the nuclei disintegrated, resulting in the death of the cells (Fig. 3). Phase-contrast time-lapse cinephotomicrography did not show evidence of proliferation and phagocytosis.

Small Epithelial Cells (SE).—Small ovoid and spindle-shaped epithelial cells were observed in the 2nd week of cultivation. Each cell was characterized by a round nucleus and a cytoplasm filled with minute droplets and fine granules (Fig. 4). The average diameter of the cell was ca. 40 μ. These cells became apparent either near the vicinity of the large epithelial cells (Fig. 6) or actively migrating from tissue fragments (Fig. 4). Occasionally myoblastlike cells (Myo) appeared simultaneously with the actively migrating small epithelial cells.

The interval between 15 and 35 days after the initial seeding was one in which the cells began to proliferate, and they continued to do so at such a rapid pace that by the time the culture was 45 days old, the cells dominated the culture field (Fig. 5). Mitotic figures were frequently seen in the outgrowth. During the 1st 3 days of their appearance, these cells were adherent to the glass and stationary. Later, they began to show oscillating movements which lasted for ca. 20 days in culture. As the culture aged, this motility decreased gradually until it finally ceased. The cells tended to pile up in some areas to form small clusters or thick discs of cells (Fig. 7). This morphological alteration during the process of monolayering of the cells on the glass occurred when growth activity reached its peak (35–45 days after the initial seeding).

Cytolysis of the cells became evident ca. 60 days into the culture period. The cells became highly refractile and vacuolated, assuming irregular forms and losing their adhesion to the glass (Fig. 8). Occasionally, floating sheets of cells consisting of both large and small epithelial cells became apparent at the surface of the culture medium. Survival time was short, lasting only 25 days.

Fibroblastlike Cells (F).—This type of cells became apparent 10 days into the culture period, migrating independently of one another from tissue fragments (Fig. 9). The cells were irregular in size and shape. Each cell had either an oval or elongated nucleus, and a cytoplasm which was drawn out into one or more processes of variable length.

The interval between 10 and 35 days after the initial seeding was characterized by continual cell migration from the explants. As cultivation proceeded, rapid cell proliferation was evident and a dense population consisting of scattered discrete fibroblastlike cells became apparent (Fig. 10).

Fibroblastlike cells migrating from explants moved readily toward free spaces in an apparently random pattern. An analysis of time-lapse phase-contrast cinephotomicrography of the movement of a cell showed that a thin fanlike membrane (Fig. 11, M) at one edge of the cell underwent a continual undulating movement, leading the cell as it moved about.

Signs of cellular degeneration were evident by the time the culture was 55 days old. There was cessation of cell movement, change in shape, increasing refractility and vacuolation, and loss of adhesion to the glass.

Polygonal Cells (P).—These cells were large, flat, polygonal, and formed a contiguous sheet around the explant (Fig. 12–14). Each cell had a well-defined, centrally placed spherical nucleus, a prominent nucleolus, and a clear, nearly granule-free cytoplasm. Each cell was ca. 80 μ diam.

This type of outgrowth became evident by the time the culture was 10 days old. Growth during the next 30 days was characterized by an increase in the number of cells from rapid cell proliferation and active migration of cells from the explants (Fig. 14). Mitosis usually occurred in the cells that were located at the periphery of the growing cell sheet. Occasionally, binucleated cells were noted with the nuclei situated close to one another (Fig. 13). In some explants, fiberlike elements migrated together with the polygonal cells in a continuous sheetlike pattern.

[4] A sequence of these time-lapse studies will be deposited in the film library of the Tissue Culture Association.

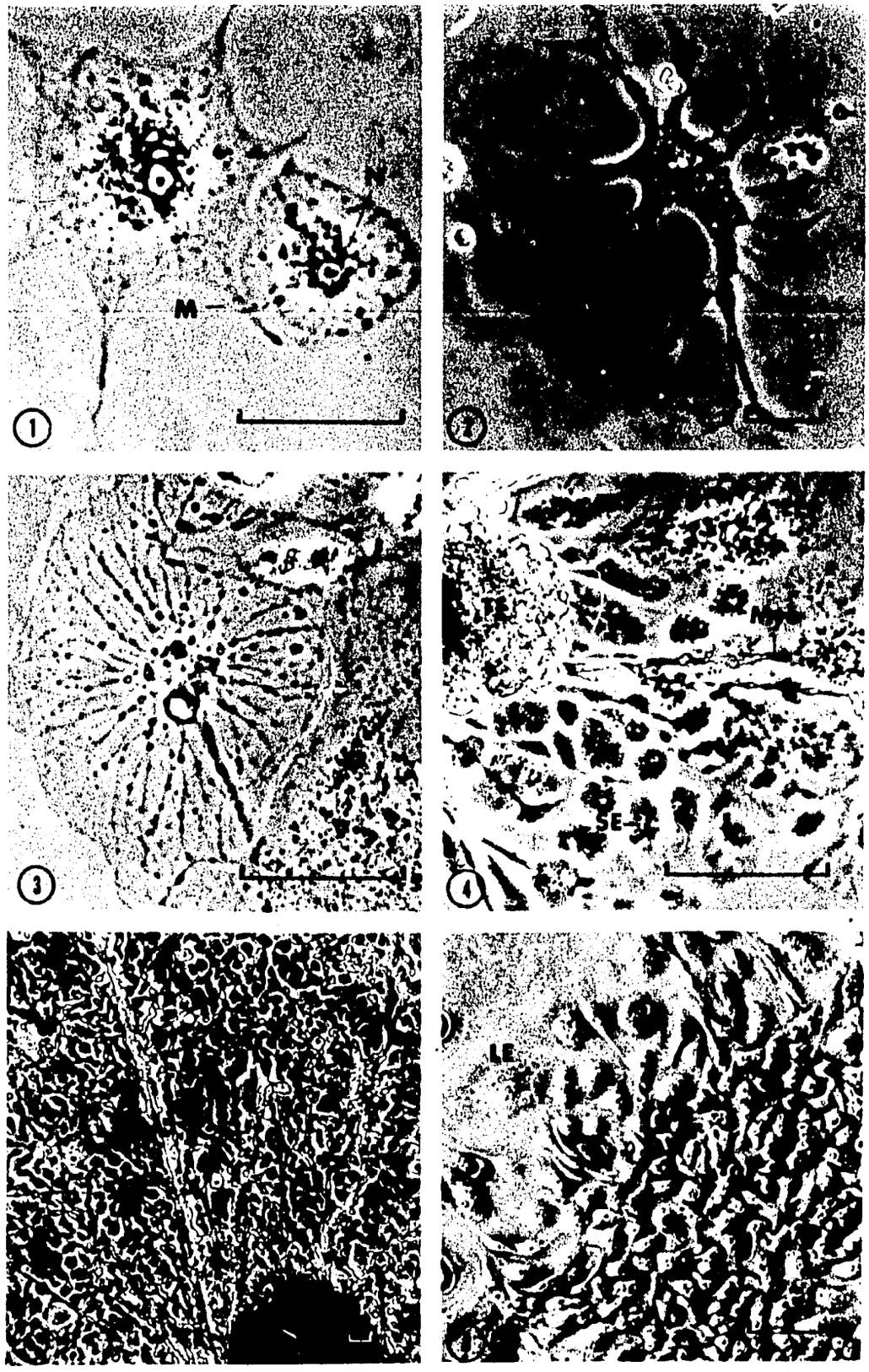

FIG. 1–6.—*A. stephensi* primary tissue culture. Phase contrast of living cells (Fig. 1–5); optical shadow casting of living cells (Fig. 6). 1, Large epithelial cells, (19 days). 2, A large epithelial cell in the process of shrinkage, (35 days). 3, Aging large epithelial cell, (45 days). 4, Small epithelial cells migrating from a thoracic fragment, (19 days). 5, A portion of the culture field showing a large population of small epithelial cells and a few myoblastlike cells, (15 days). 6, a group of large and small epithelial cells, (30 days). (Scale lines on Fig. 1–6 represent 50 μ.)

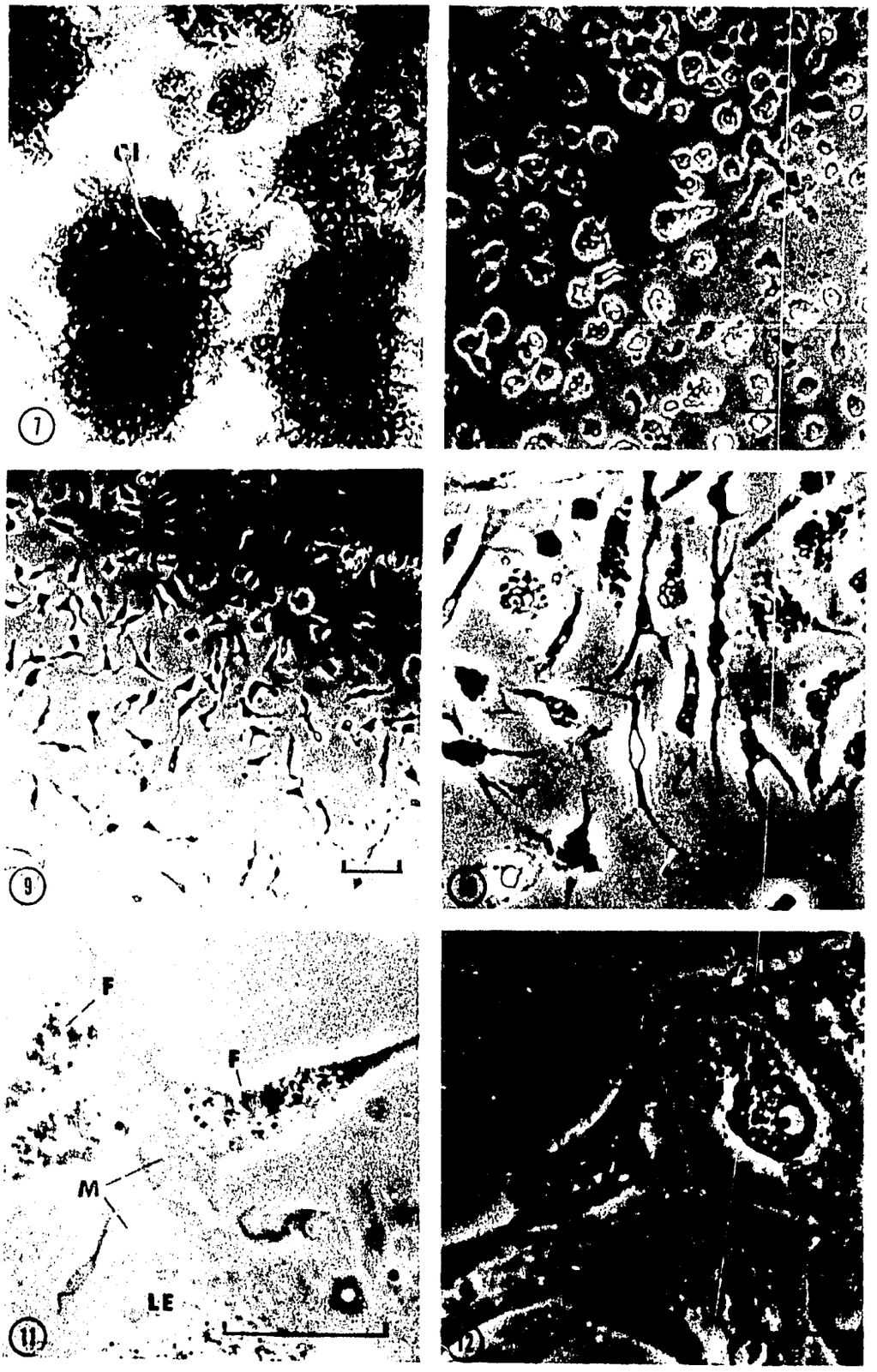

FIG. 7–12.—*A. stephensi* primary tissue culture. Optical shadow casting of living cells (Fig. 7); phase contrast of living cells (Fig. 8–12). 7, Clumping of small epithelial cells, (50 days). 8, Histolyzed small epithelial cells, (95 days). 9, Tissue fragment with migrating fibroblastlike cells, (27 days). 10, Fibroblastlike cells, (32 days). 11, Large epithelial cell and fibroblastlike cells, each type with well-defined ruffling membrane, (14 days). 12, Polygonal cells located at periphery of cell sheet, (17 days). (Scale lines on Fig. 7–10 and 12 represent 50 μ; on Fig. 11, 25 μ.)

Unusual pulsatile activity in this type of cell sheet was evident and lasted for as long as 20 days.

Time-lapse filming showed that the polygonal cells moved as a cohesive sheet along the glass. Wavelike activities of the membranes at the free edges of the marginal cells could be seen as the sheet of cells moved. Behavioral differences among the cells were observed in response to the activity of the peripheral cells. Submarginal cells in contact with the peripheral cells were markedly stretched, assuming a characteristically peduncular appearance with no specific pattern of orientation (Fig. 13). Cells which were more or less near the explant retained their polygonal forms and became radially oriented with respect to the rest of the cells (Fig. 14).

Cell degeneration started by the time the culture was 50 days old. Strings of small cytoplasmic bubbles developed at the periphery of the cell sheet. The cells became highly refractile and vacuoles formed within the cytoplasm. Adhesion of the cell sheet to the glass decreased as degeneration progressed. By the time the culture was 60 days old, a nondescript monolayer was detectable.

Fiberlike Elements.—Myoblastlike Cells (Myo).—The growth patterns of the cells showing myoblastlike characteristics were of 2 types. One type consisted of cells that were initially few in number but increased progressively with the passage of time; these cells originated from fragments of the thoracic (TF) and abdominal (AF) regions (Fig. 16). Another distinct type consisted of cells that were initially few in number and remained as such throughout the cultivation period; these cells arose from isolated fragments of the gut (GF) (Fig. 15).

In the 1st type, the cells were conspicuously long and slender. Each cell consisted of a prominent central body containing an ovoid nucleus and a cytoplasm which was drawn out into long threadlike processes. The cells, in their emergence from the explants 7 days after initial seeding, appeared either as individual cells projecting radially or as groups of cells interlaced by fibrils and extending outwardly (Fig. 16, 17). Occasionally, large epithelial cells exhibited gliding movements either between 2 myoblastlike cells or under a myoblastlike cell moving between it and the glass.

Rhythmic contractile activity independent from that of the source of origin was apparent either in an individual myoblastlike cell or an agglomeration of similar cells. Some activities were strong, others weak; however, both activities lasted for as long as 15 days. These contractile elements remained unattached to the glass at the middle of the strand during their active period. However, as activity declined, the whole strand tended to adhere to the glass surface. Degeneration was characterized by the appearance of strands of coalescing blebs (B) along the cytoplasmic extensions (Fig. 18). As the culture aged, these blebs increased in number and the cells became detached from the glass. Ultimately, the cells were seen floating on the surface of the medium.

In the 2nd type of myoblastlike cells, the outgrowth appeared as thin, membranelike elements on the 7th day of the cultured period. They extended a short distance from their origin (gut fragment, GF) in a distinctive pattern with the edges attached and the center portion unattached to the glass, as shown in Fig. 15. Slight striations and several nuclei became evident as the outgrowth aged. The gut fragments (GF) that gave rise to this type of outgrowth exhibited rapid waves of movement typical of gut peristalsis, lasting for 3 weeks in culture. As cultivation progressed, the gut fragments lost their motility as well as their integrity, becoming deformed, smaller, and darker. On the other hand, the outgrowth began to show twitching movements that lasted for a week. As the outgrowth aged, motility decreased, adhesion of the entire outgrowth to the glass became apparent and numerous minute nodules appeared throughout the outgrowth. This appearance persisted as such until the end of the cultivation period.

Neuronlike Cells (NC).—Cells of this type arose from fragments of the thorax and abdomen during the 2nd week of cultivation. Characteristically, each cell consisted of a cell body (CB) with very fine fibrillar processes (Fi) (Fig. 19). Initially, the processes were short but as growth proceeded, each process lengthened into a very thin fibril, either as an unbranched, Y-shaped, or multibranched form (Fig. 20). No evidence of pulsatile activity was observed among these outgrowths. As the culture aged, the outgrowth became dense and very complex. Cross-connections occurred between the cell bodies and between fibrillar branches of the cells. A characteristic affinity of the tip of the fibrils to neighboring large epithelial cells (LE) and myoblastlike cells (Myo) was observed during the cultivation period (Fig. 20).

These neuronlike cells survived well for an extended period of time. Degeneration which occurred on the 8th week of cultivation was characterized by the loss of cross connections between cell bodies and between fibrils, and detachments of the cells from the glass.

Vesicles.—These outgrowths became evident on the 2nd week of the cultivation period as small, hollow, transparent, spherical vesicles at the cut edges of the thoracic and abdominal fragments. The number of vesicles that originated from a specific cut edge of a fragment ranged from 1 to 5. They remained attached to the fragment throughout the cultivation period. Two types of vesicles were apparent during the culture period: cellular (CV) and noncellular (NCV) (Fig. 21, 22).

Growth of the cellular sphere was characterized by an increase in size from the rapid proliferation of the cell population. A mature vesicle consisted of a single layer of either squamous cells (Sq) (Fig. 23) or small epithelial cells (SE) interspersed with fibroblastlike cells (F) (Fig. 22). In vesicles with squamous cells, thickening of some areas which was followed by a conspicuous darkening process was apparent. As the culture aged, the cells tended to ag-

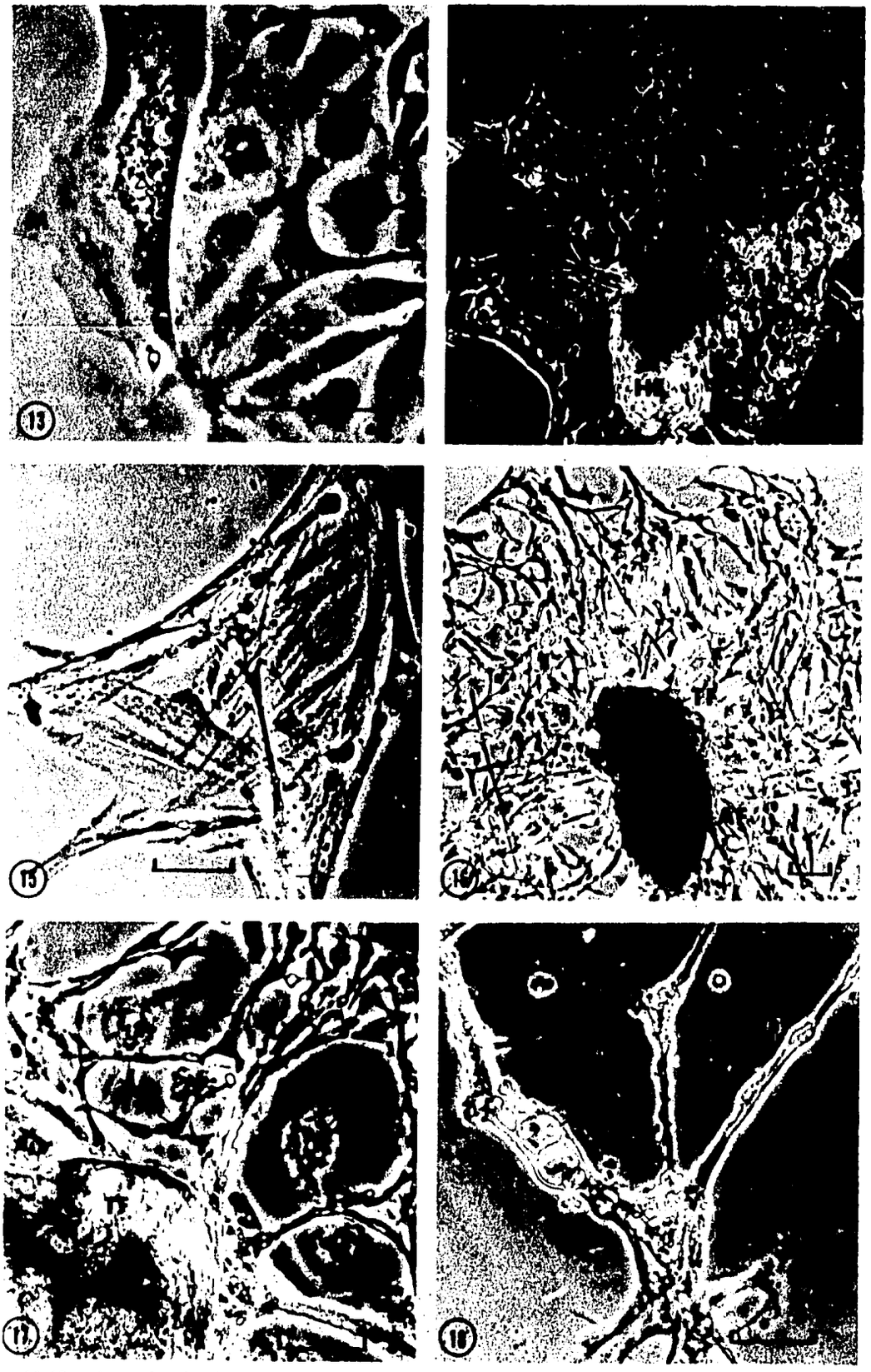

FIG. 13–18.—*A. stephensi* primary tissue culture. Phase contrast of living cells. 13, A group of polygonal cells showing binucleation and characteristic peduncular appearance, (17 days). 14, Contiguous sheet of polygonal cells from head fragment, (10 days). 15, Myoblastlike cells drawn into thin sheets, (10 days). 16, Interlacing myoblastlike cells from thoracic and abdominal fragments, (32 days). 17, Myoblastlike cells and large epithelial cells from thoracic fragments, (14 days). 18, Degenerating myoblastlike cells, (57 days). (Scale line on Fig. 13–18 represents 50 μ.)

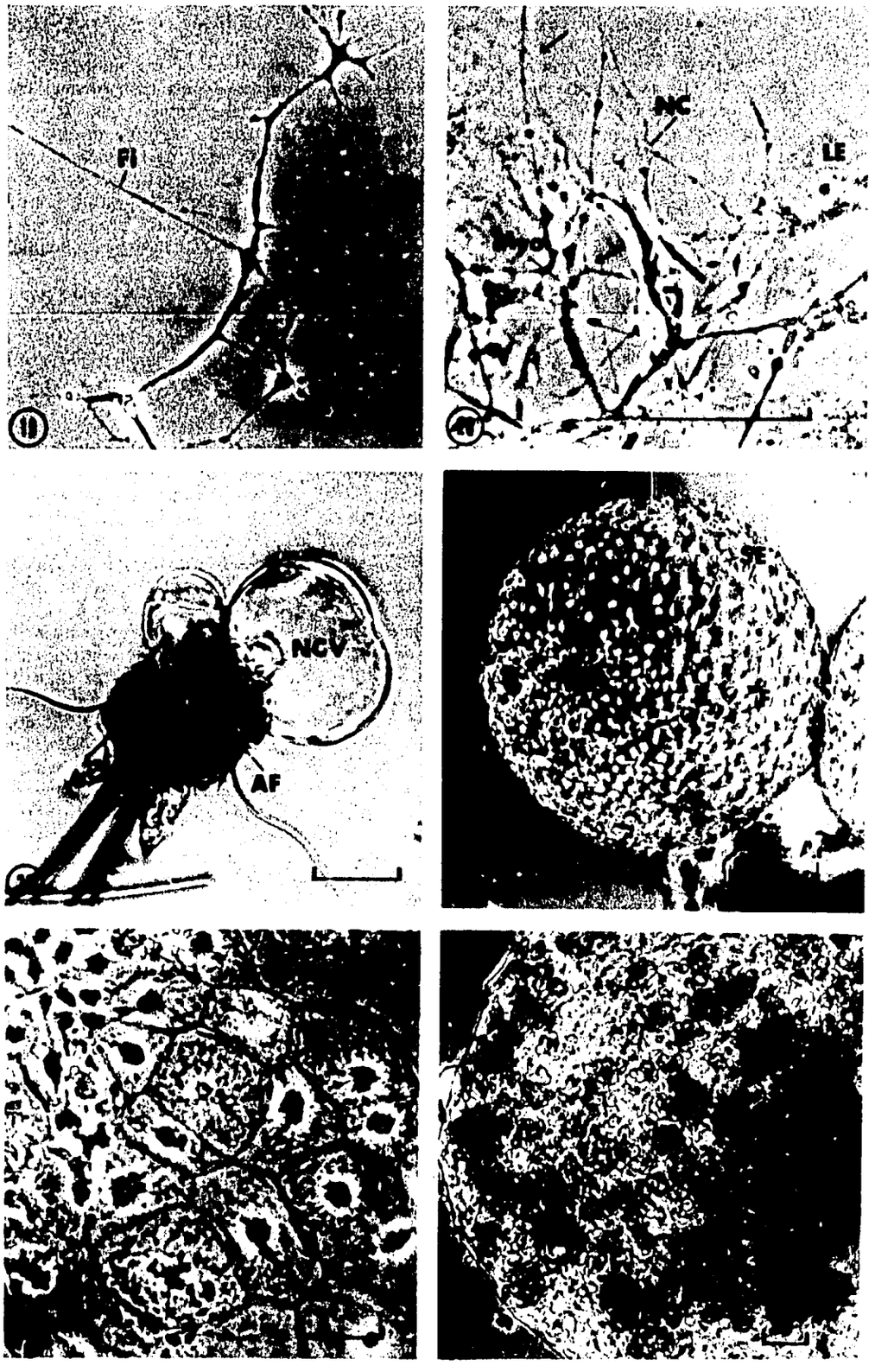

FIG. 19–24.—*A. stephensi* primary tissue culture. Phase contrast of living cells (Fig. 19–21, 23–24); optical shadow casting of living cells (Fig. 22). 19, Neuronlike cells, (30 days). 20, A group of neuronlike, myoblastlike, and large epithelial cells, (27 days); arrow points to affinity of tips of neuronlike cells to a large epithelial cell and a myoblastlike cell. 21, Abdominal fragment with noncellular vesicle (10 days). 22, Cellular vesicles from abdominal fragment, (34 days). 23, Squamous cells of a cellular vesicle, (30 days). 24, Clumping of cells of a cellular vesicle, (67 days). (Scale lines on Fig. 19–24 represent 50 μ).

gregate, giving rise to a wrinkled nontransparent cellular wall (Fig. 24). In spite of the morphological change that did occur, the vesicles remained hollow and showed no evidence of collapse throughout the cultivation period. Noncellular vesicles remained small and persisted as such throughout the cultivation period (Fig. 21).

DISCUSSION

The survey of several publications on primary insect tissue culture involving the use of different organs from donors of different species and ages as sources shows an emerging similarity of the outgrowths during the cultivation period (Trager 1938; Hirumi and Maramorosch 1964a, b; Eide and Chang 1969; Greenberg and Archetti 1969; Echalier and Ohanessian 1970; Shields and Sang 1970; Richardson and Jensen 1971). In the present study, the cellular outgrowths in the primary culture (in which newly hatched larvae of *A. stephensi* served as the sources) resembled some of the outgrowths reported by the aforementioned authors. The primary culture consists of 9 different types of outgrowths which appear at different times during the cultivation period. The outgrowths could be morphologically distinguished as large and small epithelial cells, fibroblastlike cells, neuronlike cells, 2 types of myoblastlike cells, polygonal cells, and noncellular and cellular vesicles.

A point of interest in the present study is the consistent pattern of events that has been observed in all the culture flasks examined during the cultivation period. A latent period is maintained for 2 days after explantation. On the 3rd day, some explants become attached to the glass and begin to exhibit pulsatile activities. Then a sequential growth of the different types of cells arising either from the peripheries of the explants or from a dissociated suspension of cells occurs. First to appear in the culture field are the large and small epithelial cells. They are followed by fibrillike elements among which the myoblastlike cells tend to predominate over the neuronlike cells. Then fibroblastlike cells and contiguous polygonal cells become apparent. The latter are closely followed by the appearance of noncellular and cellular vesicles. The events just described are followed by a period of active cell migration, multiplication, and in some cases, cellular differentiation. These activities reach their maximum during the 5th week of cultivation. This period is followed by a gradual decrease in activities that leads to a period of degeneration.

Degeneration is indicated by behavioral as well as morphological changes: loss of motility; loss of adhesiveness to the glass; withdrawal of cytoplasmic processes, rounding off and eventual shrinkage of cells; increased refractivity, granulation, and vacuolation; and appearance of blebs and characteristic clustering of cells. Echalier and Ohanessian (1970) reported that the clustering of cells may result from loss of contact inhibition during the monolayering of cells. Eide and Chang (1969) showed by time-lapse study that clustering is the result of rapid cell division in aggregations of morphologically similar cells. In the present study, clumping of small epithelial cells is apparent as the culture ages. It is possible that these morphological alterations during degeneration may result from nutritional depletion or toxicity caused by the accumulation of metabolic wastes. Interpretation of any apparent behavioral and morphological alteration in the primary insect tissue culture requires further critical analysis and definitely remains to be explored.

We have demonstrated that the primary culture of cells derived from newly hatched larvae can be maintained for 60 days without changing the nutrient medium. This long-term maintenance of viable outgrowths in culture is of great value because it provides an adequate "life span" which could be utilized for the in vitro development of the parasites which in vivo is completed at ca. 14 days. Presently, our efforts are being directed toward developing the primary cell-cultured substrate into an efficient one in the hope that it will support the development of the entire sporogonic cycle.

Firstly, inclusion of antibiotics in the medium to insure sterility in culture was found to inhibit the development of the parasites (Dubin et al. 1950, Terzian et al. 1952, Micks and Ferguson 1961, Chao and Ball 1964). Therefore, we omitted antibiotics from the medium and preparations of explants were done under strictly aseptic conditions. Presently, viable primary cultures grown in antibiotic-free media are being maintained in this laboratory.

Partial success in the in vitro cultivation of the sporogonic forms has been reported by several workers when supplements of adult organs were incorporated in either a cell-cultured system or chemically defined substrate (Chao and Ball 1964, Schneider 1968a, Walliker and Robertson 1970). The need, therefore, for sterile organs arises. Attempts to rear *A. stephensi* aseptically have been successful in this laboratory (manuscript in preparation) and availability of germ-free adult mosquitoes has great potential value to the present research. Organs which were taken from newly emerged aseptically reared adult male and female mosquitoes and introduced either in the primary cell-cultured substrate of varied ages or in cell-free nutrient medium were found healthy and viable for as long as 30 days without loss of integrity; there is no sign of morphological alteration and there are persistent marked behavioral activities such as gut peristalsis and ovary contractility.

It has been demonstrated that temperature influences the in vivo as well as in vitro development of the parasites (Trager 1938, Huff 1940, Ball and Chao 1957, Chao and Ball 1962); development of the mosquito phase of *Plasmodium relictum* is accelerated as temperature is increased from 18 to 31°C. Yoeli et al. (1965) showed that in vivo, the sporogonic development of a strain of *P. berghei* takes place within a narrow temperature range (18–22°C). In view of this observation, the need for primary cultures incubated within this temperature range is apparent.

Presently, viable primary cultures incubated at either 20° or 27°C are being maintained in our laboratory. It has been observed that a similar pattern of cellular outgrowths and viability of the cells exists in the cultures incubated at either temperature. However, a longer latent period after explanation has been observed in the cultures incubated at lower temperatures than in the ones at higher temperatures.

It has been shown by some workers (Micks and Ferguson 1961, Chao and Ball 1964, Schneider 1968a) that the *p*H requirement of each state of the sporogonic cycle during in vitro development is exacting. A *p*H close to 7 supports the development of early stages and a *p*H of 8 is favorable to the late sporogonic forms. In view of this observation, we are directing our efforts toward procedural modifications to maintain the *p*H of the substrate within the required limit of the parasite in cultivation. Work in progress shows that cell-cultured substrates can be established in nutrient media wherein the *p*H was initially adjusted at either 6.5, 7, 8, or 8.5. Preliminary results show that the outgrowths grow equally well within a *p*H range of 6.5–8. Furthermore, a slight fall in *p*H level of the medium from the initial level is noted on the 4th week of the cultivation period. Further work along this line is still in progress.

Exploratory experiments in the in vitro cultivation of the parasites, which are now in progress in our laboratory, show that the primary cell substrate appears to be a receptive system for the development of the parasite.

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ABBREVIATIONS USED ON FIGURES

AF, abdominal fragment
B, cytoplasmic bleb
Cl, clump of small epithelial cells
CB, cell body
CV, cellular vesicle
F, fibroblastlike cell
Fi, fibrillar process
G, granule
GF, gut fragment
HE, head explant
LE, large epithelial cell
M, membrane
Myo, myoblastlike cell
N, nucleus
Nu, nucleolus
NC, neuronlike cell
NCV, noncellular vesicle
Pe, peduncular polygonal cell
Sq, squamous cell
SE, small epithelial cell
TF, thoracic fragment

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