ESTABLISHMENT OF THREE DIPLOID CELL LINES OF *ANOPHELES STEPHENSI* (DIPTERA: CULICIDAE)

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The culture of insect tissues in vitro offers considerable potential for studying many aspects of the pathogenic cycles of protozoan, bacterial, and viral agents which have an insect vector or reservoir. Studies directed toward this end have been limited in number since the culture of insect cells has, in itself, been a very difficult task (2, 8, 13). Progress in this field was not too encouraging until the work of Grace demonstrated the feasibility of obtaining both long-term primary cultures and continuous cell lines of insect origin (5–7). Following this lead, insect cell lines have now been reported for 9 genera and 13 species (1, 3 9, 10, 12).

This report describes the establishment of 3 additional cell lines originating from larval tissues of the mosquito *Anopheles stephensi* which is the vector both in nature and in the laboratory for a number of malaria parasites.

MATERIALS AND METHODS

Anopheles stephensi Liston (India strain) eggs, 24 hr old, were surface-sterilized by immersion in 10% benzalkonium chloride for 1 hr followed by an additional 10–20 min in 70% ethanol. The eggs were rinsed thoroughly in sterile distilled water, transferred to moistened sterile filter paper in a petri dish, and placed overnight in a 22°C incubator.

First instar larvae, displaying light gray head capsules, were transferred to a depression slide and minced in Rinaldini's salt solution (RSS) (11) containing 0.2% trypsin (1:250, Difco, Difco Laboratories, Detroit, Mich.). Approximately 200 larvae were used to initiate each culture. The fragments were placed in fresh RSS-trypsin solution and incubated for 45 min. After the addition of fetal bovine serum (FBS), the fragments were centrifuged, resuspended in 1 ml of culture medium, and seeded into plastic T-30 flasks containing an additional 3 ml of medium. The cultures were maintained at 27 ± 0.5°C.

A modified Wyatt-Grace culture medium (5) was used in the present study. The concentration of the sugars and inorganic salts was altered as follows (in mg/100 ml): glucose, 100; sucrose, 1600; trehalose, 50; NaCl, 300; KCl, 110; $CaCl_2$, 40; $MgCl_2 \cdot 6H_2O$, 114; and $MgSO_4 \cdot 7H_2O$, 40. Sodium phosphate, fructose, the vitamins and organic acids were omitted. Cholesterol, dissolved in Tween 80 and 95% ethanol (4), was added at a concentration of 0.2 mg/100 ml. The medium was supplemented with 1% 10X abbreviated NCTC 135 medium (lacking the amino acids, inorganic salts and sugars; this medium is commerically available) and 15% inactivated FBS. The pH of 6.9 was monitored by the use of 0.01% phenol red.

Once established, the cell lines have been maintained by subculturing every 4–7 days. During their growth the cells become firmly attached to the bottom of the flasks. Prior to transfer they are incubated with the RSS-trypsin solution for 2–3 min followed by pipetting to dislodge them. Renewal or replacement of the medium between subcultures is not necessary, provided a minimum of 4 ml is placed in the flasks.

To facilitate cytological study, precleaned sterile 9 × 35 mm coverslips were dispersed over the bottom of a flask prior to cell seeding. Forty-eight hr later, the medium was removed and the cells were fixed with FAA (formalin, ethanol, acetic acid, 6:16:1, plus 30 parts water) and stained in situ with Ehrlich's hematoxylin. After dehydration, the coverslips were removed, placed in xylene, and mounted in Canada balsam. In addition, chromosome counts were made on cells with colchicine-blocked metaphases.

Cells from all three lines have been frozen at −68°C in the above culture medium containing either 10% glycerol or 10% dimethyl sulfoxide (DMSO) and successfully regenerated.

RESULTS AND DISCUSSION

Twenty-six primary cultures were initiated during the period of February to April, 1968. Of these, 15 followed the pattern of development given below. Six of the remaining cultures became contaminated, and five showed very limited growth or none at all. The transition to subculturing was attempted during April and May, 1968. This was successful in 11 of the 15 cultures and, of these, three were selected for continuous culture.

Hollow, cellular spheres developed at the cut ends of the fragments a few days after being placed in culture, and continued to increase in size to the extent that they soon dwarfed the original fragments. As many as 10 such spheres would issue from a single fragment edge. In healthy spheres the cells retained their transparency and grew in

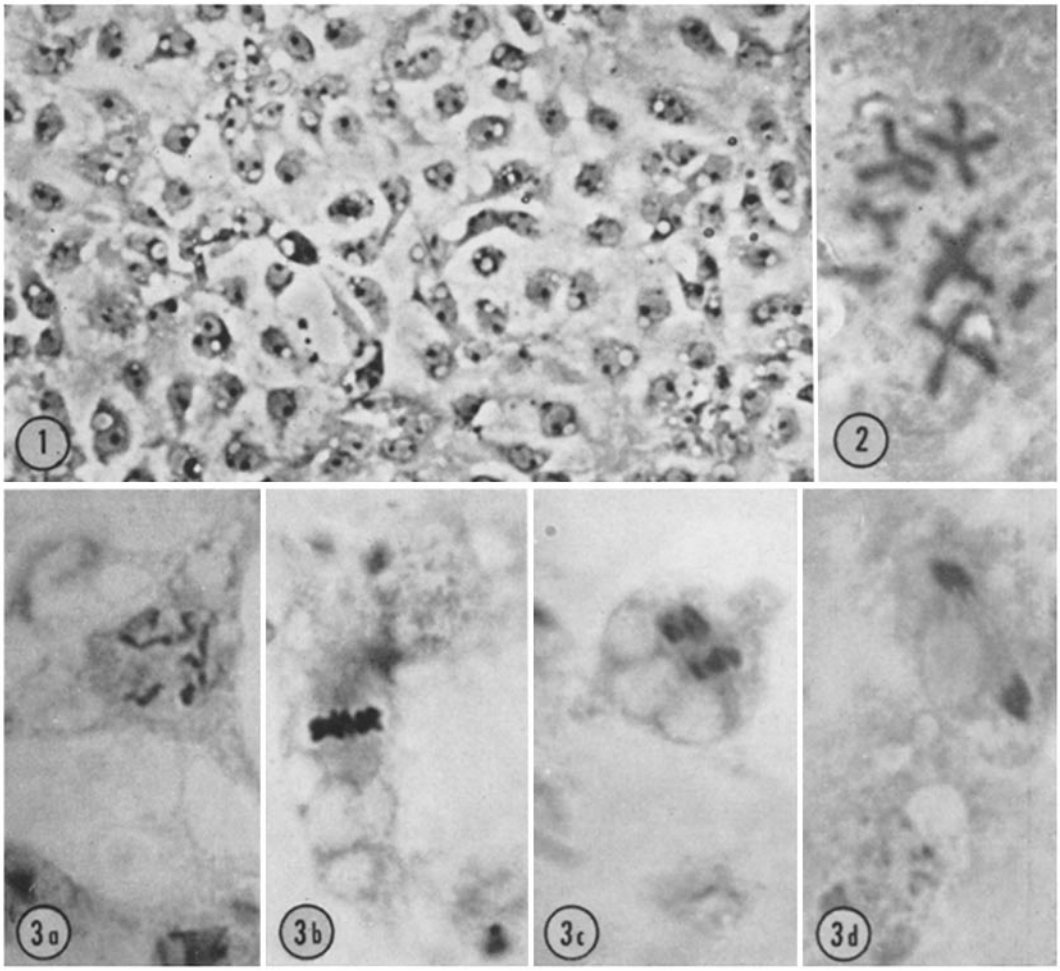

FIGURE 1 Confluent sheet of *Anopheles stephensi* cells grown in a plastic T-flask for 72 hr (25th subculture). Phase contrast. × 600.

FIGURE 2 Chromosomes of *A. stephensi* cell with colchicine-blocked metaphase (22nd subculture). Stained with aceto-orcein. × 3500.

FIGURE 3 *a–d* Cells displaying varying stages of mitosis in 24th subculture of *A. stephensi*. Stained in situ with Ehrlich's hematoxylin. × 3000.

monolayers; in unhealthy spheres there was a tendency for the cells to pile up, usually some distance from the periphery of the fragment, to become granular and eventually to degenerate. This type of growth pattern is similar to that described by Singh (12) for cultures derived from newly hatched larvae of *Aedes aegypti* L.

The medium was partially withdrawn and renewed 7 days after the cultures had been set up and every 4 or 5 days thereafter. Approximately 3 wk later, gentle pipetting of the medium dislodged most of the cell spheres from the fragments, and the former were either teased apart or placed in the RSS-trypsin solution prior to being seeded into new flasks. In either instance the cells readily attached to the floor of the flasks and resumed multiplying. One week later, the density of the cells required subculturing. Approximately 10^4 cells/ml were sufficient to start a new culture. After these initial transfers the three cell lines have

been subcultured 31, 34, and 38 times, respectively.

The majority of cells range from 4 to 9 μ in diameter and 12 to 20 μ in length. They are epithelial in appearance (Fig. 1) but do not form true monolayers as there is a tendency for the cells to pile up on one another at central foci, particularly as the cultures age. The cells are predominantly diploid, having 6 chromosomes (Fig. 2). Seven or eight chromosomes are present in approximately 5% of the cells, the highest complement seen in any one cell being 12. All stages of mitosis are readily seen in stained preparations (Fig. 3a–d). The mean mitotic index, based on a minimum count of 1000 cells, is 1.5% in 48 hr cultures and 1.6, 2.0, and 1.9% in 72, 96 and 120 hr cultures, respectively. Mitosis is normally completed within 40 min.

In contrast to the present *A. stephensi* lines and the cell lines of Singh (12), those established by Grace (5–7), Echalier and Ohanessian (3), and Landureau (9) are all highly polyploid. Cells of the former cell lines maintained a relatively steady rate of growth after the first few days of culture. Most, if not all, of the latter lines experienced an initial period of migration and growth followed by a lag or cessation of mitotic activity before active growth was resumed. This prolonged period of adaptation, in one instance requiring 4 months (5), doubtless contributed in large part to the emergence of polyploid cells in the latter lines.

With the exception of the Grace and Mitsuhashi cell lines, which were derived from late larval or pupal stages, all of the other lines have been obtained from embryos or from newly hatched larvae. Thus, the use of cells from the younger stages, while they may adapt more readily to conditions in vitro, does not necessarily guarantee the establishment of diploid lines. In the present study, neither late embryos nor older 1st instar larvae with black chitinized head capsules proved to be as suitable donors as the very young larvae. This suggests that in starting other insect cell lines it may be a matter not only of selecting the right stage but also of selecting a fairly narrow range of hours within that stage to obtain the most responsive cells.

Regardless of their origin, all of the primary and established insect cell lines have required a supplement to the culture medium, either in the form of hemolymph and/or FBS. Obviously, homologous hemolymph would be the most appropriate supplement. Since the small size of most insects precludes obtaining sufficient amounts of hemolymph, one is left with the use of either heterologous hemolymph or vertebrate sera. Unfortunately, the convenience of using the latter tends to override the fact that it probably is the less desirable of the two alternatives.

In an attempt to simplify the medium, the NCTC 135 supplement was omitted. The cells were capable of growing in such a medium for two transfers at a rate comparable to the controls. Thereafter, growth was much slower and the cells could not be maintained beyond the fifth transfer. In addition, the organic acids present in the Wyatt-Grace formulation were initially incorporated into the *A. stephensi* medium at the same concentration. Surprisingly, the omission of the organic acids stimulated rather than retarded growth.

It is fairly common in insect tissue culture literature to find an admonition concerning the use of trypsin for dissociating insect cells (8, 13). Such caution probably stems from the report of Grace (2) who initially found trypsin to be detrimental to insect tissues but who subsequently revised his opinion (5). Since trypsin has now been successfully used to disperse cells of numerous species, including those in the present report, this admonition might well be viewed with some skepticism.

Another enzyme, pronase, was occasionally used to separate the cells prior to subculturing and proved to be more effective in this respect than trypsin. The newly seeded cells also had a tendency to form better monolayers. Although the rate of multiplication was not affected at the concentration used (0.01%), the cells were somewhat elongated which may have been indicative of some injury. Here, also, judgement should be reserved until more insect cells have been subjected to this enzyme and until more definitive criteria have been used to assess its action.

Admittedly, there are at least two interrelated drawbacks to the present method of initiating cell cultures: (a) it is obviously difficult, if not impossible, to determine the origin of those cells which survive and proliferate, and (b) the cells are undoubtedly heterogeneous with respect both to the initial tissues and/or organs involved and to the fact that the donors are of both sexes. At present, few, if any, alternative methods are available for the smaller insects. It is possible that nutritional studies undertaken on cell lines so

crudely obtained, particularly early in their isolation, would lead to the design of more adequate media. This, in turn, might make it possible to initiate cell cultures with smaller inocula. The use of the feeder layer technique might also prove of value in this respect.

SUMMARY

The establishment of three diploid cell lines of *Anopheles stephensi* is described. The primary cultures were initiated between February and April, 1968 and consisted of minced tissues from early first instar larvae. Trypsinized cells from such cultures readily grew when transferred to new flasks. After these initial transfers, each of the cell lines has been subcultured a minimum of 30 times. The pertinent features of the cell lines are given and some comparisons are made with respect to the other insect cell lines now available. Some observations on insect tissue culture in general are also made.

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