GROWTH OF CELLS DERIVED FROM *CULISETA INORNATA* AND *AEDES VEXANS* IN TISSUE CULTURE A PRELIMINARY NOTE [1]

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INTRODUCTION. Many workers have attempted to initiate primary and serial passage lines of mosquito cells, with only limited success (Trager, 1938; Ball, 1954; Jones, 1962; Peleg, 1965; Kitamura, 1966). After numerous attempts Grace (1966) finally in 1963 established a cell line derived from axenically grown *Aedes aegypti* larvae, by employing hemolymph derived from *Antherea eucalypti* in the culture medium. This is considered the first true line of mosquito cells, and the finding has considerable potential value for studies of insect-borne human and animal viruses as well as in fundamental insect pathology. The practical use of these cells, until recently, has been limited because the media had to be supplemented

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with lepidopteran hemolymph which is expensive and difficult to obtain. Recently, Nagle, *et al.* (1967), Suitor (1968), and Sweet and Dupree (1968) have adapted the cell line to a medium free of hemolymph. An additional drawback, even though the cells have been cloned (Suitor, *et al.* 1966), is that the cells do not attach to the surface of the vessel but grow in suspension. This limits their usefulness for viral cytopathology observation and plaquing studies. Very recently, K. R. P. Singh of India (1967) has reported on the establishment of cell lines from larvae derived from *Aedes aegypti* and *Aedes albopictus* which do form monolayer cultures and which can be passaged and maintained on a hemolymph free media. These cultures are not generally available and they have been introduced into this country only during the past 6 months.

METHODS. Several months ago we initiated studies in an attempt to develop methods to prepare reproducible primary or continuous tissue cultures from the pupal or adult stage of mosquitoes. Three species were utilized: 1) pupae of *Aedes vexans*, 2) a newly emerged to 12 hours old adult female *Culiseta inornata,* and 3) pupae of *Aedes triseriatus*. In each case the material was washed in Earle's balanced salt solution (EBSS), sterilized by submersion in 3 percent hydrogen peroxide, followed by several washings in sterile media. The specimens were then treated as follows:

The peritrophic tissue of each pupa was removed microscopically, and the appendages and heads removed from the adults. The remaining materials were then minced with fine forceps and scissors and/or finally homogenized gently in media utilizing a 1 ml syringe and 25 gauge needle. The resulting suspensions were placed in stoppered T–15 glass flasks containing perforated cellophane, or in Leighton tubes. In addition, droplets were placed in plastic petri dishes which were then clotted with chick embryo extract-plasma mixtures, and cover slips applied.

The medium used in all cases was a modification of that described by Yunker *et al.* (1967), and is as follows:

Grace's Insect Tissue Culture Medium (GIBCO) Supplemented with	
Heat Inactivated Fetal Bovine Serum	10%
Whole Chicken Egg Ultrafiltrate	10%
Bovine Plasma Albumin (Fraction V)	1%
D(+) Trehalose	1%
Penicillin	100 units/ml
Streptomycin	100 μ g/ml
Fungizone	25 μ g/ml

All vessels contained from 2 to 5 ml of medium which was replenished or replaced at weekly intervals. All cultures were incubated at 28° C. Those prepared in open petri dishes were held in a CO_2 incubator under a moist atmosphere.

A total of 18 cultures were set up, representing 15 *Aedes vexans,* two *Culiseta inornata*, and one *Aedes triseriatus*. The cultures were observed at weekly intervals during the past five months.

RESULTS. The findings can be summarized as follows according to mosquito and type of culture:

1. *Culiseta inornata.* Growth of cells was observed in one culture only. No definite evidence of cellular multiplication was seen from any of the explants or suspended pieces of tissue for a period of a month. At this time round, unattached cells were observed. The cells ranged in size from 20μ up to 55 to 60μ in diameter. A few spindle-shaped cells 40 to 80μ by 10 to 18μ wide were observed. Many cells were bizarre in appearance; binucleation or multinucleation was common. The cells are very granular in appearance and often appear pigmented. In some respects they appear similar to the *Aedes* cells described by Grace (1966) and Suitor (1966a); however the spindle-shaped cells did not predominate, and in general *Culiseta* cells were larger in appearance. The number of cells has increased rapidly since first observed. Seven

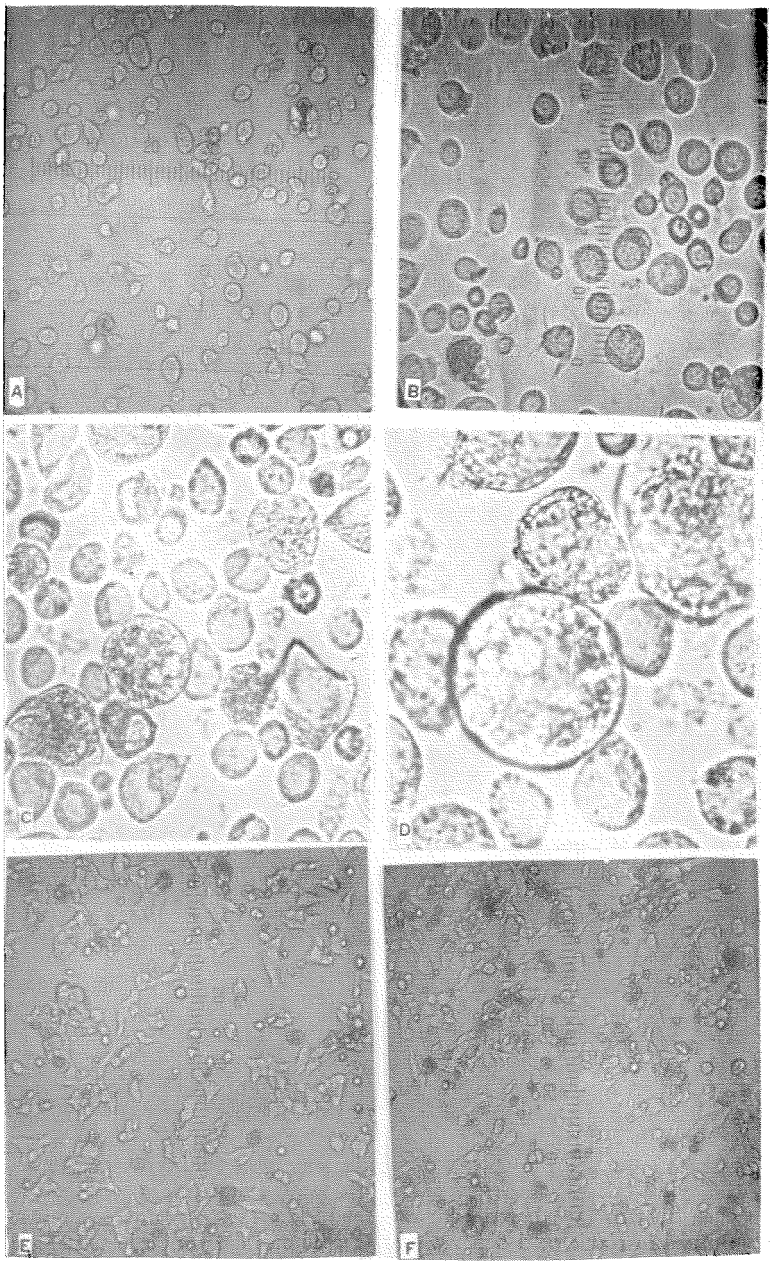

FIG. 1.—GSRI's adult mosquito line from *Culiseta inornata* sixth generation.
A—10X; B—20X; C—40X; D—60X; E—cells beginning to attach to form monolayer, 10X magnification. F—same as E.

subcultures or passages have been made and cell concentrations in the range of 8 x 10^5/ml have been achieved. (See Figures 1 and 3.)

It is not possible to determine from which tissues the cells have arisen; however, there was some indication from microscopic examination of tissue fragments that they were from the thoracic area.

2. *Aedes vexans.* Of the 15 odd cultures that were set up as explants or suspension cultures, only one or possibly two showed evidence of cellular multiplication after a 6 week period of time and only in those cultures planted in stoppered T-flasks.

Like the previous cultures, the cells were free floating; cell counts in the range of 10^3/ml were encountered. In the positive culture, the cells appeared to be growing at a much slower rate over a period of one month. In general, they were similar in appearance to those observed in the *Aedes aegypti* or *Culiseta* cultures; how-

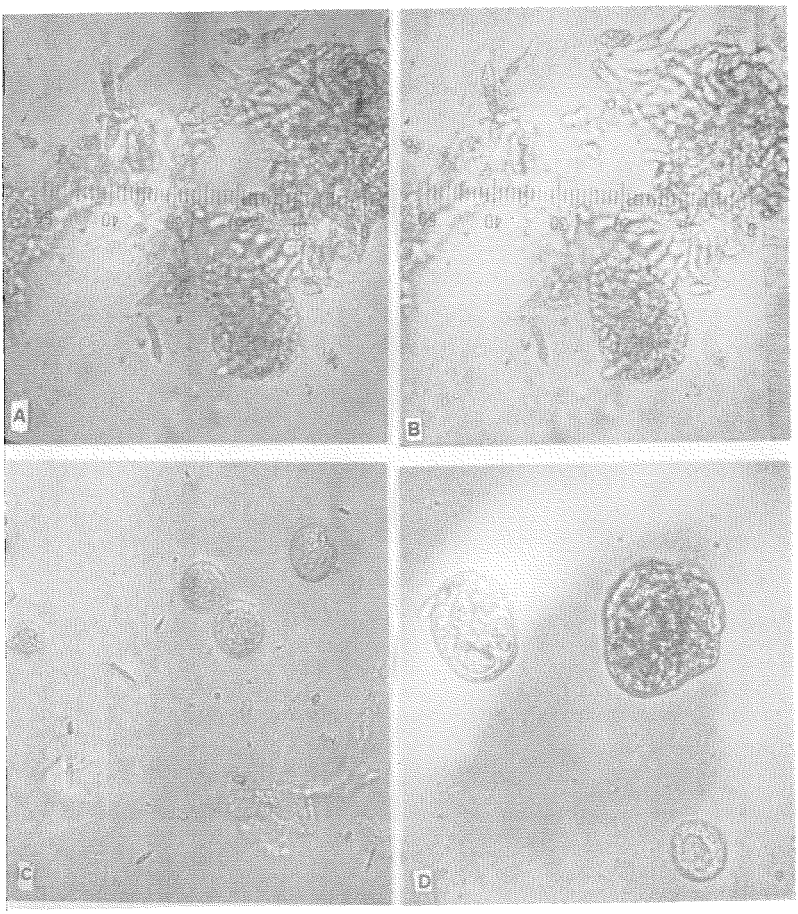

FIG. 2.—GSRI's mosquito line derived from pupae of *Aedes vexans.*
A—10X magnification explant; B—same as A; C—20X magnification of cells in suspension; D—40X magnification.

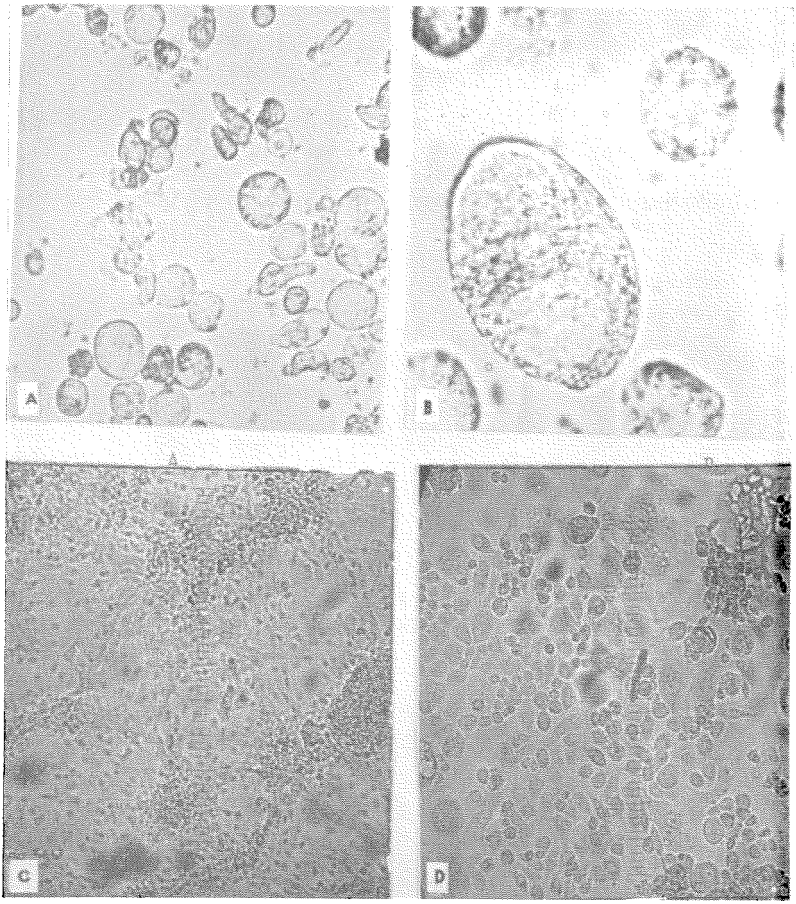

FIG. 3.—*Aedes* sp. cultures.
A—Grace's line of larval-derived *Aedes aegypti*—adapted to media without hemolymph (Sweet and Dupree), 10X magnification—unstained. B—Same as A. 60X magnification—unstained. C—Singh's monolayer of larval-derived *Aedes aegypti*. 10X magnification—unstained. D—Singh's monolayer of larval-derived *Aedes albopictus*. 10X magnification—unstained.

ever, the cells were smaller, 10 to 20μ size predominating. Passage of the cells has been only moderately successful due to the very slow multiplication rate. (See Figures 2 and 3.)

Explanted cultures which were incubated under CO_2 conditions with or without plasma clots appeared to be viable over a period of 6 to 8 weeks; however, thus far, there has been no conclusive evidence of cellular outgrowth nor have we been able to detect free floating cells in the supernatant fluids. (See Figure 2.)

3. Studies with *Aedes triseriatus* suspended cultures thus far have not yielded the results that were seen with the other two lines; however, the tissue still appears to be viable.

SUMMARY. In summary, these preliminary results appear to indicate that it may be possible to establish cell lines from adult and pupal stages of at least two species of

mosquitoes. If this is true, it will be the first instance of such cultures other than from larval tissue. These cell lines appear more similar to Grace's (1966) *Aedes aegypti* lines than to Singh's lines (1967) because they grow as free floating cells rather than as monolayers. The results also indicate that the tissue culture can be established without the use of insect hemolymph.

Should these lines become firmly established and well characterized, we will have another tool to study mammalian arboviruses more readily in their natural insect host tissue. Further, the development of mosquito tissue culture may well open up new vistas to search for natural mosquito viruses which, if found, may have the potential for use in mosquito control.

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