GROWTH OF PRIMARY MONOLAYER CELL CULTURES FROM THE MOSQUITO, *CULEX SALINARIUS* (DIPTERA, CULICIDAE)

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INTRODUCTION. The *in vitro* culture of mosquito cells offers considerable potential for study of viral pathogens which have these insects as vectors or reservoirs. Some of the few cell lines available for this purpose are discussed by various authors in recent years (Grace, 1966; Singh, 1967; Sweet and Dupree, 1968; and Schneider, 1969). During a symposium on insect tissue culture held in Washington in March, 1970, additional progress in mosquito cell culturing was reported—including a description by Schneider (personal communication, 1970) of her recent success in starting a cell line from *Culex salinarius*. Prior to this time, no culturing of cells of *C. salinarius* had been attempted except for the work reported here and the purpose of this paper is to describe the success in obtaining primary cultures from this species during 1968 and 1969.

MATERIALS AND METHODS. The mosquitoes utilized in this study were obtained from the colony of *C. salinarius* started in this laboratory for this purpose (Wallis, and Whitman, 1968). Tissues from this mosquito, required for starting tissue cultures, were provided by the axenic rearing technique described by Wallis and Lite (1970).

The culture medium used was that described by Singh (1967). It consisted of a four-to-one ratio of basic medium to heat-inactivated bovine serum. The basic medium per 100 ml contained: 20 mg $NaH_2PO_4H_2O$, 10 mg $MgCl_2GH_2O$, 20 mg KCl, 20 mg $CaCl_22H_2O$, 700 mg NaCl, 12 mg $NaHCO_3$, 400 mg d-glucose, 650 mg lactalbumin hydrolysate, 500 mg yeastolate, 78.5 ml glass distilled water, and 1.5 ml of a mixture of penicillin and streptomycin (to give 1,000 units of penicillin and 1.0 mg streptomycin per ml). Fetal bovine serum and antibiotics were added after the medium was filtered through a size 0.22 μ millipore filter. In a later portion of this study the initial medium preparation was supplemented with 16 percent of a commercial preparation T.C.-199 (with glutamine and 2X Earle's base, Cat. No. 115 EP, Grand Island Biological Co., New York). Initial pH was 7.0.

Tissues from various growth stages of *C. salinarius* were prepared for culture: newly-hatched larvae, 3rd instar larvae, 4th instar larvae, pupae and adults. Numbers of these (from 10 to 200) were placed in 0.25 ml of 0.25 percent trypsin solution in the well of a deep depression slide and minced with microdissection instruments under a dissecting microscope. Trypsin solution containing the minced tissue was pipetted into tubes containing 4 ml of medium and spun in a chilled centrifuge at 2,000 rpm for 15 minutes. The supernatant was discarded and the remaining tissue pellet diluted with 8–10 ml of fresh media and transferred to 30 ml plastic (Falcon) flasks. These were incubated at 23° C overnight, and those remaining free from contamination (as determined by lack of bacterial or mycotic growth in the nutrient medium) were then incubated at 28° C.

After initial cell cultures were started and placed in incubation, they were ex-

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amined every day. The medium was changed at various intervals whenever pH changes indicated a build-up of acidic condition, or when subculturing of growing cells was attempted. When good initial growth occurred, medium was replaced routinely twice a week. In this case, two-thirds of the old medium was replaced with fresh medium. The cells were then centrifuged from the old medium and returned to the flask. When cultures developed only budding tissue and vesicles, these were centrifuged from the old medium, redissected in 0.25 percent trypsin solution and reseeded in fresh medium.

To keep a permanent record of the stages of cell growth obtained, stained slides were prepared periodically. For this purpose, Leighton tubes containing glass slides were used. Cell suspensions in medium were transferred from the flasks to the tubes, and when monolayer growth occurred, the slides were removed and the cell sheets fixed by immersion in Bouin's solution for 15 minutes. They were then rinsed in 70 percent alcohol, dried and stained with Giemsa stain; and photomicrographs were then prepared.

A total of 125 initial cell preparations from the various stages of larvae, pupae, and adults were started. Subculturing was attempted from all primary cultures exhibiting heavy growth.

RESULTS AND DISCUSSION. Various degrees of cell growth were observed in cultures from each of the developmental stages of *C. salinarius* in newly hatched larvae, 3rd instar larvae, 4th instar larvae, pupae and adults. The various stages produced differences in both the amount and type of cell growth. One type was the formation of multiple free-floating vesicles as described by Singh (1967). Another was the formation of monolayer cell sheets adhering to the sides of the flask. When vesicles were formed they were rounded, hollow balls of cells originating from the cut ends of tissue segments. They developed much more readily from tissues derived from larvae than from those of pupae. They appeared first as very small outgrowths of the tissue. Spherical in appearance, they seemed to be completely hollow and quite rigid. However, upon being removed from the medium and punctured, they collapsed as though they were filled with fluid. These vesicles increased rapidly in volume, and upon reaching maximum size, detached from the tissue and remained floating within the medium. With proper care, the vesicles were kept alive for long periods of time—as long as 4 months. Multiplication of vesicles occurred in a regular sequence, each began to bud until two were formed, not necessarily of equal size. The daughter vesicles then detached and floated independently within the medium. Vesicle multiplication was induced by fragmentation initiated in the process of centrifugation and redissection in trypsin.

New vesicles often grew from the dissected parts of the old ones. Frequently, more than one formed from a single tissue site. It was not uncommon for three or more to bud from the small cut end of a tissue strand. According to previous reports of vesicle formation, attachment seldom occurred (Mitsuhashi, 1965). However, in this study vesicles occasionally did adhere to the bottoms of the flasks.

Growth of cells and vesicles from first-stage larvae and adults was rather slight, although some cellular activity was evident.

The most abundant cell growth developed from 3rd and 4th instar larval and pupal tissues. The monolayer type of growth patterns of cell derived from the fourth stage larvae was studied in detail. Soon after the trypsinized cell suspension was pipetted into flasks, many of small, round, independently-floating cells were observed. Within 12 hours, these cells began to group together into many small aggregates comprised of four to six cells. From one side of the cluster, one or several cells sank to the bottom of the flask, and the rest soon followed. By the end of 24 hours many small clumps of cells were secured to the side of the flask. At approximately the same time, a few spindle shaped cells also appeared to be ad-

hering to the bottom of the container. After 36 hours of incubation, while the number of spindles increased, the new growth of this type did not form a continuous patch of cells. Elongated cells within the clump developed pseudopod-like extensions which served to disseminate the groups and to align themselves to other closely attached cell aggregates. This type of development continued for another 12 to 15 hours until a peak growth rate was reached.

After a period of time cell multiplication began to dwindle, and it became obvious from the dark color of the contents of the flask that a medium change was necessary. Although fresh medium was added, the change did not induce a continuation of rapid cell reproduction. In fact, a steady decline in cell attachment began following the peak reached at 48 hours, although as long as periodic medium changes were made, the round floating cells remained healthy within the flasks. One group of such cells was kept alive *in vitro* for 8 weeks. It appeared that after the prolonged period in which these cells were kept alive at a constant population rate, cell multiplication had taken place.

Several factors seemed associated with the subsequent amount of cell growth obtained from initial tissue preparation. One related to the manner in which the tissue was minced. If the tissue was cut with sharp microscalpels, rather than torn apart with dissecting needles, more growth resulted. The best results correlated quite well with the maximum number of clean incisions performed on the larvae or pupae. Dissections of this type resulted in a minimum of cell mortality and a maximum of both vesicle formation (in the case of larvae) and primary monolayer cell growth (from pupae). Another factor observed in experimental cultures of all stages was the density of the cell population within each flask. This seemed to be an important variable in determining the primary growth rate. Flasks seeded with few cells resulted in only several widely separated patches of growth. Those flasks which were overseeded produced an abnormally large amount of toxic metabolic wastes, and resulted in premature death and sloughing off of cells. Through trial and error it was found that the best results were obtained when 15 to 18 fourth stage larvae or pupae were dissected for each flask.

Comparison of the intensity of growth in cultures from tissues of various stages of *C. salinarius* can be summarized as follows: From newly hatched larval and adult tissues only light growth was obtained; medium growth developed from tissues of third and fourth stage larvae; and heavy growth resulted in cultures of pupal tissues.

In summarizing the types of cells and growth patterns observed in cultures from the various developmental stages, these observations were made: there were many epithelial cells concentrated and attached to the bottom of culture flasks in cultures from larval stages and pupae, but only a few scattered in patches in those from adult tissues. Attached fibroblasts were not seen in cultures of adult tissues, but there were many concentrated in the ones from larvae and pupae. Many cells with pseudopod-like extensions (described by Singh, 1967) as muscle cells were observed in clusters in cultures, and none from tissues of adults. Many vesicles appeared in larval cultures, but only a few in pupal cultures and none in adult cultures. Free-floating cells were scarce in larval cultures, whereas many were seen in pupal and adult cultures.

Primary monolayer cell-sheets attached to the flasks were maintained for periods of from 2 to 6 weeks with regular medium changes, but no successful subculturing was possible with them. However, subcultures were made from those containing growing vesicles. These were harvested, redissected in trypsin solution and transplanted into new medium repeatedly. One such culture was transferred 12 times over a 4-month period. However, no luxuriant growth such as obtained by Singh with *Aedes aegypti* tissues was ever achieved, and no continuous cell line has

yet been established from tissues of *C. salinarius*.

SUMMARY. The first successful primary tissue cultures from the mosquito, *Culex salinarius* are described together with the types of cell growth and the techniques utilized. Comparison is given of the types of growth obtained from the various developmental stages of the mosquito. From larval tissues, vesicles developed and were maintained in culture for periods up to four months. From pupal tissue, primary monolayer cell-sheets attached to the flasks developed repeatedly and were maintained for periods from two to six weeks.

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OBSERVATIONS ON LOW VOLUME SPRAYING

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The concept of low volume appeared in the operating techniques of the spray planes of this District in 1955. Airplane spraying at the rate of one quart per acre started in that year. Fifteen years later applications are at the rate of 0.5 to 1.0 fluid ounce per acre.

The change has brought about considerable savings. The cost of the solvent saved in a year can be in the order of 5¢ per acre, or $5,000.00 for each 100,000 acres! Other savings, more difficult to isolate, are in the operation of the airplanes and their service vehicles. At one time we operated two, 2,000-gallon tank trucks, one for each plane. Each tanker carried water, insecticide, gasoline and oil. There were transfer pumps, recirculating pumps and gasoline pumps. There were measuring tanks and mixing tanks. Also, there were tank trucks stuck trying to reach landing strips near the scene of operations.

Fifteen years later we have a small trailer pulled by a ½ ton pick-up truck. The trailer carries insecticide solution in a 200-gallon plastic tank. An insecticide-resistant transfer pump can fill both planes in just minutes. A 100-gallon supply of gasoline is in stainless steel tanks. An explosion proof pump services the planes quickly.

New techniques were developed during the years as application rates were reduced, and we now accomplish more, with fewer men, doing less work. What we thought was to be an Utopian operation was spoiled when we awakened to the fact that we did not know our rate of application at any given time. We knew rates *before* application and *after* application but not *during* application. Sometimes a good kill was obtained in an area, but, in the next area treated, just a few miles away, there would be a poor kill.

Pressure appeared to vary for no apparent reason. Better pressure gauges showed clearly that pressure did vary. Precision pressure regulators were tried in place of