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ESTABLISHMENT OF CELL LINE FROM *CULEX* MOSQUITO*

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S. KITAMURA. *Establishment of Cell Line from Culex Mosquito**. Kobe J. Med. Sci. 16, 41-50, March, 1970——The cell line established from the mosquito, *Culex molestus*, growing well in the medium supplemented with 10 volume percent of calf serum instead of the derivation of insect fluid, has been still continuing its growth for over 87th passage at 28°C in the author's laboratory. Population doubling time of this cell line was about 30 hr and chromosomes observed in 80th passage were 2n(=6) in the main.

For the convenience of arboviruses investigations, a modification of the culture medium was made and the following results were preliminary obtained; those media supplemented with one volume percent of either bovine plasma albumin or calf serum were able to maintain the cultures for over a month without showing any changes in their appearance, suggesting its promising availability for the virological research.

The search for the cell line to be subcultured in the medium without calf serum is under investigation.

INTRODUCTION

Many attempts to obtain the cell line have been performed with the objects to investigate the behavior of arboviruses in connection with their insect hosts, and the reports on the established cell lines, obtained from *Aedes* mosquitoes, appeared successionally in the past decade (Grace 1966, Gubler 1968, Peleg 1966, 1968a, Singh 1967, Sweet 1968). These cell lines have been promptly applied to the virological investigations and much of the results were reported (Converse 1967, Filshie 1968, Peleg 1968b, Rehacek 1968, Singh 1968).

There still remains, however, a lot of practical task, such as further improvement of the medium for many other insect species and different tissues, completion of the synthetic medium for the experiments, and establishment of the cell clone appropriative to special virological approaches (Suitor 1966, 1969, Varma 1969). Much more different cell lines from the various species of insect, if obtained, would be contributive not only to the understanding of interaction between viruses and their insect hosts but also to much of the other practical studies (Ball 1954, Beckel 1956, Mitsuhashi 1964, Varma 1965). In this paper, the results are described of the establishment of cell line from the ovary tissue of mosquito, *Culex molestus*.

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MATERIALS AND METHODS

Mosquitoes: Female *Culex molestus* mosquitoes selected out of the population maintained in the laboratory condition for over 7 years were used as tissue sources.

Tissues: About 24 hr after emergence at 18 to 20°C breeding condition, ovaries were desected out of the individuals in a condition as intact as possible and transplanted into the culture vessels as the undissociated explants. The details of primary and subculture procedures were the same as those reported previously (Kitamura 1965, 1966).

Culture media: A basal culture medium (BCM) was consisted of NaCl 650 mg, KCl 50 mg, $CaCl_2 \cdot 2H_2O$ 10 mg, KH_2PO_4 10 mg, $NaHCO_3$ 10 mg, glucose 200 mg, lactalbumin-hydrolysate (Difco) 1,000 mg and glass distilled water 100 ml, and sterilized at 115°C for 15 minutes. The routinely used culture medium (RCM) was, just before use, made up by adding 2 parts of synthetic medium 199 and one part of heat inactivated calf serum (56°C, 30 minutes) to 8 parts of BCM, and adjusted to a pH 7.2 with 2% KOH.

Three different media were prepared for the other experiments: 1, A medium for cell sheet maintenance was decreased in rate of calf serum supplement to one tenth of that of the RCM. 2, A maintenance medium for virological experiments was a modified RCM containing one volume percent of bovine plasma albumin (BPA) (Fraction V, Armour) instead of the calf serum. 3. The preliminary adaptaion test of cells was conducted in another modified medium composed of BCM 80 ml, 199 solution 20 ml, bovine plasma albumin 1,000 mg, and calf serum 1 ml. The third medium tentatively named M-41 by the author was sterilized by filtration, instead of heating, just before use. The pH of the three media described above was adjusted to 7.2.

The primary culture was handled in small square tubes (1.5×1.5×4.0 cm in size), and serial culture was performed using the half flat plaque bottles (2.0×5.0 ×8.0 cm in size). Both of the culture bottles were incubated at 28°C. Cell counting for estimating generation time was made by the usual heamocytometric method and the chromosome observation was made with Colchicine-Hypotonic-Acetic acid fixation and Giemsa staining.

No antibiotic was used through the experiments.

RESULTS

As previously reported, the undissociated ovaries from the mosquitoes were explanted in a modified medium in October 1967. Cell outgrowth began from the periphery of the explants about 20 hr after the culture was started. Approximately 30 days after culture initiation, the active cell multiplication stopped and almost none of the cell outgrowth was observed during the next 5 months. The author noticed, however, that the cells adhered to the glass surface have kept their appearance without any microscopical changes through this period, renewing the medium once a week.

Six months after the culture initiation it was observed microscopically that a small number of fine epitherial-type cells began to increase in number slowly accompanying alternation of the cells. Several small cell sheet consisted of about 20 to 200 cells soon came to be observed also. The culture medium was changed twice a week during the period.

At the end of May 1968, 7 months after culture initiation, one cell colony actively growing in size was observed in one of the culture vessels and this growing cell colony finally covered almost all of the glass surface about 20 days after its growth came to be observed.

For the first subculture, the old medium was decanted, and washed by gentle pipetting twice respectively with one ml of fresh medium, and the cell suspension pooled was equally divided into two bottles with additional 2 ml of fresh culture medium each.

The cells remained in the original vessel showed still active multiplicity and they were cultivated over 8 times for about 10 weeks, when one ml of the medium was replaced once a week.

Subculture was made repeatedly thereafter at about 10 days intervals by the gentle pipetting for 6 months with the medium supplemented with 20 volume percent of heat inactivated calf serum. In the early subculture, the suspension containing about 3,000 to 5,000 cells in 3 ml of the culture medium was placed in a bottle. For about half a year, the medium with 20 volume percent of calf serum was used. Then the amount of the serum was decreased gradually to 10 volume percent and the cell growth rate, once lowered, was found to be recovered through 10 times serial passages. Cell seeding was done by a 1 to 2 split in the best condition of cell growth, but occasionally, once about every 20 passages, the way of splitting had to be modified. That is, in the culture where an obvious cell malformation or other change of appearance occured, cells from two to three bottles were pooled and seeded into one bottle to continue the subculture. Since 70th passage, however, because of a rapid cell increase in number, the subculture was made by a 1 to 3 split twice a week.

The cell sheet mainly consisted of the epitherial-type cells, a small number of fibroblast-type cells, scattering like a focus, and another type cells was observed in some bottles.

The growth rate of cells is shown in Figure 1 with an estimated population-doubling time of about 30 hr, and 10 fold cell increase in number was observed during 5 days. It was not capable to classify clearly the cells in this line, however, four types were morphologically distinguished in the culture (Photo 1, 2, 3, 4). The first type was spindle-shaped, through passages dominantly grown and loosely connected with each other. Another was a rather square shaped one, which was slightly larger in size but small in number and slow in the growth rate than the first one, being apt to show patch like appearance in the monolayer. Another was fibroblast like one and arranged in the monolayer connecting with each other at both ends of the cell; it was hard to make them detached from the glass surface either by usual gentle pipetting or by trypsinization. The last one was several-fold large in size and very few in number and frequently scattered separately in the monolayer.

Cell suspension obtained from every passage usually contained these four cell types and individual type of them showed their own growth rate through passages. Attempts to obtain cell clone(s) are under investigation.

In most of the cells, 2n chromosomes complement of 6 was observed, but in some cells, the increased number of the chromosome was observed (Photo 5).

Several passages were made using the medium M-41. Cell proliferation rate with this medium was about 5 fold lower than that of the usual, however, there were no apparent changes in their appearance.

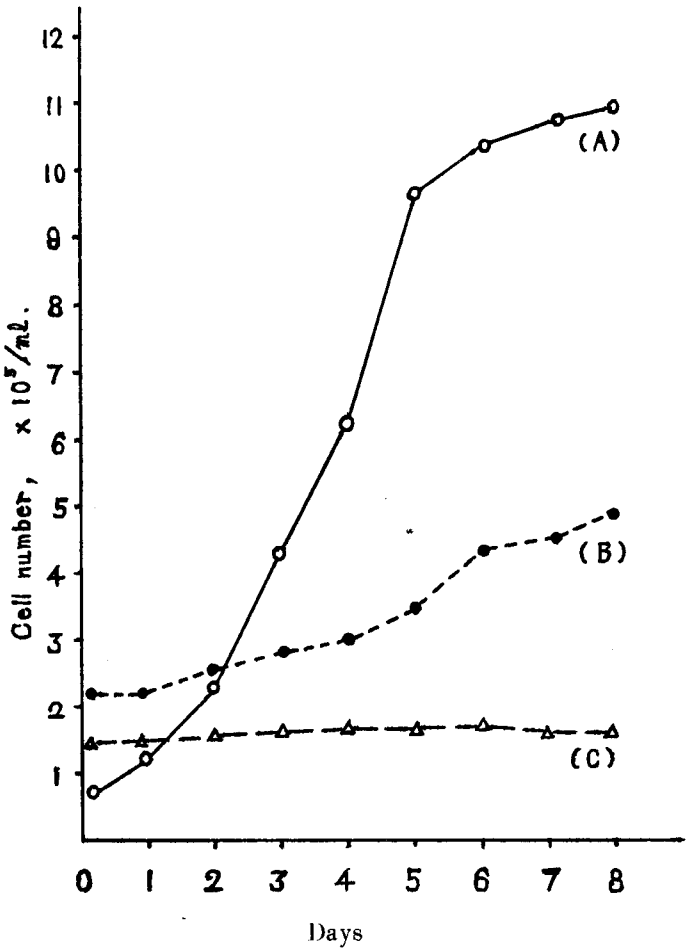

Fig. 1. The growth curves of *Culex molestus* cell line in the 3 different media. The medium (A) was supplemented with 10 volume percent of heat inactivated calf serum, (B) was contained 1,000 mg of BPA and 1 ml of calf serum, and (C) was supplemented with 1 % of BPA.

To maintain the culture for a long period without any cell proliferation, a modified RCM containing one volume percent of bovine plasma albumin (BPA) was found appropriate. This medium could keep the cells in good condition reserving their potential multiplicity. In fact, the cells, when seeded, came to show the normal growth within a few days in the usual medium.

DISCUSSION

The established cell lines from the *Aedes* mosquitoes were obtained by many investigators (Grace 1966, Singh 1967, Peleg 1968b, and Varma 1969), and these cell lines greatly contributed to the practical investigation on the insect-borne viruses (Filshie & Rehacek 1968, Yang 1969, Peleg 1968b).

It has been also required to obtain *Culex* mosquitoes cell lines for the studies on arboviruses, since *Culex* mosquitoes are important vectors of arboviruses as *Aedes* mosquitoes in nature. No previous report, however, reported the establishment of the *Culex* mosquitoes cell line.

The present paper reported the results of the establishment of a cell line from the ovary of *Culex molestus* after consecutive work on the improvement of culture medium and of the process of subculturing.

The primary active cell outgrowth was obtained from the ovary tissue of *Culex molestus* using the medium enriched with 20 percent of calf serum. The calf serum used was found to be the effective additive to the medium not only for the primary cell culture but also for successive transfer of the cells.

Therefore, this medium was used for the following subcultivation, and the results obtained indicated the successful repetition of 85 times cell transfer. Even after the subsequent cell transfer repeated for about 2 years, a remarkably morphological cell change was hardly observed. The cell population doubling time was estimated as about 32 hr during the active growth phase at 28°C, and the majority of cells at 80th passage had the normal chromosomes of $2n(=6)$.

Based on these results, it is thus concluded that the cells continuing their growth over successive transfer for long period represents the established line of *Culex* mosquito. For the practical application of this line, however, there still remain certain limits.

The first limitation is in the problem concerning the additive of the culture medium. Though the calf serum used in the present work showed both of a great advantage to promote cell growth and to continue the subculture, it limited the use of these cell to arbovirus investigation because of the possible existence of an antibody to arbovirus in it. To eliminate such a possibility in the practical application of this cell line, it is desirable to maintain the cells for a certain period in the medium free of the calf serum but enriched with the additive not containing antibody-like substance. From this reason, BPA, which is easily obtained than calf serum, was tested and found that it did permit healthy survival of cells for over a month, and the most available degree of BPA supplement to the routine maintenance of cells was decided as one volume percent.

The second is the problem that the cell population is composed of various

types of cells. It has been suggested that virus may propagate only in the susceptible cells in the insect host (Peleg 1968b). The special cell strain capable of propagating or reproducing the virus, if obtained, would be worth using practically. The selection of different cell lines from the original line using the several modified culture media is under investigation in the author's laboratory.

SUMMARY

1. A mosquito cell line was established from the ovaries of *Culex molestus*.

2. The culture medium used was composed of 80 parts of BCM, 20 parts of 199 solution and 10 parts of heat inactivated calf serum.

3. Cells of 4 types in this line have been subculturing over 87th passage at the present culture condition growing with their own growth rate respectively.

4. Sorting out of the different type of cells was not successful yet.

5. Cells survived over a month in the maintenance medium supplemented with either one volume percent of calf serum or 1% of BPA.

6. The population doubling time was about 30 hr at the active growth phase and 2n(=6) complement of chromosomes was observed in the majority of cells.

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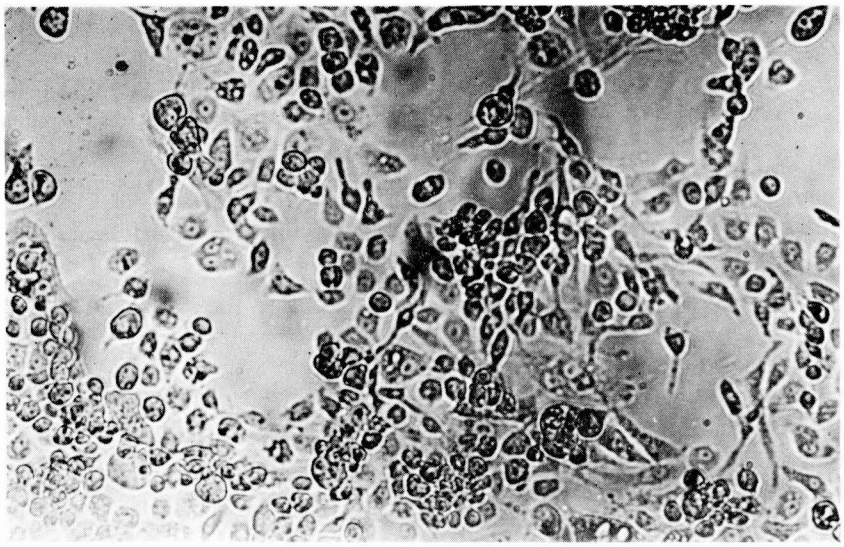

Photo 1. *Culex molestus* cell line showing four cell types at 85th subculture. Living cells, 2 days after seeding. ×100

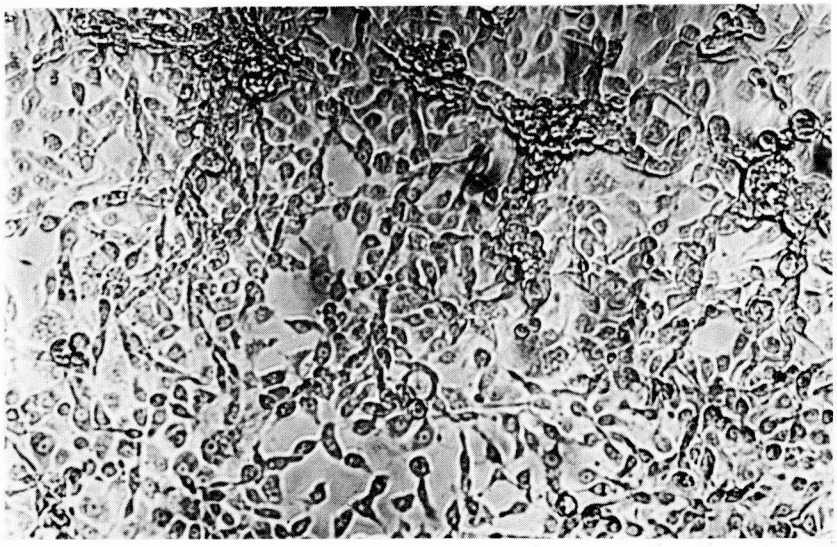

Photo 2. The spindle-shaped epitherial-type cells at 85th subculture. Living cells, 5 days after seeding. ×100

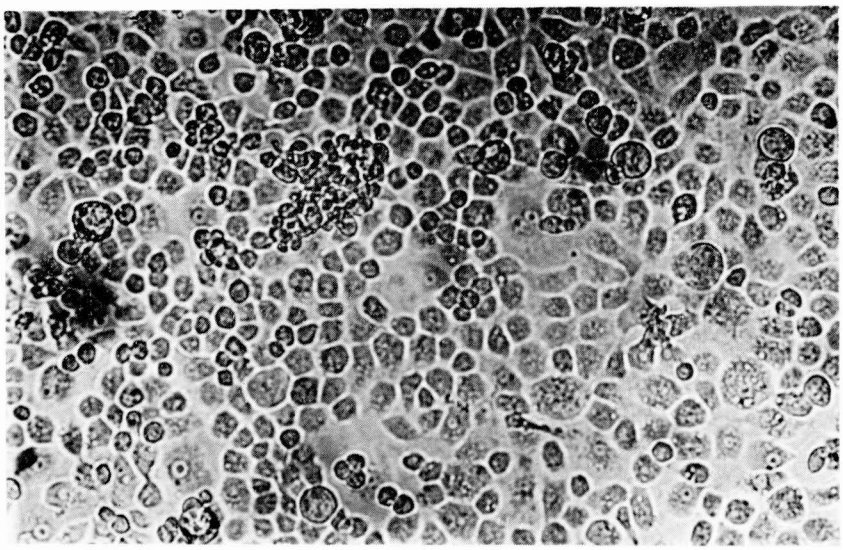

Photo 3. The square shaped epitherial-type cells and the fourth large cells at 85th subculture. Living cells, 7 days after seeding ×100

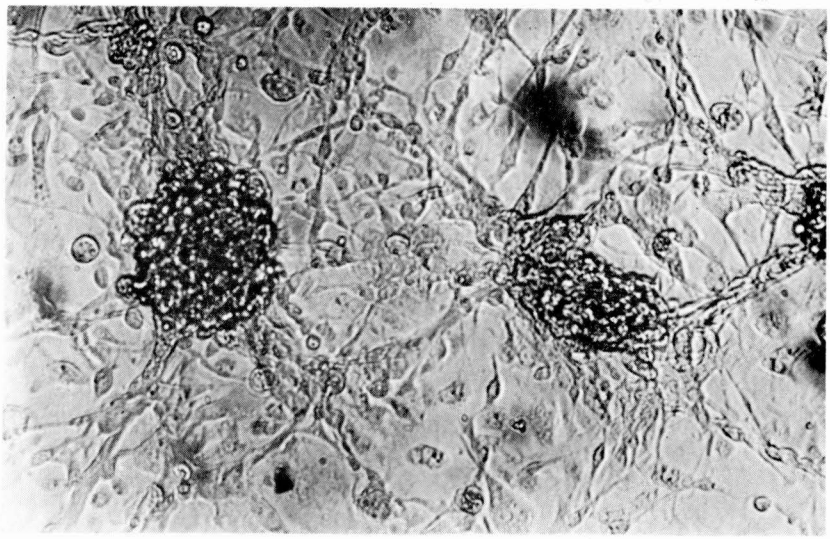

Photo 4. The fibroblast-like cells at 85th subculture. Living cells, 6 days after seeding. ×100

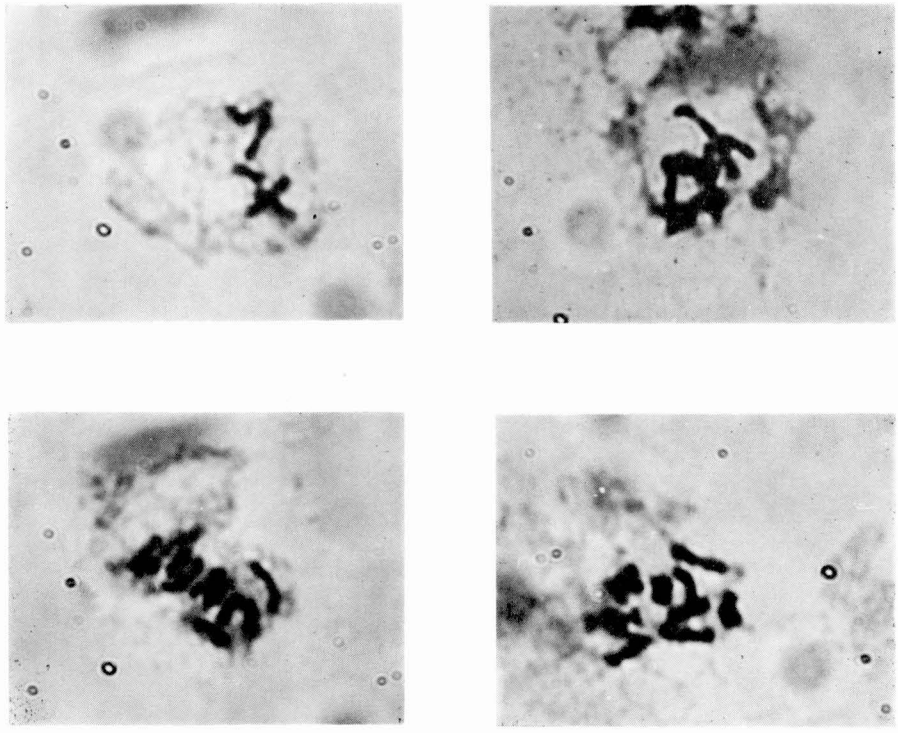

Photo 5. The chromosome number observed in the cells at 80th subculture. Cells were stained with Giemsa after colchicine-acetic acid treatment, 2 days after seeding. ×400