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Establishment of a Cell Line from Larvae of **Culex Pipiens fatigans** and its Susceptibility to Infection With Some Group C Arboviruses *

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RESUMO

Os autores relatam o estabelecimento de uma nova linhagem de células obtidas a partir de mosquitos *Culex pipiens fatigans* colonizados em laboratório. Empregando esta nova linhagem de células foi realizado um estudo comparativo de sua susceptibilidade a cinco arbovírus do Grupo C: Apeu, Marituba, Murutucu, Oriboca e Itaqui. Todos os cinco tipos de arbovírus foram capazes de infectar células, demonstrando um aumento no título de 0,5 a 2,0 unidades logarítmicas. A infectividade foi demonstrada por sub-inoculação em camundongos recém-natos e cultura de células HeLa e não pelo efeito citopatogênico das células de mosquito. Foi realizada também uma comparação entre a susceptibilidade desta nova linhagem e um estudo similar empregando células de *Aedes albopictus.*

INTRODUCTION

The availability of stable cell lines from mosquitoes has provided opportunities to study arboviruses in vector cells *in vitro.* The *Aedes albopictus* and *Aedes aegypti* cell lines established by Singh (10), em 1967, have proven to be susceptible to numerous arboviruses - Singh and Paul (11), 1968; Buckley (5), 1969; Yunker and Cory (12), 1975. Since these lines were established, many more species of mosquitoes and invertebrate cell lines have been established and their susceptibility to arboviruses has been tested - Barry (1), 1975; Barry and Conceição (2), 1977. Other utilizations of invertebrate cell lines have been noted by Maramorosch (7), in 1975.

With a plan to study the comparative aspects of susceptibility of a number of arboviruses to various dipteran cell lines as well as attempts to establish new cell lines for such testing, this present paper reports on the establishment of a new cell line derived from neonate larvae of *Culex pipiens fatigans* and its susceptibility to infection with five members of Group C arboviruses occurring in Brazil, name-

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ly Apeu, Marituba, Murutucu, Oriboca and Itaqui.

MATERIALS AND METHODS

Cells cultures: Utilizing the method of Schneider (9), in 1971, egg rafts of this species were collected over an 8 hour period and allowed to develop to the point of red eye spot formation. Rafts were separated by 2.5% sodium hypochlorite solution and surface sterilized by immersion in 10% benzalkonium chloride and by 70% ethanol. Following an over night incubation period at 27° C, the freshly hatched larvae were cut into two or three fragments and trypsinized in Rinaldini's salt solution - Rinaldini (8), in 1954, with 0,2% trypsin (1:250, Difco). Approximately two hundred larvae were treated for each primary culture attempt. Since composition of medium is a critical factor for growth, the following media were tested for their ability to support cell replication - Mitsuhashi-Maramorosch, MM/VP_{12}, Grace's, Schneider's and Hsu-721 Hink (6), 1976.

Viruses: The viruses evaluated in this study were suplied as lyophilized suckling mouse brain material from either the Institute Evandro Chagas, Belém, Pará, Brazil, or the Department of Virology, New York State Health Department, Albany, New York, USA. The viruses were reconstituted in phosphate buffered saline with 0.75% bovine albumin. The strains employed were Apeu (BeAn 848), Marituba (BeAn 15), Murutucu (BeAn 974), Oriboca (BeAn 17) and Itaqui (BeAn 12797) all having culicine mosquitoes as natural vectors and all having close antigenic relationships as determined by hemagglutination-inhibition, complement fixation and neutralization testing - Berg (4), 1975.

Inoculation of cultures: Stationary glass tube cultures were prepared with cell suspensions of stock cultures. The cells were incubated at 27° C until confluent monolayers were obtained in three days. The cultures were inoculated with 0.1 ml of a 10^{-2} dilution of the infected mouse brain stock. An adsorption period of 2 hours at 37° C was permitted, after which the cultures were rinsed with Rinaldini's salt solution. Fresh culture medium without fetal calf serum was added to the tubes. Control tubes of medium plus virus and medium plus uninfected cells were also prepared. Duplicate cultures were frozen at minus 70° C for viral assay after 0, 1, 2, 3, 4, 7, 10 and 14 days incubation at 37° C.

Viral assay: Prior to assay each pair of infected cultures and medium controls were thawed and frozen three times, pooled and centrifuged to remove cellular debris. Serial 10 fold dilutions of each sample were made in Dulbeco's phosphate buffered saline with 0.75% bovine albumin and virus was assayed in three day old suckling mice to determine an approximate titer and followed by a complete microtiter assay in HeLa cells grown in Eagle's minimum essential medium with 10% bovine serum and antibiotics.

RESULTS AND DISCUSSION

Cell line: As may have been expected, the response of these tissue fragments varied with the medium employed. In dozens of attempts the response was negative. This has been attributed to such variable as age of eggs at sterilization, age or condition of larvae at the time of mincing and trypsinization, lack of tissue adaptation or medium constituents. Ultimately, after three months, the tissue fragments had shed a sufficient quantity of cells to form a complete monolayer ($6cm^2$) on a glass cover slip within a Leighton tube. These cover slips were passed to larger vessels ($12cm^2$ and $25cm^2$) and cell proliferation occured. When complete monolayers were obtained, standard methods of passage were attempted. Gentle pipetting and trypsinization proved to be totally deleterious, but gentle scraping with a rubber policeman followed by gentle pipetting to obtain cell separation was totally effective.

Of the five types of media employed in the preparation of primary cultures, the HSU-721 supplemented with 15% fetal calf serum proved to be the medium of choice. The *Culex pipiens fatigans* cells were consideredstabilizad by the 12th generation and are routinely split in a 1:2 ratio weekly or may be maintained for over a month with 7-10 day changes of medium. The cells are somewhat less resistant do adverse conditions than are other cell lines maintained in the laboratory, such as *A. albopictus, A. aegypti, A malayensis. A pseudoscutellaris* and *Drosophila melanogaster*, and therefore must be treated more carefully.

Cell susceptibility: All of the Group C arboviruses multiplied in the *Culex pipiens fatigans* cells, but there was no production of cytopathic effect (CPE) even though these viruses ar known to produce CPE in many vertebrate cell lines. Two of these arboviruses, Marituba and Murutucu have been shown to produce plaques in *A. albopictus* cells Yunker and Cory (12), 1975.

Table 1 gives the pertinent details of the susceptibility of *C. pipiens fatigans* cells to the arboviruses tested. It is noted that all the viruses tested were capable of infecting the mosquito cells, resulting in a virus increase in the range of less than 1.0 to 2.0 dex units. This cell line must be considered to be much less sensitive to the same Group C arboviruses and VSV-Alagoas than that of other mosquito cell lines - Barry and Conceição (2), 1977; Barry & col. (3), 1979. Control tubes with medium and virus did not demonstrate viral activity at day 7. Control tubes of medium and cells without virus, as well as infected cultures, demonstrated some cell deterioration after being incubated for 14 days at 37° C. As noted above, these cells seem to demonstrate less resistance to adverse conditions, such as higher temperature, and the observation was not considered to be even the early stages of CPE.

It is of interest to note the somewhat varying degree of arbovirus infectivity amongst these antigenically closely related arboviruses. However, this may be due to the necessity of back titration invertebrate cells. It is not inconceivable that other comparative studies, which are being conducted in this laboratory with several other dipteran cell lines, will provide

TABLE 1

GROWTH OF ARBOVIRUSES IN *CULEX PIPIENS FATIGANS MOSQUITO* CELLS AS ASSAYED IN HELA CELLS

VIRUS	VIRUS INOCULUM	HIGHEST TITER IN CULTURE	DEX INCREASE
APEU	2.7	3.2	0.5
MARITUBA	3.0	5.0	2.0
MURUTUCU	2.5	3.7	1.2
ORIBOCA	2.9	4.8	1.9
ITAQUI	3.0	4.5	1.5

Titers expressed as 50% end points of infectivity per 0.1 ml.

information of value to indicate the significance of dipteran cell lines for the study of arbovirus kinetics.

SUMMARY

The authors present the results of the establishment of a new mosquito cell line of *Culex pipiens fatigans* prepared form laboratory reared material. Following the establishment of this cell line a comparative study on the susceptibility of this cell line to five members of the Group C arboviruses was conducted. All five arboviruses, Apeu, Marituba, Murutucu, Oriboca and Itaqui were capable of infecting the cells, demonstrating a virus increase in the range of 0.5 to 2.0 dex units. Infectivity was not accompanied by the demonstration of cytopathic effect. Viral assay was performed by subinoculation in suckling mice and HeLa cells. A comparison is made between the degree of susceptibility with this cell line to that of a similar study employing cells of *Aedes albopictus*.

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