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# ESTABLISHMENT OF A MOSQUITO CELL LINE FROM *HAEMAGOGUS EQUINUS* LARVAE

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## SUMMARY

The establishment and characterization of a *Haemagogus equinus* mosquito cell line (GML-HE-12) are described. The cells are diploid (2N=6) and seem to be free of contaminants. Their susceptibility to 13 arboviruses was tested. Eleven of the viruses multiplied in this cell line; six of these viruses still showed titers of 4 $\log_{10}$ plaque forming unit/ml or greater at 33 d postinoculation. No overt cytopathologic effect was observed.

*Key words:* mosquito; cell line; virus susceptibility.

## INTRODUCTION

Many cell lines have been established from mosquito tissue (1–4) and have become available for virological work. However, few of the studies have utilized cell lines originating from natural vector species of the viruses.

This report describes the establishment and characterization of a cell line from larval tissue of *Haemogogus equinus*, a natural vector of sylvan yellow fever. Yellow fever (YF) virus has been isolated from this species of mosquito many times in Panama (5–7). The ability of the GML-HE-12 cell line to support the growth of 13 arboviruses, including YF, is reported.

## MATERIALS AND METHODS

*Mosquitos.* The *Hg. equinus* eggs used in this work came from the Maje'75 colony established at the Gorgas Memorial Laboratory from mosquitoes obtained in the field station at Maje Island, Bayano Lake, Panama.

*Initiation.* Cultures were initiated from several hundred embryonated eggs in July 1981. The eggs were surface-sterilized and allowed to hatch. First-instar larvae were fragmented, resuspended in 2 ml growth medium in 25 $cm^2$ plastic flasks and incubated at 28° C. Three different media were used for the primary cultures: MM(8), MM/VP12 (9), and L15/MEM (10).

*Characterization.* The parameters used for characterizing the cell line included growth characteristics, morphology, sterility tests, karyology, isozyme analysis, and virus susceptibility.

Tests for bacterial and fungal contamination were performed in sodium thioglycollate and Sabouraud dextrose broth. Incubation was carried out at 25 and 37° C. Tests for *Mycoplasma* were done by inoculation of 0.2 ml cell culture fluid into *Mycoplasma* broth and onto agar plates. Plates were incubated anaerobically at 25° and 37° C. Tests for latent viruses were done by inoculation of Vero (African green monkey kidney) culture tubes with whole and ruptured cells.

Chromosomal counts were done at Passage 37 using 3-d-old cultures treated for 4 h with colchicine at a final concentration of 0.6 μg/ml.

The isozyme phenotypes of six enzymes were examined: malate dehydrogenase (oxaloacetate decarboxylating) (NADP+), ME-1.1.1.40; malate dehydrogenase, MDH-1.1.1.37; hexokinase, HK-2.7.1.1.; isocitrate dehydrogenase (NADP+), IDH-1.1.1.42; phosphoglucomutase, PGM-2.7.5.1; and glucosephosphate isomerase, PGI-5.3.1.9. Isozymes were separated by cellulose acetate electrophoresis according to techniques described by Brown and Knudson (11). Cell concentration was $2 \times 10^7$ cells in 50 μl electrophoresis buffer (0.1 *M* Tris/0.1 *M* maleic acid/0.1 *M* EDTA ($Na_2$)/0.1 *M* $MgCl_2$; pH 7.4) for all enzyme assays except ME and IDH, which were diluted to $4 \times 10^5$ cells in 50 μl buffer. For comparison, cells of the GML-HE-12 cell line were run simultaneously with larvae and adults

TABLE 1

GROWTH OF ARBOVIRUSES IN THE *HG. EQUINUS* CELL LINE, GML-HE-12

| Virus | Strain | Virus Titers[a] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Days Postinoculation | | | | | | | | | |
| | | Inoculum[b] | 1 | 2 | 7 | 8 | 9 | 10 | 12 | 15 | 33[c] |
| Vesicular stomatitis-New Jersey | 3566 | 3.0 | 7.7 | | 5.6 | | 4.9 | | 4.0 | | 5.1 |
| Mayaro | Bayano 407 | 2.4 | 3.5 | | 6.1 | | 5.4 | | 4.6 | | 4.1 |
| Eastern equine encephalitis | Panama 1958 | 2.7 | 3.9 | | 6.1 | | 6.9 | | 7.4 | | 4.7 |
| St. Louis encephalitis | BV-7 | 1.0 | <1.0 | | 3.9 | | 4.0 | | 3.6 | | 4.6 |
| Yellow fever-Wild | Jiménez | 2.7 | 0 | | 4.7 | | 6.7 | | 6.0 | | 4.7 |
| Dengue 1 | Jamaica 1977 | 1.0 | | 3.6 | | 6.3 | | 6.6 | | 4.9 | |
| Dengue 2 | PR-77 | 2.0 | | 3.3 | | 4.0 | | 3.4 | | 4.3 | |
| Dengue 3 | PR-38 | 1.0 | | 3.7 | | 4.8 | | 4.9 | | 4.2 | |
| Dengue 4 | H-241 | 1.6 | | 2.6 | | 3.7 | | 3.6 | | 2.5 | |
| Guaroa | Darien *Ateles* | 1.9 | 4.5 | | 5.2 | | 5.3 | | 5.8 | | 4.7 |
| Punta Toro | D-4021A | 2.0 | <1.0 | | <1.0 | | 0 | | 0 | | 0 |
| Chagres | JW-10 | 1.9 | <1.0 | | <1.0 | | 0 | | 0 | | 0 |
| Changuinolalike | Bayano 2992 | 2.0[d] | 3.0 | | 3.0 | | 3.5 | | 3.5 | | 6.0 |

[a] Virus titers expressed at $\log_{10}$ plaque forming unit (PFU)/ml.

[b] Expressed as $\log_{10}$ PFU of virus inoculated per milliliter maintenance medium in culture flask.

[c] Sample taken after cells were frozen and thawed.

[d] Titers for the Changuinola virus expressed as $TCID_{50}$/ml.

from the *Hg. equinus* laboratory colony, Maje 75, source for the GML-HE-12.

The susceptibility of the cell line to 13 arboviruses was tested. In most cases low passage virus (<10) was used. Single flasks of GML-HE-12 cells were inoculated with 0.5 ml of virus suspension and incubated at 28° C. Fluid renewal was done every 4 d using MM + 10% fetal bovine serum + antibiotics. Samples were collected periodically until Day 33, stored at −70° C for 1½ months, and then titrated by plaque assay in Linbro 96-well plates of Vero cells (12) or by assay in Vero tubes.

## Results

Although islands of cells were evident in all the primary cultures within 1 month after their initiation, only those cells maintained in L15/MEM medium continued to proliferate and went on to form a monolayer. The first subculture was done 2 months after initiation at a 1:2 split ratio using a rubber policeman to detach the cells. The evolution of primary to continuous culture did not differ significantly from that described earlier for mosquito cell lines (3,4,9).

In an attempt to improve cell growth, we tried gradual replacement of L15/MEM by MM medium. This was accomplished between Passages 17 and 19. For purpose of comparison, a subline was started on MM/VP12 medium at Passage 39. Both lines have grown well although the cells maintained in MM medium tend to become more rounded and clumped with continued growth and overcrowding. The current passage level of both is 90 and the split ratio 1:7 every 4 to 5 d. Frozen pools have been prepared at Passages 7, 12, 14, 23, 43, and 80.

Morphologically the cell line consists of a mixture of fibroblastlike and epithelial-like cells, with the latter predominating.

The sterility tests showed no evidence of bacterial, fungal, or mycoplasmal contamination. There was also no indication of latent viruses.

Chromosomal counts of 52 metaphases indicated that the cells are diploid (2N=6).

The isozyme phenotypes of the enzymes PGM and PGI were indistinguishable in cells from the cell line, larvae and adults. IDH and ME stained too intensely at $2 \times 10^7$ cells in 50 µl buffer and the cell suspension was diluted to $4 \times 10^5$ cells in 50 µl buffer to assay these enzymes. The relative mobilities of IDH, ME, and HK isozymes from *Hg. equinus* larvae and cells from the cell line were indistinguishable. On the other hand, the migration of stained bands in MDH showed that cells from the cell line were characterized by a single band that was also present in adult *Hg. equinus*, but not larvae. A second, faster migrating band was seen in both adults and larvae. These results suggest that MDH enzyme activity is more similar to that of the adult mosquito than to that of the larvae from which the cell line was derived.

The virus susceptibility studies of the GML-HE-12 cell line were carried out at passage levels 28 and 38. The results given in Table 1 show the virus titers at various intervals postinoculation. Out of 13 arboviruses tested, only the two sand fly-fever group viruses (Punta Toro and Chagres) failed to multiply in *Hg. equinus* cells. Five viruses (vesicular stomatitis, Mayaro, eastern equine encephalitis, YF, and Guaroa) multiplied to relatively high titers (about 5 logs) and even at Day 33 postinoculation showed titers of 4 logs or higher. Dengue 2 and dengue 4 were sustained by the *Hg. equinus* cells but did not show a significant increase in titer over the amounts inoculated. The titers of the Changuinolalike virus were low in the culture fluid but increased over 100-fold upon lysis of the infected cells by freezing and thawing. No obvious cytopathologic effect was observed with any of the viruses tested.

## Discussion

The work described in this paper is the first to report a cell line established from *Hg. equinus* mosquitoes.

Most of the viruses tested that are normally transmitted by mosquitoes replicated well in these cells, showing titers of 4 logs or higher even at Day 33 postinoculation. Since the virus cultures were terminated on Day 33, we have no information as to the length of time this cell line might support replication of the viruses tested.

The viruses transmitted by sand flies failed to multiply in the GML-HE-12 cell line except for the Changuinola, an orbivirus, which seemed to be cell-associated. Unlike Punta Toro and Chagres, viruses of the Changuinola group have occasionally been isolated from unengorged mosquitoes (13); this might have some relation to the ability of the Changuinola virus to multiply in the *Hg. equinus* cell line.

None of the viruses tested produced obvious CPE, which is consistent with the findings in many mosquito cell lines.

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