CULTIVATION OF MOSQUITO CELL LINES IN SERUM-FREE MEDIA AND THEIR EFFECTS ON DENGUE VIRUS REPLICATION

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SUMMARY

Seven mosquito cell lines from five species (Aedes aegypti, Ae. albopictus, Ae. pseudoscutellaris, Culex tarsalis, and Toxorhynchites amboinensis) were adapted to three kinds of serum-free media (SEM), which were composed of equal volumes of tryptose phosphate broth and of either Leibovitz (L15) medium, Eagle's minimum essential medium, or Medium 199 with Hanks' salts. Population growth rates of the cells cultivated in the SFMs were generally slower than those of original cell cultures maintained in conventional media containing bovine sera. A karyological study showed a significant shift to heteroploidy in two of the four cell lines examined. Four SFM-adapted sublines were compared with parental cultures for replication of dengue viruses. Ae. aegypti RML-12, Ae. albopictus C6/36, Ae. pseudoscutellaris AP-61, and Tx. amboinensis TRA-171 demonstrated different levels of alteration in virus replication ranging from lower titers (as in Ae. albopictus C6/36) to comparable or higher titers (as in Ae. aegypti RML-12) when they were simultaneously inoculated with four dengue serotypes.

Key words: mosquito cell culture; serum-free medium; dengue viruses; viral replication.

INTRODUCTION

Bovine serum, in particular fetal bovine serum (FBS), is an important component of growth media for most animal cell cultures. However, because it is expensive and requires strict quality control, many laboratories in countries of the tropics, where dengue is endemic, often cannot obtain good quality sera in the volume required. It is, therefore, desirable to grow cells in inexpensive serum-free media (SFM), so long as the cells are not adversely altered through cellular adaptation to SFM. In arbovirus research, the most important trait of cultured cells is susceptibility to viral infection. Although mosquito cells have been cultured previously in SMF (1-3), little is known about the changes in susceptibility to arbovirus infection in these cells. Recently, I found that a subline (TRA-284-SF) from a nonbiting mosquito, Toxorhynchites amboinensis, adapted to a SFM was as sensitive to dengue virus infection as the parental cell culture maintained in a conventional medium containing a bovine serum (4).

Another advantage of the use of SFM is the lack of interfering substances in virological or physiological experiments. For example, arbovirus antibodies may be found in bovine serum, inasmuch as some viruses are known to be naturally transmitted to bovine species. Also, bovine serum may possess enzyme activity that interferes with physiological studies of mosquito cells in vitro (5). The purpose of this study was to adapt several mosquito cell lines to SFMs and to determine whether changes in cell line characteristics occur.

MATERIALS AND METHODS

Mosquito cell cultures. The following cell lines (at more than 50 passage levels) were used for adaptation to SFMs and for virus replication study. (a) Ae. albopictus Clone C6/36 (hereafter called AAL-C6/36) (6) was maintained at 28°C in Eagle's minimum essential medium (MEM) containing 10% heat-inactivated FBS, 0.2 mM nonessential amino acids, and 0.2 mM L-glutamine. (b) Ae. pseudoscutellaris (AP-61) (7) was maintained in Mitsuhashi-Maramorosch/Varma-Pudney (MM/VP12) medium (8). (c) Ae.
Aedes aegypti (RML-12) (2) was maintained in Leibovitz’ (L15) medium containing 20% FBS and 10% tryptose phosphate broth (TPB) (C. E. Yunker, personal communication). (d) *Tx. amboinensis* (TRA-171) (9) was maintained in MM/VP12 medium. In addition, the following three cell lines were cultured in the SFMs, and the processes of adaptation were studied. These cell lines were used only for evaluating the applicability of the SFMs to other mosquito cells but not for testing the susceptibility to virus infection. Those cell lines were originally maintained in either the MM or the MM/VP12 medium. (e) *Ae. aegypti* (AGY-101) by Kuno (unpublished), (f) *Ae. aegypti* (ATP-10) by Singh (10), and (g) *Culex tarsalis* by Chao and Ball (2).

**Adaptation to SFM.** All mosquito cell lines were initially adapted to a SFM composed of equal volumes of L15 medium and TPB (hereafter called SFM-L medium). The dehydrated TPB (Difco, Detroit, MI) was dissolved in distilled water (29.5 g/l) and autoclaved before mixing. Initially, one-third of the original growth medium was replaced at irregular intervals (1 to 3 wk) with the SFM-L medium for 3 passages (with 1:2 split) until sublines completely adapted to the SFM-L medium were obtained in 3 to 12 wk. When the above procedure failed, as in the cases of AAL-C6/36, AGY-101, ATP-10, and *Culex tarsalis* cells, after two-thirds of the original media had been replaced with the SFM-L medium, the cells were subcultured at weekly intervals for 4 to 8 wk in the medium consisting of 1 part original medium and 9 parts SFM-L medium. After the original media were totally replaced with the SFM-L medium, the cell cultures were split 1:2 for several passages at irregular intervals ranging from 1 to 3 wk until the cultures could be split 1:3 to 1:5 at weekly intervals. Once the cells were subcultured in the SFM-L medium more than 15 times, they were grown in two other SFMs in which L15 medium was replaced with either MEM (hereafter called SFM-E medium) or Medium 199 with Hanks’ salts (hereafter called SFM-H medium).

**Cell line characteristics.** The changes in characteristics of cell lines after adaptation to SFM were studied with respect to cell morphology, growth curve, chromosome number, and dengue virus replication. For the growth curve study, one million cells were seeded per flask (25 cm²), and the viable cell population excluding trypan blue dye was determined daily with the use of the hemocytometer. For karyological study, cell cultures were treated with Colcemid (0.1 μg/ml) for 18 h, and chromosome numbers of 100 nuclei were counted, according to the method of Schneider (11).

**Viruses and titration.** Four serotypes of dengue viruses adapted to cell culture and kept at the San Juan Laboratories were used. They were DEN 1 (Hawaii)-one monkey, one mosquito, and 17 LLC-MK² cell passages; DEN 2 (New Guinea “C”)-24 suckling mouse, 6 LLC-MK² cell, and 2 Vero cell passages; DEN 3 (PR-6)-13 suckling mouse and 2 TRA-171 cell passages; and DEN 4 (H-54157)-2 TRA-171 cell passages. Viruses were inoculated into the parental and the corresponding sublines, cultured more than 20 times in either SFM-L or SFM-E medium at a multiplicity of infection of 0.1 plaque-forming unit (PCU)/cell, and incubated at 28°C for 7 d with 5 ml of maintenance medium. The maintenance medium used for the parental cultures was the conventional medium containing 2% FBS, whereas that for the cultures adapted to SFM was the SFM-L or SFM-E medium.

Extracellular virus titers in the supernatant fluids collected on the 7th d after inoculation were plaque-assayed in the rhesus monkey (LLC-MK²) cell cultures, according to the method of Eckels et al. (12). Two replicate cultures were prepared per virus per cell line (or subline), and the test was conducted twice.

**RESULTS**

**Adaptation to SFM.** Adaptation to the SFM-L medium was most rapid in the TRA-171 cell line, which required less than 5 wk before weekly subculture was possible. Adaptation of the RML-12 and AP-61 cells took 8 to 10 wk, respectively. The remaining cell lines (AAL-C6/36, ATP-10, AGY-101, and *Culex tarsalis*) all required periods ranging from 3 to 6 months for adaptation. In general, the growth rate of most cell lines dropped considerably between the 3rd and 8th passage in the SFM, making weekly or twice-weekly 1:2 splitting difficult. During the above slow growth phase, weekly change of spent media was essential for the survival of cells. The four cell lines (AAL-C6/36, AP-61, RML-12 and TRA-171) adapted to SFM were subcultured more than 40 times, and the remaining cell lines were subcultured 16 to 25 times.

**Morphological changes.** The cells of the AAL-C6/36 and RML-12 cell lines adapted to the