IN VITRO Vol. 19, No. 9, September 1983

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

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(Received January 14, 1983; accepted March 14, 1983)

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 SMFs 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 SMF-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 SMF 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 SMFs 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 m*M* nonessential amino acids, and 0.2 m*M* L-glutamine. (b) *Ae. pseudoscutellaris* (AP-61) (7) was maintained in Mitsuhashi-Maramorosch/Varma-Pudney (MM/VP12) medium (8). (c) *Ae.*

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, APT-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 cm^2), 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-MK_2 cell passages; DEN 2 (New Guinea "C")-24 suckling mouse, 6 LLC-MK_2 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-MK_2) 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

SFM-L medium (hereafter called AAL-SFL and RML-12-SFL sublines, respectively) were larger in size (Fig. 1 *B*, *F*) than those in the corresponding parental cultures (Fig. 1 *A*, *E*).

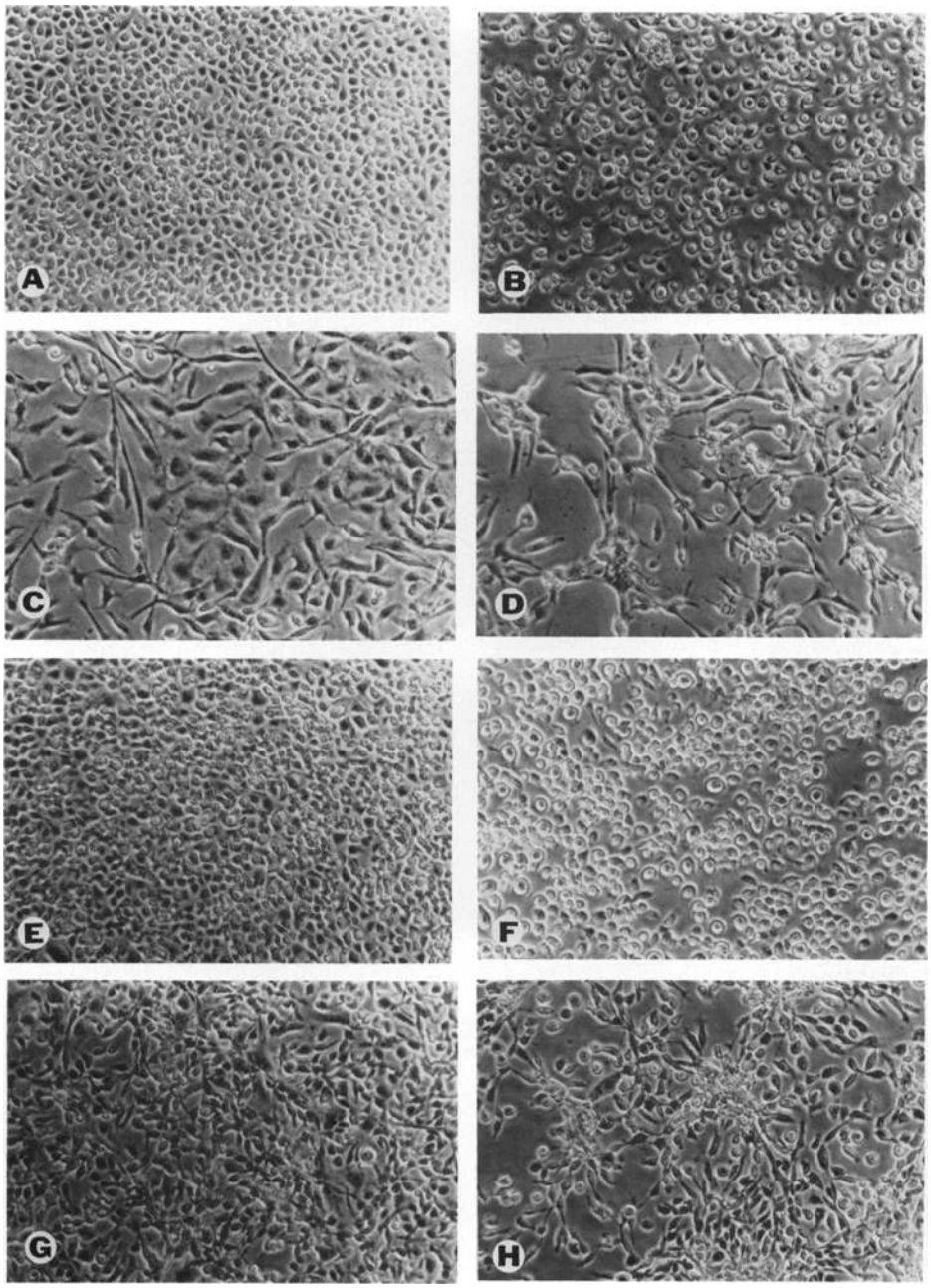

FIG. 1. Morphology of four mosquito cell lines and their sublines grown in a serum-free medium. Phase contrast: ×60. *A*, *Aedes albopictus* (C6/36) cells in a conventional medium; *B*, a subline of the C6/36 cells in a serum-free medium (SFM-L); *C*, *Aedes pseudoscutellaris* (AP-61) cells in a conventional medium; *D*, a subline of the AP-61 cells in a serum-free medium (SFM-L); *E*, *Aedes aegypti* (RML-12) cells in a conventional medium; *F*, a subline of the RML-12 cells in a serum-free medium (SFM-L); *G*, *Toxorhynchites amboinensis* (TRA-171) cells in a conventional medium; *H*, a subline of the TRA-171 cells in a serum-free medium (SFM-L).

The cells in the AP-61 and TRA-171 cell lines adapted to the SFM-L medium (hereafter called AP-61-SFL and TRA-171-SFL sublines, respectively) were predominantly composed of shorter fibroblastlike cells (Fig. 1 *D*, *H*) than those in the corresponding parental cultures (Fig. 1 *C*, *G*). Furthermore, single-cell vesicles were produced approximately tenfold more frequently in the AP-61-SFL and TRA-171-SFL cultures than in the parental cultures. For each cell line, no morphological differences were found among the three kinds of sublines (hereafter called SFL, SFE, and SFH sublines, respectively) maintained in the SFM-L, SFM-E, or SFM-H medium.

In the AGY-101 and *Culex tarsalis* cell lines adapted to the SFM-L medium, numerous vesicles that did not appear in the parental cultures developed in the SFM-adapted cultures.

Growth curve. Growth rates of only four cell lines (AAL-C6/36; AP-61; RML-12; and TRA-171) were studied. As shown in Fig. 2, growth of all SFM-adapted cells was slower than that of the corresponding parental cells, and the highest cell populations in the former cells never reached those in the latter cells. Between the SFL and SFE sublines, basically similar growth rates were obtained for three cell lines (Fig. 2 *A*, *B*, *D*). In the RML-12 cell line, a higher cell population was consistently obtained after 4 d in the SFE subline (Fig. 2 *C*). The initial drop in cell population was consistently observed in the AP-61-SFL cells (Fig. 2 *B*).

Chromosome number. To determine whether the cells adapted to the SFMs were different from the cells in the parental cultures, chromosome numbers of four cell lines were analyzed. As shown in Table 1, basically little change was noted in the AAL-C6/36 and AP-61 cell lines, whereas a shift to greater heteroploidy was found in the RML-12 and TRA-171 cell lines.

Viral replication. The results of comparative dengue virus replication in parental cell lines and the corresponding SFL sublines are shown in Table 2. Virus titers were mostly lower for all dengue serotypes in the AAL-C6/36-SFL and AP-61-SFL sublines than the titers obtained in the parental cultures. In the RML-12 cell line, however, the virus titers in the SFL subline were comparable to those in the parental cell line. In the TRA-171 cell line, the virus titers of DEN 1 and DEN 3 in the two cultures were comparable, whereas titers of DEN 2 and DEN 4 were lower in the SFL cultures than in the parental cultures. The difference in virus titer in a given parental cell culture varied by as much as one log from test to test, whereas that in a given SFL subline varied by as much as 2.7 log (Table 2).

When SFM-E medium was used instead of SFM-L medium, again virus titers of all dengue serotypes in the SFE subline were lower than the values in the parental cultures in the AAL-C6/36 cell line (Table 3). Similarly, virus titers in the SFE sublines of the AP-61 and TRA-171 cell lines were either comparable or lower, depending on the serotype. The RML-12 cell line, on the other hand, had DEN 1 and DEN 2 virus titers, which

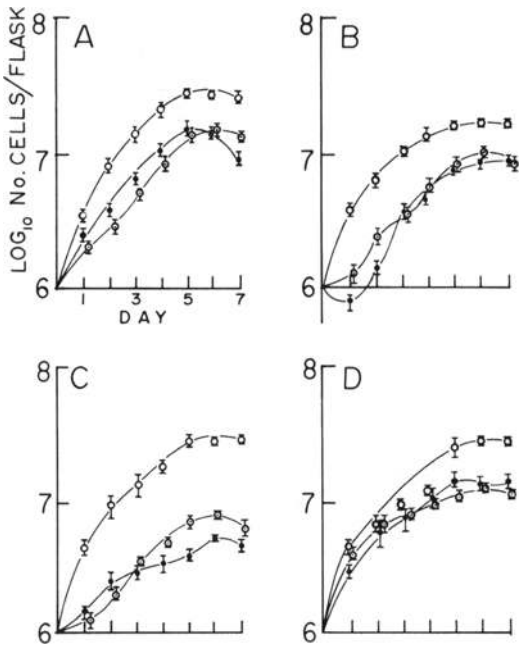

FIG. 2. Growth curves of four mosquito cell lines and their sublines adapted to serum-free media. *A*, *Aedes albopictus* (C6/36) cells. AAL-C6/36 (—○—) — Parental cells grown in a conventional medium; AAL-C6/36-SFL (—●—) — cells grown in a serum-free medium containing L15 (SFM-L); AAL-C6-36-SFE (—⊙) — cells grown in a serum-free medium containing MEM (SFM-E). *B*, *Ae. pseudoscutellaris* (AP-61) cells. AP-61 (—○—) — Parental cells grown in a conventional medium; AP-61-SFL (—●—) — cells grown in the SFM-L medium; AP-61-SFE (—⊙—) — cells grown in the SFM-E medium. *C*, *Ae. aegypti* (RML-12) cells. RML-12 (—○—) — Parental cells grown in a conventional medium; RML-12-SFL (—●—) — cells grown in the SFM-L medium; RML-12-SFE (—○—) — cells grown in the SFM-E medium. *D*, *Toxorhynchites amboinensis* (TRA-171) cells. TRA-171 (—⊙—) — Parental cells grown in a conventional medium; TRA-171-SFL (—●—) — cells grown in the SFM-L medium; TRA-171-SFE (—⊙—) — cells grown in the SFM-E medium. Each point indicates geometric mean titer and *vertical bar* indicates standard error of mean (n=4).

TABLE 1

CHROMOSOME NUMBER OF FOUR MOSQUITO CELL LINES CULTURED WITH A CONVENTIONAL MEDIUM OR WITH A SERUM-FREE MEDIUM CONTAINING L15 MEDIUM (SFM-L)

Cell Line or Subline[a]	Percentage of Nuclei With the Following Number of Chromosomes											
	6	8	9	10	12	14	16	18	20	22	24	24
AAL-C6/36[b]	98				2							
AAL-C6/36-SFL Subline	96				4							
AP-61[c]	12	1	10	2	25	8	17	10	1	5	4	5
AP-61-SFL Subline	30	1	6		26	1	6	18			6	6
RML-12[d]	92				6						2	
RML-12-SFL Subline	46	7	4	3	28	1		3			7	1
TRA-171[e]	84		2		8			6				
TRA-171-SFL Subline	62		6		28			2			2	

[a] All parental cell lines were grown in conventional media containing FBS. All sublines were grown in a serum-free medium (SFM-L).
[b] Igarashi's Clone C6/36 of Singh's *Aedes albopictus* cells.
[c] *Ae. pseudoscutellaris* cells by Varma and Pudney.
[d] *Ae. aegypti* cells by Bhat.
[e] *Toxorhynchites amboinensis* cells by Kuno.

were tenfold higher in the SFE sublines and titers of DEN 3 and DEN 4 which in turn were comparable to those in the parental cell cultures. The difference in virus titer in a given parental cell culture varied by as much as 2 log from test to test, whereas that in a given SFE subline varied by as much as 3.5 log.

DISCUSSION

One approach to developing SFM is to substitute animal sera with essential growth factors or protein fraction such as bovine albumin, or both. In mammalian cell cultures, some of the growth factors in bovine serum, such as insulin, transferrin, and selenium, have been identified and used successfully in SFMs (13). However, the effects of the above growth factors on insect cells are not well known. On the other hand, the importance of bovine albumin on insect cell growth has been studied (14), and it has been used as a component of SFM for other mosquito cell lines (1). Other less expensive materials, such as lactalbumin hydrolysate, peptone, TPB, and yeastolate, have also been used in the preparation of

TABLE 2

DENGUE VIRUS REPLICATION IN FOUR MOSQUITO CELL LINES CULTURED WITH A CONVENTIONAL MEDIUM (CM) OR WITH A SERUM-FREE MEDIUM CONTAINING L15 MEDIUM (SFM-L)

Cell Line	Type of Medium	Extracellular Virus Titer (Log PFU/ml)			
		DEN 1	DEN 2	DEN 3	DEN 4
AAL C6/36[a]	CM[b]	6.3 (5.8-6.7)[c]	5.2 (4.8-5.5)	3.2 (2.8-3.5)	6.5 (6.1-6.9)
Subline (SFL)	SFM-L	4.0 (3.0-5.0)	4.2 (3.9-4.6)	2.0 (2.0-3.0)	4.9 (4.2-5.5)
AP-61[d]	CM[e]	6.3 (6.2-6.6)	5.8 (5.3-6.4)	3.9 (3.4-4.1)	6.1 (5.3-7.2)
Subline (SFL)	SFM-L	4.9 (4.5-5.3)	5.0 (4.6-5.2)	2.9 (2.4-4.2)	5.1 (4.1-5.9)
RML-12[f]	SM[g]	5.5 (5.2-5.7)	5.7 (5.4-6.5)	3.4 (2.7-3.8)	5.4 (4.1-6.5)
Subline (SFL)	SFM-L	5.5 (4.5-6.0)	5.9 (4.7-7.2)	3.7 (3.3-4.3)	4.4 (3.7-5.1)
TRA-171[h]	CM[e]	5.8 (5.2-6.4)	5.8 (5.4-6.2)	4.5 (4.0-4.6)	6.5 (6.3-6.9)
Subline (SFL)	SFM-L	5.4 (5.2-5.7)	4.4 (3.0-5.7)	4.6 (3.2-5.4)	4.9 (4.0-5.6)

[a] Igarashi's Clone C/6/36 of Singh's *Aedes albopictus* cells.
[b] Eagle's MEM supplemented with 10% FBS, 0.2 m*M* nonessential amino acids, and 0.2 m*M* L-glutamine.
[c] Geometric mean log titer in four replicate cultures in two tests. The numbers in parentheses indicate ranges.
[d] *Aedes pseudoscutellaris* (AP-61) of Varma and Pudney.
[e] MM/VP12 medium containing 15% FBS.
[f] *Aedes aegypti* (RML-12) of Bhat.
[g] L15 medium containing 20% FBS and 10% tryptose phosphate broth.
[h] *Toxorhynchites amboinensis* (TRA 171) of Kuno.

TABLE 3

DENGUE VIRUS REPLICATION IN FOUR MOSQUITO CELL LINES CULTURED WITH A CONVENTIONAL MEDIUM (CM) OR A SERUM-FREE MEDIUM CONTAINING EAGLE'S MEM (SFM-E)

Cell Line	Type of Medium	Extracellular Virus Titer (Log PFU/ml)			
		DEN 1	DEN 2	DEN 3	DEN 4
AAL-C6/36[a]	CM[b]	6.3 (5.8-6.7)[c]	5.6 (5.1-5.9)	3.4 (3.2-3.9)	7.3 (6.1-7.8)
Subline (SFE)	SFM-E	5.5 (4.8-6.0)	3.8 (3.1-4.1)	2.0 (2.0-2.0)	4.3 (2.5-6.0)
AP-61[d]	CM[e]	6.0 (5.5-6.5)	5.3 (4.8-5.8)	3.5 (2.4-3.9)	5.8 (5.1-6.7)
Subline (SFE)	SFM-E	5.0 (4.2-5.6)	5.3 (4.4-5.8)	1.4 (2.0-2.8)	5.7 (5.1-6.5)
RML-12[f]	CM[g]	4.2 (4.0-4.5)	4.0 (3.9-4.2)	4.2 (3.3-5.3)	5.5 (5.0-6.0)
Subline (SFE)	SFM-E	5.4 (4.9-5.6)	5.0 (4.6-5.4)	4.1 (3.3-4.9)	5.5 (4.8-5.9)
TRA-171[h]	CM[e]	5.3 (5.1-5.5)	5.1 (4.9-5.2)	3.8 (3.5-4.3)	5.9 (5.2-7.1)
Subline (SFE)	SFM-E	4.2 (4.1-4.5)	4.2 (3.9-4.6)	3.7 (2.8-4.4)	5.4 (4.2-6.4)

[a] Igarashi's Clone C6/36 of Singh's *Aedes albopictus* cells.
[b] Eagle's MEM supplemented with 10% FBS, 0.2 m*M* nonessential amino acids, and 0.2 m*M* L-glutamine.
[c] Geometric mean log titer in four replicate cultures in two tests. The numbers in parentheses indicate ranges.
[d] *Aedes pseudoscutellaris* (AP-61) of Varma and Pudney.
[e] MM/VP12 medium containing 15% FBS.
[f] *Aedes aegypti* (RML-12) of Bhat.
[g] L15 medium containing 20% FBS and 10% tryptose phosphate broth.
[h] *Toxorhynchites amboinesis* (TRA 171) of Kuno.

SFM for insect cells (2,3). Exact cost comparison of media using different bovine serum substitutes is difficult because many factors (formulation, economic conditions, and commercial practices) are variable. In a recent study, however, it was found that TPB was less expensive than either albumin or bovine sera for the preparation of the same volume of growth medium for mosquito cell culture (4). Because the cells adapted to the SFM-L medium were readily grown in the other SFMs by the substitution of L15 medium with other common mammalian cell culture media, it may be possible to reduce the cost of mosquito cell culture media further by adapting the cells to SFMs containing less expensive basal media than those studied in this report.

This study has shown that even though mosquito cell lines are easily adapted to SFM, cell growth is slower, a disadvantage if a rapid cell growth is desirable. For arbovirus replication, however, rapid cell growth is not always essential, as evidenced by the common practice of using maintenance media containing reduced amount of bovine sera, after infection. Therefore the slower cell growth in the SFM-adapted mosquito cells is not necessarily unfavorable for virus studies.

With respect to the level of virus replication in mammalian cells grown in SFM, Weiss et al. (15) reported that several enteroviruses, adenoviruses, and herpes viruses replicated to titers comparable to those obtained in conventionally grown cells, using primary baboon kidney cells. Similarly, shaker cultures of BHK-21 cells adapted to a SFM were found to support the replication of DEN 2 virus to high titers (16). Although there exist at least a few mosquito cell lines adapted to SFM (1-3), most of the studies on viral replication in insect cell cultures adapted to SFM have been conducted in moth cells using nuclear polyhedrosis viruses (17). Recently, a SFM containing bovine albumin was used as a maintenance medium for the propagation of dengue viruses in the AAL-C6/36 cells (18), but the long-term effects of that SFM on the cell growth and viral replication are not known. A recent study showed that a subline (TRA-284-SF) from a nonbiting mosquito, *Tx. amboinensis*, grown in the SFM-L medium was as equally effective for dengue virus replication as the parental cell cultures (4). In the present study, a similar result was obtained in the RML-12 cell line. However, not all attempts to adapt mosquito cell lines to SFM result in retention of the same levels of dengue virus replication, because this study demonstrated various degrees of reduction in the viral replication in the other SFM-adapted mosquito cell lines, depending on cell line and virus serotype. Furthermore, greater variations in virus titer obtained in the SF sublines from test to test suggest inconsistency in viral replication at least in some of the sublines tested. Whether or not the aforementioned changes in the levels of viral replication are due to instability in physiological conditions of cells by adaptation to SFM, heterogeneity of cell popula-

tion with respect to susceptibility to virus infection, or selection of groups of cells with virus susceptibility different from that of parental cell cultures is not known.

Although the periods for adaptation varied considerably, essentially all cell lines could be adapted to the SFM-L medium. Inasmuch as many mosquito cell lines are maintained in the conventional media used in this study or the media similar to them, it is possible that many other mosquito cell lines not used in this investigation are adaptable to the SFMs prepared in this study. On the other hand, inasmuch as the changes in other cell characteristics, such as cell morphology, chromosome number, and susceptibility to virus infection, were recognized in some cell lines after adaptation to SFM, care must be exercised before using SFM-adapted mosquito cell cultures for experimentation to ascertain that the important traits for a particular study were not adversely affected through the process of adaptation.

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I thank Mr. A. Oliver of the San Juan Laboratories for his assistance in chromosome preparation. Also, I am grateful to Drs. S. M. Buckley and R. Tesh of Yale University School of Medicine, New Haven, Connecticut; Dr. M. Race of Caribbean Epidemiology Centre, Port of Spain, Trinidad; Dr. C. E. Yunker of the Washington State University, Pullman, Washington; and Dr. J. Hardy of the University of California, Berkeley, California, for the gifts of APT-10, AAL-C6/36, AP-61, RML-12, and *Culex tarsalis* cells, respectively.

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