Arbovirus Replication in Mosquito Cell Lines (Singh) Grown in Monolayer or Suspension Culture (34793)

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The two mosquito cell lines originally derived by Singh (1) from minced larvae of *Aedes aegypti* and *Aedes albopictus* are capable of growing in medium free of insect hemolymph. The latter cell line in particular has been shown to support the replication of several arboviruses (2–4).

The experiments outlined in this paper were designed to determine to what extent Singh's cell lines could be handled by a variety of standard tissue culture and virological techniques and to substantiate by chromosome analysis that the insect nature of the cell line has been maintained after continuous *in vitro* cultivation.

Materials and Methods. Cells. The *Aedes albopictus* and *Aedes aegypti* cell lines of Singh (1) were obtained from Dr. Sonja M. Buckley, Yale Arbovirus Research Unit and Dr. Arthur E. Greene, Institute for Medical Research, Camden, N.J. *A. albopictus* cells had been subcultured at least 50 times, and *A. aegypti* 30 times, prior to these experiments. Stock cultures were maintained in 32-oz glass medicine bottles (Brockway). For experiments, monolayer cultures were grown in plastic tissue culture petri dishes or flasks (Falcon) and stirred suspension cultures in Bellco (Vineland, N.J.) "spinner" assemblies. Unless otherwise noted, all stationary cultures were incubated at 28–30°, all spinner cultures at room temperature.

Media. Stock growth medium (M-M medium) was that of Mitsuhashi and Maramorosch (5) in which Singh (1) established the lines. It was prepared according to Buckley (4). The concentration of heat-inactivated (56°, 30 min) fetal calf serum was reduced to 10%. The *A. albopictus* cells were also grown in basal medium of Eagle (BME) (6) enriched with 1% tryptose phosphate (Gibco) and 10% fetal calf serum. For spinner cultures, M-M medium was used routinely. Petri dish cultures were maintained in a humidified 5% CO_2 atmosphere.

Virus. Sindbis virus grown in chick embryo fibroblasts was received from Dr. Boyce Burge, Albert Einstein College of Medicine, and adapted to growth in baby hamster kidney cells (BHK-21) maintained in the HT medium of Rouse *et al.* (7). The virus used was derived from the eighth passage in BHK-21 cells and had a titer of 10^8 plaque-forming units (pfu) per ml. Dengue-2 (New Guinea B strain) virus was from the tenth passage in KB cells and had a titer of 5×10^7 pfu/ml. It was derived from a plaque-purified stock as described previously (8). Unless otherwise noted, mosquito cell monolayers approximately 80% complete were inoculated at multiplicities of 1 or 2 pfu/cell and incubated for 1–1.5 hr at 28°. The inoculum was removed, the cells washed two or three times with phosphate-buffered saline (PBS), and an appropriate volume of medium was added. Virus-containing medium was harvested at 24 or 48 hr. None of the experiments reported here used virus that had been previously passed through mosquito cells.

Dengue virus was assayed by plaque count in BHK-21 monolayers. The procedure was as outlined previously (9) except that 0.9% agar and 0.05% DEAE–dextran were incorporated in the overlay medium instead of methyl cellulose. Sindbis virus was assayed by plaque count in BHK-21 monolayers.

Chromosome counts. The method for chromosome counting was that of Mellman (10) with some modifications appropriate for the culture system used. Heavily grown, attached cultures of *A. albopictus* in which the cells had started to pile up in characteristic clus-

ters were used. Such clusters are still actively growing, though they can no longer be classified as monolayers. 10^{-6} *M* colchicine was added and the cells were incubated for 4 hr at 28°. Cells were scraped into a 10-ml volume of medium, pipetted gently for dispersion, and centrifuged in siliconized tubes. After removal of the supernatant medium, 6 ml of 20% calf serum in distilled H_2O was added, and the cells were suspended and incubated at 28° for 12 min. Cells were centrifuged (5 min at 1000 rpm) and the supernatant fluid removed. Two milliliters of fresh fixative (3 parts methanol:1 part glacial acetic acid) were added slowly down the side of the tube; the cells were resuspended and centrifuged. The supernatant fluid was replaced with fresh fixative, and the resulting suspension was used for smears. One drop of cell suspension was added to a damp slide that had been alcohol-cleaned and soaked in cold water. The fixative was ignited by passing the slide through a burner with a small flame, and the slide was allowed to dry completely before staining with Giemsa.

Results. Monolayer cultures. Newly seeded *A. albopictus* cells which have settled and made contact with plastic or glass containers average 10–15 μ in diameter. They grow in a monolayer initially, individual cells enlarging and stretching out on the subsurface to 30–40 μ average diameter. Aggregates of small cells soon appear, which become the dominant feature of the culture within a few days and clearly represent the most actively growing segment of the population. A single large nucleolus is visible in each nucleus of the underlying stretched cells, which have a marked tendency to become multinucleate. Cultures seeded at a density of 2×10^5 cells/cm^2 are complete monolayers by 48 hr. In such cultures, there are always some cells which do not attach to the substrate. Indeed, cells are regularly observed to form monolayers floating on the surface of the growth medium. These become thick, multilayered "membranes" if not disturbed during refeeding manipulations.

Spinner cultures. Cells grown in actively stirred suspensions[1] ("spinner" cultures) retain a fairly uniform size of 10- to 15-μ diameter, although occasional larger cells are seen. Although most spinner cells are round, some cells retain a spindle shape. (This form and type of growth is typical for all cells in the *A. aegypti* cell line of Grace [11].) Unlike Buckley (4), we have found that monolayers and cell clumps are not readily dispersed with trypsin (0.25% in PBS lacking Ca^{2+}) or in a 1:1 mixture of 0.25% trypsin and 1:5000 ethylenediaminetetraacetic acid (EDTA) particularly when grown in plastic vessels. The adherence of cells to each other and to glass or plastic is loose enough so that a combination of scraping and pipetting can be used for dispersion.

To establish spinner cultures, monolayer cultures were scraped and seeded in spinner flasks at concentrations of approximately 5×10^5 cells/ml. The best growth medium in these experiments was unaltered M-M medium containing 10% fetal calf serum. After a lag of 24–48 hr, cells grew readily, doubling in number every 24–30 hr. Figure 1 shows typical cell growth curves at 23 and 28°. Cultures maintained at 37° were not viable. In the rich (and chemically ill-defined) M-M medium, cell densities exceeding 7×10^6 cells/ml were occasionally reached, although cultures were routinely maintained at densities of $0.75–2.5 \times 10^6$ cells/ml by addition of fresh medium at appropriate intervals. Daily dispersal of clumps by pipetting up and down enabled the spinners to be carried in log phase growth over long periods.

Chromosome number. Chromosome counts on 96 well-spread mitotic figures revealed 72 figures having 6 chromosomes, 23 having 12 (5 of these estimated at 10–13), and one having 24. From the arrangement of the chromosomes in the last pattern, it seemed likely that this figure was formed from two cells. Figure 2A and B shows typical 6- and

[1] The *Aedes aegypti* cell lines of Grace (11) is sometimes referred to as growing in suspension, since the cells do not attach to the substrate. We have adopted the term "spinner" cultures to distinguish cells which must be actively stirred to prevent attachment to each other and to the substrate from the nonadhering, unstirred cells of Grace's line.

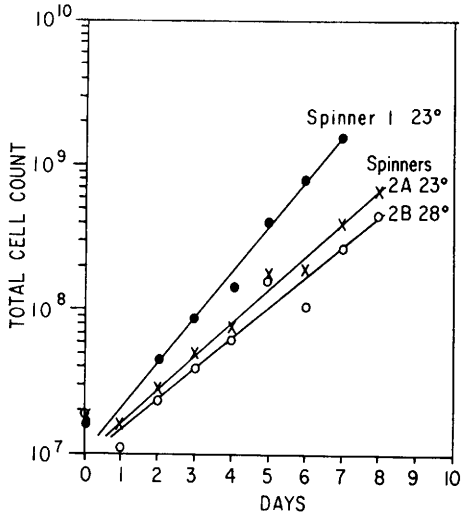

FIG. 1. Growth curves of *A. albopictus* cells in spinner cultures. Spinner 1 was established from cells scraped from a monolayer culture and was incubated at 23°. Spinners 2A and B were similarly established from monolayer cultures which were pooled and divided into two spinners incubated at 23 and 28°. All spinners were fed with M-M medium containing 10% heat-inactivated fetal calf serum. Total cell counts for each point were calculated with correction for the dilution factors introduced by daily feeding of the cells.

12-chromosome spreads, all chromosomes being approximately mesocentric. Approximately 12% of the cells remaining on the slides after the spreading procedures were found to be at some stage in mitosis. This estimate was based on observation of 20 low-power (100×) microscope fields containing a total of 853 cells, 105 of which were in mitosis.

Arbovirus growth curves. Monolayer cultures in 6-cm diameter plastic petri dishes, having 5×10^6 cells each, were infected with dengue-2 or Sindbis virus at multiplicities of 1 or 2 pfu/cell, respectively. Figure 3 shows the growth of dengue-2 virus in *A. albopictus* cells. We were not able to show productive infection of the *A. aegypti* cell line with this virus. Although maximum titers of dengue-2 virus from the *A. albopictus* cultures were quite low, the latent period and time to maximum yield were comparable with those observed in vertebrate cells (12).

The growth curves obtained with Sindbis virus in the *A. aegypti* and *A. albopictus* cell lines are shown in Fig. 4. After a latent period of about 3 hr, the release of Sindbis virus reached a maximum by about 25 hr

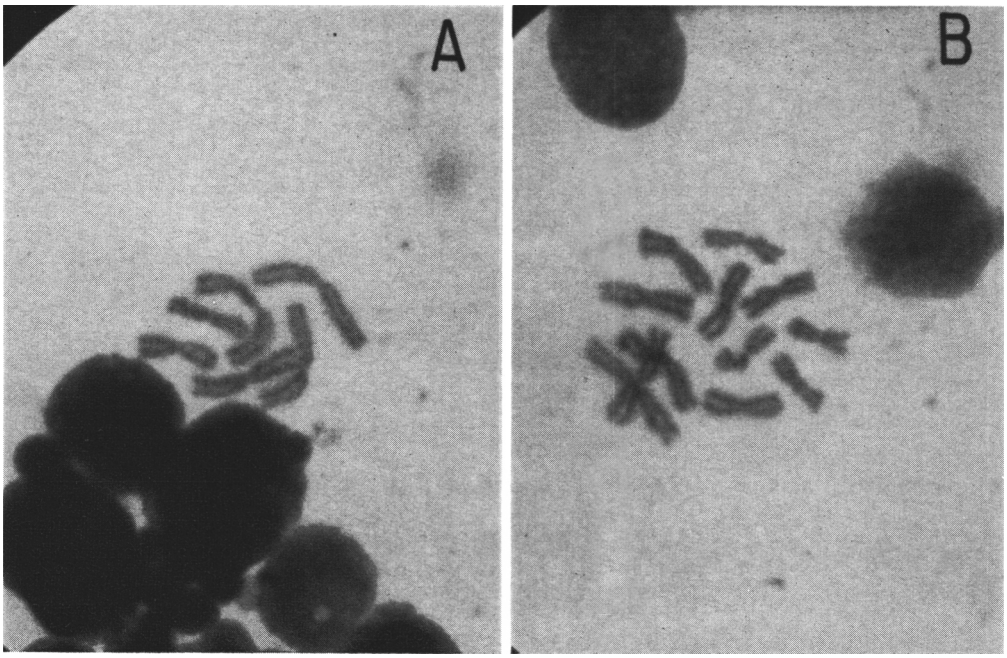

FIG. 2. Chromosomes of *A. albopictus* cells from monolayer tissue culture. Six chromosomes (A) were observed in about 75% of figures and 12 chromosomes (B) in approximately 25%.

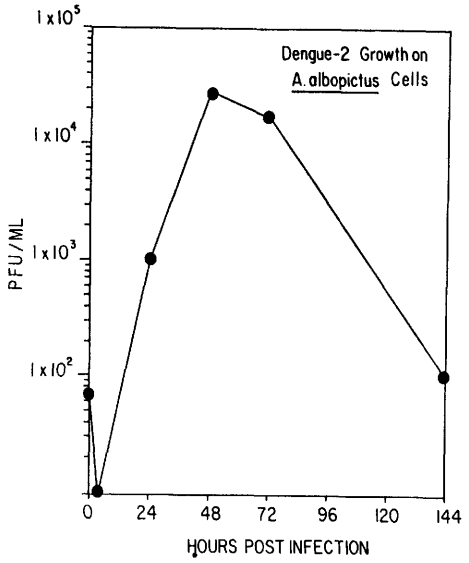

FIG. 3. Growth curve of dengue-2 virus in *A. albopictus* cell line. Replicate monolayer cultures in 6-cm diameter plastic petri dishes were inoculated at a multiplicity of 1 pfu/cell. After an adsorption period of 90 min, inocula were removed, the cell layers were washed three times to reduce unadsorbed virus, and M-M medium was added. Incubation temperature was 28°. Samples were frozen at —70° for simultaneous plaque titration.

after infection. The change in slope after 10 hr (Fig. 4) suggests that all cells were not simultaneously infected at the virus multiplicity used and that more than one cycle of release may have been involved. No cytopathic effect was observed, which is in accord with the observation of Singh and Paul (2).

Chronically infected cells. Cultures of *A. albopictus* cells infected with Sindbis virus as above were carried in monolayer culture in M-M medium for 8 weeks, being divided and fed at weekly intervals. Samples of the growth medium taken just prior to refeeding have shown titers ranging between 1×10^7 and 10×10^7 pfu/ml. No alteration in appearance or growth rate of the cells has been observed.

These persistently infected cells were transferred from monolayer cultures to spinner flasks 6 weeks after infection. Their growth pattern at room temperature was indistinguishable from that of uninfected spinner cells, doubling time being 24 hr after a 2-day period of adjustment to the spinner environment. Figure 5 shows the cell counts and virus titers corrected for the dilution factors resulting from daily feeding of the cells. Virus was released at a rate of approximately 1 pfu/cell/day.

Actinomycin treatment of normal and infected cells. Actinomycin D in concentrations up to 0.1 μg/ml had little or no effect on either cell viability or Sindbis virus production over a period of 24 hr, as shown in Table I. In the presence of concentrations in excess of 0.1 μg/ml, cytopathic effect (CPE) increased with a concomitant reduction in virus yield.

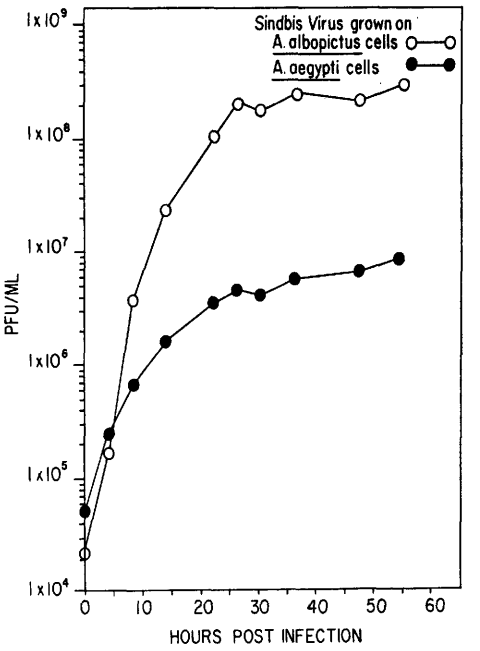

FIG. 4. Growth curves of Sindbis virus in A. *albopictus* and *A. aegypti* cell lines. Monolayer cultures were inoculated at a multiplicity of 2 pfu/cell. The procedure was as described for Fig. 3.

Discussion. A generalization worthy of note regarding the *A. albopictus* cells is the extreme ease with which they can be handled and their adaptability to a variety of media, temperatures, and methods of maintenance. In M-M medium containing 10% heat-inactivated fetal calf serum the cells can be

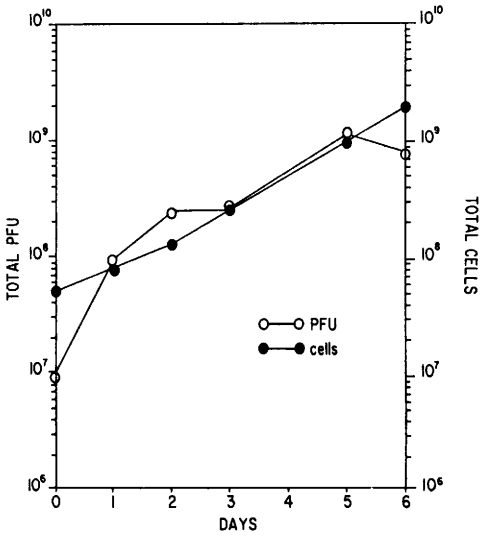

FIG. 5. Sindbis virus production by chronically infected *A. albopictus* cells grown in spinner cultures. After 6 weeks of culture as infected monolayers, cells were introduced into spinners. By the end of Day 3 they were showing a doubling time of 24 hr and a virus release rate of approximately 1 pfu/cell/day.

grown rapidly and efficiently at temperatures ranging from 23 to 30° in tightly closed glass or plastic flasks. They can also be grown readily in a humidified 5% CO_2 atmosphere in plastic petri dishes.

Although cultures were maintained for prolonged periods and cells appeared normal in several variations of Eagle's medium (6, 13) both in monolayers and spinners, cell growth generally proceeded less rapidly than in M-M medium. It appears that these cells respond unusually well to the spinner environment and are probably maximally adaptable to a variety of media in spinners.

The consistent finding of six chromosomes, or low multiples thereof, tends to confirm the mosquito origin of the cells. *A. albopictus*, like other mosquito species, is reported (14) to have six chromosomes. Vertebrates having such low chromosome numbers are unknown. Indeed *Drosophila* and mosquito species are the only animals reported to have fewer than ten chromosomes (14). Dr. Arthur E. Greene (personal communication) has also reported finding only six chromosomes in this cell line. On the other hand, Grace (11) counted 96 chromosomes (32n) as the most common number in his cell line derived from *A. aegypti*, and Suitor *et al.* (15) confirmed this as an average number in a cloned line derived from Grace's cells.

Productive infection of the *A. albopictus* cell line by Sindbis and dengue-2 viruses has been reported by Singh and Paul (2). Although cell numbers are not specified, these appear to have been low multiplicity infections since maximum virus production was reached only after 3–4 days. The experiments reported here indicate that both *A. albopictus* and *A. aegypti* cell cultures enable Sindbis virus to replicate at 28° with kinetics similar to its replication in vertebrate cell cultures incubated at 37°. A possible implication of this observation is that, in both insect and vertebrate cells, the rate of replication of virus is controlled largely by the function-

TABLE I. Effect of Actinomycin D on the 24-hr Yield of Sindbis Virus from *A. albopictus* Cells.[a]

	Actinomycin D concentration (μg/ml)					
	0.00	10^{-4}	10^{-3}	10^{-2}	10^{-1}	1
Total cells/flask	2.5×10^6		3.0×10^6	2.5×10^6	1.5×10^6	$<2 \times 10^5$
Virus yield (pfu/ml)	2.5×10^7	4.5×10^7	3.5×10^7	3.0×10^7	2.2×10^7	2.6×10^6
Cytopathic effect	—	—	—	—	—	+++

[a] Replicate monolayer cultures in 30-ml plastic tissue culture flasks were infected and inocula removed after virus adsorption. They were then fed 5 ml of M-M medium containing various concentrations of actinomycin D. The inhibitor was present in the medium for the entire 24-hr period before harvest. Cells were scraped and counted; released virus was assayed by the plaque method. Cytopathic effect was observed only at the highest concentration of actinomycin (1 μg/ml).

ing of the enzymatic machinery or overall metabolism of the host cell rather than by the viral genome.

Bannerjee and Singh (16) have reported on chronic infection of this cell line with Japanese encephalitis, West Nile, and Chikungunya viruses. Infection with Sindbis virus, as with these, produces no cytopathic effect and induces chronic shedding of virus into the medium. Chronically infected monolayer cells which had undergone six passages in monolayers after infection were transferred to the spinner environment. They behaved like normal cells (Fig. 1) but continued to shed virus at a rate approximating 1 pfu/-cell/day. Whether this reflects a low level of production by all cells or indicates that only a few cells in the population are infected is unknown at this time.

The actinomycin experiments described herein were preliminary to experiments on virus-specific RNA isolated from infected mosquito cells. The dose-response experiments were comparable to those of Stollar *et al.* (17) in dengue-2 infected KB cells and the results (Table I) were quite similar both in terms of effect on Sindbis virus production and in terms of the amount of actinomycin causing cell damage. Experiments to be reported elsewhere are being carried out to delineate the types of RNA found in infected and uninfected mosquito cells in the presence and absence of actinomycin D. Preliminary results indicate that higher levels of actinomycin than those reported here, acting for shorter periods of time, are effective in eliminating more than 95% of cellular RNA synthesis while permitting the synthesis of virus-specific RNA species similar to those observed in vertebrate cells (18). Residual cellular RNA synthesis is largely confined to a species with a sedimentation coefficient of 12S.

Summary. Singh's mosquito cell line derived from larval *A. albopictus* was grown in spinner cultures with a doubling time of 24–30 hr at 23 or 28°. It was found to be adaptable to various partially defined growth media. The typical chromosome count was 6, with 12 chromosomes appearing in 25% of figures. Infection of the cells with Sindbis virus or type 2 dengue virus at multiplicities of 1 or 2 pfu/cell and maintenance at 28° yielded viral growth cycles similar in timing to those seen in infected vertebrate cells at 37°. Cells chronically infected with Sindbis virus yielded virus at the rate of 1 pfu/cell/-day 7 weeks after infection. Sindbis virus production by cells exposed to actinomycin-D was unaffected by noncytotoxic doses. CPE due to actinomycin occurred at concentrations similar to those affecting vertebrate cells.

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