of identity could occur in the antigenic profiles of some MCA-induced tumors. Similarly, there may be some overlapping between BP-induced tumor antigens and the antigens in Ad12 and HET cells.

Summary and Conclusions. A significant retardation in the appearance and growth of benzo[*a*]pyrene-induced tumors was noted in hamsters pretreated with unpurified type 12 adenovirus (active or heated-inactivated) and also with sonicated virus-free Ad12-transformed hamster embryo cells (HET). Maternal immunization with active Ad12 or sonicated HET similarly retarded the development of benzypyrene-induced tumors in the mature offspring, when the first of three injections of the chemical was given at the age of 3 weeks.

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Susceptibility of the *Aedes albopictus* and *A. aegypti* Cell Lines to Infection with Arboviruses (33940)

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In 1967, Singh (1) successfully established cell lines from larvae of *Aedes albopictus* and *A. aegypti,* using a growth-promoting medium that Mitsuhashi and Maramorosch (2) described in 1964 for the propagation of leafhopper tissue culture.

Subsequently, Singh and Paul (3, 4) investigated the susceptibility of these *Aedes* cell lines to infection with various arboviruses. They observed a cytopathic effect (CPE) with Japanese B, West Nile, and dengue types 1, 2, and 4 viruses in the *A. albopictus* but not the *A. aegypti* cell line. Mosquito-borne viruses multiplied in at least one of the two cell lines; two tick-borne viruses, Kyasanur Forest disease and Kaisodi, failed to multiply in either.

The purpose of the present study was to determine whether the *A. albopictus* and *A. aegypti* cell lines could efficiently serve as *in vitro* assay systems for arboviruses. Twenty-three different arboviruses were screened for their ability to produce CPE in or to infect these two cell systems. As a base line for the study, the same viruses were simultaneously tested in two mammalian cell lines, baby hamster kidney (BHK-21) (5) and Vero, both employed routinely in this laboratory for the propagation of arboviruses.

Materials and Methods. Tissue culture. The BHK-21 cell line was maintained according to procedures already described (6). Transfers 12–22 were used in the experiments. The Vero cell line used was obtained as transfer 100 from Dr. N. Wiebenga in 1965, through the courtesy of the Laboratory of Tropical Virology, National Institutes of Health, Bethesda, Maryland. This continuous line of African green monkey (*Cercopithecus aethiops*) kidney cells was originally propagated by Dr. Y. Yasamura at Chiba University, Japan. Confluent monolayers in Roux bottles were dispersed with a mixture of

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equal parts of trypsin (0.25% and Versene (1:5000), and suspended in 90% Eagle's basal medium (7) supplemented with 10% calf serum. Stationary tube cultures were prepared, inoculated, and maintained as described for BHK-21 cultures (6). Transfers 139–142 were used in the experiments.

The *A. albopictus* and *A. aegypti* cell lines, obtained through the courtesy of Dr. Singh, Virus Research Centre, Poona, India, were transferred according to his procedure (1) with some modifications. The growth-promoting medium, which for convenience is here designated M-M because described by Mitsuhashi and Maramorosch (2), was prepared in two main steps. First, solutions A and B were made up as follows (per liter of 10x solution: Solution A (10x conc): $NaH_2PO_4 \cdot H_2O$, 2.5 g; $MgCl_2 \cdot 6H_2O$, 1.25 g; KCl, 2.5 g; $CaCl_2 \cdot 2H_2O$, 2.5 g; NaCl, 87.5 g; and demineralized water to 1000.0 ml. Solution B (10x conc): $NaHCO_3$, 1.5 g; and demineralized water to 1000.0 ml. The 10x concentrated stock solutions A and B were kept at 4°.

Second, the M-M medium was completed by dissolving 5.0 g of D-glucose, 8.15 g of lactalbumin hydrolysate, and 6.25 g of yeastolate in 800.0 ml of demineralized, distilled water, and then adding 100.0 ml each of solutions A and B. The M-M medium was next sterilized by filtration through a Seitz-EK pad, after which the following were added per 790.0 ml of filtered M-M medium: 200.0 ml of heat-inactivated (56°, 30 min), filtered fetal bovine serum, and 10.0 ml of an antibiotic mixture containing 10,000 units of penicillin and 10,000 μg of streptomycin/1.0 ml. Different preparations of the M-M medium, stored at 4°, varied in pH from 6.9 to 7.0.

Confluent monolayers of the *A. albopictus* and *A. aegypti* cell lines in French square bottles (160 ml) were dispersed with trypsin–Versene (as described for the Vero cell line). Cells were suspended in M-M medium supplemented with 20% inactivated fetal bovine serum. Cultures were transferred weekly, *A. albopictus* by a 1:20 split and *A. aegypti* by a 1:6 split. Culture bottles containing 10 ml of cell suspension in growth medium were incubated at 28°. The growth-promoting medium was changed once between transfers. Transfers 49–56 (*A. albopictus*) and 39–59 (*A. aegypti*) were used.

Stationary tube cultures were prepared with cell suspensions representing a 1:20 split (*A. albopictus*) or a 1:6 split (*A. aegypti*) in M-M medium with 20% inactivated fetal bovine serum. Each tube was seeded with 0.5 ml of cell suspension and incubated at 28°. Confluent monolayers were obtained within 3–7 days, the day depending on the number of cells present in the bottle cultures ($5–15 \times 10^6$/ml).

During infection experiments, *Aedes* cell cultures were maintained as follows. Prior to virus inoculation, the growth-promoting medium for each culture was replaced with 0.9 ml of maintenance medium, composed of 97% M-M medium and 3% inactivated fetal bovine serum. Twenty-four hr postinoculation (p.i.), the maintenance medium was changed and 2.0 ml of the same medium substituted per culture. Thereafter, fluids were changed at 3–4-day intervals. Cultures were incubated at 30 and 35°.

Virus inoculation. Lyophilized or wet-frozen, infected mouse-brain virus stocks were obtained from the World Health Organization International Reference Centre, Yale Arbovirus Research Unit (YARU), New Haven. The 23 arboviruses tested are listed in Table I, according to the sero-group classification system employed at YARU in 1968.

Titrations of single-virus pools, using serial 10-fold dilutions, were carried out on the same day in cultures of all 4 cell lines. Each dilution was inoculated into 3 tubes, 0.1 ml/tube. Control cultures were inoculated with 0.1 ml of diluent, which consisted of 0.75% bovine albumin in phosphate-buffered saline, pH 7.2. The CPE was graded as described elsewhere (6). Cultures were examined for development of CPE for up to 25 days p.i., using a light microscope with a magnification of 100×. Titration end points were calculated by the method of Reed and Muench (8) and are expressed either as tis-

TABLE I. List of Arboviruses Tested.

Group	Virus[a]	Strain
A	Chikungunya	Ross
	EEE	TenBroeck
	Semliki	Original
	VEE	TR donkey
B	Cowbone Ridge	Original
	Langat	TP 21
	Modoc	M 544
	St. Louis	Parton
	West Nile	Eg 101
	Yellow fever	Asibi
		Couma
Vesicular stomatitis	VSV-N.J.	Hazelton
	VSV-Indiana	Ind. lab.
Phlebotomus fever	Sicilian SFF	Sabin
	Naples SFF	Sabin
Hughes	Farallon	AR 846
Kaisodi	Silverwater	M 3737
Kemerovo	Chenuda	Eg Ar 1152
Quaranfil	Johnston Atoll	Johnston
Qalyub	Qalyub	Eg Ar 370
	Bandia	RV 611
Ungrouped	C 5581	—
	URB-TMA 1381	—
	Colorado tick fever (CTF)	65-68

[a] EEE = eastern equine encephalitis; VEE = Venezuelan equine encephalitis; SFF = sandfly fever. Three of the above arboviruses, namely, Bandia (supplied by the Institut Pasteur, Dakar, Senegal), C 5581 (by the Queensland Institute for Medical Research, Brisbane, Australia), and URB-TMA 1381 (by the University of Illinois, Urbana), are not yet reported in the literature and this mention is not intended to serve as formal description or to imply endorsement of the designation.

sue-culture cytopathic dose$_{50}$ (TCD_{50}) per ml or as infectious dose$_{50}$ (ID_{50}) per ml. The ID_{50} titers were determined by subinoculation of either undiluted fluid phase or undiluted, combined fluid and cell phases into an appropriate *in vitro* or *in vivo* host system.

During the second and third week after inoculation, *A. albopictus* and *A. aegypti* cultures inoculated with low virus doses (5–500 ID_{50}) were titrated individually in BHK-21 or Vero tube cultures to ascertain the amount of virus produced in the insect cells.

Storage of A. albopictus *and* A. aegypti *cells.* Cells from confluent cultures were dispersed (trypsin/Versene) and resuspended in storing medium at a cell count of 5–10 $\times$ 10^6 cells/ml. The storing medium consisted of 40% inactivated fetal bovine serum, 50% M-M medium, and 10% dimethyl sulfoxide. Cell suspensions were dispersed by a syringe with an 18-gauge needle into Cryules[2] (1.0 ml/Cryule), which were immediately glass-sealed and placed in cotton wool in an ice-cream box made of expanded polystyrene. The box was then stored in a Revco freezer at —65° for at least 16 hr, after which the Cryules were quickly transferred to a Linde liquid-nitrogen refrigerator.

For recovery of viable cells, each Cryule was thawed rapidly at 37°, and the contents were withdrawn by syringe, added to the growth-promoting medium (1.0 ml of stored cells / 9.0 ml of medium), and centrifuged at 1500 rpm for 5 min. The cell sediment was then resuspended in 4.0 ml of growth-promoting medium, dispensed into a 1-oz bottle, and incubated at 28°. With the longest storage period tested thus far, 3.5 months, viable cells of both *Aedes* lines have been recovered.

Results. In preliminary experiments, *A. albopictus* cells were studied quantitatively, and *A. aegypti* cells qualitatively, at 30 and 35° incubation under maintenance conditions. Tubes seeded with 100,000 cells/ml were grown in growth-promoting medium at 28° for 4 days, at the end of which time confluent monolayers were present; the cultures of each line were then divided into 2 groups, of which one was incubated at 30° and the other at 35°. In each *A. albopictus* group a cell count was made on 5 randomly selected cultures on days 9, 11, 15, 21, 25, 29, and 33 after seeding (Fig. 1); on these same days, fluids were changed in the remaining tubes. Although a slightly lower number of cells was present at the higher temperature of incubation, in both *A. albopictus* groups cells multiplied between days 4 and 15; thereafter, both growth curves entered a stationary phase that lasted through

[2] Wheaton Glass Co., Millville, N. J.

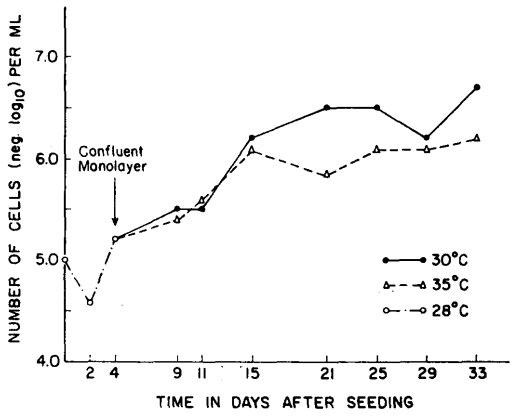


FIG. 1. Growth curve of *A. albopictus* cells at 30 and 35° under maintenance conditions.

day 30. No difference in cellular appearance was observed between the 2 groups of *A. aegypti* cultures. In view of these results, subsequent studies were carried out at 35°.

The findings in these studies may be briefly stated, as follows. The CPE in *Aedes* cells was produced only by West Nile virus and only in *A. albopictus* cells; all 23 agents caused CPE in BHK-21 cells, and all but Cowbone Ridge and Modoc did so in Vero cells. Ability to infect both *Aedes* cell lines was demonstrated for the 7 mosquito-borne viruses of groups A and B tested (Chikungunya, eastern equine encephalitis, Semliki, Venezuelan equine encephalitis, St. Louis, West Nile, and yellow fever) and for the Indiana and New Jersey strains of vesicular stomatitis virus (VSV). Colorado tick fever (CTF) virus, an ungrouped tick-borne agent, multiplied in *A. albopictus* but not *A. aegypti* cells, and the remaining 13 viruses failed to multiply in either mosquito cell line. These 13 comprised Cowbone Ridge and Modoc, 2 group B viruses with unknown vectors; Sicilian and Naples sandfly fever viruses; and the tick-borne agents Langat (group B), Farallon, Silverwater, Chenuda, Johnston Atoll, Qalyub, Bandia, C 5581, and URB-TMA 1381.

Table II shows the titers obtained in the different cell lines with 4 representative viruses; eastern equine encephalitis (EEE), West Nile, VSV-Indiana, and CTF.

Except for VSV-Indiana, the viruses that infected both *Aedes* cell lines had consistently higher ID_{50} titers in *A. albopictus* cells, and these titers compared favorably with TCD_{50} titers of these viruses in BHK-21 cells (see EEE virus, Table II). In addition, infected fluid phases of individual *A. albopictus* and *A. aegypti* cultures inoculated with dilutions of these viruses close to the ID_{50} end point (5–500 ID_{50}) showed as much as a 10,000-fold virus increase when titrated in BHK-21 or Vero cultures. VSV-Indiana had consistently higher titers in *A. aegypti* cells (Table II), a finding confirmed in triplicate experiments.

With West Nile virus, the only one to cause CPE in mosquito cells, the titration end point was reached within 7 days after inoculation, and testing of the cultures by subinoculation of the infective fluid phase into BHK-21 cells showed the TCD_{50} and ID_{50} titers to be identical (Table II). The CPE began 48 hours p.i. and consisted initially of multinucleated giant cells, located mainly at the periphery of the sheet. Subsequently, fusion of the small cells occurred, resulting in formation of syncytial areas. On days 3 and 4 p.i., these areas and the multinucleated giant cells became necrotic and detached from the glass; in addition, cells with elongated processes (fibroblast-like) appeared, and pyknosis of groups of cells or single cells was prominent. On day 5 p.i., cultures inoculated with 10^{-2} to 10^{-9} dilutions of virus showed clear-cut CPE, which subsequently reached grades 3+ and 3–4+ in those inoculated with terminal dilutions. Cultures inoculated with diluent failed to display either multinucleated giant cells or fusion of cells during the experimental period. Development of CPE was specifically inhibited in an *A. albopictus* culture inoculated with a mixture of West Nile virus and a homologous immune mouse ascitic fluid (Figs. 2, 3).

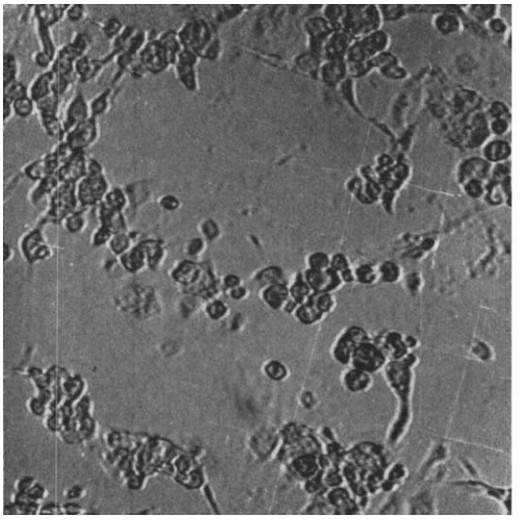
Although yellow fever virus (2 strains) and SLE virus produced transient as well as sporadically occurring CPE in *A. albopictus* cultures, they were nevertheless rated negative because the changes were not consistent from dilution to dilution within a single titration.

TABLE II. Behavior of 4 Representative Arboviruses in BHK-21, Vero, *A. aegypti*, and *A. albopictus* Cell Lines.

Virus	Mouse brain passage level	Cell line	TCD_{50}/ml[a] ($-\log_{10}$)	Titration end point read on day	ID_{50}/ml[a] ($-\log_{10}$)	Day of subinoculation	Host of subinoculation
EEE	100's	BHK-21	>11.3	4	—	—	—
		Vero	11.5	4	—	—	—
		A. aegypti	0	—	9.3	7	BHK-21
		A. albopictus	0	—	11.3	7	BHK-21
West Nile	10	BHK-21	>9.5	4	—	—	—
		Vero	10.3	7	—	—	—
		A. aegypti	0	—	8.5	8	BHK-21
		A. albopictus	10.5	7	10.5	8	BHK-21
VSV-Indiana	5	BHK-21	8.7	3	—	—	—
		Vero	8.3	2	—	—	—
		A. aegypti	0	—	>9.3	14	Vero
		A. albopictus	0	—	4.5	14	Vero
CTF	3	BHK-21	8.5	11	—	—	—
		Vero	6.5	6	—	—	—
		A. aegypti	0	—	0	11	BHK-21
		A. albopictus	0	—	5.7	11	BHK-21

[a] TCD_{50} = tissue-culture cytopathic $dose_{50}$; ID_{50} = infectious $dose_{50}$, determined by subinoculation.

Discussion. The present findings, that mosquito-borne viruses—as well as 2 VS viruses—infected both *Aedes* cell lines whereas viruses with other vectors (ticks and sandflies) did not multiply in either, agree with those of Singh and Paul (3, 4). These results suggest that the *A. albopictus* and *A. aegypti* cell lines could be important tools for screening arboviruses to determine their possible potential for multiplying in mosquito



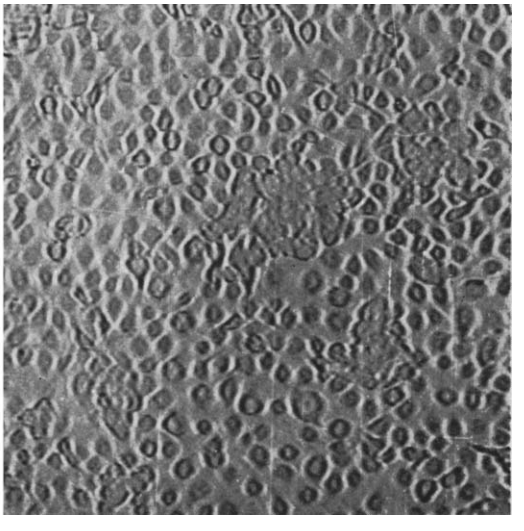


FIG. 2. Unstained *A. albopictus* cells 6 days after inoculation with 300 TCD_{50} of West Nile virus (Eg 101); ×160.

FIG. 3. Unstained *A. albopictus* cells 6 days after inoculation with a mixture (equal parts) of 300 TCD_{50} of West Nile virus (Eg 101) and undiluted West Nile immune ascitic fluid; ×160.

cells. The almost complete failure of the 23 arboviruses tested to produce CPE in the *Aedes* cell lines is hardly surprising, since mosquito-borne viruses multiply in the vector in nature without causing death.

The use of inactivated, rather than unheated, fetal bovine serum proved essential for obtaining satisfactory growth and maintenance media. Samples of unheated serum contained a heat-labile, cytotoxic factor that induced granulation and rounding of cells in both *Aedes* cell lines, especially the *A. albopictus*. Likewise, dispersion of cells with a mixture of Versene and trypsin proved important; use of this method provided cell suspensions in which single cells predominated, and such were superior to those obtained with 0.1% trypsin as described (1).

Both *Aedes* cell lines have now undergone over 40 transfers in this laboratory, and their morphology has remained unchanged from that originally described by Singh (1).

Summary. Twenty-three different arboviruses were tested for ability to produce CPE in or to infect cell lines from larvae of *Aedes albopictus* and *A. aegypti*. Only one virus, mosquito-borne West Nile, produced CPE, and only in *A. albopictus* cells. Four group A viruses (Chikungunya, eastern equine encephalitis, Semliki, and Venezuelan equine encephalitis), 3 group B viruses (St. Louis, West Nile, and yellow fever), and the Indiana and New Jersey strains of vesicular stomatitis virus infected both *Aedes* cell lines. Colorado tick fever virus multiplied in the *A. albopictus* cell line only. Thirteen viruses failed to multiply in either *Aedes* cell line under the experimental conditions: Cowbone Ridge and Modoc, group B viruses with unknown vector; Sicilian and Naples sandfly fever viruses; Langat, a tick-borne agent in group B; and 8 tick-borne agents not of group B.

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