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Susceptibility of *Aedes albopictus* C6/36 Cells to Viral Infection

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The susceptibility of the C6/36 clone of *Aedes albopictus* monolayer cell cultures was determined with 46 prototype viruses passed through three subcultures. Viral growth was confirmed by titration of the passage material in other susceptible host systems. Nineteen viruses demonstrated good growth in C6/36 cells: coxsackievirus group A type 10 and group B types 2, 3, 4, and 5; enterovirus 69; mumps virus; poliovirus types 1 to 3; reovirus types 1 to 3; vaccinia virus; dengue virus type 2; eastern equine encephalomyelitis virus; La Crosse virus; Rocio virus; and St. Louis encephalitis virus. Ten viruses did not adapt to growth in the C6/36 cultures. Seventeen other virus strains displayed only limited growth which was primarily restricted to the initial C6/36 passage or was detected by hemagglutinin reactions without observable cell degeneration. Of the 46 viruses, 33 (72%) were capable of initiating infection with a demonstrable cytopathic effect in the initial C6/36 passage. Hemagglutination or complement fixation titers or both were obtained with dengue virus type 2, eastern equine encephalomyelitis virus, La Crosse virus, mumps virus, reovirus types 1 to 3, and Rocio, St. Louis encephalitis, and vaccinia viruses.

Singh and Paul (14) first reported the isolation of dengue viruses in *Aedes albopictus* cell cultures. Ajello (1) described the replication of eastern equine encephalomyelitis virus (EEE), and the yellow fever, West Nile, Ilesha, and Flanders viruses in *A. albopictus* cell cultures. In recent years, several other continuous mosquito cell lines have also been developed that are susceptible to infections by flaviviruses (9, 10, 12). The high degree of susceptibility of the C6/36 clone of *A. albopictus* cells to dengue viruses has also been documented (7, 10). Igarashi et al. (8) described the isolation of Japanese encephalitis virus in C6/36 cells. An improved method of isolation and identification reported by Tesh (15) incorporated the use of C6/36 cells and the indirect fluorescent antibody technique. He also reported that antigen development occurred at a faster rate and with a marked cytopathic effect (CPE) in dengue virus type 2 (DEN-2) cultures incubated at 32°C. Gubler et al. (5) improved the sensitivity of this procedure by using serotype-specific, anti-dengue virus monoclonal antibodies in both the direct and indirect fluorescent antibody tests.

For years our laboratory has prepared reference and diagnostic viral reagents (3). We have investigated numerous primary and continuous cell cultures for support of viral replication. Chappell et al. (2) reported that *A. albopictus* cells were more susceptible to arbovirus infection than were newborn mice. Ajello (1) described progressively increasing amounts of complement-fixing antigen occurring with time in *A. albopictus* cultures infected with several different arboviruses. However, neither of these investigators used the C6/36 clone, which has been described as one of the more sensitive mosquito cell lines to arbovirus infection (7, 10). The use of C6/36 cells has centered around the isolation and identification of dengue viruses from mosquito pools and from human sera collected in the acute stage of febrile illnesses. Little is known about the comparative value of these cells for assay and cultivation of other viruses.

The C6/36 clone of *A. albopictus* cells was initially introduced into our laboratory to determine the efficacy of these cells for preparing reference complement fixation (CF) and hemagglutination inhibition (HAI) reagents for arboviruses indigenous to the United States. Also of interest was the susceptibility of C6/36 cells to infection by viruses which might occur in low concentrations in viremic sera.

MATERIALS AND METHODS

Viruses. Forty-six prototype virus strains were evaluated for growth in C6/36 cultures: adenovirus types 2 and 7a; coronavirus OC-43; cytomegalovirus AD-169; coxsackievirus group A types 2, 4, 5, 7, 9, 10, and 16; coxsackievirus group B types 2, 3, 4, and 5; echovirus types 3, 4, 6, 7, 9, and 11; enteroviruses 69 (strain Toluca-1) and 70 (strain J670/7); herpes simplex virus types 1 (strain MacIntyre) and 2 (strain MS); influenza virus A/Philippines/2/82; measles virus Philadelphia 26; mumps virus Enders; parainfluenza virus types 1 (strain C-39) and 3 (strain C-243); poliovirus types 1 (strain Mahoney), 2 (strain Statler), and 3 (strain Saukett); reovirus types 1 (strain Lang), 2 (strain Jones), and 3 (strain Dearing); rotavirus SA-11; respiratory syncytial virus Long; rubella virus Gilchrist; vaccinia virus Lister; varicella-zoster virus VZ-10; the alphavirus EEE New Jersey 60; the flaviviruses DEN-2 New Guinea C, Rocio virus (ROC) SPH-34675, and St. Louis encephalitis virus (SLE) TBH-28; and the bunyavirus La Crosse virus (LAC) Original. Since C6/36 infection by other strains of EEE, DEN-2, and SLE has been reported, these viruses were considered controls for the host system.

Host systems. A monolayer culture of *A. albopictus* cells, clone C6/36 in passage 30, was obtained from N. Karabatsos, Division of Vector-Borne Viral Diseases, Center for Infectious Diseases, Centers for Disease Control, Ft. Collins, Colo.

The C6/36 cells were grown in Eagle minimum essential medium (EMEM) containing 10% inactivated fetal calf serum. The maintenance medium consisted of EMEM with 0.4% bovine serum albumin fraction V. Monolayer cultures were grown in plastic 75- and 150-cm^2 flasks. Stock C6/36 cultures split at a 1 to 8 ratio and seeded at 5×10^5 cells per ml generally formed complete monolayers within 96 h. The cultures were incubated at 29°C.

Other cell culture host systems sensitive to infection by the viruses examined were used as detection hosts for confirming the viral infection of the C6/36 cells. Monolayer cultures of primary rhesus kidney (PrRhK) cells and contin-

uous lines of human embryonic lung fibroblastlike (HLF), rabbit kidney (RK-13), human lung, diploid (MRC-5), and African green monkey kidney (Vero) cells were used. These monolayers were grown in screw-cap culture tubes (16 by 125 mm) at 36°C in EMEM with 10% inactivated fetal calf serum. EMEM with 2% calf serum served as the maintenance medium.

The detection system for arboviruses was 2- to 3-day-old ICR suckling mice inoculated intracerebrally.

Experimental procedure. Kuno et al. (10) reported that detection of dengue virus infection in C6/36 cells was directly related to the virus titer. They reported 7 days as the minimum incubation period necessary for detecting infection by 10^2 50% tissue culture infective doses ($TCID_{50}$) per ml of virus. In an attempt to simulate low levels of infectious virus, as might be expected from field isolates or in viremic serum specimens, we diluted all virus seed cultures to between 10^2 and 10^3 $TCID_{50}$ per ml. A 6-ml virus inoculum was transferred onto the C6/36 monolayer in 75-cm^2 flasks. The inoculum was adsorbed for 2 h at 36°C and decanted, and 25 ml of maintenance medium was added. Cultures were incubated at 32°C with daily observation for evidence of CPE. All cultures were harvested after 10 days of incubation, or earlier if a 3 to 4+ CPE was observed. At the time of harvest, infected cells were removed from the surface by shaking and divided into 2-ml portions for assay. Harvested cultures were stored at −70°C.

C6/36 passages 2 and 3 were accomplished after thawing the frozen material from the previous passage, centrifuging at 150 × *g* for 5 min, and adding 5 ml of the undiluted supernatant to the monolayer culture in 75-cm^2 flasks. After adsorption for 2 h at 36°C, 20 ml of maintenance medium was added. The cultures were incubated at 32°C with periodic observation for CPE. If 150-cm^2 flasks were used, the inoculum was increased to 10 ml, and 30 ml of maintenance medium was used.

Infectivity titration of viruses. Viral infection of the C6/36 cultures was confirmed by titrating virus from each passage in a host system of proven susceptibility. The detector cell cultures were incubated at 36°C. Arbovirus specimens were titrated in suckling mice. Virus titers were calculated by the method of Reed and Muench (13) as either 50% mouse lethal doses or $TCID_{50}$ per milliliter of the original virus suspension.

Serologic assay. Specimens of the supernatant fluids from the cell passages were examined in CF and in hemagglutination (HA) and HAI tests for viral reactivity. The microtiter CF test described by Palmer et al. (11) and the standardized viral HA-HAI tests of Hierholzer et al. (6) were used. Arbovirus HA-HAI tests were performed by the procedure of Clarke and Casals (4). The titers of the crude C6/36 products were compared with those of reference reagents prepared in either infected suckling mouse brain suspensions or in cell cultures. Standard and modified extraction procedures described by Chappell et al. (3) were used routinely in the preparation of the reference reagents.

RESULTS

A. albopictus C6/36 monolayer cultures were tested for susceptibility to infection by 46 prototype virus strains. Ten viruses (22%) did not adapt to growth and were not infectious to C6/36 cells after three passages. The noninfectious viruses were coronavirus, cytomegalovirus, coxsackievirus group A types 2, 4, 5, and 7, parainfluenza virus type 1, respiratory syncytial virus, rotavirus SA-11, and varicella virus. The C6/36 passage materials were not infectious in the detector host systems.

Test results are shown (Table 1) for 14 viruses that demonstrated replication in the C6/36 host in all three subcultures. The titers of C6/36 passages 1 and 3 are reported and indicate that the degree of susceptibility varied with each virus examined. The supernatant fluids of passage 3 infected with mumps virus, vaccinia virus, and reovirus types 1, 2, and 3 gave positive HA reactions. The mumps virus culture gave a minimum HA titer and was not tested for HAI. The HA titers of the vaccinia virus and three reovirus cultures were confirmed as specific in the HAI test with reference antiserum. The other viruses shown in Table 1 were not tested for hemagglutinin reactivity.

The results for a second group of 14 viruses which demonstrated only limited growth in C6/36 cells are shown in Table 2. These viruses caused obvious CPE and cellular degeneration in the initial C6/36 passage. Limited growth became evident in passage 2 when only 5 of the 14 viruses demonstrated infectivity. The $TCID_{50}$ per milliliter determined to the detector cells are shown. No reactivity was observed in passage 3 with these viruses. The HA assay of the passage 1 rubella virus culture was negative.

The supernatant fluids from C6/36 passage 1 of influenza A/Philippines/2/82, measles virus, and parainfluenza virus type 3 gave low-titered HA reactions without observable CPE (Table 3). When transferred to the detection host system, CPE and high-titered HA reactions were observed with C6/36 passage 1 of the influenza virus and parainfluenza virus type 3 cultures. Measles virus passage 1 demonstrated a high multiplicity of infection but low HA titer. Specific HAI titers were obtained with the influenza virus and parainfluenza virus type 3 cultures with reference antiserum, whereas the measles virus HAI test was inconclusive. C6/36 passages 2 and 3 for these three viruses were negative for CPE and by serologic assay.

TABLE 1. Viral growth in *A. albopictus* C6/36 cells

Virus (strain)	Titer[a] of C6/36 passage:		HA titer[b] of passage 3
	1	3	
Coxsackievirus			
Group A, type 10 (Kowalik)	≥3.5	5.5	
Group B			
Type 2 (Ohio)	3.0	2.5	
Type 3 (Nancy)	≥3.5	<1.5	
Type 4 (Van Benshoton)	≥3.5	1.8	
Type 5 (Faulkner)	2.5	3.5	
Enterovirus 69 (Toluca-1)	2.5	5.5	
Mumps (Enders)	≥3.5	3.5	2
Poliovirus			
Type 1 (Mahoney)	≥3.5	2.2	
Type 2 (Statler)	≥3.5	1.8	
Type 3 (Saukett)	≥4.5	2.5	
Reovirus			
Type 1 (Lang)	≥3.5	5.5	128
Type 2 (Jones)	4.5	6.8	1,024
Type 3 (Dearing)	4.2	3.0	16
Vaccinia (Lister)	≥4.5	2.5	128

[a] Log_{10} per $TCID_{50}$ per milliliter.
[b] Reciprocal of dilution.

Extensive CPE was observed in cells infected with EEE and ROC, less CPE was observed in cells infected with SLE and LAC, and only slight CPE occurred in cells infected with DEN-2 (Table 4). Each passage was titrated in suckling mice. The 50% lethal doses of the initial C6/36 passages with the five viruses were comparable to the infectivity titers of the mouse brain seed material used as the initial inoculum. This is of interest because the DEN-2 cultures showed a minimum CPE. A slight decline was observed in the infectivity titer of EEE passage 3; however, the infectivity of the other virus passages remained stable.

The CF and HA titers of the unprocessed passage materials are also shown in Table 4. Only the final passage of each virus was tested for CF reactivity. A minimum CF titer of 1:4 was obtained with four of the viruses, whereas the LAC passage gave a CF titer of 1:64. The EEE and ROC materials gave HA titers of 1:64. SLE and DEN-2 gave minimum (1:10) HA titers in all passages, and titers for LAC were negative throughout. The HA titers for EEE and ROC were confirmed as specific by the HAI test with reference antiserum. As controls in the host system, cultures of EEE, DEN-2, and SLE reacted as expected.

DISCUSSION

Of the 46 viruses examined for growth potential on C6/36 cells, 36 (78%) gave evidence of replication in the initial passage. By passage 3, the number of viruses which continued to infect C6/36 cells had decreased to 19 (41%). Ten viruses (22%) were not adaptable to growth in C6/36 cells.

The best growth potential was observed with the five arbovirus strains. The infectivity titers of these cultures were comparable to those of the reference seed cultures grown in suckling mouse brain. The three reoviruses showed good replication in C6/36 cells and also produced hemagglutinins. All of the 18 enteric viruses displayed infectivity in the initial passage. However, only the polioviruses, enterovirus 69, and the coxsackievirus group B viruses persisted through passage 3. Other enteric viruses displayed a declining growth potential on passage. Mumps and vaccinia viruses also showed growth in C6/36 cells.

Serologic assay in the HA-HAI and CF tests (Tables 1 and 4) gave further evidence of viral replication. These assays were performed on crude materials, and acceptable titers were obtained with reovirus, vaccinia virus, EEE, LAC, and ROC. As described by Chappell et al. (3) the production of diagnostic reagents usually requires concentration and extraction procedures. Taking this into consideration, we believe that satisfactory high-titered HA reagents can be prepared for several of the viruses tested. Our results indicate that C6/36 cells may be most valuable in the preparation of reagents for arboviruses.

Only established prototype viruses were used in these studies. I acknowledge that wild-type viruses from isolation materials may react differently. However, the results indi-

TABLE 2. Viruses with limited growth in *A. albopictus* C6/36 cells

Virus (strain)	Titer[a] of C6/36 passage[b]: 1	2	Detector cell culture
Adenovirus			
Type 2 (Adenoid 6)	2.5	–	PrRhK
Type 7a (S-1058)	3.5	–	PrRhK
Coxsackievirus			
Group A, type 9 (Bozek)	≥3.5	5.5	PrRhK
Group A, type 16 (G-10)	3.5	–	PrRhK
Echovirus			
Type 3 (Morrisey)	2.2	–	PrRhK
Type 4 (Pesascek)	≥3.5	5.5	PrRhK
Type 6 (D'Amori)	4.3	–	PrRhK
Type 7 (Wallace)	2.8	–	PrRhK
Type 9 (Hill)	6.5	2.8	PrRhK
Type 11 (Gregory)	3.8	2.8	PrRhK
Enterovirus 70 (J670/7)	3.5	–	MRC-5
Herpes simplex virus			
Type 1 (MacIntyre)	4.2	1.5	Vero
Type 2 (MS)	1.5	–	Vero
Rubella (Gilchrist)	3.5[c]	–	Vero

[a] Log_{10} per $TCID_{50}$ per milliliter. –, Negative.
[b] Passage 3 for all viruses was negative.
[c] Determined by echovirus type 11 challenge.

TABLE 3. Viruses showing hemagglutinin production without visible C6/36 cell infectivity

Virus (strain)	Passage no.	C6/36 cells: $TCID_{50}$/ml	C6/36 cells: HA titer[a]	Cells	Detection system: $TCID_{50}$/ml	Detection system: HA titer
Influenza (A/Philippines/2/82)	1	–[b]	8	PrRhK	2.8	256
	2 and 3	–	<2	PrRhK	–	<2
Measles (Philadelphia 26)	1	–	2	HLF	≥2.5	4
	2 and 3	–	2	HLF	–	<2
Parainfluenza type 3 (C-243)	1	–	4	PrRhK	2.8	128
	2 and 3	–	<2	PrRhK	–	<2

[a] Reciprocal of dilution.
[b] –, Negative.

TABLE 4. Growth of arboviruses in *A. albopictus* C6/36 cells

Virus (strain)	Test	Titer of C6/36 passages: 1	2	3
EEE (NJ/60)	ID[a]	8.3	8.5	7.0
	HA[b]	64	64	32
	CF[b]	—[c]	—	4
ROC (SPH-34675)	ID	9.0	8.8	8.6
	HA	32	64	64
	CF	—	—	4
SLE (TBH-28)	ID	7.8	7.3	7.1
	HA	10	<10	10
	CF	—	—	4
DEN-2 (New Guinea C)	ID	5.5	6.1	6.1
	HA	10	10	10
	CF	—	—	4
LAC (Original)	ID	6.3	5.5	6.6
	HA	<10	<10	<10
	CF	—	—	64

[a] ID Infectivity titer; 50% suckling mouse lethal dose per milliliter.
[b] Reciprocal of dilution.
[c] —, Not tested.

cate that several viral agents may be cultured in C6/36 cells; therefore, isolates from viremic blood specimens must be carefully identified.

C6/36 cells are easy and economical to maintain in the laboratory. They can be stored at room temperature (25 to 28°C) for approximately 2 weeks without a change of medium. The cells have a high split ratio of 1 to 8 to 1 to 10 and grow to a complete monolayer in 3 to 4 days when seeded at 5×10^5 cells per ml. These cells should be usable in most laboratories.

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