Replication of Dengue Virus Type 2 in *Aedes albopictus* Cell Culture

PANTIPA SINARACHATANANT AND LLOYD C. OLSON

Department of Microbiology, Faculty of Science, Mahidol University, and The Rockefeller Foundation, Bangkok, Thailand

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The replication of type 2 dengue (D-2) virus in *Aedes albopictus* (Aal) mosquito cell cultures differed from that in vertebrate (LLC-MK2) rhesus monkey kidney cells. Virus readily replicated in Aal cells at either 30 or 37 C, but had no apparent effect on the host cell. Persistent infection was established with continual virus production for at least 6 months, although the virulence of progeny virus for both suckling mice and LLC-MK2 cells became attenuated. Density gradient analysis of infected Aal cell supernatant products indicated that only complete virus was released, in contrast to infected LLC-MK2 cells which also released incomplete virus. The surface antigens of the virus produced in Aal cells appeared to be considerably modified in that antiserum to vertebrate cell-produced D-2 virus did not block hemagglutination, whereas anti-Aal cell antiserum did. Virus infectivity could be neutralized by the antiserum to D-2 virus grown in vertebrate cells, however. Virus produced in LLC-MK2 cells did not demonstrate a similar host-cell modification. These results may reflect a difference in the mechanism by which D-2 virus matures in Aal cells.

The arboviruses represent an example of broad adaptation to diverse hosts in that they replicate both in poikilothermic invertebrates as well as in homothermic vertebrates. Replication in the vertebrate host is generally characterized by destruction of the infected cell, in contrast to the apparent lack of damage to the infected arthropod host (2, 33). This suggests a difference in the mechanism of replication or maturation insofar as it affects the two kinds of cells.

The production of dengue viruses in mammalian cells has been extensively investigated (3-5, 24, 25, 27, 28). Studies of dengue virus replication in mosquito cells reported to date have been concerned with the net production of infectious virus (14, 20, 21, 26), of complement-fixing antigens (22), and with the gross effect of virus replication on the host cell (7, 15, 20-22, 30). In the present study, we have examined the products of dengue-2 virus replication in a continuous cell line derived from *Aedes albopictus* mosquitoes (19). The data indicate (i) that the replication of dengue-2 (D-2) virus differs from that in mammalian cells and (ii) that during maturation in mosquito cells, certain surface antigens of the virion are modified, apparently as the result of incorporated cell membrane components.

MATERIALS AND METHODS

Cell cultures. *A. albopictus* (Aal) cells (Singh) were cultivated in modified Mitsuhashi and Marasmus medium (13) composed of salt solution (0.01% $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 0.005% $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.015% KCl, 0.0086% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.35% NaCl, and 0.2% D-glucose), 0.325% lactalbumin hydrolysate (Gibco), 10% heat-inactivated (56 C, 30 min) fetal bovine serum (FBS; Microbiological Associates, Inc.), penicillin (100 U/ml), and streptomycin (100 $\mu\text{g}/\text{ml}$). Maintenance medium (Aal-MM) consisted of the same salt solution and antibiotics supplemented with 0.024 mM/ml of L-glutamine (Gibco), amino-acid mixture for minimal Eagle medium (Microbiological Associates, Inc.), and tissue culture vitamins (Difco). Both media were adjusted to pH 7.2 with NaHCO_3 . Confluent monolayers of Aal cells were obtained after 2 to 4 days of incubation at 30 C. Passage was effected by dispersing the culture with a pipette and transferring the cell suspension to new bottles at a splitting ratio of 1:6 or 1:8.

The LLC-MK2 line of continuous rhesus monkey kidney cells was grown in medium 199 (M199; Gibco) containing 20% FBS, penicillin (100 U/ml), streptomycin 100 $\mu\text{g}/\text{ml}$, and NaHCO_3 and incubated at 37 C. The maintenance medium (LLCMK2-MM) consisted of M199 with 5% inactivated FBS.

Viruses. The prototype strain of dengue virus type 2 (New Guinea "C") was obtained from the Virus Department, SEATO Medical Research Laboratory, Bangkok. It had been passaged 27 times in suckling mice followed by 7 passages in LLC-MK2 cells.

Concentrated virus was prepared in LLC-MK2 cells by infecting the cultures with D-2 virus at a multiplicity of 0.001 to 0.01 PFU per cell. Supernatant fluids were harvested after 5 to 7 days of incubation at 37 C and clarified by centrifugation at $12,000 \times g$ for 30 min. Virus was pelleted by centrifugation at $105,000 \times g$ (Spinco Ti 60 rotor) for 2.5 h. The pellet obtained from 250 ml of supernatant fluid was suspended in 0.5 ml of borate saline, pH 9.0, containing 0.2% bovine plasma albumin (BABS9). The concentrated virus suspension was treated by ultrasonic vibration for two 1-min cycles at 10 kcycles (Raytheon sonic oscillator, model DF101) before gradient analysis.

Eight 16-oz (ca. 480 ml) bottles of 1-day-old Aal cell monolayers were inoculated with D-2 virus, 0.001 to 0.01 PFU/cell, and then were incubated at 30 C under Aal-MM. The supernatant fluid harvested 2 to 5 days later was clarified, pelleted, resuspended, and sonically treated as described above.

Suckling mouse brain suspensions of dengue virus were produced by inoculating 1- to 2-day-old mice with virus and aseptically removing the brains when the mice showed advanced morbidity. The brains were triturated in a mortar with sand and made up to a 20% suspension in M199 and 50% FBS, pH 7.2. The suspension was clarified by centrifugation at $12,000 \times g$ for 30 min, and the supernatant fluid was stored at -70 C.

Sucrose gradient analysis. Samples (0.5 ml) of concentrated virus were layered on linear gradients of 5 to 25% (wt/vol) sucrose in BABS9 (ca. 14 ml) and centrifuged at $63,000 \times g$ for 3 h (Spinco SW40 rotor). Fractions (0.5 ml) were collected from the top by using an ISCO model-D density gradient fractionator and assayed appropriately.

Virus assay. For plaque assays, 0.3 ml of virus samples were allowed to adsorb onto 3- to 4-day-old monolayer cultures of LLC-MK2 cells for 1 h at room temperature and for 1 h at 37 C. Cells were then overlaid with nutrient agar medium consisting of 0.9% Noble agar, 5% heat-inactivated FBS, and 0.02% DEAE-dextran (Pharmacia) in basal Eagle medium (Gibco), adjusted to pH 7.2 with NaHCO_3 . After incubation at 37 C for 7 days, the monolayers were stained with Hanks balanced salt solution containing 0.9% Noble agar and 1:100,000 neutral red. Plaques were counted after overnight incubation at 37 C.

Mouse assays were performed by inoculating 2- to 3-day-old mice intracerebrally and by observing them for 21 days after inoculation. The mouse mean lethal dose (50% mortality end point [LD_{50}]) was computed by the method of Reed and Muench (16). Hemagglutinin (HA) activity of virus in gradient fractions was determined in microtiter plates by the method of Clarke and Casals (8) by using goose erythrocytes. Hemagglutination-inhibition tests were performed with acetone-extracted antisera (8) against 4 to 8 units of HA antigen. Complement-fixing (CF) antigens were assayed by microtiter methods (11) by using 2.5 units of complement and 2 units of mouse antiserum to D-2 virus; the units were selected from the results of "checker board" titration. The antigen-serum-complement mixtures were incubated over-

night at 4 C, the standard hemolytic system was added, and the titers were read after 30 min of incubation at 37 C.

Neutralizing antibody titers were determined by the plaque reduction neutralization test as described by Russell et al. (17); 50% plaque reduction end points were calculated by the method of Cutchins et al. (9).

Antisera. Reference monkey hyperimmune serum and mouse hyperimmune ascitic fluid against D-2 virus (New Guinea "C" strain) were obtained from the Virus Department, SEATO Medical Research Laboratory, Bangkok. Mouse hyperimmune serum was prepared by injecting weanling mice intraperitoneally or intravenously with 10^3 to 10^4 PFU of D-2 virus (New Guinea "C") prepared in suckling mouse brains. Immunization was repeated three times at 2-week intervals, and the serum was collected 7 days after the last injection.

Rabbit antiserum was prepared against Aal and LLC-MK2 cell antigens by injections of 10^8 washed cells, half of which had been treated by ultrasonic vibration for two 1-min intervals at 10 kc. Aal cells were mixed in an equal volume of complete Freund adjuvant (Difco) and 0.25 ml was injected into each foot pad. Seven days later, 0.5 ml was injected subcutaneously. Serum was harvested 1 week later. LLC-MK2 cells were similarly suspended in adjuvant and injected into the foot pads on day 0. Booster injections were given on days 8 (intramuscular), 28 (intraperitoneal), 88 and 95 (subcutaneous), and 109 (intramuscular). The serum was collected 2 weeks after the final injection. Both Aal and LLC-MK2 cell antisera produced multiple precipitin bands when reacted with the homologous cell sonic extract by the agar-gel double-diffusion technique.

RESULTS

Replication of D-2 virus in Aal cells. D-2 virus was inoculated into Aal cell monolayer cultures (0.003 PFU/cell) and Aal suspension cultures (0.001 PFU/cell), and the cultures incubated at 30 C. The supernatant fluids were assayed daily for infectious virus by plaquing in LLC-MK2 cells (Fig. 1). After 7 days of incubation, infected monolayer cultures were suspended, diluted 6-fold in fresh growth medium, and subpassaged. The subpassaged cultures were incubated at 30 C for 3 to 7 days, at which time the amount of supernatant fluid virus was determined by titration, and passage was repeated. Virus production continued for the first 34 passages tested (representing approximately 6 months in culture), although the titers gradually declined from approximately 10^6 to 10^7 PFU/0.3 ml in early passages to $10^{4.3}$ PFU/0.3 ml by the 34th passage. The supernatant fluid from the 41st passage produced no plaques in LLC-MK2 cells when tested undiluted or at 10-fold dilutions up to a 10^{-3} dilution.

The gradual decline and eventual disappearance of plaque-forming virus in the supernatant

fluid of chronically infected Aal cells was due in part to the attenuation of virulence of progeny

FIG. 1. Titer of D-2 virus in the supernatant fluid of Aal cell cultures after infection with 0.003 PFU per cell in monolayer cultures (O) and 0.001 PFU per cell in suspension cultures (●).

virus for LLC-MK2 cells. We could not, however, exclude a concomitant reduction in the absolute amount of infectious virus produced. The original D-2 virus (New Guinea "C") readily produced large (5 to 8 mm), clear plaques in LLC-MK2 cells within 7 days after incubation under agar. Virus from the 34th passage of chronically infected Aal cells, however, produced small (1 to 2 mm), hazy plaques only after 21 days of incubation in LLC-MK2 cells under agar (Fig. 2). Despite the lack of plaque-forming virus in supernatant fluid from the 41st passage culture, assays by intracerebral inoculation of suckling mice indicated that infectious virus ($10^{2.3}$ LD₅₀/0.01 ml) was present. That attenuation of virulence for mice had also occurred was suggested by the prolonged incubation period (8 to 16 days) of the latter, compared with a normal incubation period of 4 to 8 days.

Although Aal cells readily supported D-2 virus replication, we were unable to detect cell damage in infected cultures. Infected monolayers appeared morphologically normal even when grown in plastic vessels (30). Infected Aal cell suspensions and monolayers, both under liquid and agar medium, uniformly retained the ability to take up vital stain (neutral red). These

FIG. 2. LLC-MK2 cell monolayers incubated under agar and stained with neutral red: uninfected (left); 7 days after infection with D-2 virus produced in LLC-MK2 cells (center); and 21 days after infection with D-2 virus derived from the 34th passage of chronically infected Aal cells (right). Large divisions on the scale = 5 mm.

experiments used D-2 virus both in its initial passage and after six passages in Aal cells. It is possible, however, that cell changes occurred, but did not result in cell death. Sutor and Paul (30) have, for example, reported the induction of syncytia formation in D-2-infected Aal cells. Although we never observed this phenomenon by microscope, we attempted to exclude it by comparing the cell volume distribution of infected and uninfected cell suspensions. Uninfected cells, chronically infected cells (30th passage), and acutely infected cells (5 days after inoculation of Aal cells with D-2 virus, 0.001 PFU/cell) had identical cell volumes as determined with a Coulter counter, model B, coupled with a model J automatic size distribution plotter (Coulter Electronics, Inc.). These results suggested that syncytia formation was not significant during D-2 virus infection under these conditions.

Under natural and laboratory conditions, D-2 virus replicates at different temperatures in vertebrate cells than in mosquito cells. To examine the possibility that cell changes produced by D-2 virus are temperature dependent, the growth rates of infected Aal cells at 30 and at 37 C were determined. The 12th passage of chronically infected Aal cells was used. Approximately 10^4 cells were seeded into 1-oz (ca. 30 ml) prescription bottles, and incubated at 30 or at 37 C. Control, uninfected cells were similarly incubated. Thereafter, the number of cells in each of two bottles of each series was enumerated in a counting chamber and averaged. The results indicated that there was no significant difference in the growth rate of infected versus uninfected Aal cells at either temperature and that Aal cells grew only slightly slower at 37 than at 30 C. Microscope examination and neutral red staining failed to reveal evidence of cell damage in infected cultures incubated at either temperature.

Production of virus and virus products by infected Aal cells. Concentrated supernatant fluids from D-2-infected Aal and LLC-MK2 cells were centrifuged through linear sucrose gradients, and the distribution of HA activity was determined. A representative result is shown in Fig. 3. The distribution of HA activity from LLC-MK2 cells was similar to that reported previously (5, 24, 25, 27, 28). The rapidly sedimenting HA peak (RHA) represents complete virions, and the more slowly sedimenting HA (SHA) a form of incomplete virus (25, 27). The HA activity from Aal cells sedimented as one peak, coinciding with the RHA peak of cocentrifuged LLC-MK2-produced virus. These results were reproducible in a series of runs.

SHA could not be detected in gradient fractions of D-2 virus produced either from low or high passage in Aal cells. To confirm that the pattern shown in Figure 3 did not represent incomplete separation, concentrated Aal-grown virus was applied to a series of sucrose gradients and subjected to centrifugation for increasing periods of time before the tubes were fractionated and the fractions were assayed. The HA activity sedimented as a single homogeneous component (Fig. 4).

These results suggested that infected Aal cells released HA exclusively associated with complete virions. The failure of infected Aal cells to produce SHA may reflect the lack of cell damage to these cells by replicating D-2 virus. The SHA that is produced by the infected LLC-MK2 cells probably represents virus subunits that are released into the medium from disintegrating cells, i.e., these particles were released before complete assembly and maturation. Alternatively, the lack of release of SHA from infected Aal cells may be the result of inhibition at low temperature of a temperature-dependent process. To investigate this latter possibility, the production of virus by Aal and LLC-MK2 cells incubated at the alternate temperature was determined. LLC-MK2 cells were infected

FIG. 3. Distribution of hemagglutinin after rate zonal centrifugation of concentrated D-2 antigens derived from LLC-MK2 cells (top) and Aal cells (bottom). Sucrose gradients (5 to 25%) were centrifuged for 3 h at $63,000 \times g$. RHA, rapidly sedimenting hemagglutinin; SHA, slowly sedimenting hemagglutinin.

FIG. 4. Distribution of hemagglutinin after rate zonal centrifugation of concentrated D-2 antigens produced in Aal cells. Sucrose gradients (5 to 25%) were centrifuged at $63,000 \times g$ for the length of time indicated.

with D-2 virus at a multiplicity of 0.01 PFU/cell and incubated at 30 C, and chronically-infected Aal cells (12th passage) were diluted 1:6 in fresh growth media and incubated at 37 C. Virus replication was not inhibited by the change of incubation temperature in either cell type (Table 1). In LLC-MK2 cells, cytopathological effect was first noted 1 to 2 days later, during incubation at 30 C rather than at 37 C. The HA produced by infected cells incubated at both 30 and 37 C was also analyzed by sucrose density gradient fractionation. The results from Aal cells again showed a single peak located in the RHA region from cells incubated at either 30 or 37 C. The LLC-MK2 cells incubated at the two temperatures produced substantial amounts of both RHA and SHA at 30 C, although the amount of RHA produced at 30 C was proportionately greater than at 37 C.

LLC-MK2 cells infected with D-2 virus release a soluble CF antigen in addition to the RHA and SHA fractions which also fix comple-

ment in the presence of D-2 virus antibody (5, 24). The distribution of CF activity in the sucrose gradient fractions of infected LLC-MK2 and Aal cell products was determined. Mouse anti-D2 virus antiserum was the antibody. The Aal virus was the product of the 31st passage, chronically infected cultures. Both cell types produced soluble CF antigen that was not associated with HA activity (Fig. 5); the RHA

TABLE 1. Production of D-2 virus by infected Aal cells incubated at 37 C and of LLC-MK2 cells incubated at 30 C

Day of incubation	Virus titer (PFU/ml)	
	Aal cells	LLC-MK2 cells
1	5×10^5	0
2	4×10^6	0
3	ND ^a	3×10^4
4	9×10^5	6×10^4
5	4×10^5	9×10^5
6	2×10^5	8×10^5
7	2×10^6	5×10^5

^a Not determined.

FIG. 5. Distribution of hemagglutinin (HA) and complement-fixing (CF) antigens after rate zonal centrifugation of concentrated D-2 antigens derived from LLC-MK2 cells (top) and Aal cells (bottom). Sucrose gradients (5 to 25%) were centrifuged for 3 h at $63,000 \times g$. SCF, soluble CF antigen; RHA, rapidly sedimenting hemagglutinin; SHA, slowly sedimenting hemagglutinin.

and SHA fractions from LLC-MK2 cells also fixed complement. The failure to find CF activity associated with Aal-cell RHA was surprising, and was confirmed in two additional gradient analyses of the products of both low- (Aal-3) and high- (Aal-37) passage virus.

Host cell modification of D-2 virus surface antigens by Aal cells. The suggestion that the CF antigens on the surface of the virions (RHA) produced in Aal cells were not reacting with antibody to D-2 virus produced in suckling mouse brain prompted further investigation of the immunological reactivity of Aal-cell virus. Pooled RHA fractions from both the 3rd and the 37th passage of chronically infected Aal cells were used as HA and were reacted with mouse antiserum prepared against mouse brain D-2 virus and with rabbit anti-Aal cell and anti-LLC-MK2 cell antisera. The hemagglutination-inhibiting titer of each serum was determined against 4 to 8 HA units of the test virus and compared with the titer obtained against 4 HA units of the D-2 virus produced in LLC-MK2 cells. Titers are shown in Table 2 and indicate that virus HA produced in Aal cells was not inhibited by D-2 virus antiserum, but was inhibited by antiserum prepared against Aal cells. In contrast, D-2 virus HA derived from LLC-MK2 cells was blocked by anti-D-2 antiserum, but not by anti-LLC-MK2-cell antibody. HA produced in Aal cells has a pH optimum (pH 6.0) different from the HA prepared in either LLC-MK2 cells or suckling mouse brain (pH 6.4).

Infectivity titrations of gradient fractions showed that infectious virus (10^6 to 10^7 PFU/0.1 ml) was associated only with RHA fractions for both LLC-MK2 and Aal virus. Infectivity was completely neutralized by the mouse anti-D-2 antiserum, although this antiserum had a low neutralization titer (1:20) to both LLC-MK2 and Aal virus.

Concentrated suspensions of infected and of normal Aal cells were disrupted by sonic treatment, and debris was removed by centrifuging at

$12,000 \times g$ for 30 min. The supernatant fluid was centrifuged through sucrose as before. No HA activity could be detected in any fraction. Thus, isolated cellular material did not by itself contribute to the hemagglutination observed in association with RHA and infectious virus produced in Aal cells.

The antigenic alteration of D-2 virus was host-cell specific. D-2 virus from 3rd-passage-infected Aal cells (Table 2) was passed into LLC-MK2 cells. Progeny virus was subjected to rate zonal centrifugation, and RHA fractions were used as HA antigen. The profile of HA activity was characteristic of LLC-MK2 virus, and both the RHA and SHA fractions fixed complement after reacting with mouse anti-D-2 antibody. Hemagglutination by RHA fractions was also inhibited by dilutions of anti-D-2 mouse ascitic fluid to the same titer as homologous HA antigen prepared from infected mouse brain (Table 2). These results indicated that the immune reactivity of D-2 virus was considerably modified by replication in Aal cells.

In order to characterize the immunological character of the viral antigens involved in neutralization reactions (N antigens), plaque reduction neutralization test assays were performed. Virus from 3rd-passage-infected Aal cells was passed three times in LLC-MK2 cells, and the infectious progeny were used as the virus for plaque reduction neutralization test assays. Infectious virus derived from the 3rd and the 5th passage of chronically infected Aal cells and the D-2 prototype (New Guinea "C") grown in LLC-MK2 cells were also used. Hyperimmune monkey antiserum with a high neutralizing antibody titer against D-2 virus was the antiserum. The results (Table 3) indicated little or no difference in the N antigens of the LLC-MK2 or of the Aal-produced virus that was subsequently passaged in LLC-MK2 cells. Anti-Aal cell antiserum did not significantly neutralize third-passage Aal cell D-2 virus. Fifth-passage D-2 virus was neutralized by Aal cell antiserum to some degree, although without an alternate

TABLE 2. *Inhibition of D-2 hemagglutinin prepared in LLC-MK2 and in Aal cells by antidengue and anti-cellular antisera*

Antiserum	Hemagglutination inhibition titer against D-2 HA prepared in:			
	LLC-MK2 cells	Aal cells, 3 passage	Aal cells, 37 passages	Aal 3 → LLC-MK2
Anti-D-2 mouse ascitic fluid	1:1280	1:20	1:20	1:1280
Rabbit anti-Aal cells	<1:10	1:320	1:320	<1:10
Rabbit anti-LLC-MK2 cells	<1:10	<1:10	<1:10	<1:10
Normal rabbit serum	<1:10	<1:10	<1:10	<1:10

TABLE 3. *Neutralization of D-2 virus produced in LLC-MK2 and Aal cells by ant dengue and anti-cellular antisera*

Antiserum	Passage history of D-2 virus			
	LLC-MK2-7	Aal-3	Aal-5	Aal-5 → LLC-MK-3
Monkey D-2	1:640 ^a	1:370	1:350	1:300
Rabbit anti-Aal cell	<1:10	<1:10	1:80	<1:10
Rabbit anti-LLC-MK2 cell	<1:10	<1:10	<1:10	<1:10
Normal rabbit serum	<1:10	<1:10		

^a Neutralizing antibody titer assayed by plaque reduction neutralization test.

means of quantitating the potency of this antiserum, the full implication of the relatively low (1:80) titer cannot be assessed.

DISCUSSION

A number of observations reported herein indicate that the replication characteristics of D-2 virus in Aal mosquito cell differs considerably from that in LLC-MK2 vertebrate cells. Significant differences in this regard include (i) the apparent lack of damage to the Aal host cell by replicating D-2 virus, (ii) the failure to find incomplete virus released by infected Aal cells, and (iii) the production in Aal cell of D-2 virions with altered immunological reactivity. These different manifestations of D-2 virus replication in mosquito cells may ultimately relate to a single underlying mechanism, i.e., the process of maturation and release of the virions from the Aal cells.

Our failure to note cell damage stands in contrast to previous reports (7, 14, 15, 30) that D-2 virus produces distinctive cytopathological effects in Aal cells. This lack of cytopathogenicity is consistent with the situation in nature, wherein mosquitoes infected with arboviruses characteristically remain infected for life without apparent ill effects. As has been emphasized by Ginsberg (10), the provision of optimal nutritional requirements, including serum factors, is one prerequisite for establishing and maintaining persistent infection in vitro. The use of an enriched maintenance medium during initial infection and of modified growth medium to maintain chronically infected Aal cells may have favored cellular metabolism and provided balanced conditions in vitro that approximated natural conditions of dengue-infected mosquito cells. Presumably, then, neither the synthesis of

viral components nor the release of mature virus are detrimental to the infected Aal cell.

The HA produced by Aal cells appears to be exclusively associated with complete virus (RHA), whereas LLC-MK2 cells produce both RHA and SHA. The latter has been extensively characterized (4, 5) and is a form of incomplete virus particle. The accumulation of SHA in the medium of infected LLC-MK2 cultures thus may reflect the release of virion subparticles or maturation products from virus-damaged cells (18).

Both the cytopathogenicity and accumulation of SHA in vertebrate cells, and the lack thereof in infected Aal cells, were characteristics of the host cell and not of the incubation temperature. Stollar et al. (29) have also found differences in replication intermediates of Sindbis virus released from infected vertebrate and mosquito cells; whereas mosquito cells released predominately 20S double-stranded RNA; vertebrate cells in addition released a variety of smaller molecules. These investigations suggest that D-2 replication intermediates are toxic to vertebrate, but not Aal, cells. In the case of vertebrate cells, this would resemble the situation found in vaccinia-infected HeLa cells (32), wherein a virus replication product is cytotoxic, a situation which, moreover, probably exists in other virus-host systems (23).

Antiserum produced against D-2 virus antigens prepared in mouse brain readily reacted with a supernatant fluid-soluble CF antigen from Aal cultures, but did not fix complement in the presence of Aal cell-derived D-2 virus. This is a relatively insensitive assay system, and the gradient fractions may have contained insufficient amounts of antigen. However, the nonsedimenting soluble CF fractions produced in Aal cells contain much higher titers of antigen than that produced in LLC-MK2 cells. This suggests that CF activity in the RHA region is less reactive, although this contention cannot be quantitatively assessed. The data concerning the immunological characteristics of the HA are less ambiguous. The HA activity of Aal-cell D-2 virus is not blocked by anti-D-2 (mouse brain) antibody, but is efficiently blocked by anti-Aal cell antiserum. In addition, the pH profile of the HA produced in Aal cells is slightly, but significantly, shifted. The strict dependence of altered immunological reactivity on host-cell modification was demonstrated by characterizing the HA at each passage from virus produced consecutively in LLC-MK2, Aal, and LLC-MK2 cells. Only the virions from Aal cells failed to react with anti-D-2 antibody in

CF and HA inhibition tests. This virus, when passaged back into LLC-MK2 cells, produced progeny that once again possessed the characteristics of vertebrate-cell-produced virus. These results suggest that, during assembly or maturation in Aal cells, the surface structure of the virion is modified by Aal-cell components that are either incorporated into the virion or remain closely associated with it. This in itself is not unique; host-cell membrane components can be detected in the virions of herpes simplex virus (12) and of vesicular stomatitis virus (6), for example. However, in neither of these examples nor in the D-2 virus produced in LLC-MK2 cells is the antigenic structure so extensively altered. Because the surface antigens are virus determined, we assume that these are fully expressed during replication in Aal cells, but that the presence of Aal-cell antigens interferes sterically with the normal antibody-combining site(s) on the HA, but not on the N antigens.

The apparent incorporation by D-2 virus of cellular antigens during the release of virus from mosquito cells has implications with respect to infection of vertebrates. For example, Alger et al (1) have recently reported that sporozoites of *Plasmodium berghei* incorporate normal mosquito antigens into its surface structure; mice immunized with mosquito salivary gland proteins thus are protected against infection. Whether the same phenomenon will be true for mosquito-cell-produced dengue virus remains to be investigated. However, our results suggest that anti-Aal cell antibody may partially neutralize such virus in vitro. Host-cell modification of dengue virus also bears on the question of repeated dengue infection with heterologous serotypes (31). Heterotypic antibody seems to form against endogenously replicated dengue virus derived from the vertebrate host. Reinfection with mosquito-produced virus may present this host with virions possessing modified, less reactive, surface antigens, and thereby reduce the efficiency of neutralization by host antibody.

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