GROWTH AND PERSISTENT INFECTION OF AFRICAN HORSE-SICKNESS VIRUS IN A MOSQUITO CELL LINE (*)

H. Mirchamsy, A. Hazrati, S. Bahrami, and A. Shafyi,

SUMMARY

Two strains of type 9 African horse-sickness (AHS) virus, adapted to mouse brain or to mouse brain and in monkey kidney stable (MS) cell line, were readily propagated in a mosquito (*Aëdes albopictus*) cell line. The peak titers of cell-released and cell-associated virus were observed 96 to 120 hours after cells were infected. In the growth-curve studies, the cell associated viral titer was nearly the same as or slightly higher than that of cell-released virus. Cytopathic changes were not observed in infected mosquito cell cultures, but viral antigen was detected in cytoplasm and around the nuclei of infected cells by fluorescent antibody technique and by acridine orange staining. The persistent infection of *A. albopictus* cell line with AHS virus and maturation of complete virus in cell generation were observed.

Transmission of AHS by various species of arthropods was suspected, without adequate experimental evidence, long before 1944 when Du Toit[3] produced the disease by inoculating horses with a suspension of culicoides caught in the field.[1,6,21]

Mosquitoes, as possible vectors of AHS virus, were studied by several workers. Nieschulz *et al.*[8] and Nieschulz and Du Toit,[9] however, indicated that mosquitoes of the genus *Aedes,* although they harbored the virus for one week after experimental feeding, could not transmit the disease. Recently (1965 and 1966), Ozawa *et al.*[11,12] transmitted AHS by means of the bite of *Anopheles stephensis* and *Culex pipiens* which had engorged infected horse blood 15 to 22 days previously, and the bite of *Aedes aegypti* which had been fed with viral suspension. Results of further investigations by Ozawa *et al.*[13] indicated that the virus remained in *A. aegypti* for more than 5 weeks, providing further evi-

dence of the possible contribution of mosquitoes in natural transmission of the disease.

Since the *in vitro* susceptibility of an arthropod cell line to infection with a virus may reflect the vector-virus relationship, the purpose in the present report was to study the multiplication of AHS virus in a newly established cell line from *A. albopictus*.[17]

Materials and Methods

Viral Strains.—Two strains of type 9 vaccinal AHS virus designated S2 [5] were used to adapt the virus to the host cell system: One strain was at its 104th passage in mouse brain (S2-104), and the other at the 7th passage in MS cell culture (S2-MS7).

Cell Line.—The original mosquito (*A. albopictus*) cell line was supplied as a 54th passage in the medium of Mitsuhashi and Maramorosch[7] that contained 20% calf serum. This cell line underwent 40 additional passages in this laboratory by using lactalbumin hydrolysate (LAH) medium which consisted of 7.18 mg. of NaCl, 0.4 mg. of KCl, 0.335 mg. of CaCl2. 2H2O, 0.2 mg. of MgSO. 7H2O, 0.142 mg. of NaH2PO4. 2H2O, 2.2 mg. of glucose, 6.5 mg. of LAH, 0.05 mg. of yeast extract, 0.001 mg. of phenol red, 0.12 mg. of NaHCO3,100 units of penicillin/ml., and 100 μg. of streptomycin/ml. The final pH was 6.5. The medium was supplemented with 5% heat-inactivated calf serum and was filtered • (pore size, 0.22 mμg).

Each week a subculture of *A. albopictus* cell sheet was made in Roux bottles by treating the cell sheet with a solution of 0.25% trypsin [b] in phosphate-buffered saline (PBS) solution. The growth-promoting capability of this medium was enhanced when bicarbonate was omitted and the working pH was 6.7. The incubation of the cultures at 24 to 26 C., instead of 28 C. as suggested by Singh,[17] was another factor which increased the outgrowth of this cell. By applying these 2 changes, a complete sheet of cells was regularly formed in 3 days.

Infection of Cells.—The medium of monolayer culture prepared in 4-0z. medical flat bottles was discarded, and the cell sheets were washed once with the growth medium (pH 7.0) and then inoculated with 1 ml. of virus per bottle. The inoculated bottles were incubated at 30 C. for 60 minutes, and then 12 ml. of the same medium containing 2% calf serum was added to each bottle. The inoculated bottles were reincubated at 30 C.

For the 1st passage, fluid of infected MS cell culture or a 10% suspension

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a   Millipore Filter Corporation, Bedford, Mass.
b   Difco Laboratories, Detroit, Mich.

of infected mouse brain in the medium that was centrifuged at 3,000 r.p.m. for 15 minutes was used as inoculum. Nine other passages were performed in the same manner with the fluid obtained from previously infected cells after they were frozen and thawed and then centrifuged at 3.000 r.p.m. for 15 minutes.

All the viruses grown in *A. albopictus* cell cultures were stored at — 70 C. throughout the experiment.

Study of Persistent Infection.—For investigation of the possibility of persistent infection of *A. albopictus* cells with AHS virus and establishment of a viral carrier subline of this cell, the cultures of three 4-oz. medical flat bottles were inoculated with AHS virus adapted to MS cells at its 7th passage in *A. albopictus* cells. The multiplicity of infection was approximately 1.

After an adsorption period of 60 minutes at 30 C., the cell sheet of each inoculated bottle was washed 3 times with warm PBS solution, 12 ml. of LAH medium containing 2% type-specific AHS rabbit antiserum was added, and bottles were incubated at 30 C. After 5 days, the supernatant fluid was discaided, and cells were washed 3 times with PBS solution and were collected by a rubber policeman in 12 ml. of LAH medium containing 2% calf serum. Three bottles were cultured with harvested cells of 1 bottle. The cells were grown in LAH medium with 5% calf serum at 24 C. At one-week intervals, the cell sheet became confluent, and the fluid of each bottle was saved and stored frozen at—70 C. The cultures of 2 bottles were washed 3 times with PBS solution and were sub-cultured as before. The cells of the 3rd bottle were suspended in 12 ml. of LAH medium, sonicated, and centrifuged as will be indicated in the paragraph on growth curve studies, and the preparation was stored at —70 C. for viral titration.

Viral Titration.—Viral samples originated from MS-adapted virus were titrated in MS cell culture tubes as described in a previous report.[10] Serial tenfold dilutions of the virus were made with LAH containing 2% calf serum. Four cell culture tubes were used for each dilution, each tube being given 0.1 ml. of the viral dilution. The final results were recorded 7 days after inoculation, and the viral titer was calculated by the method of Reed and Muench.[14]

Viral samples, of mouse brain origin, were titrated simultaneously in MS cell culture tubes and in adult mice. The technique of titration in mice has been described.[5,10]

Growth Curve Studies.—The cell sheets of cells prepared in twenty-five 4-oz medical flat bottles were washed once with PBS solution and then inoculated with the virus at a multiplicity of infection of approximately 10. An adsorption period of 60 minutes at 30 C. was allowed, and the cell sheets were washed 3 times, each time with 10 ml. of warmed medium before 12 ml. of the medium

containing 2% calf serum was added to each bottle. The bottles were immediately reincubated at 30 C.

Samples were collected at postincubation intervals of 0, 12, 24, 48, 96, 120, 144, 192, 216, 240, 264, and 336 hours to determine viral content both in the fluid and the cells. Two bottles were examined at random at each time interval.

Pooled fluid of bottles was centrifuged at 3,000 r.p.m. for 10 minutes, and the supernate was kept at—70 C. for later titration of the cell-released virus.

The bottles were washed once with 10 ml. of PBS solution, and then 12 ml. of LAH medium with 2% calf serum was added to each bottle. The bottles were kept at —70 C. Immediately before titration, these bottles were quickly thawed, and the pooled cell suspension, which was in a glass container kept in an ice bath, was subjected to 3 minutes of ultrasonication at 20 kc./second in a cell disruptor, c tuned to 2.5 amp. This preparation was centrifuged at 3,000 r.p.m. for 10 minutes, and the supernate was used to determine the cell-associated virus.

Cytologic Studies.—Monolayers of the cells on coverslips in Leighton tubes were inoculated with the virus. At appropriate intervals, the inoculated and noninoculated coverslips were fixed and then stored at 4 C. after they were covered by PBS solution. The coverslips were stained with hematoxylin and eosin stain as described by Reissig *et al.*[15] or they were stained with a 0.01% solution of acridine orange dissolved in citrate buffer (PH 3.6 to 3.8) for 5 minutes. The coverslips stained with acridine orange were examined with a fluorescent microscope d after being washed in citrate buffer and mounted on a glass slide in the same buffer.

Fluorescent Antibody Staining.—The inoculated and noninoculated cells on coverslips prepared as described in the previous paragraph were washed twice with PBS solution, fixed 10 minutes with cold acetone, washed with PBS solution, and stained overnight at 4 C. with a conjugate prepared from hyperimmune rabbit type 9 AHS virus antiserum. The stained coverslips were rinsed in 3 changes of PBS solution, mounted in 10% glycerine in PBS solution, and examined as those stained by acridine orange.

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c Branson Sonifier Cell Disrupter, Branson Instruments, Inc., Stamford, Conn.

d Zeiss microscope, Carl Zeiss, Inc., New York; with Osram HBO 200-W. high-pressure mercury arc lamp, BG 12 exciter filter, and UG5(47/50) barrier filter, Aloe Scientific Company, Silver Spring, Md.

TABLE 1—Infective Titers of Different Passages of African Horse-Sickness Virus in *Aëdes albopictus* Cells

Viral strain	Titrated in	Passage levels								
		1	2	3	4	5	6	7	8	10
S2-MS7°	Monkey kidney stable cells	5.0†	5.0	4.5	5.0	5.0	5.0	4.0	4.5	4.0
S2-104°°	Monkey kidney stable cells	0	0	0	2.0	3.0	3.5	2.5	2.5	2.5
	Mouse brain	5.5‡	5.0	4.0	4.2	4.6	4.0	3.0	N.D.	N.D.

° Seventh passage of strain S2 in monkey kidney stable cells was used for the 1st passage of the virus into mosquito cells. °° One hundred and fourth passage of strain S2 in mouse brain was used for the 1st passage of the virus into mosquito cells. † Log TCID50/ml. ‡ Log LD50/ml.
N.D. = Not done.

Results

Viral Adaptation to Mosquito Cell Line.—Titers of the virus during passages are given (Table 1). The cells inoculated with MS-adapted viral strain had a high titer from the 1st passage. In mouse brain-adapted virus, within 4 passages virus was not demonstrable when it was titrated in MS cell culture tubes, although the cell suspensions had high-titer viral content when they were titrated in mice. In both strains during the 10 passages, the original inoculums were diluted far beyond the extinction points of the original infectivity, ensuring the adaptation and replication of the virus in the new host system.

Nonstained inoculated cells, examined microscopically, did not differ from noninoculated cells within 7 days after inoculation. An easily definable cytopathic effect could not be observed in the mosquito cells inoculated with AHS virus.

TABLE 2—Neutralization Test of African Horse-Sickness Virus Strain S2 Grown in *Azdes albopictus* Cells with Rabbit S2 Antiserum.

Items	−Log viral dilution mixed with serum					
	0	1	2	3	4	5 —
Normal rabbit serum	4*	4	4	4	1	0
Rabbit S2 antiserum	0	0	0	0	0	0

* Number of monkey kidney stable culture tubes showing cythopathic effect. Four tubes were used for each vira dilution.
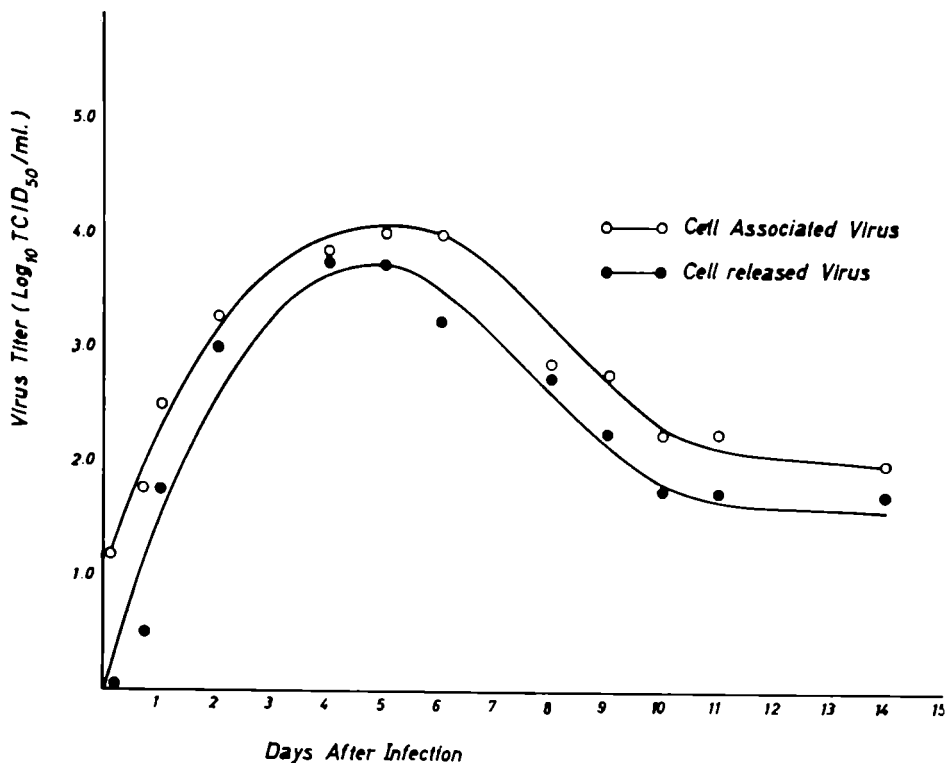
Fig. 1—The growth of type 9 African horse-sickness virus (strain S2-MS7) in *Aëdes albopictus* cell culture.

The nuclei of cells infected for 3 days, however, were somewhat larger and some times pyknotic when the preparations were stained with hematoxylin and eosin stain.

Identity of Virus.—The virus was neutralized by the specific antiserum (Table 2), as confirmed by the fact that the same antiserum inhibited the growth of the virus in *A. albopictus* cells when 1 ml. of the antiserum was added to the cells immediately after they were infected.

Growth Curve Studies.—The titers of cell-released and cell-associated virus were nearly the same (Fig. 1 and 2). Little virus was detected before the 20th hour postinoculation, and in both cases the virus reached its maximal titer on the 4th day postinfection. This was confirmed when the inoculated cells on coverslips collected at regular intervals were studied by acridine orange staining or by fluorescent antibody staining technique. By acridine orange staining, small round bodies were observed in cytoplasm 24 to 48 hours after inoculation. The bodies with distinguished reddish orange color were well defined in the

cytoplasm 96 to 120 hours postinoculation. Immunofluorescent antigen in infected cells was seen after the appearance of the small round bodies.

Persistent Infection in Aedes albopictus Cells.—After 5 consecutive passages of AHS type 9 virus in *A. albopictus* cells, the amounts of cell-released and cell-associated virus were $10^{1.25}$ and $10^{2.75}$ TCID50/ml., respectively.

So far, the weekly subculture of the subline kept in antibody-free medium at 4 C. does not have any cytopathic effect as a consequence of chronic infection of the cells by AHS virus.

Discussion

Since the beginning of the 20th century, most insects and some ticks prevalent during warm wet seasons have been incriminated, without supporting experimental evidence, as possible carriers and transmitters of AHS virus.[16,20,21]
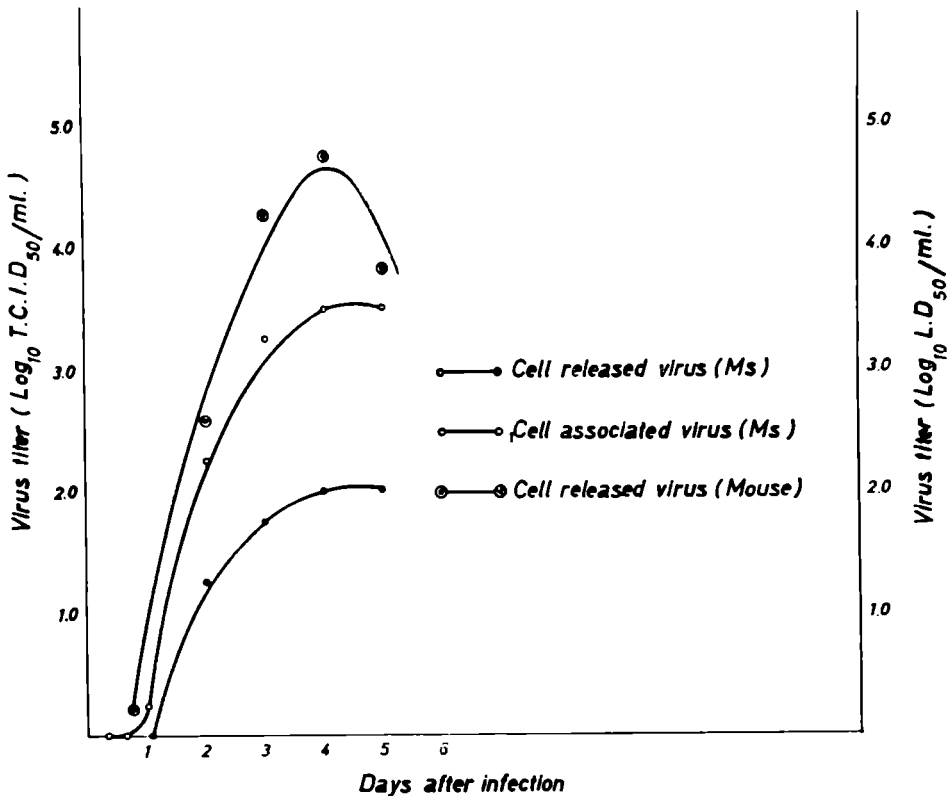

Fig. 2—The growth of type 9 African horse-sckness virus (strain S2–104) in *Aëdes albopictus* cell culture. Titration was done in monkey kidney stable and mouse brain cells.

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On an experimental basis, on the other hand, investigators were unable to agree on a definite role in transmission by a known vector. In this regard, although South African authors denied any significant role of mosquitoes of the genus *Aedes* in the transmission of AHS virus,[8,9] Ozawa *et al.*[11,12] were able to transmit the disease by means of the common mosquitoes, *C. pipiens, Anoph. stephensis,* and *A. aegypti.*

In most transmission experiments so far published by various workers, the lack of evidence for the biological transmission of the virus by a given vector is possible. As a matter of fact, AHS virus was shown to be highly resistant against heat; e.g., it was found that a suspension of virus can be kept for 40 days at 25 C. without great loss of its titer.[6] Thus, isolation of virus or its transmission to horses by means of a suspension of mosquitoes contaminated with a high-titer infective virus cannot necessarily be attributed to a biological transmission, but rather to a simple mechanical passage. The occurrence of the disease in the early summer of 1961 in hot and wet areas of the southern part of Iran where different types of insects were in abundance was attributed to the mechanical transport of virus by most of the insects.

To illustrate the definite biological transmission of AHS virus by a vector, it may be essential to demonstrate the susceptibility of the cells of the vector to this virus *in vitro.*

Recently (1968 and 1969), the growth of several arboviruses in established lines of insect cells was demonstrated.[18,19] Most investigators used the cell cultures of *Aedes* in their *in vitro* studies. Converse and Nagle[2] adapted yellow fever virus to Grace's cell line of *A.aegypti.*[4] Several arboviruses were similarly adapted by Singh and Paul[18] to the *A. albopictus* cell line. To our knowledge, the report of the present experiment is the first report on multiplication of AHS virus in insect cell culture. Both strains adapted to mouse brain and MS cell culture were easily adapted at 30 C. in the 1st passage to mosquito cell culture (Table 1). In the growth curve studies, the maximal yield of both strains were obtained 96 to 120 hours after infection. The titer of cell-associated virus was always slightly higher than that of the cell-released virus (Fig. 1 and 2). The identity of virus grown in the cells was also demonstrated by seroneutralization test with typespecific homologous AHS antiserum (Table 2). Cytopathic changes due to AHS virus on *A. albopictus* cells were not observed when inoculated cultures were compared with noninoculated ones. This observation is similar to that of Yunker and Cory[22] who adapted Colorado tick fever virus to the same strain of *A. albopictus* cells without any detectable cytopathogenic effect being caused by the virus. It was, however, possible for us to demonstrate the presence of mature virion in cytoplasm and around the nuclei of the infected cells by fluorescent antibody technique and by acridine orange staining.

An important observation in the present study was the persistent infection of mosquito cells by AHS virus. When virus was first grown in the *A. albopictus* cell line in the presence of specific antibody, and subsequent populations of cells comprising a number of generations were grown in antibody-free medium, synthesis of rather large amounts of complete virion inside the cells was evident. The absence of cytopathic effect following viral synthesis indicated that, apparently, cellular repressor blocking viral synthesis or maturation does not exist and mosquito cells may be viral carriers for a long time without having detectable cellular changes. This phenomenon may lead to a better understanding of the fate of AHS virus in interepizootic periods.

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