Studies of Interferon Production in *Aedes albopictus* Mosquito Cells[1] (36946)

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The interferon antiviral system has been well documented in mammalian and avian vertebrate species. Antiviral activity with many of the characteristics of interferon has also been reported in lower vertebrates such as fish (1–3), tortoise cells (4), and in the garter snake (Stringfellow and Glasgow, personal communication). The evidence favors the existence of the interferon defense system throughout the vertebrates.

It is unclear whether viral interference or interferon exist in animal species lower than vertebrates. Peleg (5) reported that cell culture fluids obtained from Semliki Forest virus infected *Aedes aegypti* mosquito cells had no interferon activity. Libikova and Buckley (6) reported similar results with Kemerovo virus infection of *Aedes albopictus* cells. However, in both studies only 1 or 2 time periods of induction of interferon were studied, and residual virus was inactivated by relatively harsh acid or heat treatment. Peleg (5) and Libikova and Buckley (6) also indicated that there was no interference between viruses in infected mosquito cells. Yunker and Baron (personal communication) have also found that in general there is no viral interference in *A. albopictus, A. aegypti,* or Hsu's *Culex quinqefaciatus* cells. However, in two instances they did observe low level interference (av reduction of 1.5 $\log_{10}$) with Sindbis virus growth—in West Nile virus infected *A. aegypti* cells and polyriboinosinic-cytidylic acid treated *A. albopictus* cells. Bergold (7) has also reported that polyriboinosinic-cytidylic acid reduced the growth of vesicular stomatitis virus in mosquitos and mosquito cells. In addition to these observations with mosquitos, Garzon and Kurstak (8) have indicated that both interference and interferon occur in the wax moth. However, no characterization of the endotoxin-induced material as an interferon was described.

Thus, it is currently a question whether interferon occurs in invertebrates. The current experiments were designed to detect interferon in Singh's *A. albopictus* mosquito cell line under the following conditions: (i) infection with viruses known to produce and be sensitive to the action of vertebrate interferons, (ii) incubation of cells at both 28 and 36° to determine if temperature affected interferon production, and (iii) inactivation of viruses in interferon samples by ultraviolet light and antiserum treatment in order to preserve possibly labile invertebrate interferon-like substances.

Materials and Methods. Cells. Singh's *A. albopictus* cell line (9) was used between passages 176 and 210. Stock cultures were grown at 28° in 32 oz prescription bottles in the modified medium of Mitsuhashi and Maramorosch (10), and maintained in the same medium but with 3% heat-inactivated fetal calf serum. The cells were harvested by scraping monoloyers from the surface with a rubber policeman and dispersing cell clumps by repeated pipetting. For interferon production and virus growth curve studies, *A. albopictus* cells were cultured in 1 or 8 oz powder jars. For virus titrations, primary chicken embryo fibroblasts were prepared from 9 to 13 day old embryos (Spafas, Inc., Biglersville, PA) as previously described (11).

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Viruses. Sindbis Ar 1055 virus was cultured in chicken embryo cells. West Nile Ar 248 virus was cultured by intracerebral inoculation of suckling mice and stock virus was prepared as a 10% suckling mouse brain suspension. Virus titers were determined as the number of plaque forming units (PFU) per milliliter in agar overlays of chicken embryo cells.

Virus growth and interferon production. For virus growth and interferon production studies, confluent monolayers of *A. albopictus* cells were infected with either virus and maintained at 28° or, for some experiments, at 36°. Sindbis or West Nile virus cell culture fluids were harvested between 1 and 10 days after infection for titration of virus yields and for assay of interferon activity. For interferon production assays, virus in fluids was partially removed by ultracentrifugation twice at 100,000*g* for 1 hr at 4°. Residual virus remaining in the supernatant following centrifugation was inactivated by ultraviolet (uv) irradiation or immune ascitic fluid (IAF) treatment. Ultraviolet irradiation was performed at 140 ergs mm^{-2} sec^{-1} for 90 sec, 10 cm from 2 germicidal lamps (General Electric G15T8, 15 W). Immune ascitic fluid was prepared in Dublin ICR mice by the method of Hammon and Sather (12). For virus inactivation, undiluted IAF was reacted with equal parts of supernatant fluids overnight at 4°. Uninfected cell culture fluids were processed identically to infected fluids to determine if toxic inhibitory substances appeared in incubated medium. In several instances it was noted that these processed uninfected cell culture fluids reduced the control virus yield by as much as 1 $\log_{10}$. For some early experiments, virus was inactivated by treatment of cell culture fluids with acid (dialysis against pH 2 buffer of 0.15 *M* HCl–KCl for 2 days at 4°) or heat (56° for 1 hr). Samples from all fluids were tested for effectiveness of virus inactivation before use in interferon assays. All samples were frozen at —50 to —70° until assayed, and assayed within 3 wk of collection.

Interferon assay. The *A. albopictus* cells were treated with virus-free or uninfected cell culture fluids overnight at 28°, washed 3 times with Earle's balanced salt solution, then infected with either homologous or heterologous virus (m.o.i. = 0.1–10). After 1 hr of adsorption, cells were washed 3 times, maintenance solution was added (13), and the cultures were incubated 24 hr at 28°. Supernatant fluids from duplicate cultures were pooled and frozen at —70° until the virus yield from each sample collected during one entire experiment could be determined simultaneously by virus titration on chicken embryo cells. A unit of interferon activity was designated as the reciprocal of the dilution of an infected cell culture fluid which decreased the virus yield by 0.5 $\log_{10}$ from the control virus yield.

Results. Comparative growth of Sindbis virus in A. albopictus *and chicken embryo cells.* Sindbis virus grew to a titer of 5×10^8 PFU/ml in chicken embryo cells. Because Sindbis virus does not produce cytopathic effects or plaques in *A. albopictus* cells, the following experiment was done to determine the virus titer in these cells. The stock virus was diluted and used to infect stationary tube cultures of *A. albopictus* cells. After 24 hr of incubation at 28°, the cell culture fluids from individual tubes were harvested and subcultured onto chicken embryo cells. The titer (most probable number of effective particles) of the virus in *A. albopictus* cells was then calculated. By this method Sindbis virus titered 3×10^7 effective particles/ml in *A. albopictus* cells. Thus the efficiency of plating of Sindbis virus in chicken embryo cells was approximately 10-fold greater than in *A. albopictus* cells. The titer of Sindbis virus in *A. albopictus* cells was used in determining the multiplicity of infection of this virus in interferon experiments.

Persistent inapparent infection of A. albopictus *cells.* West Nile virus replicated in *A. albopictus* cells at 28° to a maximum titer of 10^6–10^7 PFU/ml of extracellular virus, and levels of 10^6 PFU/ml were measured through 9 days after infection (Fig. 1). Thus, infection appeared to be persistent, as has been repeatedly shown for *A. albopictus* cells infected with arboviruses (13, 14). Infection was also generally inapparent. In only one experiment, in which cells were maintained for 14 days with media changes every 3 days,

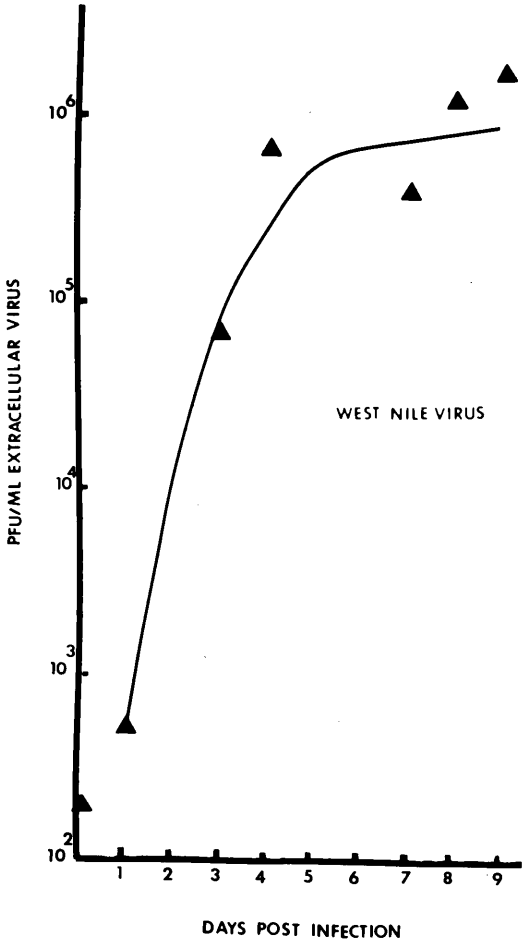

FIG. 1. Infection of *A. albopictus* cells with West Nile virus. Confluent monolayers of *A. albopictus* cells were infected with 2 × 10^2 chicken embryo PFU of West Nile virus, and incubated at 28°. Supernatant fluids were removed at intervals for virus titration.

was some cytopathology seen with West Nile virus infection on Days 11–14 after infection.

Sindbis virus also produced a persistent inapparent infection in *A. albopictus* cells when cells were maintained at either 28 or 36°. Virus yields persisted at levels of 10^7–10^8 PFU/ml from Days 2 through 10. Extracellular titers of virus from cells maintained at 36° were lower than the titers observed in the same cells maintained at 28° (Fig. 2).

Interferon assays. Table I shows the results of interferon assays that were conducted in nine different experiments in which *A. albopictus* cells were infected and maintained at 28°, and fluids were harvested at intervals for virus titration and interferon assay. When virus in cell culture fluids was inactivated by acid or heat treatment, undiluted fluids exhibited no consistent interferon-like activity. When fluids were assayed against the homologous virus, occasionally a sample would reduce the yield of virus from 0.5 to 1.0 $\log_{10}$ (Expts 1, 9, Table I). However, there was no clear cut pattern of interferon production activity. These results are similar to those of Peleg (5) and Libikova and Buckley (6).

In order to avoid the possibility of destroying a labile invertebrate interferon, milder methods of ultraviolet light and IAF treatment to inactivate residual virus were employed. In addition, in some experiments, infected cells were maintained at 36° to augment possible interferon synthesis (15). In one experiment at 36°, in which uv-treated

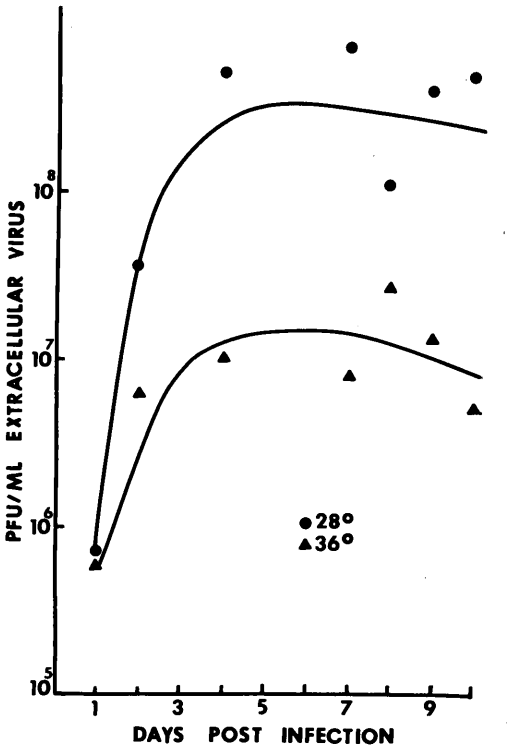

FIG. 2. Infection of *A. albopictus* cells with Sindbis virus. The *A. albopictus* cells were grown to confluency at 28°. Monolayers were infected with Sindbis virus (0.3 m.o.i.), and incubated at either 28 or 36°. Supernatant fluids were removed at intervals for titration of extracellular virus.

TABLE I. Production of Interferon in *A. albopictus* Cells.

Expt	Inducing virus/ challenge virus	Incubation temp (°)	Virus inactivation[a]	Interferon production[b] 6 hr	1	2	3	4	5	6	7	8	9	10
1	Sindbis/Sindbis	28	Acid	—	±	—								
2	Sindbis/Sindbis	28	Acid		—	—	—	—						
3	Sindbis/Sindbis	28	Heat		—	—	—	—						
4	Sindbis/Sindbis	28	uv		—	—	—	—		—	—			
5	Sindbis/Sindbis	36	uv		±	—	±	±	±		±			
6	Sindbis/West Nile	28	IAF		—	—	—	—		—	—			
7	Sindbis/West Nile	28	uv		—	—		—			—	—	—	—
8	Sindbis/West Nile	36	uv		—	—		—			—	—	—	—
9	West Nile/West Nile	28	Acid		±	±	—	±						
10	West Nile/Sindbis	28	IAF		—	—	—	—						
11	West Nile/Sindbis	28	uv		—		—	—			—	—	—	

[a] Viruses inducing interferon were inactivated by acid (pH 2), heat (56° for 1 hr), uv (ultraviolet light), IAF (immune ascitic fluid).

[b] Interferon production was measured by yield reduction of the challenge virus, with dilutions of supernatant fluids or cells and supernatant fluids taken at intervals (6 hr, 1–10 days) after infection of cells with inducing fluids. — = no yield reduction with undiluted fluids. ± = inconsistent low level reduction (0.5–1.0 $\log_{10}$) in fluids undiluted and diluted 1:2, 1:4.

fluid from Sindbis virus infected cells was assayed for ability to reduce the yield of the homologous virus, some inhibitory activity (1–4 units) was detected (Expt 5, Table I). However, in all other experiments in which residual virus was inactivated by the mild ultraviolet or IAF treatments, no interferon activity was observed. No activity was observed whether infected cells were maintained at 28 or 36°, or whether cell culture fluids were assayed against either homologous or heterologous viruses.

Discussion. Sindbis and West Nile viruses grew well in *A. albopictus* cells, reaching titers of 10^6–10^7 PFU/ml. Virus titers remained high through the 10 day observation period although no cytopathic effect was observed. The lack of cytopathology with West Nile Ar 248 virus infection is in contrast to other reports (13, 14). Apparently not all strains of West Nile are capable of readily producing cytopathology in *A. albopictus* cells.

Although the viruses grew well and reached titers similar to those observed in infected chicken embryo cells, no significant interferon production was detectable in fluids harvested throughout the 10 days of incubation. These results confirm those of Peleg (5) with Semliki Forest virus and Libikova and Buckley (6) with Kemerovo virus, who found that heat- or acid-inactivated materials taken 1–5 days after infection had no reproducible interferon activity. However, Peleg (5) and Libikova and Buckley (6) used only 28° incubation and 1–2 time periods of interferon collection. In our studies we were unable to show clear evidence of interferon production in *A. albopictus* cells incubated at 36 or 28°, in fluids obtained after prolonged cell incubation for up to 10 days, or by using mild procedures to inactivate residual virus.

There are several possible reasons for the inability to detect interferon production by *A. albopictus* cells: (i) very low levels of interferon might be present and undetectable by the present procedures. We feel this is not likely. Sindbis and West Nile viruses are known to be good interferon inducers in vertebrate systems (15). Sufficient levels of these inducers (10^6–10^8 PFU/ml) were present to produce interferon in vertebrate cells. In the study with Kemerovo virus, the virus only grew to levels of 10^3–10^4 PFU (6). Sufficient time (1–10 days) was allowed for possible slow synthesis of an interferon. The possibility that an invertebrate interferon might be labile was considered. Collected materials were frozen rapidly at —50 to —70° and assayed within 3 wk of collection. Residual virus was inactivated by mild methods to avoid interferon destruction. The assay chosen for detection of interferon activity

(yield reduction) is one of the most sensitive assays for interferon (16). Thus we feel it is most likely that low levels of interferon activity were not present. Indeed, Libikova and Buckley (6) concentrated materials 5-fold and still found no consistent interferon activity. In addition, in some of our early experiments cell + cell culture fluids were assayed together and no interferon activity was found. Thus any interferon does not appear to be primarily cell associated.

ii. The possibility exists that *A. albopictus* cells may not be able to produce interferon, yet may be active to its action. This phenomenon has been observed for certain sublines of VERO cells (17). We tested uninfected and Western encephalitis virus infected *Culex tarsalis* mosquito extracts (courtesy of Dr. W. D. Sudia, CDC) for interferon activity on *A. albopictus* cells. No activity was observed.

iii. The alternative possibility, that *A. albopictus* cells can produce interferon but are not sensitive to its action, cannot be eliminated at present. Certain cell lines that have been in culture for extended passages do appear to lose some sensitivity to interferon, and the *A. albopictus* cells were used in passages 176–210. It would be interesting to pursue these studies in *A. albopictus* cells closer to origin, or in primary mosquito cells.

iv. From the available data, it appears most likely that Singh's *A. albopictus* mosquito cells can neither produce nor be sensitive to the action of interferon. This raises the intriguing possibility that the interferon antiviral defense system may have evolved with the vertebrates, similar to the immune system. The interferon system has recently been shown to be closely linked to the immune system (18) in regard to the similar cells involved in interferon and antibody synthesis, and in the production of interferon by sensitized lymphocytes on contact with their specific sensitizing antigen.

Summary. Singh's *Aedes albopictus* mosquito cell line was infected with Sindbis and West Nile viruses in order to assess the ability of these invertebrate cells to produce interferon. The viruses grew well in the mosquito cells, producing titers of 10^6–10^8 PFU/ml throughout 10 days of incubation. Temperature affected virus growth; Sindbis virus grew approximately 10-fold more at 28° than at 36°. Culture fluids from 1 to 10 days of incubation at either temperature were assayed for interferon activity by the yield reduction assay method. Virus in fluids was inactivated by ultraviolet light or immune serum treatment in order to preserve any labile invertebrate interferon activity. No consistent reduction of yields of homologous or heterologous viruses were obtained at either incubation temperature of 28 or 36° at any of the times periods studied through 10 days after infection. *Aedes albopictus* mosquito cells apparently produce no or very little interferon on infection with Sindbis or West Nile viruses.

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