Role of Extracellular Virus in the Maintenance of the Persistent Infection Induced in *Aedes albopictus* (Mosquito) Cells by Sindbis Virus

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Sindbis virus infection of cultured mosquito cells was found to have no effect on the growth of these cells; instead, a persistent infection of the culture followed an initial acute phase of rapid virus synthesis. Nearly all of the cells in the acute stage of infection were found to actively release virus in an infectious-center assay and to contain significant amounts of virus antigen as determined by immunofluorescence. Cells in the persistent phase of infection released few virions into the media, and only a small percentage of the cultured cells could be demonstrated to contain detectable amounts of virus antigen by immunofluorescence assay. In spite of the fact that nearly 100% of the cells in the persistent phase of infection were found to be virus negative by the two assays described above, the culture as a whole totally excluded the expression of superinfecting virus, as did cells in the acute phase, suggesting that most of the persistently infected cells did, indeed, contain virus information. Prevention of reinitiation of the cells in the persistent phase by eliminating extracellular virus resulted in a curing of the culture such that it responded to infection by added virus much as would an uninfected culture.

The alphatogavirus Sindbis belongs to a general grouping of viruses that alternatively grow in cells of both vertebrate and invertebrate hosts, the arboviruses (arthropod-borne viruses). Because cell cultures of representative vertebrate and invertebrate hosts are available, it is possible to examine the interaction of the arboviruses with cells of both the vertebrate host and invertebrate vector in the laboratory at the biochemical, molecular-biological, and morphological levels. This system, therefore, provides a unique opportunity to examine the expression of a single piece of genetic information, the viral RNA, in two biochemically and genetically unrelated environments.

Vertebrate cells respond very differently from invertebrate cells to Sindbis virus infection (2, 5, 10, 14). Although the onset of virus production, rate of virus release, and yield of virus produced are similar in cultured cells of both hosts, the virus-infected mosquito cells do not show the cytoidal effects of infection observed in the vertebrate cells. Sindbis virus-infected mosquito cells enter a state of persistent infection in which the culture continuously produces viruses for indefinite periods of time (5, 10, 14). It has been shown previously that the process of Sindbis virus development in mosquito cells differs from that observed in cultured vertebrate cells (5). The budding of virions from the infected cell surface, which is characteristic of infected vertebrate cells, was rarely found in infected mosquito cell cultures. The bulk of Sindbis virions produced in mosquito cells were detected in internally located vesicles and were released later by fusion of the vesicle with the cell surface. Viral nucleocapsids were found in the cytoplasmic vesicles in various stages of envelopment, suggesting that virus assembly could also take place in these internalized structures. Gliedman et al. (5) suggested that the localization of some aspects of the virus reproductive process to restricted areas of the cell cytoplasm might protect the invertebrate cell from toxic agents that result from virus infection and cause the death of infected vertebrate cells.

The persistent infection is established in the infected mosquito cells without loss of cells from the culture or any obvious alteration in any of the growth characteristics of the infected culture (10, 14; this study). The classically proposed mechanisms for the maintenance of persistent infections in some virus-vertebrate cell systems (6, 7, 17) do not seem to be responsible for the maintenance of the persistent infection of mosquito cells by Sindbis virus. Igarashi and Stollar (8) have elegantly demonstrated that
neither defective interfering particles nor their truncated RNAs are found in persistently infected mosquito cells. These authors further demonstrated that defective interfering particles produced by serial passage of Sindbis virus in vertebrate cells could not interfere with normal Sindbis virus growth in the invertebrate cell cultures. A single passage of a vertebrate-produced population of viruses rich in defective particles in mosquito cells was sufficient to free that virus of defective interfering particles. Temperature-sensitive mutants (which have also been implicated in the maintenance of some persistent infections [17]) are produced in Sindbis virus-infected mosquito cells but only at times significantly later than the onset of the persistent state (13).

We have investigated the role that extracellular virus plays in the maintenance of the persistent state of infection by studying persistently infected mosquito cell cultures under conditions that either allowed or prevented the reinfection of cells by progeny virions released into the surrounding medium.

MATERIALS AND METHODS

Cells, virus, and media. Aedes albopictus cells were provided by Sonya Buckley (Yale Arbovirus Research Unit, New Haven, Conn.) and were cultured in the medium of Mitsuhashi and Maramarosch (9) as described previously (12). BHK-21 cells were provided by Peter Faulkner (Queens University, Ontario, Canada) and were grown in Eagle medium (4) supplemented with 10% calf serum. The heat-resistant strain of Sindbis virus, which serves as wild-type virus in this laboratory, as well as the temperature-sensitive mutant ts-20 were provided by Elmer Pfefferkorn (Dartmouth Medical School, Hanover, N.H.). Virus stocks were prepared and titrated in BHK cells as described previously (12).

Infection of mosquito cell cultures for the experiments described in this study were carried out as described by Renz and Brown (12).

Infectious-center assay. An assay of the percentage of A. albopictus cells capable of releasing virus after infection with Sindbis virus was carried out by a modification of the procedure described by Bishop and Koch (1). Infected mosquito cells were removed from the monolayer, washed three times in cold phosphate-buffered saline, and suspended in cold mosquito medium (the low temperature was used to minimize virus production). The concentration of cells was determined, and the cells were serially diluted in cold medium. A 0.5-mL portion of each dilution was mixed with 1.0 mL of freshly trypsinized and washed BHK-21 cells (ca. 10^6 cells) in Dulbecco medium (3) containing 10% tryptose phosphate broth and 10% fetal calf serum. This mixture was brought to room temperature, and 1.0 mL of 1.4% agarose (Sigma Chemical Co., St. Louis, Mo.) in complete Dulbecco medium, at 42°C, was added to it. This mixture was quickly poured into petri dishes (60 by 15 mm) containing a preformed base consisting of 1.0% agarose in the Dulbecco medium described above. The agarose was allowed to harden for 30 min at room temperature, and the plates were placed at 32°C. Plaques forming in the cell culture were stained with neutral red and counted 3 days later.

Although our A. albopictus cells cannot be continuously cultured in either Eagle or Dulbecco medium, we found that infected mosquito cells that are transferred into Dulbecco medium produce approximately the same amount of virus (2.4 x 10^6 PFU/mL) as an equivalent number of cells cultured in mosquito medium (4.8 x 10^6 PFU/mL).

Preparation of anti-Sindbis serum. Sindbis virus that had been grown in BHK-21 cells was purified by three consecutive sedimentations to equilibrium and one velocity sedimentation in potassium tartrate gradients, dialyzed against phosphate-buffered saline, and mixed with an equal volume of Freund complete adjuvant. A sample of this preparation (containing about 100 µg of viral protein) was injected subcutaneously into a rabbit, and this injection was repeated 10 days later. After 3 weeks, an additional injection of Sindbis virus was given intravenously. A 1:10,000 dilution of the resulting antiserum inactivated over 99% of virus infectivity in 15 min at room temperature. In an indirect immune precipitation assay, this antiserum was found to react with all three viral structural proteins (data not shown).

Immunofluorescence assay. The immunofluorescent detection of Sindbis virus antigens in the infected mosquito cells was carried out essentially as described by Vogt (16). Anti-Sindbis serum was prepared against BHK-grown virus as described above and served as a primary label in an indirect immunofluorescence assay, after first being adsorbed exhaustively against uninfected mosquito cells.

The infected mosquito cells, growing on cover slips, were washed and fixed in acetone, as described by Vogt (16), and covered with a 1:5 dilution of the anti-Sindbis serum. The cover slips and antibody were incubated for 30 min at 37°C in a moist chamber and then carefully washed with phosphate-buffered saline and distilled water. The cells were then reacted with a 1:5 dilution of fluorescein-conjugated anti-rabbit gamma globulin (Cappel Laboratories, Downingtown, Pa.), which also had been adsorbed against uninfected mosquito cells for a period of 30 min at 37°C. The cover slips were then carefully washed in distilled water, rinsed in isopropyl alcohol, treated with xylol, and embedded on microscope slides in Eukitt (Dr. Hirtz and Co, Cologne, West Germany). The slides were then examined with a Zeiss microscope with UV optics, and the percentage of cells clearly fluorescing was determined.

RESULTS

Growth of Sindbis virus in cultured mosquito cells. The growth of Sindbis virus in A. albopictus was examined for a period of 23 days after infection with 50 to 100 PFU of virus per cell by assaying the total amount of virus in the
culture medium at regular intervals (see Fig. 1). The relative amount of virus recovered in the medium increased until about 21 h postinfection and then fell to a lower level, where it remained throughout the rest of the examination period.

Effect of Sindbis virus infection on the rate of growth of A. albopictus cells. The rate of growth of cultured A. albopictus cells is shown in Fig. 2 and is compared to the growth rates of newly infected and persistently infected cells cultured at the same time at the same initial cell density. The doubling time of the uninfected cells was found to be 14 to 16 h. Infection of the cells with Sindbis virus did not affect the rate of growth of the cells, regardless of whether the infection was carried out at the time of subculturing or the cells had been infected for a period in excess of 90 days. The delay in the onset of cell growth shown for two of the cultures in Fig. 2 was not found to be characteristic of those cells, but rather reflected some difference in the physiological state of the cells at the time of subculturing. In repeating this experiment a number of times, it was found that none of the cultures consistently demonstrated such a lag before the onset of logarithmic growth. Cells examined at any time after virus infection were not distinguishable morphologically from uninfected cells (data not shown).

Release of virus from individual cells after infection with Sindbis virus. The percentage of cells in the infected culture capable of releasing virus at given times after infection was determined by using the infectious-center assay described in Materials and Methods. The number of cells registering as virus positive was determined over a period of 41 days after infection with 500 PFU of virus per cell (Fig. 3). The higher multiplicity of infection was used to be certain that all of the cells were infected, even though in a previous study we demonstrated that all of the cells in a mosquito culture could be infected by 50- to 100-PFU multiplicities under the conditions used for infection in this study (12).

A maximum number of infectious centers was obtained in the first time point taken at 17 h postinfection. At 24 h after infection there was a decrease in the number of infectious centers, and after 4 days the number was reduced to 18% of the total cells in the culture. After the initial depression, the number of infectious centers increased to about 65% and decreased again. At 24 days after infection a much smaller increase was found, and then the culture stabilized with about 1% of the total cells assayable as infectious centers and remained at this level for the rest of the examination period. This infected culture has been assayed from time to time since the period shown in Fig. 4 and has remained at a level of about 1% of the total cell number producing infectious centers for over 100 days.

The values obtained in the infectious-center assay, which exceeded 100% early in the period
of infection, probably result from an inability to completely prevent the release of virus from the infected cells during preparation for the suspended cell culture used in the infectious-center assay. It has been demonstrated that the mode of virus release from the infected mosquito cells is via the fusion of virus-containing vesicles with the cell plasma membrane, resulting in the simultaneous release of large numbers of virions (5). It would only be necessary for one of these release events to take place before the cells are fixed in the agarose to result in an increase in the value obtained in this assay. For this reason, the infectious-center assay can be considered only an indicator of the relative number of cells releasing virus during the

**Fig. 3.** Percentage of Sindbis virus-infected A. albopictus cells actively releasing virus as determined by an infectious-center assay. Cells were infected and assayed as described in the text. Symbols: (●) infected cells cultured in normal medium; (○) infected cells cultured in medium containing anti-Sindbis serum. Percent infected cells means those cells determined to be virus positive by this assay procedure.

**Fig. 4.** Release of virus from Sindbis virus persistently infected A. albopictus cells grown in medium with anti-Sindbis serum after transfer into normal medium. The arrows indicate the points at which the infected cells were removed from the antiserum, washed, and placed in normal medium. The infected cells were assayed as in Fig. 3. Symbols: (▲) cells removed from antiserum containing medium at 14 days; (●) cells removed from antiserum containing medium at 25 days; (○) persistently infected cells maintained in normal medium.
"acute" phase of infection, when virus production is maximal.

Experiments carried out with BHK cells, using the same procedure as that used with the mosquito cells, produced the expected 100% value after infection with a high multiplicity and corresponding lower percentages when multiplicities less than 1 PFU/cell were used (data not shown). As the infected mosquito cells enter the persistent phase of infection, the reduction in the amount of virus released into the medium suggests that the process of emptying of the virus-containing vesicles from the infected mosquito cells is less frequent. We expect, therefore, that the values for the percentage of cells releasing virus become a more accurate representation of the real situation as the culture enters the persistent phase.

Effect of anti-Sindbis serum on the number of infectious centers. The number of detectable as actively releasing virus by the infectious-center assay was also determined in an experiment in which reinfection of the mosquito cells by extracellular virus was prevented by including anti-Sindbis serum (1%, by volume) in the cell culture medium. The inclusion of antiserum in the growth medium at a high concentration did not result in any clumping of the infected cells (either in the acute or the persistent phase of infection), nor did it in any way alter the rate of growth of the culture (Fig. 2). This observation is consistent with the notion that the surfaces of mosquito cells infected with, and producing, Sindbis virus are not extensively modified by virus protein (5). Infected cells were placed in antiserum directly after the initial period of infection and were taken from the same infected culture as that represented in Fig. 3 as non-antiserum treated. As was found with cells in normal medium, a maximum number of infectious centers was obtained in the presence of anti-Sindbis serum at 17 h post-infection (Fig. 3). The number of infectious centers then fell rapidly to very low values, and the culture did not show the later increases demonstrated by the cells cultured in the normal medium. The number of infectious centers fell to a low value of 0.001% in the 41 days covered in this study, and subsequent assays have indicated that the culture remained at this level for over 100 days. The inability to completely eliminate the virus-infected cells from this culture in the presence of antiserum probably results from an inability of the antiserum to inactivate all virions in the medium. A few particles may escape inactivation and maintain the infected status of the culture.

Return to the acute phase of infection by persistently infected cells grown in antiserum after removal of the antibody. The state of infection of persistently infected cells grown in the presence of antiviral serum was followed after the removal of the antibody by the infectious-center assay and by assaying for the presence of virus-specific antigens in the cells by the fluorescent-antibody procedure described in Materials and Methods. Antibody was removed from the infected culture at four different times after infection. Each time the culture was washed exhaustively with phosphate-buffered saline and given fresh normal medium. The removal of the antiserum at 14 days postinfection resulted in a rapid increase in the number of infectious centers and a return to a low level in a period of about 5 days (Fig. 4). Removal of antiserum at 25 days after infection (same culture) resulted in a lag of 4 days before the increase in number of infectious centers was detected (Fig. 4). The maximum number of infectious centers obtained when the antiserum was removed at the later time was also higher than that obtained when the antibody was removed after 14 days (Fig. 4). The maximum percentage of infectious centers was obtained at 7 days after the removal of the antiserum, and then the level decreased to that expected of a persistently infected culture. The entire cycle required 15 days for completion. The delay in the increase in percentage of infectious centers when the antiserum was removed late, compared to the rapid increase seen when the antiserum was removed early, may result from the low level of infection of the culture at the late time. The limited number of infected cells remaining must produce enough virus to reinfect the large number of "cured" cells in the culture. At 14 days after infection, enough of the cells are still producing virus to achieve this in a short period of time. On the other hand, the more nearly complete "curing" of the culture achieved by the longer incubation time in antiserum allows for a greater production of virus and a higher percentage of infectious centers.

In an experiment in which the number of cells containing virus-specific antigens was determined by an immunofluorescence assay, it was found that an increase in the number of cells that contained significant amounts of virus antigens was detected before a major increase in the percentage of infectious centers (Fig. 5), suggesting that a certain number of cells can contain significant amounts of virus without releasing amounts of virus that can be detected in the infectious-center assay. When the antibody was removed from the same culture at 86 days after infection, a lag of 5 days was observed before the number of cells containing significant amounts of viral antigen
increased. The number of cells determined to be virus positive increased for a 3-day period to a maximum of 65% and then decreased in 2 days to the level determined before the removal of the antiserum (Fig. 6). The failure of the antiserum-"cured" culture to achieve a value of 100% fluorescence-positive cells after the removal of the antiserum (newly infected cells do so in 24 h, Fig. 6) most likely reflects the lack of synchrony with which the cured cells are infected by the small amounts of virus released after the removal of the antiserum. Some of the cured cells are infected early and have gone through the acute phase of virus production before the bulk of cells becomes infected, whereas others may only enter the acute phase after the majority of the cells are entering the persistent phase. The observed lag in the increase of fluorescence-positive cells can be eliminated and the number of fluorescence-positive cells can be increased to nearly 100% by simply adding virus to the culture after removal of the antiserum (Fig. 6).

Persistently infected cells maintained under the same conditions in normal serum showed levels of fluorescence throughout this study comparable to those of uninfected control cells (Fig. 6).

Ability of antiserum-treated and untreated cells to exclude superinfecting Sindbis virus. We have previously demonstrated that the ability of a culture of infected mosquito cells to exclude a second superinfecting virion can be used as an indication of the percentage of the cells that were infected by the first virus (12). The exclusion of the superinfecting virion is complete when the culture is completely infected with the first virus. The ability of the antibody-treated and untreated, persistently infected cells to exclude superinfecting Sindbis virus was tested by reinfesting the cultures with the Sindbis mutant ts-20. ts-20 is particularly suitable for an experiment of this type because of its low reversion index (12) and because our isolate of this mutant produces a plaque that is easily distinguishable, morphologically, from that produced by virus released from the persistently infected cells. The result of this experiment is shown in Table 1. Very
little temperature-sensitive virus was released from the superinfected, persistently infected cells maintained in normal medium, and this virus produced plaques with the morphology of the endogenous virus released from the persistently infected culture. The cells treated with antiviral serum produced as much virus as the morphological and temperature-sensitive characteristics of ts-20 as an equivalent number of uninfected control cells. This result shows that although the number of infectious centers and the antibody-binding capability of the persistently infected cells is very low, nearly all cells contain the viral information required for the exclusion of superinfecting virions. Persistently infected cells grown in the presence of antiviral serum have lost this viral function.

When the Sindbis virus-persistently infected mosquito cells were superinfected with West Nile virus (a flavito-gavirus), they produced as much progeny virus as an equal number of uninfected mosquito cells (Table 1). This observation is in agreement with the work of Stollar and Shenk (15), who found that Sindbis virus-persistently infected mosquito cells excluded superinfecting Sindbis virions but not Eastern equine encephalitis virus (an alphatogavirus). The interference phenomenon thus seems to be specific for the virus inducing the persistent infection and not due to some generally acting antiviral agent such as interferon.

**DISCUSSION**

Cultured mosquito cells infected with Sindbis virus at a high multiplicity were found to pass through an initial "acute" phase of infection in which large amounts of virus were released into the culture medium and entered a "persistent" phase of infection in which the yield of extracellular virus was much lower. At no point in this process of infection could any differences be detected in the growth rate or the general morphology of the infected cells compared with uninfected cells. The infected culture in the persistent phase of infection differed from cultures in the acute phase in that very few of the cells could be demonstrated to contain virus structures either by an infectious-center assay or by immunofluorescence. Cells in the persistent phase of infection were, however, found to completely exclude the expression of superinfecting virus, suggesting that they contained at least those virus-induced factors responsible for the homologous interference phenomenon. The persistent phase of infection was found to be dependent upon the presence of extracellular virus for its maintenance. When persistently infected cultures were grown in the presence of antiviral serum, the culture became "cured" of virus infection to the extent that it responded to the addition of superinfecting virus as would an uninfected culture.

The combined capabilities of the Sindbis virus-infected mosquito cells to prevent the expression of superinfecting virions (homologous interference) and to isolate some of the biochemical events related to virus replication to restricted areas of the cell cytoplasm provide a possible explanation for how the persistent infection is maintained in this invertebrate system. When mosquito cells are infected with Sindbis virus, the cells enter a stage of maximum virus synthesis (acute phase). A large number of the virus-producing vesicles are generated in the cell cytoplasm, each being some-

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**Table 1. Exclusion of superinfecting virus by persistently infected *A. albopictus* cells**

<table>
<thead>
<tr>
<th>Infected cells</th>
<th>Cells releasing virus (%)*</th>
<th>Virus produced at 24 h (PFU/ml)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>28°C</td>
</tr>
<tr>
<td>PI* (36 day) cells in normal medium superinfected with ts-20</td>
<td>1.23d</td>
<td>4.5 x 10^6</td>
</tr>
<tr>
<td>PI (36 day) cells in medium with anti-Sindbis serum superinfected with ts-20</td>
<td>0.001d</td>
<td>1.1 x 10^6</td>
</tr>
<tr>
<td>Cells newly infected with ts-20</td>
<td></td>
<td>2.0 x 10^6</td>
</tr>
<tr>
<td>Cells newly infected with heat-resistant Sindbis virus</td>
<td>160.0</td>
<td>9.0 x 10^6</td>
</tr>
<tr>
<td>PI (200 day) in normal medium superinfected with West Nile virus*</td>
<td></td>
<td>3.1 x 10^6</td>
</tr>
<tr>
<td>Cells newly infected with West Nile virus*</td>
<td></td>
<td>2.9 x 10^6</td>
</tr>
</tbody>
</table>

* Equal numbers of cells were infected or superinfected with 50 to 100 PFU of Sindbis virus, mutant Sindbis virus ts-20, or West Nile virus per ml.  
* Determined by infectious-center assay.  
* PI, Persistently infected.  
* Determined before superinfection.  
* Assay of virus produced after 24 h of incubation at 28°C was carried out in Vero cells at 37°C.
what isolated biochemically and genetically from the other and the host. The biochemical isolation of virus synthesis in the cytoplasm is indicated by the observation that many of the temperature-sensitive mutants that readily complement in vertebrate cells fail to do so in cultured mosquito cells (12). The observation that defective interfering particles can neither be propagated nor demonstrate their inhibitory action in mosquito cells (8) suggests that normal particles cannot provide the necessary helper function for these virions in the mosquito cells. This may reflect the isolation of the replication of individual infecting virions in the cytoplasm of infected cells.

The virus-containing vesicles produced early after infection may be released from the cells at varying time intervals. The large amount of virus released during the acute phase suggests that many of these structures are lost from the infected cell in the first 48 h after infection. As the virus-induced vesicles were released into the surrounding medium, the cell would have less virus-synthesizing capability and contain less virus antigen. The presence of a single virus-containing vesicle in the cytoplasm of a cell might not be detectable in either the infectious-center assay or the immunofluorescence assay but might be adequate to maintain the cell's immunity to superinfection. The progressive loss of virus-induced vesicles from the infected cells coupled with the normal process of cell division would result in the appearance of uninfected cells in the culture. These cells would then be susceptible to reinfection by virions in the medium, and the cycle of acute to persistent phase of infection would be repeated for these particular cells. The experiments presented here suggest that at any given time only a small percentage (ca. 1%) of the cells in the persistently infected culture are cured and reinfected and enter the acute phase. The presence of antivirus serum in the growth medium can prevent the reinfestation of the cells as they are cured and thus allows the culture as a whole to become greater than 99% free of infected cells and to respond to infection by added virus as would an uninfected culture.

Although these data do suggest that the maintenance of the persistent state of infection in the cultured mosquito cells is dependent upon reinfestation of the cells by extracellular virus, the decrease in the rate of synthesis of virus components (detectable by immunofluorescence) suggests that the persistently infected cells possess some mechanism for specifically retarding virus production without interfering with normal cell function. The demonstration that persistently infected cultures are readily infected by, and will allow the production of, a superinfecting nonhomologous virus such as West Nile virus (this study) or Eastern equine encephalitis virus (15) indicates that the suppression of virus synthesis is not the result of a classical interferon-like agent.

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LITERATURE CITED


