Exclusion of Superinfecting Homologous Virus by Sindbis Virus-Infected Aedes albopictus (Mosquito) Cells

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The infection of tissue-cultured Aedes albopictus (mosquito) cells by an alphavirus ultimately results in a persistently infected cell population which can be maintained in the laboratory for years. One characteristic of this culture is that it will not support the replication of superinfecting homologous virus. We have shown that mosquito cells persistently infected with Sindbis virus produce an antiviral agent which when applied to uninfected mosquito cells suppresses Sindbis virus replication. The exclusion of virus replication in the antiviral-agent-treated cells is similar to the phenomenon of homologous interference described in alphavirus-infected vertebrate cells. In this study we examined the expression of homologous interference in three lines of mosquito cells and compared the expression of homologous interference to the effects of the antiviral activity. The cell lines were found to differ in their ability to express homologous interference, and evidence suggests that the mosquito cells may suppress replication by homologous interference or by the action of the antiviral agent.

MATERIALS AND METHODS

Cells, virus, and media. A. albopictus cells were obtained from various sources. The u4.4 cell line was derived in our laboratory from the original larval isolate of Singh (29) which was provided by Sonya Buckley (Yale Arbovirus Research Unit, New Haven, Conn.). The C6/36 clonal isolate originally prepared by Igarashi (13) was provided by Kenneth Ekels (Walter Reed Army Institute of Research, Washington, D.C.). The C710 A. albopictus line derived from the C6/36 cell line was kindly provided by V. Stollar (Rutgers Medical School, New Brunswick, N.J.). Baby hamster kidney (BHK-21/13/F) cells were passaged as described previously (24). All cells (vertebrate and invertebrate) were cultured in Eagle minimal essential medium (8) supplemented with 10% fetal calf serum, 2 mM glutamine, and 10% tryptose phosphate broth as described previously (24, 28).

In this study, the heat-resistant strain of SV (SVHR) which served as wild-type virus and the temperature-sensitive mutant ts-23 were both obtained from the collection of Elmer Pfeiffer virus (Dartmouth Medical School, Hanover, N.H.) and were prepared as previously described (23). Mutant ts-23, which belongs to complementation group D, is an RNA-positive mutant with a defect in protein transport and processing which results in an accumulation of immature virus membrane proteins in the Golgi apparatus of the vertebrate cell (7, 9, 10, 30, 34).

Homologous interference assay. Mosquito cell monolayers were infected with the primary virus, ts-23, at a multiplicity of infection (MOI) of 100 PFU/cell by allowing the virus to adsorb for 90 min at room temperature. The infected cell monolayers were then washed with fresh medium and incubated at 28°C for 2, 10, 24, 48, 96, 144, or 168 h (depending on the experiment) before superinfection with SVHR at the same MOI for an identical adsorption time. After superinfection with SVHR, infected cells were incubated for an additional 24 h at 28°C. Virus production was then determined by plaque assay at both permissive (28°C) and...

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nonpermissive (37°C) temperatures as previously described (24).

Superinfection exclusion assay. Virus production in three persistently infected u4.4 cell lines was determined at 28°C by plaque assay. The cell lines used were p14.8.78, p30.8.78, and p28.6.77. Each of these lines represents u4.4 cells initially infected with SVHR on the indicated date. After determination of the initial level of virus production by these cells, cell monolayers were superinfected with SVHR as described. Wild-type virus production was determined 24 h later by plaque assay at 28°C. Wild-type virus could be readily distinguished from endogenous virus by plaque morphology.

Assay of the SV-induced antiviral agent produced by three persistently infected cell lines of u4.4. Medium from three SVHR persistently infected u4.4 cell lines was clarified by centrifugation and applied to uninfected u4.4 cells for 48 h at 28°C. Medium from uninfected u4.4 cells served as mock-treatment controls. Cells were infected with SVHR at an MOI of 100 PFU/cell and incubated at 28°C in medium harvested from either the persistently infected or the uninfected cells. Virus production was determined at 24 and 48 h postinfection by plaque assay.

RESULTS

Production of SVHR in vertebrate and invertebrate cells. To investigate the establishment of homologous interference by SV in A. albopictus cells, the RNA-positive, temperature-sensitive mutant ts-23 of complementation group D was used with SVHR in A. albopictus cells. Three uninfected cell lines of A. albopictus which differ in their response to SV infection were selected: u4.4, C6/36, and C710. Neither the u4.4 cell line derived from the original larval isolate of Singh (29) nor the C6/36 cell line demonstrates a cytopathic effect (CPE) upon infection with SV. In contrast, the C710 cell line derived from the C6/36 cell line displays a strong CPE upon infection with SV when placed in medium containing low concentrations of calf serum (27, 32). These cells revert to a normal phenotype if the calf serum content is subsequently raised. Furthermore, both CPE+ and CPE− cells enter a persistently infected state with equal efficiency following an acute phase of infection.

Wild-type virus production in each of the cell lines is shown in Table 1. As seen, virus production in the C710 cells was found to be consistently higher than in the other cell lines tested. Generally, virus production in the C710 parent line, C6/36, was found at an intermediate level when compared with production in u4.4 and C710 cells. Thus, the cell lines varied not only in their response to virus infection as determined by CPE development (BHk and C710, CPE+; C6/36 and u4.4, CPE−) but also in the average amount of virus produced per cell.

Establishment of homologous interference in A. albopictus cell lines. The expression of homologous interference at various times postinfection has been reported in both A. aegypti and A. albopictus cells (23, 33). In both cases, interference with the replication and production of superinfecting viruses has been found during both the acute and the persistent phases of infection with SV. In this study, an attempt was made to determine the time at which homologous interference was established in three different cell lines of A. albopictus cells. Cells were initially infected with ts-23 and challenged with SVHR at various times postinfection. Virus production was assayed at 24 h after superinfection at both permissive and nonpermissive temperatures. As seen in Fig. 1, yields of superinfecting SVHR (virus capable of forming a plaque at the nonpermissive temperature) from both C6/36 and C710 cells were reduced when superinfection was performed at 10 and 24 h after the initial infection with ts-23 while no interference with the replication of the superinfecting SVHR was seen when the challenging virus was added at 2 h postinfection. In contrast, the u4.4 cell line failed to demonstrate homologous interference even when the superinfecting virus was added as late as 24 h postinfection. The difference observed between virus assayed at permissive and nonpermissive temperatures following a mixed, simultaneous infection of the u4.4 cells may be viewed as insignificant since other experiments (data not shown) failed to demonstrate this occurrence. Both in this and in other experiments, only titers showing a >10-fold difference are considered significant.

To determine the time at which homologous interference occurred in the u4.4 cells, the cells were infected with ts-23 and superinfected with SVHR after longer time intervals. Times for superinfection were selected which would include times during the acute and persistent phases of infection. Virus production was assayed 24 h after the addition of the superinfecting virus. As shown in Fig. 2, homologous interference could not be demonstrated in the u4.4 cells even at 1 week (168 h) postinfection.

It has been demonstrated that an MOI of 50 to 100 PFU/cell is sufficient to infect all A. albopictus cells in a population (25); however, to ensure that the absence of homologous interference in the u4.4 cells was not due to a subpopulation of uninfected cells, the MOI at which both the initial virus, ts-23, and the challenging virus, SVHR, were added was increased from 100 to 500 PFU/cell. The increase in MOI had no effect on the establishment of homologous interference (data not shown).

Establishment of superinfection exclusion. To determine whether the u4.4 cell line is ever capable of excluding superinfecting homologous virus, three cell lines derived from u4.4 which were persistently infected with SVHR (p14.8.78, p30.8.78, and p28.6.77) were examined for their ability to permit replication of SV. Each of these lines had been persistently infected with SV for several years. Virus production was assayed before superinfection with SVHR. As shown in Fig. 3, the level of virus production seen prior to superinfection was characteristic of the levels found in persistently infected cells (ca. 0.05 PFU/cell) and was of a distinctive, small-plaque-forming variety. When these cells were challenged with SVHR, only very low levels of wild-type virus production were seen at 24 h after superinfection (Fig. 3). Thus, although homologous interference did not occur in these cells oreter the acute phase or early in the persistent phase of infection, the u4.4 cells could exclude superinfecting virus when persistently infected for extended periods of time.

Production of the antiviral agent by the three persistently infected cell lines. We have demonstrated previously that the production of SVHR in u4.4 A. albopictus cells can be over

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TABLE 1. SVHR growth in vertebrate and invertebrate cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>CPE (+/−)</th>
<th>SVHR yield (PFU/ml)</th>
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<tbody>
<tr>
<td>BHK-21</td>
<td>+</td>
<td>1.8 × 10^6</td>
</tr>
<tr>
<td>u4.4</td>
<td>−</td>
<td>2.3 × 10^6</td>
</tr>
<tr>
<td>C6/36</td>
<td>−</td>
<td>9.4 × 10^6</td>
</tr>
<tr>
<td>C710</td>
<td>+</td>
<td>2.6 × 10^6</td>
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* Cell monolayers were infected with 100 PFU of SV per cell and incubated at 28°C, and virus production was assayed 24 h later.
90% suppressed if the cells are treated with medium containing the SV-induced antiviral activity for 48 h before infection (26). This same cell line does not exclude superinfecting virus even when challenged with a second virus at 1 week after the initial infection. The A. albopictus C710 cells exclude superinfecting virus at 10 h after initial infection. The u4.4 cell line, therefore, does not express homologous interference since this phenomenon is defined in cultured vertebrate cells. This implies that the exclusion of superinfecting virus in the u4.4 cells occurs by an alternative mechanism. The SV-induced antiviral agent previously described in persistently infected u4.4 cells (26) has been shown to interfere only with the replication and production of superinfecting homologous virus. To test whether the superinfection exclusion seen in the persistently infected u4.4 cells used in this assay was due to the antiviral agent, the activity of the antiviral agent in medium removed from cultured, persistently infected cells was examined. Uninfected cells were placed in medium collected from each of the three persistently infected cell lines (used in the superinfection exclusion assay) and incubated for 48 h. The cell monolayers were then challenged with SV. After adsorption of the virus, the monolayers were washed and the medium being tested for the antiviral agent was replaced on the monolayers. As shown in Table 2, medium removed from each of the three persistently infected cell lines demonstrated an antiviral activity in the u4.4 cells upon infection with SV. Levels of virus production by u4.4 cells treated with the antiviral-agent-containing medium were characteristic of levels seen in established, persistently infected cell lines. When the CPE+ C710 cells were treated with medium from pi28.6.77 cells in a similar assay, virus production at 48 h postinfection was 60% that of control cultures (data not shown). As discussed above only titers showing differences 10-fold or more are considered significant in the assay for antiviral activity. The inhibition of virus production seen with the C710 culture treated with the antiviral factor may result from the varied cellular composition of A. albopictus cultures. These cultures are composed of mixtures of larval cells. The difference in the levels of inhibition of virus production seen with the u4.4 and C710 cells upon treatment with the antiviral agent could result from different percentages of the cells which are sensitive to the agent. These data support the contention that the superinfection exclusion observed in the u4.4 cell line is due to the action of the virus-induced antiviral factor and not to the homologous interference typically seen in vertebrate cells or found in the C710 cells.

**DISCUSSION**

Homologous interference has been demonstrated in both vertebrate and invertebrate systems (2, 15, 23, 33). In the vertebrate cell, homologous interference is the first assayable event following infection with SV and is a virus-
encoded function. In the invertebrate cell, a similar phenomenon has been reported to occur during both the acute and the persistent phases of infection. From the data presented here, it seems that superinfecting homologous virus can be excluded from the mosquito cell by at least two mechanisms. One of these mechanisms may be similar to homologous interference since that phenomenon has been described for alphavirus-infected vertebrate cells. Homologous interference requires an initial infection with virus to establish the conditions within the cell which result in the failure to support the replication of the superinfecting homologous virus. In tissue-cultured vertebrate cells, these conditions are met within 15 min of infection with the first virus. In cultured mosquito cells, the expression of homologous interference is delayed in cells capable of demonstrating the phenomenon and apparently absent in other cell types. Both the C710 and the C6/36 cell lines demonstrated interference with superinfecting virus production by 10 h after infection with the primary virus. In contrast, the u4.4 cell line failed to demonstrate homologous interference during both the acute (2, 10, 24, and 48 h postinfection) and the early-persistent (96, 144, and 168 h postinfection) phases of infection. Interference with the production of homologous superinfecting virus in the u4.4 cells was only found when the antiviral activity was detected (Fig. 3 and Table 2). Unlike homologous interference, interference by the antiviral agent (i) requires a 48-h treatment to set up the conditions which block SV replication in u4.4 cells, (ii) does not require an initial infection with virus particles, and (iii) is cell specific to the extent that it does not occur in C710 cells.

It has been proposed that homologous interference in SV-infected cultured vertebrate cells is the result of the formation of replication complexes containing SV plus strand RNA, nonstructural proteins, and a host component which is available in limited quantities (1, 2). When a sufficient number of complexes are formed such that all of the host component is consumed, the replication of additional (superinfecting) genomes cannot be supported. There is also evidence that host cell functions participate in the replication of SV in cultured insect cells. In the invertebrate cell, treatment with actinomycin D (cell lines u4.4 and C6/36) and enucleation (u4.4) have been shown to interfere with SV production (10, 28). Differences in the participation of the host in the virus replication in the three invertebrate cell lines could explain their variability with regard to virus production, as well as the differences seen in the establishment of homologous interference and superinfection exclusion.
The three mosquito cell lines studied here display many differences in their response to SV infection when compared with each other and with vertebrate cells. In this report the establishment of homologous interference and superinfection exclusion were examined. Other reports have demonstrated differences in virus maturation routes (6, 11, 32), production and effects of defective interfering particles (14, 16), ability to demonstrate cytopathic effects (26, 32), complementation of temperature-sensitive mutants (24), effects of various drugs on virus production (19, 28, 31), and the effect of enculcation of cells on virus growth (10). Examination of whole insects and specific tissues derived from insects has revealed that differences do exist in the expression of virus functions and in the total virus production within the tissues of the insect (12, 20). Thus, it is possible that the different A. albopictus cell lines examined here may represent different partially differentiated tissue types derived from the mosquito embryo.

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LITERATURE CITED