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Superinfection Exclusion of Alphaviruses in Three Mosquito Cell Lines Persistently Infected with Sindbis Virus

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Three *Aedes albopictus* (mosquito) cell lines persistently infected with Sindbis virus excluded the replication of both homologous (various strains of Sindbis) and heterologous (Aura, Semliki Forest, and Ross River) alphaviruses. In contrast, an unrelated flavivirus, yellow fever virus, replicated equally well in uninfected and persistently infected cells of each line. Sindbis virus and Semliki Forest virus are among the most distantly related alphaviruses, and our results thus indicate that mosquito cells persistently infected with Sindbis virus are broadly able to exclude other alphaviruses but that exclusion is restricted to members of the alphavirus genus. Superinfection exclusion occurred to the same extent in three biologically distinct cell clones, indicating that the expression of superinfection exclusion is conserved among *A. albopictus* cell types. Superinfection of persistently infected C7-10 cells, which show a severe cytopathic effect during primary Sindbis virus infection, by homologous virus does not produce cytopathology, consistent with the idea that cytopathology requires significant levels of viral replication. A possible model for the molecular basis of superinfection exclusion, which suggests a central role for the alphavirus *trans*-acting protease that processes the nonstructural proteins, is discussed in light of these results.

Alphaviruses are enveloped viruses which contain a single-stranded, positive-sense RNA genome of approximately 12 kb (28). They are arboviruses and in nature alternate between replication in higher vertebrates and in hematophagous arthropods, usually mosquitoes. For Sindbis virus, the prototype alphavirus, the principal arthropod hosts are mosquitoes of the genus *Culex* and the primary vertebrate hosts are passerine birds (20). The virus does infect mammals, however, and strains of Sindbis virus are known that cause epidemic disease in humans characterized by arthralgia, including polyarthritis, and rash. Infection of mammals often results from transmission by *Culiseta* or *Aedes* mosquitoes. The recent introduction of *Aedes albopictus* into the United States, where it has undergone a rapid range expansion, as well as the spread of this mosquito in other areas of the world has raised concern that it might serve as a vector for the transmission of alphaviruses, including Sindbis virus, or of other arboviruses to humans or other mammals. *A. albopictus* has been shown to be an efficient vector for Sindbis virus (5), and these mosquitoes have already been shown to harbor the highly pathogenic alphavirus eastern equine encephalitis virus in Florida (18). Further studies of the replication of alphaviruses in *A. albopictus* are clearly warranted (19).

Alphavirus infection of mammals is acute and may result in an illness whose symptoms can include fever, arthralgia, or encephalitis, but which, if not fatal, results in recovery in due course (4). In mosquitoes, infection with alphaviruses results in a persistent infection that lasts the lifetime of the mosquito but that leads to relatively little cytopathology (2, 32, 33). The characteristics of Sindbis virus infection of tissue culture cells mimic what is observed in vivo. Infection of most vertebrate cell lines results in massive cell death within 12 to 24 h, whereas in mosquito cells the infection begins with an early acute phase, during which large amounts of virus are shed into the medium and cell death may occur, followed by a prolonged persistent phase in which virus production is maintained at low levels and the cells continue to grow and divide through many passages (3).

Superinfection exclusion. Cells, whether vertebrate or invertebrate, infected with one alphavirus cannot be productively infected with the same or a closely-related alphavirus at some later time. This phenomenon, called superinfection exclusion or homologous interference, is not well understood, and it has been suggested that a number of factors, including competition for host cell receptors or intracellular host factors, the production of interferon or interferon-like substances by the infected host cell, the production of defective interfering viral genomes from the first infecting virus, or the production of a *trans*-acting protease by the first virus, might contribute to this phenomenon. In the case of Sindbis-infected vertebrate cells, only the translation of the nonstructural genes of the first virus is required to establish homologous exclusion, and once homologous exclusion is established a superinfecting genome is translated but not replicated (1). In Sindbis virus-infected *A. albopictus* cells, less is known about homologous exclusion, but Stollar and Shenk have shown that the RNA of the superinfecting virus fails to replicate (26). This block to replication is intracellular and not at the level of attachment or penetration, because transfected viral RNA also will not replicate in infected mosquito cells (11).

Conflicting results have been obtained regarding the ability of alphaviruses to exclude other species of alphaviruses in persistently infected mosquito cells. An uncloned line of *A. albopictus* cells infected with Sindbis virus failed to replicate superinfecting Sindbis virus but would produce the heterologous alphavirus eastern equine encephalitis virus (26). However, Eaton (6) later reported that Sindbis virus-infected *A. albopictus* cells efficiently excluded several heterologous alphaviruses (chikungunya, Una, and Semliki Forest viruses) if the

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cells were superinfected at early times after primary Sindbis infection but excluded them much less efficiently if superinfection occurred after long-term persistent infection. The interpretation of these results is complicated by the use of different alphaviruses in the two studies and by the use of uncloned cell lines, as the original cell line derived from mosquito larvae by Singh (25) is now known to contain a number of distinct cell types which are variable in their responses to alphavirus infection (11, 16, 17, 23, 31). These cellular distinctions include the appearance of cytopathology during acute-phase infection (16, 23, 31), the route of viral maturation (7, 16), and the distribution of viral proteins within the infected cell types (17). We have now reexamined the exclusion phenomenon by using three well-characterized *A. albopictus* cell lines which were clonally derived from the original line of Singh and have compared virus yields in each line with that for the same line after persistent infection with Sindbis virus for many months.

Exclusion of homologous virus. Three cloned lines of *A. albopictus* cells (U4.4, C6/36, and C7-10) were infected with the HR strain of Sindbis virus and maintained in continuous cell culture for over 1 year as described previously (16). Uninfected lines of the same clones were maintained in parallel. To test the abilities of these cells to exclude homologous virus, the persistently infected line and the uninfected line of each clone were inoculated with a number of different strains of Sindbis virus and the release of infectious virus into the culture fluid was measured at various times postinfection by plaque assay on monolayers of secondary chicken embryo fibroblasts. Prior to superinfection, the persistently infected cultures produced Sindbis virus at very low levels (less than 10^3 PFU/ml/day) and the virus produced after many passages in mosquito cells was temperature sensitive and formed minute plaques on vertebrate cells, so that the virus produced as the result of superinfection could be easily distinguished from that arising from the persistent infection (data not shown). Four strains of Sindbis virus were used for superinfection: Sindbis virus HR, the virus used to establish the persistent infection; WT, an AR339 strain from the Strauss laboratory; an AR339 isolate from the American Type Culture Collection; and Toto1101, the virus produced from a full-length cDNA clone that has been extensively used in laboratory studies (21). These four strains of virus were all derived from the original AR339 isolate of Taylor et al. (30) but differ in their passage history. The HR strain has been sequenced in its entirety (27). A comparison of complete or partial sequences of various strains of Sindbis virus derived from AR339 has shown that each differs from HR and from one another in only a few amino acids or noncoding nucleotides, but these differences, which were presumably selected during passage, affect the growth rates of the viruses in cultured cells and their virulence in mice (15).

Following infection, the culture medium was changed after 24, 48, and 72 h, and the yield of virus released into the culture fluid during the preceding 24 h was determined by plaque assay. The results from the three time periods gave comparable results, and for simplicity only the titers of the second harvest (virus produced between 24 and 48 h) are shown in the figures. All three uninfected cell lines produced high titers of the different strains of Sindbis virus (Fig. 1, dark bars). The relative yields of the different strains were somewhat dependent upon the cell line but the yield of Toto1101 was consistently 10- to 100-fold less than those of the other three strains. In contrast, all three cell lines exhibited strong homologous interference after long-term persistent infection, with the yield from the persistently infected cells being reduced by as much as 5 orders of magnitude (Fig. 1, light bars). No consistent trend in the relative ability to exclude the different strains was noted.

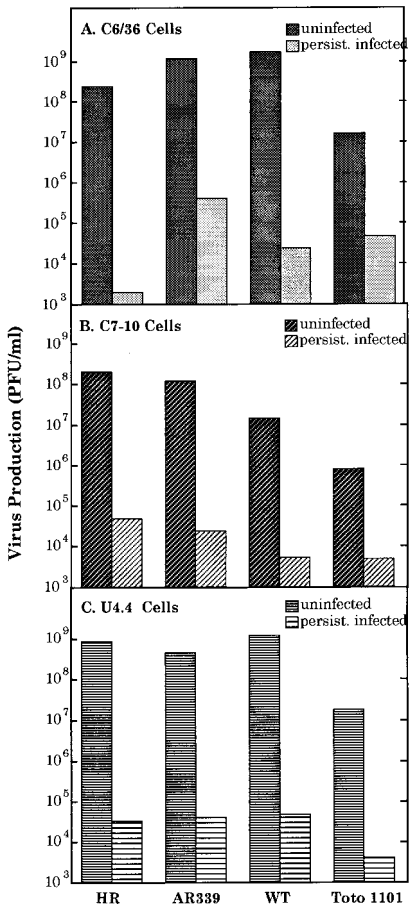

FIG. 1. Replication of various strains of Sindbis virus on three clonal cell lines of *A. albopictus*, C6/36, C7-10, and U4.4, and in cultures of these cells persistently infected with Sindbis virus HR strain. C6/36 and C7-10 cells were grown in Earle's minimal essential medium containing 10% fetal calf serum, nonessential amino acids, glutamine, and antibiotics. The U4.4 cell line was grown in Earle's minimal essential medium containing 10% fetal calf serum, 5% tryptose phosphate broth, glutamine, and 10 mg of gentamicin sulfate per ml. The cells grew as a monolayer but were only loosely attached to the plastic. Cells were grown at 28°C in a 5% carbon dioxide incubator with the caps screwed on tight. Cells were infected or superinfected with the various stocks of Sindbis virus at a multiplicity of 100 in phosphate-buffered saline containing 1% fetal calf serum and divalent cations. One hour postinfection the inoculum was removed and replaced with the same medium in which the cells were grown. The medium was replaced at 24 and 48 h, and at 72 h a final harvest was made. Virus produced at these various times was assayed by plaque assay on chicken embryo fibroblast secondary monolayers. Only the titers of the 48-h harvest (virus produced between 24 and 48 h) are shown.

Exclusion of heterologous alphaviruses. We tested the ability of the same six cell lines to exclude heterologous alphaviruses by infecting the cells with Aura, Semliki Forest, and Ross River viruses and measuring virus production as before (Fig. 2). These viruses were chosen for use in the heterologous superinfections because they represent widely divergent alphaviruses and compose the best-characterized members of the genus (28). Semliki Forest virus was assayed by plaque assay on chicken embryo fibroblasts, whereas the titers of Aura and Ross River viruses, which fail to produce plaques on chicken cells, were determined on BHK-21 (baby hamster kidney) cells. The three uninfected cell lines produced different relative amounts of these three viruses, but the yields were between 10^7 and 10^9 PFU/ml in the 24- to 48-h harvest in all cases (Fig. 2, dark bars). Yields from each of the persistently infected cell

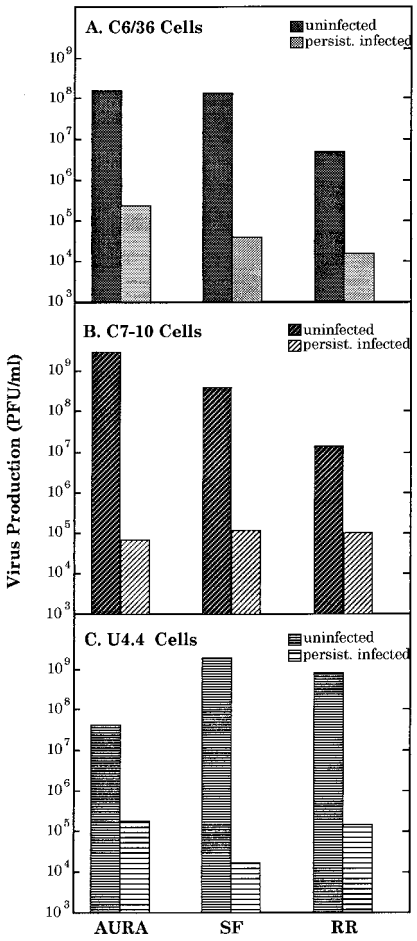

FIG. 2. Replication of Aura, Semliki Forest (SF), and Ross River (RR) viruses in three clonal cell lines of *A. albopictus*, C6/36, C7-10, and U4.4, and in cultures of these cells persistently infected with Sindbis virus HR strain. Cells were grown and infected as described in the legend to Fig. 1. In the case of Semliki Forest virus, the yield (titer) was determined on chicken embryo fibroblast monolayers, but the yields for both Aura and Ross River virus were determined on monolayers of BHK-21 cells. Only the titers of the 48-h harvest (virus produced between 24 and 48 h) are shown.

clones were markedly reduced, i.e., by 2 to 5 orders of magnitude (Fig. 2, light bars). No consistent trend in the ability to exclude any of the heterologous alphaviruses was noted, and the level of exclusion is comparable to that found for Sindbis virus strains.

Yellow fever virus is not excluded. To test the generality of the exclusion phenomenon and to ensure that the exclusion observed was not due to some trivial effect that arose from keeping the cells in culture for long periods of time, we infected these six cell lines with the unrelated flavivirus yellow fever virus. The 24- to 48-h yields of yellow fever virus were very similar in the infected and uninfected cultures of each clone (Fig. 3). We conclude that exclusion is specific to alphaviruses and that long-term maintenance of the persistently infected cells has not depressed their ability to replicate flaviviruses.

Cytopathology. We wanted to determine whether superinfection of persistently infected C7-10 mosquito cells would result in a cytopathic effect. During the acute phase of primary infection with Sindbis virus, C7-10 cells display a severe cytopathology, with morphological changes clearly visible by both light and scanning electron microscopy (16, 23). This acute infection is also accompanied by significant cell death as measured by trypan blue exclusion. Figure 4 shows the appearance in the light microscope of C7-10 cells acutely infected with the HR strain of Sindbis virus and of persistently infected C7-10 cells superinfected with the same stock of virus at the same multiplicity. At 12 h postinfection, no cytopathology is seen in either the acutely infected (Fig. 4a) or superinfected (Fig. 4c) cells. At 60 h postinfection, however, severe cytopathology is apparent in the acutely-infected C7-10 cells (Fig. 4b) but not in the superinfected cells (Fig. 4d). In a parallel series of experiments we quantitated the survival rates of superinfected and acutely infected cells. At 72 h postinfection only 40% of the acutely infected C7-10 cells survive, as determined by the ability to exclude trypan blue (Fig. 4e). In contrast, the persistently infected C7-10 cells exhibit little cell death, and in fact it is difficult to tell the mock-superinfected cells from the Sindbis virus HR-superinfected cells by this assay. These results support previously reported experiments in which cytopathology in mosquito cells was correlated with high levels of viral RNA or infectious virus production (29, 31).

Mechanism of exclusion. We have found that three distinct *A. albopictus* cell lines persistently infected with Sindbis virus exclude the replication of both homologous and heterologous alphaviruses but not of the unrelated flavivirus yellow fever virus. Although these three mosquito cell lines vary in their response to acute infection by Sindbis virus (C7-10 cells show strong cytopathic effect, C6/36 cells show a moderate level, and U4.4 cells exhibit little cytopathology) (16), all three lines produced comparable titers of alphaviruses during acute infection and, when persistently infected, all three lines appeared to exclude the homologous and heterologous viruses to very much the same extent. The mechanism by which infected arthropod cells exclude superinfecting virus is currently unclear, nor is it known whether it is the same as the mechanism of superinfection exclusion in vertebrate cells. One possible mechanism that is consistent with all known facts about superinfection exclusion is the presence in the infected cell of sufficient amounts of the *trans*-acting nonstructural alphavirus protease to destroy the replicase required for minus-strand replication. In studies in mammalian cells, we have shown that

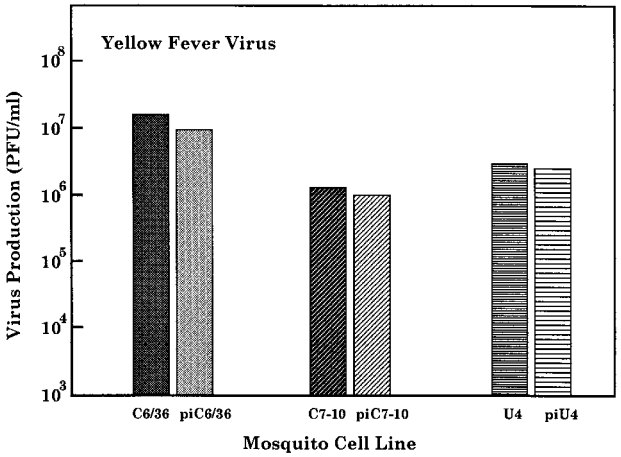

FIG. 3. Replication of yellow fever virus in three clonal cell lines of *A. albopictus*, C6/36, C7-10, and U4.4, and in cultures of these cells persistently infected with Sindbis virus HR strain. This experiment was performed in parallel with those shown in Fig. 1 and 2. This figure illustrates the yield of yellow fever virus produced between 24 and 48 h and assayed by plaque assay on BHK-21 cells. pi, persistently infected.

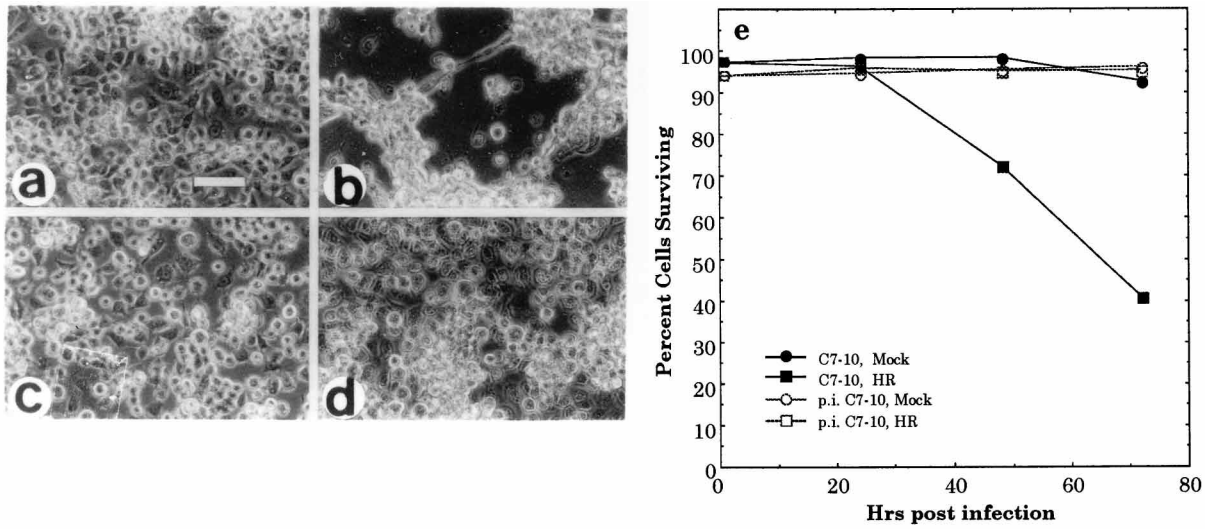

FIG. 4. Cytopathology of mosquito cells infected with Sindbis virus strain HR. Panels a through d illustrate the appearance by light microscopy of C7-10 cells after acute and persistent infection. Panels a and b show C7-10 cells during the acute phase of primary infection with Sindbis strain HR, at 12 and 60 h postinfection, respectively. Panels c and d show persistently infected C7-10 cells superinfected with Sindbis strain HR at 12 and 60 h postinfection, respectively. Panel e illustrates the percentages of C7-10 cells surviving as a function of time after infection or superinfection, as measured by trypan blue exclusion. Uninfected C7-10 cells were either mock infected or acutely infected with Sindbis strain HR, and persistently infected C7-10 cells were either mock superinfected or superinfected with Sindbis strain HR. Bar = 50 μm.

the uncleaved nonstructural precursor called P123 is required for the production of minus-strand RNA templates and that if P123 is cleaved too rapidly by the protease present in nonstructural protein nsP2, minus-strand synthesis does not take place (13, 14, 24). Since minus strands are the first templates required for RNA replication, this effectively means that no new RNA replication can take place after establishment of the infection and the appearance of sufficient *trans*-acting nsP2 protease to rapidly cleave P123, although existing minus strands can continue to be used as templates to produce plus-strand RNA. This mechanism is thought to have evolved in order to regulate RNA replication such that minus-strand RNA is only produced early in infection but may also serve to exclude superinfecting virus. If the persistently infected mosquito cells in the experiments reported here contain sufficient protease, not only would superinfecting virus be excluded but the presence of this protease could also be responsible for the downregulation of yields of virus that occurs upon establishment of the persistently infected state. Upon primary infection of mosquito cells, vigorous viral replication occurs in all cells and large amounts of virus are shed into the medium (22). After this early phase, virus replication is downregulated and virus production falls to low levels. During the persistent infection, only a fraction of the cells are actively replicating virus at any time, as shown by the fact that only a small percentage of the cells contain sufficient viral structural proteins to be detectable in an immunofluorescence assay and by the fact that upon cloning of individual cells from the persistently infected population, only a fraction of the cell clones produce virus (11, 22). Thus, it appears that upon virus infection of mosquito cells, all cells initially support virus replication but that individual cells subsequently stop replicating virus. At first, such nonproducing cells may remain resistant to superinfection, but ultimately the cells may be cured of infection and may become sensitive to reinfection by virus in the medium or by residual viral RNA in the cell, and in this way the culture remains persistently infected. A model in which a buildup of viral *trans*-acting protease leads to the shutoff of minus-strand RNA synthesis followed by the decay of viral replicases producing plus-strand RNA could account for this phenomenon. Several previous studies on superinfection exclusion in vertebrate cells are consistent with this model. First, it has been found that production of only the nonstructural genes, which include the protease gene, is required to establish exclusion (1, 12). Second, for BHK cells it has been shown that the superinfecting virus is translated into the nonstructural proteins but fails to replicate (1), consistent with the hypothesis that the superinfecting replicase complex is rapidly processed by proteolysis. Third, superinfection exclusion can be established in BHK cells by infection with RNA^- mutants ts6 and ts7 at either the permissive or nonpermissive temperature but can only be established after infection with RNA^- mutant ts24 at the permissive temperature (1, 12). It is known that ts6 has a lesion in nsP4, the RNA polymerase, and that in the double mutant ts7, although one of the lesions is in nsP2, it has little effect on proteolysis. Thus, both of these mutants produce an active protease at both the permissive and nonpermissive temperatures; however, ts24 produces a temperature-sensitive nsP2 protease such that an active protease is only produced at the permissive temperature (8–10). Although these data are consistent with the hypothesis that a functional *trans*-acting protease is required to establish exclusion, other models cannot be excluded.

It is notable that superinfection exclusion extends to viruses which are among the most widely divergent in the alphavirus genus. Sindbis and Semliki Forest viruses have 64% amino acid identity in the nonstructural proteins and 48% amino acid identity in the structural proteins. Sindbis and Aura viruses are more closely related (73% amino acid identity in the nonstructural proteins and 62% identity in the structural proteins), yet the extent of exclusion appears to be very similar for these two viruses. If exclusion does involve the nonstructural protease, our results imply that the Sindbis protease can process the Semliki Forest virus minus-strand replicase and that experimental tests of this aspect of the model are possible.

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