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Heterologous Interference in *Aedes albopictus* Cells Infected with Alphaviruses

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Maximum amounts of 42S and 26S single-stranded viral RNA and viral structural proteins were synthesized in *Aedes albopictus* cells at 24 h after Sindbis virus infection. Thereafter, viral RNA and protein syntheses were inhibited. By 3 days postinfection, only small quantities of 42S RNA and no detectable 26S RNA or structural proteins were synthesized in infected cells. Superinfection of *A. albopictus* cells 3 days after Sindbis virus infection with Sindbis, Semliki Forest, Una, or Chikungunya alphavirus did not lead to the synthesis of intracellular 26S viral RNA. In contrast, infection with snowshoe hare virus, a bunyavirus, induced the synthesis of snowshoe hare virus RNA in both *A. albopictus* cells 3 days after Sindbis virus infection and previously uninfected mosquito cells. These results suggested that at 3 days after infection with Sindbis virus, mosquito cells restricted the replication of both homologous and heterologous alphaviruses but remained susceptible to infection with a bunyavirus. In superinfection experiments the alphaviruses were differentiated on the basis of plaque morphology and the electrophoretic mobility of their intracellular 26S viral RNA species. Thus, it was shown that within 1 h after infection with either Sindbis or Chikungunya virus, *A. albopictus* cells were resistant to superinfection with Sindbis, Chikungunya, Una, and Semliki Forest viruses. Infected cultures were resistant to superinfection with the homologous virus indefinitely, but maximum resistance to superinfection with heterologous alphaviruses lasted for approximately 8 days. After that time, infected cultures supported the replication of heterologous alphaviruses to the same extent as did persistently infected cultures established months previously. However, the titer of heterologous alphavirus produced after superinfection of persistently infected cultures was 10- to 50-fold less than that produced by an equal number of previously uninfected *A. albopictus* cells. Only a small proportion (8 to 10%) of the cells in a persistently infected culture was capable of supporting the replication of a heterologous alphavirus.

Several reports have described the induction of homologous interference in alphavirus-infected vertebrate and mosquito cells. Johnston et al. (12) showed that by 1 h after infection with a variety of temperature-sensitive (*ts*) mutants of Sindbis virus (SIN), vertebrate cells were resistant to superinfection with the homologous wild-type virus. Homologous interference was also induced in *Aedes albopictus* cells within 1.5 h after infection with a *ts* mutant of SIN (20). When tested weeks or months after SIN infection, persistently infected mosquito cells are resistant to superinfection with the homologous virus (21, 24). It is not known whether the homologous interference in persistently infected cultures is mediated solely by interfering *ts* variants and defective interfering (DI) particles which are released from the cells at that time (6, 10).

The induction of heterologous interference in vertebrate cells was described by Zebovitz and Brown (25), who showed that preinfection of chicken embryo fibroblast (CEF) cells with Venezuelan equine encephalitis virus interfered with the replication of superinfecting Eastern equine encephalitis virus. Preliminary experiments (R. L. Regnery and B. T. Eaton, unpublished data) have shown that at several days after infection with Semliki Forest virus (SFV), *A. albopictus* cells restrict the replication of the closely related alphavirus Ross River virus. The heterologous resistance of such recently infected cells is in marked contrast to the susceptibility of persistently infected mosquito cells to superinfection with a heterologous alphavirus (24).

In this report, I show that the infection of *A. albopictus* cells with an alphavirus leads, within 1 h, to the induction of a state in which the cells

resist superinfection with both homologous and heterologous alphaviruses. However, cells remain sensitive to superinfection with a bunyavirus. The results indicate that the heterologous refractory state lasts for approximately 8 days, and after that time a small proportion of the cells in the infected culture is able to support the replication of heterologous alphaviruses.

MATERIALS AND METHODS

Viruses. Heat-resistant wild-type SIN was plaque purified in primary CEF cells and grown at a multiplicity of 0.01 PFU/cell on CEF cells. SIN stocks had titers ranging from 5×10^9 to 2.0×10^{10} PFU/ml at 33, 37, or 41°C. SFV, Una (strain 29494), Chikungunya (strain ATCC VR64) (CHIK), Mayaro (strain 15537), and snowshoe hare virus (strain R2929) (SSH) were obtained from H. Artsob, Department of Medical Microbiology, Banting Institute, Toronto, Ontario, Canada, plaque purified on BSC-1 cells, and grown at a multiplicity of 0.1 PFU/cell on either *A. albopictus* cells for 48 h at 28°C or BSC-1 cells at 37°C for 24 h. Virus stocks had titers ranging from 5.0×10^8 to 1.5×10^{10} PFU/ml at 33 or 37°C in BSC-1 cells. A heat-resistant variant of Una (Una-HR) was obtained by serial passage of Una three times in CEF cells at 41°C. After plaque purification in CEF cells, stocks of Una-HR were prepared by growth of the virus in either *A. albopictus* cells at 28°C or CEF cells at 37°C. SIN $ts_{p.i.}$ is a *ts* RNA^+ mutant isolated in this laboratory from *A. albopictus* cells at 89 days after infection with SIN. SIN $ts_{p.i.}$ was plaque purified on CEF cells and grown at a multiplicity of 0.01 PFU/cell in CEF cells at 33°C.

Cells. Both uninfected and persistently infected *A. albopictus* cells were grown at 28°C in AI medium (11) containing 10% fetal calf serum and 50 μg of gentamicin (Schering Corp., Kenilworth, N.J.) per ml and were passed at weekly intervals by seeding 10×10^6 cells in 75-cm^2 Corning plastic tissue culture flasks. Primary CEF cultures and BSC-1 cells were grown at 37°C in minimal essential medium (MEM) containing 10% fetal calf serum and 50 μg of gentamicin per ml.

Labeling of viral RNA in infected cells. *A. albopictus* or BSC-1 cells were infected with either SSH or an alphavirus at multiplicities ranging from 5 to 100 PFU/cell as described below. Viral RNA in infected mosquito cells was labeled with 25 to 50 μCi of [5-^{3}H]uridine per ml in MEM containing nonessential amino acids and 1.5 μg of actinomycin D per ml. After a 5- to 6-h labeling period, usually 24 h postinfection, RNA was extracted with phenol and precipitated with ethanol at −20°C (6).

Mitochondrial transcription in *A. albopictus* cells is not inhibited by actinomycin D but is sensitive to ethidium bromide (7). Control experiments have shown that ethidium bromide does not reduce the amount or alter the proportion of SIN viral RNA species synthesized in actinomycin-treated mosquito cells. In some experiments, therefore, viral RNA in infected mosquito cells was labeled in the presence of both actinomycin D and 5 μg of ethidium bromide per ml.

Infected vertebrate cells were labeled with 10 to 25 μCi of [5-^{3}H]uridine per ml in the presence of 1.5 μg of actinomycin D per ml from 2 to 7 h postinfection, and viral RNA was extracted with phenol.

rRNA in CEF cells was labeled during a 24-h incubation in MEM containing 1 μCi of [U-^{14}C]uridine per ml and extracted with phenol. The molecular weights of 28S and 18S marker rRNA were taken as 1.7×10^6 and 0.7×10^6, respectively.

Labeling of proteins synthesized in SIN-infected *A. albopictus* cells. Confluent mosquito cell monolayers in 25-cm^2 flasks were infected at 4°C with SIN at a multiplicity of 100 PFU/cell. After 1 h, monolayers were washed with prewarmed AI medium and incubated in this medium at 28°C. At different times thereafter, infected and uninfected control cultures were washed with methionine-free MEM (E-MEM) containing nonessential amino acids and proteins labeled by addition of 5 to 12 μCi of [^{35}S]methionine per ml in E-MEM for 40 min. Cells were lysed in sodium dodecyl sulfate-denaturing mix (0.625 M Tris-hydrochloride [pH 6.8], 4% sodium dodecyl sulfate, 10% 2-mercaptoethanol, 5% glycerol) and heated for 2 min in a boiling water bath.

Infectious-center assay. Monolayers of *A. albopictus* cells in 25-cm^2 flasks were infected with CHIK at a multiplicity of 10 PFU/cell, and at various times postinfection (1 h and 3, 10, and 56 days) cell monolayers were superinfected for 1 h with SIN (multiplicity, 5 PFU/cell). An uninfected monolayer was also infected with SIN at the same multiplicity. Monolayers were washed with AI medium and incubated in this medium at 28°C. At 6 h after infection, cells were removed from the flasks by a brief trypsinization and resuspended in AI medium. Cells were washed twice in AI medium, resuspended in 2 ml of AI medium, and layered over 10 ml of fetal calf serum. After centrifugation, the cells were removed directly from the pellet with a pipette, counted, and diluted in AI medium. They were adsorbed to BSC-1 or CEF cell monolayers for 30 min, overlaid with AI medium containing 1% agarose, and incubated at 33°C for 48 h.

Purification of 26S viral RNA. *A. albopictus* cells were infected with SIN at a multiplicity of 25 PFU/cell, and the viral RNA was labeled with 50 μCi of [5-^{3}H]uridine per ml in the presence of 1.5 μg of actinomycin D per ml and 5 μg of ethidium bromide per ml from 18 to 24 h postinfection. The RNA was extracted and separated into 42S and 26S components by centrifugation through a 10 to 40% sucrose gradient in 10 mM Tris-hydrochloride (pH 7.4)–10 mM NaCl–1.5 mM EDTA–0.5% sodium dodecyl sulfate in an SW27 rotor at 24,000 rpm for 18 h. The appropriate fractions were pooled, and 26S RNA was precipitated with 2 volumes of ethanol. The RNA was further purified in another sucrose gradient, and the peak and leading edge of the 26S RNA fractions were pooled. Gel analysis showed there to be little 20 to 22S double-stranded RNA present in the 26S RNA preparation.

Gel electrophoresis. [^{3}H]uridine-labeled RNA from infected mosquito or vertebrate cells was analyzed by electrophoresis in either 2.8% or gradient 2.5 to 5.0% polyacrylamide slab gels 13 cm in length, using the buffer described by Levin and Friedman (17). Electrophoresis was usually at 35 V (SSH RNA) or 50 V (alphavirus RNA) for 16 h. Gels were prepared for fluorography (1) and dried with a Bio-Rad slab gel

dryer. They were exposed to sensitized (16) Kodak SB-5 film at −70°C for 17 h and developed. Proteins were analyzed on a 10% acrylamide–0.25% bisacrylamide slab gel overlayered by a 3% acrylamide–0.08% bisacrylamide stacking gel, using the discontinuous buffer system of Laemmli (15). After electrophoresis for 16 h at 11 mA (constant current), gels were processed for fluorography, dried, and exposed to Kodak SB-5 film at −70°C for 24 h, and the film was developed.

Determination of the sensitivity of the gel assay procedure for detection of 26S viral RNA. Doubling dilutions of purified [^{3}H]uridine-labeled 26S viral RNA were made, a sample of each dilution was assayed for acid-precipitable counts, and 10-μl amounts of each dilution were added to seven wells of a 2.5 to 5.0% gradient polyacrylamide gel. After electrophoresis the gel was processed for fluorography, dried, and exposed to sensitized film at −70°C for 17 h, and the film was developed. A Joyce-Loebel Chromoscan was used to trace the grain absorbance of the film onto graph paper. The areas under the 26S RNA peaks were determined either by counting the number of square millimeters on the graph paper or by cutting out and weighing each peak. Figure 1 shows the relationship between the amount of purified 26S viral RNA added to the gel and the area encompassed by the 26S RNA peaks. The data show that, with the procedure outlined here, it is not possible to detect fewer than approximately 2,000 cpm of 26S RNA.

Quantitation of 26S RNA synthesis in infected mosquito cells. To compare the amount of 26S RNA synthesized in alphavirus-infected *A. albopictus* cells with that in cells superinfected with an alphavirus at 3 days after SIN infection (*A. albo*$_{SIN-3d}$ cells), the following procedures were adopted. Approximately 5.5 × 10^6 *A. albopictus* and *A. albo*$_{SIN-3d}$ cells were infected at the same multiplicity with either SIN, CHIK, Una, or SFV, and viral RNA was labeled with [^{3}H]uridine from 24 to 30 h postinfection. The same proportion (usually one-fourth or one-fifth) of the RNA extracted from infected *A. albopictus* and *A. albo*$_{SIN-3d}$ cells was analyzed by electrophoresis in 2.5 to 5.0% gradient polyacrylamide gels, and the labeled RNA species were detected by fluorography, using sensitized film. It has been shown that mitochondrial RNA synthesis in *A. albopictus* cells is unaffected either by SIN virus infection (see below) or by actinomycin D (7), and preliminary experiments showed that equal amounts of labeled mitochondrial RNA from the two cell types were added to the gels under these conditions. The amount of 26S RNA in *A. albopictus* and *A. albo*$_{SIN-3d}$ cells was quantitated in the following way. The areas encompassed by the 26S RNA peaks were measured with the Joyce-Loebel Chromoscan, and from the graph shown in Fig. 1 the amount of radioactivity in each 26S peak was estimated. The amount of 26S RNA in alphavirus-infected *A. albopictus* cells ranged from approximately 6,000 cpm for CHIK-infected cells to approximately 60,000 cpm for Una-infected cells. No alphavirus 26S RNA peak was detected in superinfected *A. albo*$_{SIN-3d}$ cells, indicating that the amount of radioactivity in this fraction was less than 2,000 cpm (Fig. 1). By comparing the number of counts per minute in the 26S RNA peak from infected *A. albopictus* cells with 2,000 cpm, the minimum amount of radioactivity detectable, it is possible to obtain a minimum value for the degree of inhibition of 26S RNA synthesis in *A. albo*$_{SIN-3d}$ cells compared with that in *A. albopictus* cells.

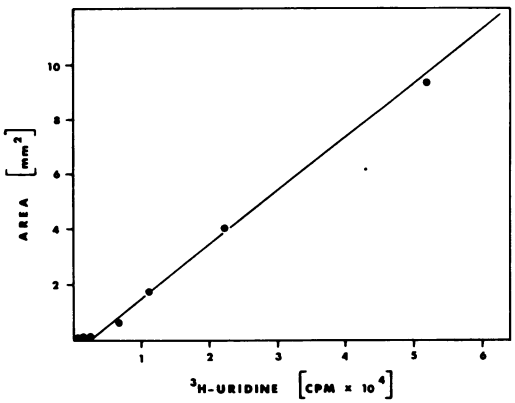

FIG. 1. *Relationship between the amount of 26S RNA subjected to electrophoresis and the area encompassed by the 26S peak. Purified [^{3}H]uridine-labeled SIN 26S viral RNA was diluted, and amounts ranging from 200 to 52,000 cpm were subjected to electrophoresis in a 2.5 to 5.0% gradient polyacrylamide gel. After fluorography with sensitized film (16), the areas under the 26S RNA peaks were quantitated as described in the text.*

RESULTS

Vertebrate and invertebrate cells infected with alphaviruses contain two major (42S and 26S) and two minor (38S and 33S) single-stranded viral RNA species. The 42S and 26S RNA forms have molecular weights of approximately 4.3 × 10^6 and 1.6 × 10^6, respectively, and the 38S and 33S RNA species migrate in polyacrylamide gels with apparent molecular weights of 3.1 × 10^6 and 2.4 × 10^6, respectively (8, 17, 19). The 38S and 33S RNAs are conformational variants of the 42S and 26S RNA species, respectively (14, 23).

Superinfection of mosquito cells with homologous and heterologous alphaviruses 3 days after SIN infection. The data in Fig. 2 indicate the species of SIN RNA synthesized in infected *A. albopictus* cells at different times postinfection. The molecular weights of the two major single-stranded RNAs synthesized from 6 to 11 h postinfection (Fig. 2, lane B) were calculated to be 3.0 × 10^6 and 1.6 × 10^6 and may therefore be designated as 38S and 26S RNA, respectively. The rate of synthesis of these RNAs reached a maximum at 24 h postinfection (Fig. 2, lane C). At 48 h postinfection, the rate of synthesis of 26S RNA was reduced, and by 72 h it was not detectable (Fig. 2, lane E). Significant inhibition of 38S RNA synthesis occurred at 72

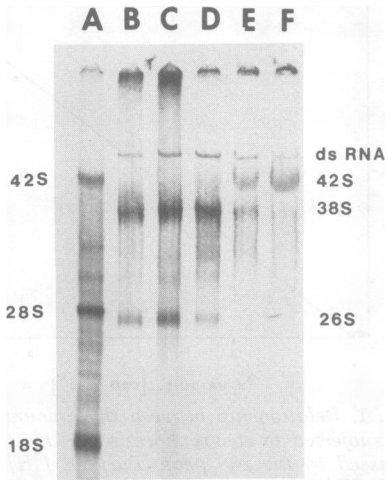

FIG. 2. *Species of viral RNA synthesized at different times after SIN infection of mosquito cells. Monolayers of A. albopictus were infected with SIN (multiplicity, 50 PFU/cell) and incubated at 28°C. RNA was labeled with [^{3}H]uridine in the presence of actinomycin D and ethidium bromide for 5 h at 6 h (B), 24 h (C), 48 h (D), 72 h (E), and 96 h (F) postinfection. RNA was analyzed by electrophoresis in a 2.8% polyacrylamide gel. Control experiments showed the bands designated 42S, 38S, and 26S to be RNase sensitive and therefore single stranded and the band designated as ds RNA to be RNase resistant and therefore double stranded. The molecular weights of intracellular viral RNA species were calculated from the positions of marker [^{14}C]uridine-labeled 28S and 18S rRNA and SIN 42S virion RNA (A).*

h postinfection. At that time, 42S RNA became detectable, and this species became the major single-stranded RNA by 96 h postinfection.

Two experimental observations indicated that 38S RNA was a conformer of the 42S RNA form. First, the large single-stranded alphavirus RNA extracted from *A. albopictus* cells up to 48 h postinfection occasionally migrated, not as a 38S species, but with the electrophoretic mobility expected of a 4.3×10^6-molecular-weight species (see Fig. 6, lanes A, B, and C). Second, analysis of viral RNA from SIN-infected *A. albopictus* cells by polyacrylamide gel electrophoresis in 7 M urea indicated that the large RNA migrated at the same rate as virion 42S RNA and 42S RNA from infected vertebrate cells (B. T. Eaton and R. Ward, unpublished data). The reason for the variation and the alteration in electrophoretic mobility of the large single-stranded RNA with time after infection of *A. albopictus* cells is unknown.

The reduced levels of viral RNA in mosquito cells 3 days after SIN infection made it possible to monitor the growth of superinfecting alphaviruses in these cells by checking for the appearance of new intracellular 26S viral RNA. At 3 days after infection with SIN, monolayers of *A. albopictus* cells (*A. albo*$_{SIN-3d}$ cells) were superinfected with SIN, SFV, CHIK, or Una. Four monolayers of previously uninfected cells were also infected. The viral RNA in the infected cells was labeled with [^{3}H]uridine at 24 to 30 h postinfection. Figure 3 shows that SIN induced the synthesis of 26S RNA in previously uninfected cells (lane C) but did not stimulate new detectable 26S RNA synthesis in *A. albo*$_{SIN-3d}$ cells (lane B). Identical results were obtained with SFV (lanes D and E), Una (lanes F and G), and CHIK (lanes H and I). Thus, by 3 days postinfection, SIN-infected *A. albopictus* cells restricted the replication of other alphaviruses. Variation in the amount of 26S RNA detected in *A. albopictus* cells infected with different alphaviruses is probably due to the multiplicities of infection used with each virus. In the case of CHIK-infected *A. albopictus* cells, in which 26S RNA is just detectable (Fig. 3, lane I), a higher multiplicity of infection and an increased concentration of [^{3}H]uridine during labeling lead to the detection of larger amounts of CHIK 26S RNA (see Fig. 5, lane A).

It is unlikely that the reduction in [^{3}H]uridine incorporation into superinfecting viral RNA species (Fig. 3) is due to a decrease in the uptake of uridine by infected mosquito cells because it has been shown that SIN infection of *A. albopictus* cells has no effect on the incorporation of [^{3}H]-uridine into actinomycin D-insensitive mitochondrial RNA species (B. T. Eaton, *In* E. Kurstak and K. Maramorosch, ed., *Viruses and Environment*, in press). Davey and Dalgarno (5) have also shown that SFV infection does not alter the amount of actinomycin D-resistant RNA species in *A. albopictus* cells.

With the procedure outlined above, it was calculated that the levels of 26S RNA synthesis in SIN-, SFV-, Una-, and CHIK-infected *A. albo*$_{SIN-3d}$ cells were, at most, 6, 16, 3, and 35%, respectively, of the levels found in infected *A. albopictus* cells.

Further evidence for viral interference was obtained from the following two sources. (i) The 24-h yields of virus from *A. albopictus* cells and *A. albo*$_{SIN-3d}$ cells infected with either SIN, SFV, or CHIK were determined. By assaying the progeny virus in BSC-1 cells, it was possible to differentiate SIN plaques (5 to 6 mm in diameter) from those of SFV and CHIK (3 to 4 and 2 mm in diameter, respectively). Table 1 shows that superinfection of *A. albo*$_{SIN-3d}$ cells with either SIN, SFV, or CHIK did not result in virus titers significantly greater than that of mock-infected *A. albo*$_{SIN-3d}$ cells. In addition, the size of the plaques produced by virus from the superin-

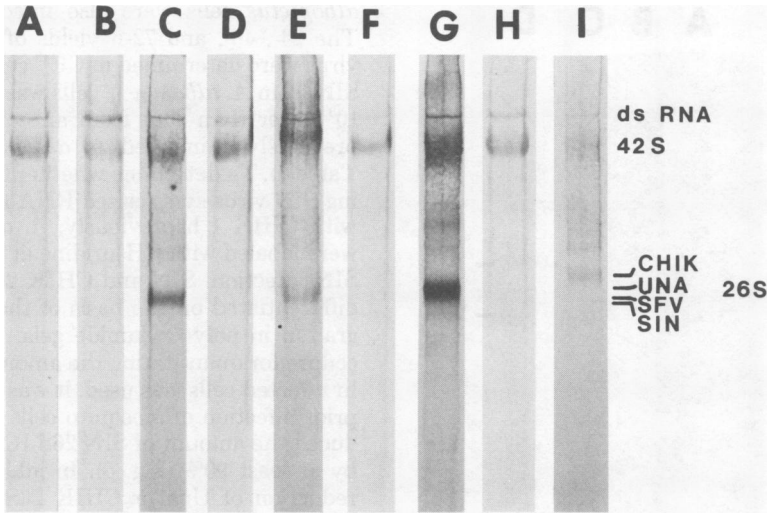

FIG. 3. *Viral RNA synthesis in A. albopictus and A. albo$_{SIN-3d}$ cells after infection with homologous and heterologous alphaviruses. Monolayers of mosquito cells were infected with SIN (50 PFU/cell). After 3 days, these cells (B, D, F, and H) and an equal number of previously uninfected A. albopictus cells (C, E, G, and I) were infected with either SIN (100 PFU/cell) (B and C), SFV (20 PFU/cell) (D and E), Una (100 PFU/cell) (F and G), or CHIK (10 PFU/cell) (H and I). A monolayer of A. albo$_{SIN-3d}$ cells was mock infected (A). Viral RNA was labeled with 25 μCi of [^{3}H]uridine per ml at 24 to 30 h postinfection and analyzed by electrophoresis in a 2.5 to 5.0% gradient slab gel.*

TABLE 1. *Production of alphaviruses in A. albopictus and A. albo$_{SIN-3d}$ cells*

Infecting virus	Cell	Titer[a]	Plaque size (mm in diam)
	A. albo$_{SIN-3d}$	6.0×10^7	5–6
SIN	*A. albo$_{SIN-3d}$*	6.2×10^7	5–6
CHIK	*A. albo$_{SIN-3d}$*	7.1×10^7	5–6
SFV	*A. albo$_{SIN-3d}$*	6.6×10^7	5–6
SIN	*A. albopictus*	9.6×10^9	5–6
CHIK	*A. albopictus*	1.2×10^{10}	1–2
SFV	*A. albopictus*	9.5×10^9	3–4

[a] Released virus was titrated in BSC-1 cells at 37°C.

fected cells was characteristic not of the superinfecting viruses, but of the persisting SIN. The large plaques induced by SIN prevented an accurate estimation of the titer of SFV and CHIK produced by superinfected *A. albo$_{SIN-3d}$* cells. The yield from mosquito cells undergoing a primary infection with either SFV or CHIK was at least 10^2-fold higher than that from superinfected *A. albo$_{SIN-3d}$* cultures. (ii) Because of the uncertainty in differentiating viruses on the basis of small differences in their plaque morphology and the difficulty in measuring the titer of the heterologous virus released by superinfected *A. albo$_{SIN-3d}$* cells, another system was investigated. *A. albopictus* cells were infected with CHIK (multiplicity, 10 PFU/cell), and 3 days later these cells (*A. albo$_{CHIK-3d}$* cells) were superinfected with SIN (multiplicity, 60 PFU/cell). A control monolayer was infected with SIN alone. Virus progeny at 24, 48, and 72 h postinfection were titrated on CEF cells. In these cells SIN produces large plaques (5 to 6 mm in diameter) within 48 h, whereas those of CHIK are undetectable at that time. The results indicated that the titer of SIN from *A. albo$_{CHIK-3d}$* cells was 10^3-fold less than that from *A. albopictus* cells (see Table 3).

Analysis of the RNA profiles in Fig. 3 suggested that alphavirus 26S RNAs may differ in their migration rates during electrophoresis in polyacrylamide gels. This was confirmed, and results indicated that the 26S RNAs can be arranged in order of increasing mobility as follows: CHIK, Una, SFV, SIN, and Mayaro (strain 15537) (data not shown).

SSH replicates in mosquito cells infected with SIN. The next experiment was done to determine whether mosquito cells infected with SIN restricted the replication of a virus other than alphaviruses. The bunyavirus SSH was cloned three times in BSC-1 cells, and a stock was prepared by infecting these cells at a multiplicity of 0.1 PFU/cell. Four SSH RNA species are found in infected BSC-1 cells and are labeled 1 to 4 in Fig. 4 (lane D). These are RNase sensitive and, therefore, single stranded (data not shown). Although Clewley et al. (4) described the presence of only three single-stranded RNA species in SSH (molecular weights, 2.9×10^6, 1.8×10^6, and 0.4×10^6), four

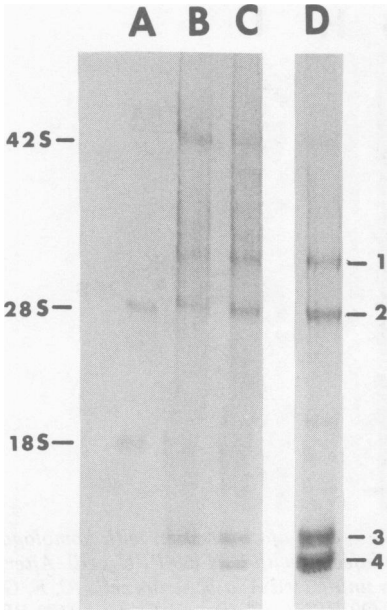

FIG. 4. *Species of SSH RNA synthesized in previously uninfected and SIN-infected mosquito cells. A monolayer of A. albo$_{SIN-3d}$ cells (B) and an equal number of previously uninfected A. albopictus cells (C) were infected with SSH (multiplicity, 25 PFU/cell), and the intracellular viral RNA species were labeled with 25 μCi of [^{3}H]uridine per ml in the presence of actinomycin D and ethidium bromide at 24 to 30 h postinfection. A monolayer of BSC-1 cells (D) was also infected with SSH (40 PFU/cell), and viral RNA was labeled in the presence of actinomycin D from 3 to 8 h postinfection. RNA was analyzed in a 2.5 to 5.0% gradient slab gel. [^{14}C]uridine-labeled marker rRNA is shown in (A). The position of SIN 42S RNA was obtained from (B). Single-stranded SSH RNA species are labeled 1 to 4.*

single-stranded RNA segments have been detected in BHK-21 cells infected with Bunyamwera virus (13). In the experiments described here, the relative intensity of band 3 varied in different experiments, and it may be associated with the replication of DI particles of SSH (R. J. Kascsak and M. J. Lyons, Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, S30, p. 218). In any event, SSH RNA species are present both in *A. albo*$_{SIN-3d}$ cells and in previously uninfected *A. albopictus* cells (Fig. 4, lanes B and C), indicating that prior infection with an alphavirus does not inhibit SSH replication.

When do alphavirus-infected mosquito cells become resistant to superinfection with heterologous and homologous alphaviruses? Mosquito cells were infected with CHIK (10 PFU/cell), and 1 h later these cells (*A. albo*$_{CHIK-1h}$ cells) were superinfected with SIN (50 PFU/cell). Previously uninfected *A. albopictus* cells were also infected with SIN. The 24-, 48-, and 72-h yields of superinfecting virus were determined in CEF cells. The titer of SIN from *A. albo*$_{CHIK-1h}$ cells was approximately 10^3 lower than that from an equal number of previously uninfected *A. albopictus* cells (see Table 3). To determine whether the superinfecting SIN virus synthesized RNA in cells infected with CHIK 1 h previously, *A. albo*$_{CHIK-1h}$ cells were labeled with [^{3}H]uridine at 24 to 30 h after SIN infection. SIN and CHIK 26S RNAs were differentiated on the basis of their rates of migration in polyacrylamide gels. When the procedure for quantitating the amount of 26S RNA in infected cells was used, it was estimated that prior infection of mosquito cells with CHIK reduced the amount of SIN 26S RNA synthesized by at least 90% (Fig. 5). In addition, a similar reduction of Una or CHIK 26S RNA was detected in *A. albopictus* cells which had been infected 1 h previously with SIN (data not shown). Similar experiments revealed that infection of either BSC-1 or CEF cells with either SIN or CHIK also leads, within 1 h, to a refractory state in which the cells are resistant to superinfection with SIN, CHIK, SFV, and Una.

Homologous interference was also induced in mosquito cells within 1 h of infection. *A. albopictus* cells were infected for 1 h with SIN $ts_{p.i.}$ (15 PFU/cell) at 22°C. Immediately thereafter,

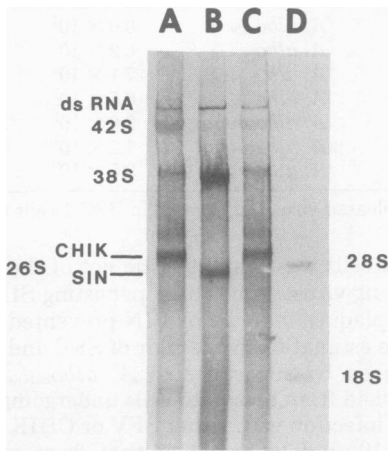

FIG. 5. *Viral RNA species in CHIK-infected mosquito cells after superinfection with SIN. Two A. albopictus cell monolayers were infected with CHIK (25 PFU/cell) (A and C). After a 1-h adsorption at 28°C, one of them (C) was superinfected for 1 h with SIN (10 PFU/cell). Previously uninfected cells were similarly infected with SIN (B). Viral RNA was labeled with 75 μCi of [^{3}H]uridine per ml at 24 to 30 h after SIN infection and analyzed in a 2.8 to 5.0% gradient slab gel. [^{14}C]uridine-labeled 28S and 18S rRNA markers are shown in (D).*

these cells and control monolayers were infected with either wild-type SIN (80 PFU/cell) or Una-HR (40 PFU/cell). The titer of the superinfecting virus was determined 24 h postinfection in CEF cells at 40°C, the nonpermissive temperature for SIN $ts_{p.i.}$. Table 2 shows that *A. albopictus* cells infected with SIN $ts_{p.i.}$ resist superinfection with both the homologous wild-type virus and the heterologous Una. The data also show that UV irradiation of SIN $ts_{p.i.}$ before infection destroys its ability to induce interference.

Replication of SIN in mosquito cells at different times after infection with CHIK. Stollar and Shenk (24) have shown that *A. albopictus* cells persistently infected with SIN are susceptible to superinfection with the heterologous alphavirus Eastern equine encephalitis virus. The next experiment determined the time after infection when infected cells lose their ability to restrict the replication of heterologous alphaviruses. *A. albopictus* cells were infected with CHIK (20 PFU/cell), and at 1 h and 3, 6, 8, 10, and 27 days postinfection they were superinfected with SIN (50 PFU/cell). On each occasion an equal number of uninfected mosquito cells was also infected with SIN. Table 3 shows that the ability of *A. albo*$_{CHIK}$ cells to support SIN replication increased significantly at 8 to 10 days after CHIK infection. However, even 27 days after CHIK infection, *A. albo*$_{CHIK}$ cells failed to yield SIN titers as high as those of previously uninfected *A. albopictus* cells. Table 4 lists the 48-h yields of CHIK, SIN, SFV, and Una produced by previously uninfected *A. albopictus* cells, *A. albo*$_{CHIK\text{-}41d}$ cells, and cells which had been infected with SIN 18 months previously (*A. albo*$_{SIN\text{-}18m}$ cells). CHIK did not replicate in *A. albo*$_{CHIK\text{-}41d}$ cells, and *A. albo*$_{SIN\text{-}18m}$ cells failed to produce detectable quantities of superinfecting SIN virus even after superinfection at multiplicities of approximately 400 PFU/cell. The yields of heterologous alphaviruses from persistently infected cultures (*A. albo*$_{CHIK\text{-}41d}$ and *A. albo*$_{SIN\text{-}18m}$) were 15- to 50-fold less than that from previously uninfected *A. albopictus* control cultures. The reduced yield of Una and SFV in *A. albo*$_{SIN\text{-}18m}$ cells (1.5 and 7.0% of control values, respectively) was correlated with a decrease of approximately 94% in the level of superinfecting viral 26S RNA in *A. albo*$_{SIN\text{-}18m}$ cells compared with that found in previously uninfected mosquito cells (Fig. 6). Superinfection of *A. albo*$_{SIN\text{-}18m}$ cells with heterologous alphaviruses leads to a stimulation of synthesis of small double- and single-stranded (putative DI) RNA (24).

Infectious-center experiments were done to determine whether the reduced yield of superinfecting heterologous virus from persistently infected cultures was due to either a reduced yield from all the cells in the population or a

TABLE 2. *Production of SIN and Una-HR in A. albopictus cells, A. albopictus cells previously infected with SIN $ts_{p.i.}$, and A. albopictus cells previously infected with UV-irradiated SIN $ts_{p.i.}$*

Virus initiating primary infection	Superinfecting virus	Titer of superinfecting virus[a]
	SIN	2.7×10^9
SIN $ts_{p.i.}$	SIN	1.6×10^7
UV-irradiated SIN $ts_{p.i.}$	SIN	2.1×10^9
	Una-HR	8.0×10^8
SIN $ts_{p.i.}$	Una-HR	5.1×10^6
UV-irradiated SIN $ts_{p.i.}$	Una-HR	8.9×10^8

[a] SIN and Una-HR were titrated in CEF cells at 40°C.

TABLE 3. *Effect of previous infection with CHIK on the yield of SIN from A. albopictus cells*

Time of superinfection with SIN (h or days after CHIK infection)	SIN virus yield[a]	
	A. albo$_{CHIK}$	*A. albopictus*
1 h	8.1×10^7	1.1×10^{11}
1 h	6.0×10^7	3.0×10^{10}
1 h	7.5×10^7	7.5×10^{10}
3 days	7.0×10^7	6.5×10^{10}
6 days	1.0×10^8	6.0×10^{10}
8 days	1.0×10^9	5.2×10^{10}
10 days	1.5×10^9	4.5×10^{10}
10 days	2.0×10^9	3.0×10^{10}
27 days	2.3×10^9	4.0×10^{10}

[a] The 48-h yields of SIN were determined in CEF cells. CHIK plaques were not detectable in these cells after 2 days of incubation at 37°C.

TABLE 4. *Yield of SIN, Una, CHIK, and SFV from previously uninfected A. albopictus cells and A. albopictus cells persistently infected with CHIK (41 days postinfection) or SIN (18 months after infection)*

Virus	Virus yield[a]		
	A. albopictus	*A. albo*$_{CHIK\text{-}41d}$	*A. albo*$_{SIN\text{-}18m}$
SIN	1.1×10^{11}	5.0×10^9	4.1×10^6[b]
Una	6.0×10^{10}	2.1×10^9	9.2×10^8
CHIK	3.0×10^9	$<5.0 \times 10^5$	1.7×10^8
SFV	3.0×10^9	ND[c]	2.1×10^8
		$<5.0 \times 10^5$	2.0×10^6[b]

[a] SIN and Una were titrated in CEF cells at 37°C. SFV and CHIK were titrated in BSC-1 cells. The persisting virus from *A. albo*$_{SIN\text{-}18m}$ cells was differentiated from superinfecting viruses on the basis of its small-plaque morphology (<1 mm in diameter).

[b] Plaque size was less than 1 mm in diameter.

[c] ND, Not determined.

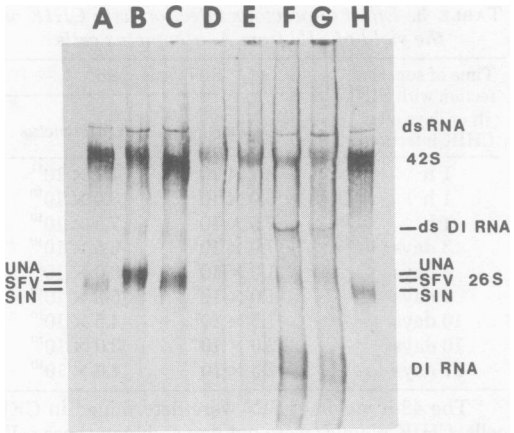

FIG. 6. *Species of viral RNA in A. albopictus and A. albo$_{SIN-18m}$ cells infected with SIN, Una, or SFV. Mosquito cells were infected with SIN (A and H), Una (B), or SFV (C) at multiplicities ranging from 20 to 50 PFU/cell. At the same time, A. albo$_{SIN-18m}$ cells were similarly infected with either SIN (E), SFV (F), or Una (G). Viral RNA in uninfected A. albo$_{SIN-18m}$ cells (D) and in infected cells was labeled with [^{3}H]uridine 24 to 30 h after virus infection and analyzed in a 2.8 to 5.0% gradient slab gel. 26S RNA synthesis in Una- and SFV-infected A. albopictus and A. albo$_{SIN-18m}$ cells was quantitated as described in the text.*

normal yield from a limited number of superinfected cells. Monolayers of *A. albo*$_{CHIK-1h}$ cells, *A. albo*$_{CHIK-10d}$ cells, *A. albo*$_{CHIK}$ cells 56 days postinfection (*A. albo*$_{CHIK-56d}$ cells), and previously uninfected mosquito cells were infected with SIN at a multiplicity of 5 PFU/cell. The proportion of cells capable of producing SIN plaques (5 to 6 mm in diameter) and CHIK plaques (1 to 2 mm in diameter) in BSC-1 cells was determined at 6 h after SIN infection. Table 5 shows that, whereas 85% of *A. albopictus* cells yield infectious centers after SIN infection, only 8 to 10% of *A. albo*$_{CHIK-10d}$ and *A. albo*$_{CHIK-56d}$ cells are capable of supporting SIN replication. Superinfection of *A. albo*$_{CHIK-56d}$ cells with SIN did not alter the proportion of these cells giving small, presumably CHIK, plaques. To ensure that the large plaques and small plaques from SIN-infected *A. albo*$_{CHIK-56d}$ cells were produced by SIN and CHIK, respectively, 10 isolated plaques of each size were picked, and the virus was grown in monolayers of mosquito cells for 3 days. The progeny virus was titrated in BSC-1 cells, and it was seen that virus from small-plaque clones had a small-plaque morphology. Similarly, virus from large-plaque clones gave rise to large plaques. In addition, large-plaque virus and small-plaque virus synthesized 26S RNA of the size characteristic of SIN and CHIK, respectively (data not shown). These data indicate that SIN replicated in a proportion of the cells in the *A. albo*$_{CHIK-56d}$ culture that were not releasing CHIK.

Steps in the development of a persistent alphavirus infection of *A. albopictus* cells. In an attempt to understand the biphasic nature of heterologous interference in alphavirus-infected mosquito cells, experiments were done to monitor the establishment and development of a persistent SIN infection of *A. albopictus* cells by examining the species of viral RNA and structural protein synthesized at different times postinfection.

A. albopictus cells infected with plaque-purified wild-type SIN yielded maximum amounts of infectious virus by 24 to 48 h postinfection and thereafter over a period of 2 weeks fell to a level approximately 10^3 lower than maximum titer. The species of viral RNA synthesized after infection are shown in Fig. 2. The data in Fig. 7 indicate the protein species synthesized in SIN-infected mosquito cells at selected times postinfection. The virus did not inhibit cellular protein synthesis, and although capsid (C) protein was easily detected from 6 h postinfection (lane C), it was often more difficult to identify PE2 and E1 proteins. The position of E1 protein was confirmed with SIN grown in mosquito cells (data not shown). Figure 7 shows that, unlike the C protein, PE2 and E1 proteins in *A. albopictus* cells (lane D) migrate a little faster than their counterparts in infected CEF cells (lane A). E1 and E2 proteins from mosquito-grown SFV migrate faster than E1 and E2 proteins from hamster cell-grown SFV (18). The rate of synthesis of C, PE2, and E1 proteins in SIN-infected mosquito cells reached a maximum at 24 h postinfection (Fig. 7, lanes D and E). After that time the synthesis of viral proteins was inhibited, and by 72 h postinfection little, if any, C protein was detected. As the amount of viral C protein decreased, there was increased synthe-

TABLE 5. *Effect of time of previous CHIK infection on the proportion of A. albopictus cells capable of being superinfected with SIN*

Virus	Cell	% of cells giving infectious centers[a]	
		SIN	CHIK
SIN	*A. albopictus*	85.0	0
SIN	*A. albo*$_{CHIK-1h}$	0.1	72.0
SIN	*A. albo*$_{CHIK-10d}$	10.0	5.1
SIN	*A. albo*$_{CHIK-56d}$	8.0	4.0
	A. albo$_{CHIK-56d}$	0	4.5

[a] SIN and CHIK plaques in BSC-1 cells were 5 to 6 mm in diameter and 1 to 2 mm in diameter, respectively, after 48 h at 37°C.

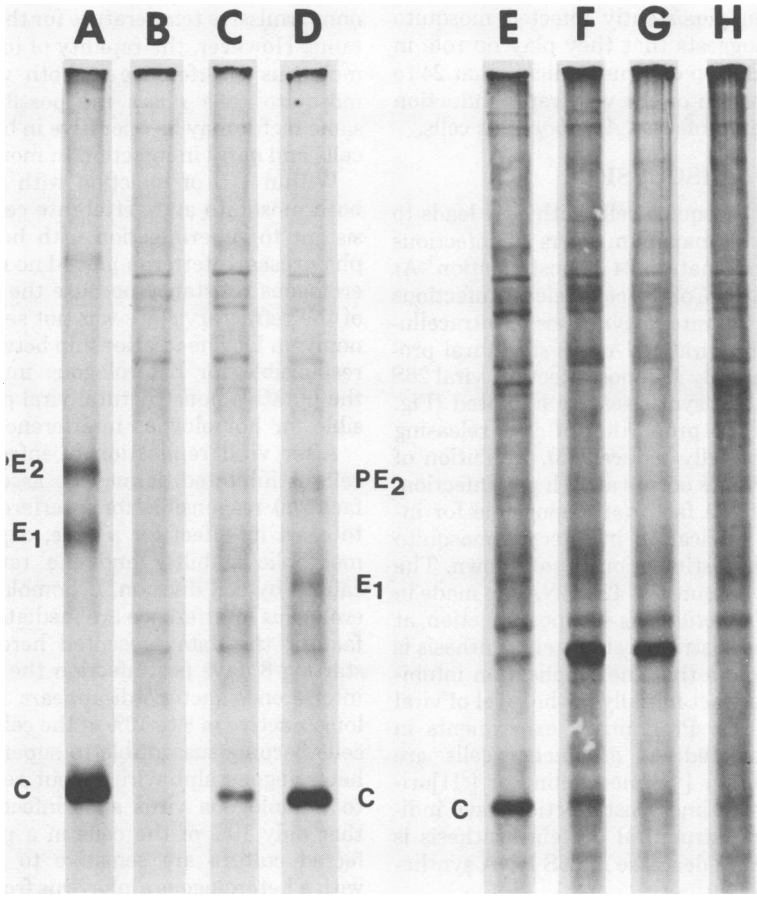

FIG. 7. *Proteins synthesized in mosquito cells during establishment of a persistent SIN infection. Monolayers of 4.0 × 10⁶ mosquito cells were infected with SIN and labeled with 10 μCi of [³⁵S]methionine per ml for 40 min at 6 h (C) and 24 h (D) postinfection. A. albopictus cells (B) were similarly labeled 6 h after mock infection. Proteins labeled in SIN-infected CEF cells for 40 min at 5 h postinfection are included as markers (A). In a different experiment, 5.0 × 10⁶ A. albopictus cells were infected with SIN and labeled for 40 min with 10 μCi of [³⁵S]methionine per ml at 24 h (E), 48 h (F), and 72 h (G) postinfection. A. albopictus cells were similarly labeled 72 h after mock infection (H). When the positions of PE2, E1, and C proteins (molecular weights, 62,000, 49,000, and 30,000, respectively) in (A) were used as markers, the molecular weight of the major protein in (F) and (G) was calculated to be approximately 40,000. The difference in the amount of label incorporated into control cells in (B) (6 h after mock infection) and (H) (72 h after mock infection) can be accounted for by the increased number of cells labeled in the later case.*

sis of a protein with a molecular weight of approximately 40,000 (Fig. 7, lanes F and G). Its origin and role in the inhibition of viral replication are presently being investigated.

There was no evidence for the synthesis of DI particles by persistently infected cells until 48 days postinfection. At that time, virus in the culture medium of persistently infected cells induced the synthesis of a double-stranded RNA species smaller than RF III (22) in infected CEF cells. The appearance of such small RNAs is good evidence for the synthesis and release of DI particles from infected cells (6). Increasing time postinfection leads to the evolution of smaller DI RNA species (B. T. Eaton, manuscript in preparation). Increasing serial high-multiplicity passage of SIN in vertebrate cells also results in the selection of progressively smaller DI specific RNA species (9). Titration of the virus in the culture medium of persistently infected mosquito cells in CEF cells at 33 and 40°C has shown that *ts* variants are detectable 7 to 8 weeks postinfection (10; B. T. Eaton, *In* E. Kurstak and K. Maramorosch, ed., *Viruses and Environment*, in press). Such variants have been shown to interfere with the replication of wild-type virus in vertebrate and mosquito cells (10). The time of appearance of *ts* variants and

DI particles in persistently infected mosquito cell cultures suggests that they play no role in either the inhibition of virus replication at 24 to 48 h after infection or the very rapid induction of interference in infected *A. albopictus* cells.

DISCUSSION

Infection of mosquito cells with SIN leads to the synthesis of maximum titers of infectious virus by approximately 24 h postinfection. At that time up to 85% of the cells release infectious virus (5), and the rate of synthesis of intracellular 42S and 26S viral RNA and structural proteins is maximal. By 48 h postinfection, viral 26S RNA and protein syntheses are inhibited (Fig. 2 and 7), and the proportion of cells releasing virus is dramatically reduced (5). Inhibition of 42S RNA synthesis occurs at 72 h postinfection. The nature of the factor(s) responsible for inhibiting viral replication in infected mosquito cells after 24 h postinfection is not known. The fact that large amounts of 42S RNA are made in infected *A. albopictus* cells 48 h postinfection, at a time when viral structural protein synthesis is inhibited, suggests that the "replication inhibition factor" may act initially at the level of viral protein synthesis. Preliminary experiments in which SIN-infected *A. albopictus* cells are pulsed with either [^{35}S]methionine or [^{3}H]uridine at different times postinfection have indicated that viral structural protein synthesis is inhibited before a decrease in 26S RNA synthesis is detected.

Within 1 h of infection with an alphavirus, vertebrate and mosquito cells are resistant to superinfection with the homologous virus (12, 20). The data of Johnston et al. (12) suggest that a nonstructural viral protein is responsible for the homologous interference induced by SIN in vertebrate cells. This conclusion was based on the finding that, while one RNA$^-$ mutant of SIN, *ts*6, induced homologous interference at both permissive and nonpermissive temperatures, another RNA$^-$ mutant, SIN *ts*24, induced the homologous refractory state only at permissive temperatures. At nonpermissive temperatures, SIN *ts*24-infected cells contain a large protein, p200 (2), which may be the precursor of some, if not all, of the nonstructural proteins encoded by the virus (3). Thus, p200 may contain a "homologous interference protein" in a form which is active only after proteolytic cleavage. Unfortunately, it is not possible to do experiments similar to those described by Johnston et al. (12) with SIN *ts*6 and SIN *ts*24 in mosquito cells. Preliminary experiments have shown that wild-type SIN does not replicate efficiently in *A. albopictus* cells at 40°C, the nonpermissive temperature for these RNA$^-$ mutants. However, the rapidity of induction of homologous interference in both vertebrate and mosquito cells raises the possibility that the same factor may be operative in both vertebrate cells and early in infection in mosquito cells.

Within 1 h of infection with an alphavirus, both mosquito and vertebrate cells are also resistant to superinfection with heterologous alphaviruses. Interferon played no role in the heterologous resistance because the establishment of the refractory state was not sensitive to actinomycin D. The relationship between the factor responsible for heterologous interference and the putative nonstructural viral protein responsible for homologous interference is unknown.

After viral replication in infected mosquito cells is inhibited, it may be assumed that the factor(s) responsible for interference continues to exert its effect for a time, depending on its metabolic stability and the rate of dilution caused by cell division. If homologous and heterologous interference are mediated by different factors, the data presented here suggest that starting 8 days postinfection the "heterologous interference factor" disappears from or is no longer active in 8 to 10% of the cells. Thus, these cells become susceptible to superinfection with heterologous alphaviruses but remain immune to homologous virus superinfection. The fact that only 10% of the cells in a persistently infected culture are sensitive to superinfection with a heterologous alphavirus from 8 days postinfection suggests that loss of the "homologous interference factor" occurs soon after disappearance of the heterologous interference factor. Cells will then become susceptible to reinfection with the homologous virus (10, 21). In a persistently infected culture, the presence of virus in the medium ensures the rapid reinfection of virus-free cells and their consequent return to a homologous and heterologous refractory state.

If homologous and heterologous interferences are mediated by the same factor, it may be suggested that the amount of factor required to maintain heterologous interference is greater than that required to maintain homologous interference. Thus, by 8 days postinfection the level of interference factor may have fallen below that which is required to maintain heterologous interference. Further loss of the factor renders the cells susceptible to superinfection with the homologous virus and their return to a totally refractory state.

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