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Homologous Interference Induced by Sindbis Virus

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Homologous interference during Sindbis virus infection has been investigated. Prior infection of either chicken embryo fibroblast or BHK_{21} cell cultures results in reduced yields of progeny virions of the superinfecting genotype. This reduction in yield results from a reduction in the number of cells in the cultures capable of producing the superinfecting genotype. The development of interference parallels the attachment kinetics of Sindbis virus. Interference requires an active viral genome since the activity is sensitive to inactivation by ultraviolet light, and an RNA^- mutant, ts-24, fails to induce interference under nonpermissive conditions. However, ts-6, an RNA^- mutant belonging to a different complementation group, and the RNA^+ mutants, ts-2 and ts-20, interfere at both permissive and nonpermissive temperatures.

The inability of an animal virus to replicate in a previously infected cell or animal is termed interference. Interference during viral infection was first described by Hoskins (11) who discovered that simultaneous infection by a neurotropic strain of yellow fever virus protected monkeys against infection by the lethal viscerotropic strain. Subsequently, many examples of interference have been reported to occur both in vivo and in vitro.

Noninterferon-mediated interference phenomena may be broadly divided into two categories: those which affect the interaction of the superinfecting virus with the cell surface and those which are mediated intracellularly. The first interference category is typified by the avian leukosis viruses (20, 21) and strains of Newcastle disease virus (3, 4). Typical of the second interference category are those systems which apparently involve intracellular competition, such as the interference described by Bellett and Cooper (1), in which defective particles of vesicular stomatitis virus (VSV) interfere with the replication of infectious virus. It has been suggested (19, 16) that the shorter RNA found in the defective virions replicates intracellularly at the expense of the longer RNA contained in the infectious virus.

The competitive hypothesis which has been proposed to explain interference by defective VSV has been expanded to include not only competition for replicase but also competition for necessary substrates, viral structural proteins and/or nucleic acid replication sites. It is in this expanded form that the hypothesis has been applied to virus interference systems in which defective particles are not involved, such as interference between picornavirus strains (9) and between various togaviruses (24). According to this hypothesis, interference results when the interfering virus has a multiplicity or temporal advantage over the superinfecting virus so that excess-interfering virus RNA successfully competes with superinfecting-virus RNA for replication sites, replicase and/or substrates. However, intracellular competition involving RNA replication may not account for all the interference observed in these systems. Pohjanpelto and Cooper (15) demonstrated that certain RNA^- temperature-sensitive mutants could interfere with the replication of wild-type virus even under nonpermissive conditions, and that inhibition of interfering viral RNA synthesis with guanidine did not inhibit interference.

In this study, homologous interference during Sindbis virus infection has been investigated. It has been demonstrated that prior infection results in reduced production of progeny virions of the superinfecting genotype and that this interference is a function of the viral genome. Our results also suggest that a single PFU is capable of inducing interference in a given host cell and that interference may be induced in the absence of interfering-virus RNA replication.

MATERIALS AND METHODS

Virus and cell cultures. Sindbis virus, strain AR339 (23), was obtained from the American Type Culture Collection, was plaque purified, and has been maintained by low-multiplicity passage (0.01 PFU/cell) in primary chicken embryo (CE) cells at 37 C. Wild-type virus was quantitated by plaque assay at 37 C (10). The temperature-sensitive mutants of

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Sindbis used in this study (ts-2, 6, 20, and 24) were obtained from E. R. Pfefferkorn. Ts-2, 6, and 24 have been characterized in his laboratory (5–8, 17, 18) and ts-20 has been characterized in this laboratory (12). The mutants were grown and assayed in CE cells at 29 C, the permissive temperature, and maintained by low-multiplicity passage. The reversion rates were monitored by assay at the nonpermissive temperature, 41.5 C. Reversion rates for ts-2, 6, 20, and 24 were 10^{-4}, 10^{-6}, $<10^{-7}$, and $<10^{-7}$, respectively. Primary CE cells were maintained in Eagle minimal essential medium with Hanks salts (MEM) (GIBCO) plus 5% newborn calf serum. Baby hamster kidney (BHK_{21}) cells were maintained in MEM supplemented with 10% tryptose phosphate broth and 10% fetal calf serum. For growth of virus stocks, the medium contained Actinomycin D (Mann Research Laboratory) at 1 μg/ml.

Infectious center assays. Appropriate dilutions of infected cells in MEM containing 5% newborn calf serum were allowed to adsorb in 0.5-ml samples onto CE monolayers for 1.5 h at 37 C. Two milliliters of agar overlay medium (0.9% Ionagar in MEM plus 5% serum) was added to each culture. After this initial overlay had solidified, an additional 6 ml of overlay was added. For determination of wild-type infectious centers in the presence of cells infected with ts mutants, the cultures were incubated at 41.5 C. The efficiency of this assay with respect to a plaque assay with extracellular virus was approximately 50%.

Inactivation of virus with UV light. Virus stocks were diluted 1:10 in Ca^{2+}- and Mg^{2+}-free phosphate buffered saline (PBS). These were exposed to a General Electric germicidal lamp (emitting 2.0×10^5 ergs per cm^2 per s) at 30 cm. At intervals, samples were removed for assay and use in interference experiments.

RESULTS

Demonstration of interference in CE cells. The design of an experimental system to demonstrate and characterize a homologous interference phenomenon requires that the interfering and superinfecting viruses be distinguishable and that the degree of interference be amenable to quantitation. In this study the distinction has been made by using ts mutants as interfering viruses and wild type as the superinfecting virus. The use of ts mutants to demonstrate interference provides two advantages. Plaque assay at nonpermissive temperature permits the quantitation of yields of superinfecting wild-type virus in the presence of an excess of mutants. In addition, by appropriate choice of mutants, specific viral genes may be selectively inactivated and their effect on the establishment and maintenance of interference observed.

Figure 1 shows the results of an experiment in which CE cell cultures were infected at 29 C with ts-24 (an RNA-minus mutant) at a multiplicity of infection (MOI) of 10 to 25 PFU per cell. After 1 h, the inoculum of interfering virus was removed, and the cultures were superinfected with wild type at a MOI of 0.5 PFU/cell. After a 30-min adsorption period, the superinfecting virus inoculum was removed, medium was added, and the cultures were incubated at 29 C. At the indicated time intervals after incubation, samples were removed and the growth of superinfecting wild-type virus was determined by plaque assay at 41.5 C. Growth of wild-type virus in cultures which previously had been infected with the mutant was both delayed and reduced compared to the growth of wild type in previously uninfected controls. Under these conditions the growth of the interfering virus was unaffected (data not shown).

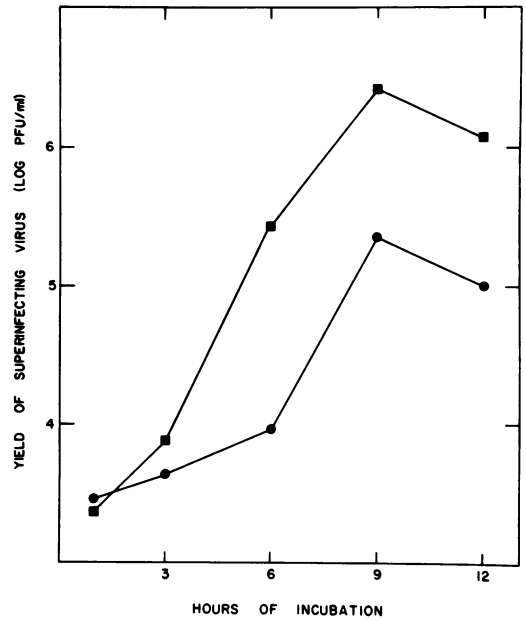

FIG. 1. *Delayed and reduced yield of superinfecting virus in CE cultures. Cultures were infected with ts-24 (10 to 25 PFU/cell) at 29 C. After 1 h, they were superinfected with wild type (0.5 PFU/cell). Cultures previously infected with ts-24 (●); control cultures which had not been previously infected (■).*

Interferon does not appear to be involved in the interference depicted in Fig. 1. Actinomycin D prevents interferon-mediated interference (22). The continued presence of Actinomycin D (1 μg/ml) during a 2-h pretreatment period, primary, and superinfection, had no effect on the degree of interference. In addition, interference activity cosediments with infectivity in rate zonal sucrose gradients (data not shown).

The reduction of superinfecting virus yields in interfered cultures could have resulted from a reduction in the yield of superinfecting virus from each individual cell or from a reduction in

a number of cells in the culture capable of producing the superinfecting genotype. To distinguish between these alternatives, cultures were either infected with ts-6 (29 C) or mock infected (29 C) at a MOI of 10 PFU/cell. After 60 min, the interfering inocula were removed, and the cultures were superinfected with wild type (0.1 PFU/cell) for 30 min. The cultures were washed twice with MEM, and the cells were removed by trypsinization, pelleted at 1000 × *g* for 10 min, and resuspended in MEM. The number of cells capable of producing superinfecting virus was determined by infectious center assay at 41.5 C (Table 1). The reduction in the yield of wild-type virus per culture may be ascribed to a reduction in the number of cells capable of being productively superinfected.

Establishment of interference as a function of time. In the experiments described above, the interfering virus was allowed 1 h to establish interference before the cultures were superinfected. Figure 2 shows that the development of interference corresponds approximately to the attachment kinetics of Sindbis virus (2) with maximal interference having developed by 60 min after primary infection. It is likely, therefore, that the time interval necessary for the development of maximal interference reflects the time necessary for maximal infection of the culture by the interfering virus.

Interference in BHK_{21} cells. Interference is also expressed in BHK_{21} cells. Figure 3 shows the results of an experiment analogous to that shown in Fig. 1. The virus yields were assayed in CE cells at 41.5 C. The growth of the superinfecting virus was delayed and reduced as compared to its growth in control BHK_{21} cultures.

Determination of the interfering dose. To determine the virus dose necessary for the induction of interference in a given cell, cultures were infected with dilutions of a freshly grown stock of interfering virus (ts-20), and the growth of superinfecting wild type in these cultures was determined for each dilution (Fig. 4). The fraction of cells in an interfered culture which has been productively superinfected is reflected in the yield of the superinfecting wild type

TABLE 1. *Infective center assay for superinfecting wild-type virus in previously infected cultures*

Interfering virus	Superinfecting virus	Infective center per culture	
		Expt 1	Expt 2
None	+	4.9×10^4	2.2×10^4
ts-6	+	4.6×10^3	2.8×10^3

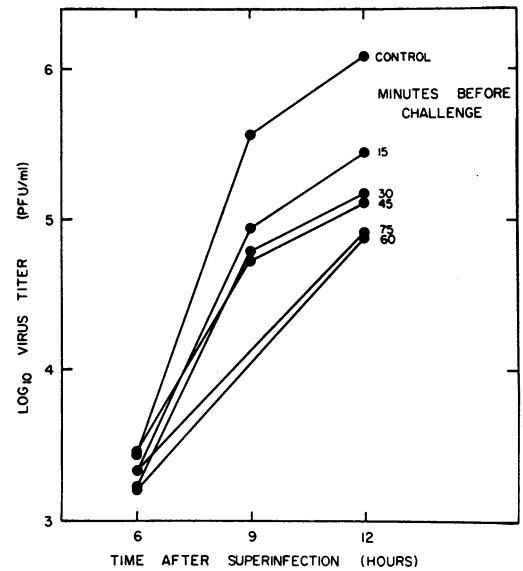

FIG. 2. *Development of interference with time. Cultures were infected with ts-24 (10 to 25 PFU/cell) at 29 C and were superinfected with wild type (0.5 PFU/cell) at the times indicated in the figure. The growth of superinfecting wild type under each condition is compared to the growth of wild type in control cultures which had not been infected with ts-24.*

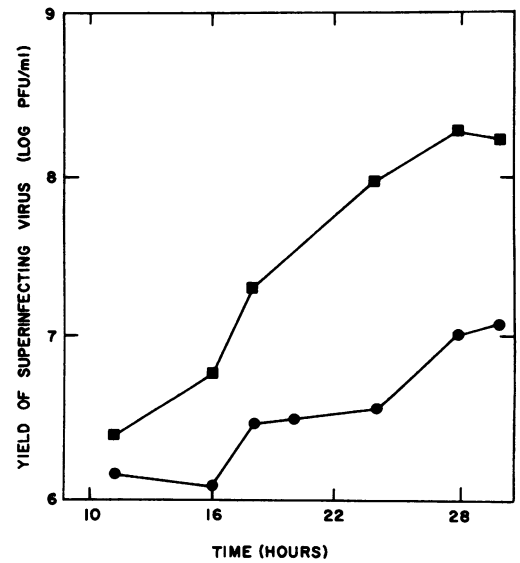

FIG. 3. *Interference established in BHK_{21} cultures. Cultures were infected with ts-20 (10 to 25 PFU/cell) at 29 C. After 1 h, they were superinfected with wild type (0.5 PFU/cell). Cultures previously infected with ts-20 (●); control cultures which had not been previously infected (■).*

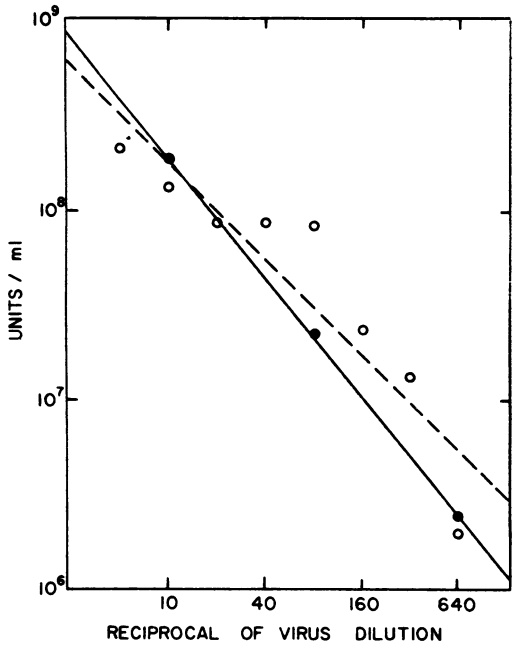

FIG. 4. *Induction of interference as a function of interfering virus dose. Cultures were infected with serial twofold dilutions of ts-20 (1:5 through 1:640; ts-20 MOI in 1:5 dilution was 9.5 PFU/cell). The 1:10, 1:80, and 1:640 dilutions were assayed independently to illustrate the dilution of PFU (●). After 1 h at 29 C, the infected cultures were superinfected with wild type. The yield of wild type was used to calculate the number of interfering doses (O) present in each dilution of ts-20 as described.*

expressed as a fraction of the yield from wild-type-infected control cultures. This fraction represents those cells in the culture which did not receive an interfering dose sufficient to make them refractory to superinfection. The fraction of cells which are not refractory to superinfection should correspond to the zero term of the Poisson distribution, e^{-m}, where m is the number of interfering doses per cell present in each dilution of ts-20. The value of m may then be used to calculate the concentration of interfering doses (open circles) in each dilution by multiplying m by the total number of cells in the interfered cultures and dividing by the volume used for the primary infection. A line through these points, calculated by the least-squares method, indicates that dilution of interfering doses corresponds approximately with the dilution of PFU (closed circles). The concentration of interfering doses in the original ts-20 stock was calculated to be 6.2×10^8/ml as compared with a titer of infectious virus of 8.5×10^8 PFU/ml. This experiment suggests that the interfering particle is the virion itself and that infection of a cell by a single PFU is sufficient to induce interference.

Inactivation of interference with UV light. A freshly grown stock of ts-20 was diluted 1:10 in PBS and inactivated with UV light. At intervals during the inactivation, samples were removed and assayed for their ability to interfere with the replication of superinfecting wild-type virus. The concentration of interfering doses in each sample was calculated as in the previous section. Figure 5 shows the inactivation of infectivity (circles) as compared to inactivation of interference (squares). The ratio

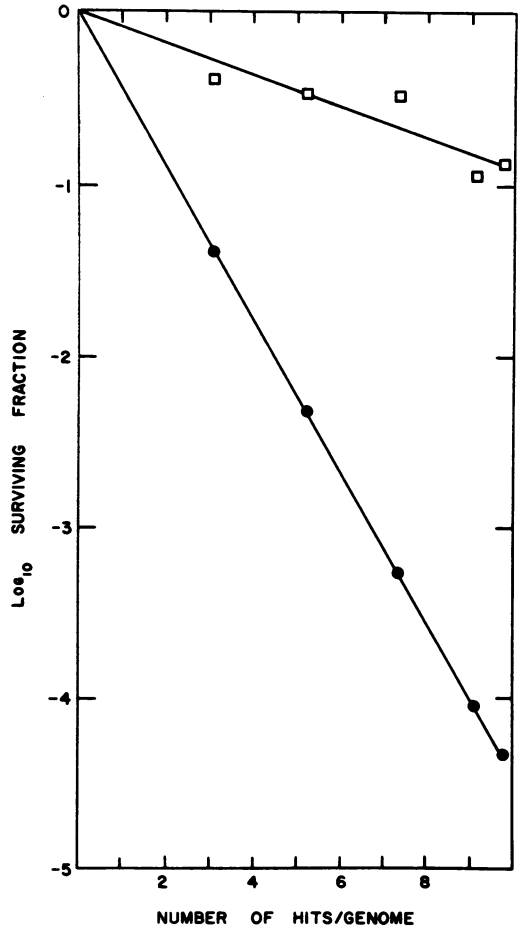

FIG. 5. *Inactivation of interference with UV irradiation. A freshly grown stock of ts-20 was diluted 1:10 in PBS and inactivated with UV light. At intervals during the inactivation, samples were removed and assayed for infectivity and for their ability to interfere with the replication of superinfecting wild type. Inactivation of infectivity (PFU), (●); inactivation of interfering activity (interfering doses), (■).*

of the slopes of these lines indicated that the induction of interference is one-fifth as sensitive to UV inactivation as infectivity.

Identification of an interference defective mutant. If interference is mediated through the activity of a viral gene as the UV inactivation results indicate, then interference-defective mutants should exist. Accordingly, several ts mutants of Sindbis were examined for their ability to interfere at nonpermissive temperatures (Table 2). After equilibration at 41.5 or 29 C, cultures at each temperature were mock infected or infected with one of the following mutants: ts-2, (capsid negative), ts-6, (RNA^-, complementation group B), ts-20, (maturation negative), or ts-24, (RNA^-, complementation group A). After 1 h for adsorption, the interfering inoculum was removed and the cultures were superinfected with wild-type Sindbis. After 30 min for the adsorption of the superinfecting virus the inoculum was removed, medium was added, and the cultures were incubated at 29 C. The titers given in Table 2 have been corrected for the growth of revertants. ts-2, ts-6, and ts-20 interfere to the same extent at both permissive and nonpermissive temperatures. Moreover, growth of superinfecting wild type was delayed relative to wild-type growth in controls under both permissive and nonpermissive conditions. ts-24, however, does not interfere as strongly at 41.5 C as at 29 C (Table 2). In addition, the growth of superinfecting wild type was not delayed as compared with control (Fig. 6).

TABLE 2. *Interference by temperature-sensitive mutants*

Interfering virus[b]	12-h yields of superinfecting wild type[a]	
	29 C	41.5 C
ts-2	1.2×10^8 (8.0)[c]	3.1×10^8 (9.7)
None	1.5×10^8	3.2×10^8
ts-6	2.8×10^8 (18.7)	2.3×10^8 (13.5)
None	1.5×10^7	1.7×10^7
ts-20	4.2×10^7 (2.1)	4.8×10^7 (3.7)
None	2.0×10^9	1.3×10^9
ts-24	1.0×10^8 (8.3)	6.2×10^8 (47.8)
None	1.2×10^8	1.3×10^8

[a] MOI equals 0.5 PFU/cell. The variation in the yields of virus reflects differences in the plating efficiency of the CE cultures over the time period in which these experiments were performed. In each individual case, however, control and interference experiments were performed in parallel on duplicate cultures and the interference expressed as a fraction of control was very reproducible.

[b] MOI equals 10 to 25 PFU/cell.

[c] Percent of control.

DISCUSSION

In this report, we have described an homologous interference phenomenon which is expressed during the replication of Sindbis virus. The phenomenon is analogous to genetic exclusion in bacteriophage systems in that previously infected cells are refractory to superinfection, and virions of the superinfecting genotype are excluded from the progeny. The interference reported in this study is not the result of defective particles in the Sindbis virus preparations. The virus stocks used in these studies were plaque purified and passaged less than four times at low multiplicity. These conditions do not lead to the production of defective interfering arbovirus particles in CE cultures (13).

The expression of Sindbis virus interference requires that the interfering virus possess an active genome. Induction of interference is one-fifth as sensitive to UV inactivation as infectivity. Also, ts-24, a mutant from one of the RNA^- complementation groups, fails to interfere at

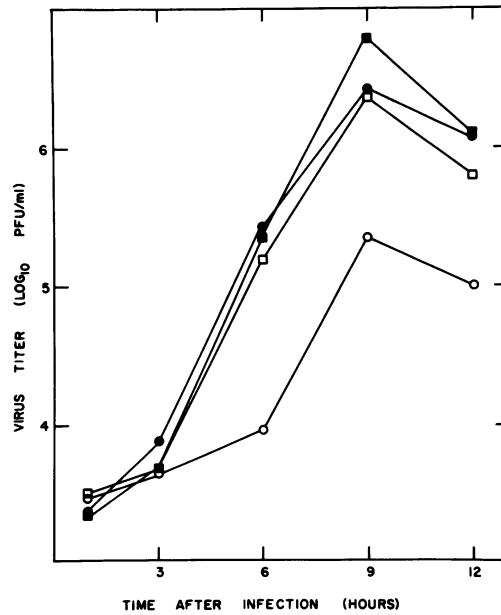

FIG. 6. *Growth of superinfecting wild-type virus in cultures previously infected with ts-24. Cultures were infected with ts-24 (10 to 25 PFU/cell) at either 29 C or 41.5 C, superinfected with wild type (0.5 PFU/cell), and incubated at 29 C. Growth of wild type in cultures infected with ts-24: at 29 C (○); at 41.5 C (□). Growth of wild type in cultures which had not been infected previously: 29 C (●); 41.5 C (■).*

the nonpermissive temperature. These experiments suggest that approximately 20% of the viral genetic material is required for the induction of interference, and that the activity of a single cistron may be responsible for interference.

Although ts-24 is defective in RNA synthesis, RNA synthesis per se is apparently not required for the induction of interference. Although ts-6 is also incapable of RNA synthesis at nonpermissive temperature, it retains the capacity to interfere under conditions which prevent the induction of interference by ts-24. This does not reflect a difference in leakiness between the two RNA$^-$ mutants since equivalent viral yields are obtained when ts-6 or ts-24 are grown at the nonpermissive temperature.

The induction of interference occurs at an early stage of the viral replication cycle. The development of interference parallels the attachment kinetics of Sindbis virus (2). Since interference may be induced in the absence of RNA synthesis, induction of interference may occur before RNA synthesis in the wild-type infection. The refractory state is established within 60 min postinfection (Fig. 2). If interference occurs before or in the absence of interfering viral RNA synthesis, then the homologous interference we have observed is not likely to be mediated by intracellular competition between interfering and superinfecting RNA molecules. Our results are consistent with those of Pohjanpelto and Cooper (15) who found that competition does not account for all of the observed homologous interference in the poliovirus system: some RNA$^-$ mutants are capable of interference under nonpermissive conditions and guanidine does not reduce the degree of interference as measured by infective centers.

The interference described here may be analogous to those interference systems in which the surface membranes of infected cells are apparently altered in such a way as to prevent a normal interaction between these cells and superinfecting virions. We have shown (R. Johnston and H. Bose, unpublished data; Bacteriol. Proc., p. 195, 1972) that prior infection prevents the irreversible attachment of superinfecting Sindbis virions, and that this inhibition of attachment occurs only under those conditions which allow the expression of interference. Palma and Huang (14) have observed a similar surface interference in VSV-infected cells. The attachment of [^{3}H]uridine labeled infectious (B) particles to cells which had been previously coinfected with B and defective interfering particles was inhibited as compared with attachment to uninfected controls. Interference observed during virus infection may represent the combined effect of abnormal processing of superinfecting virions at the cell surface as well as intracellular competition.

ACKNOWLEDGMENTS

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