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Interference Among Group A Arboviruses

EUGENE ZEBOVITZ AND ARTHUR BROWN
Biological Sciences Laboratories, Fort Detrick, Frederick, Maryland 21701

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Interference among group A arboviruses is described which does not involve the mediation of interferon. Interference was observed only if the interfering virus had an advantage over the challenge virus, either in time or in multiplicity of infection. Adsorption, penetration, and uncoating of challenge virus did not appear to be inhibited, but the synthesis of infectious viral ribonucleic acid of the challenge virus was significantly retarded. It was shown with temperature-sensitive viruses or mutants that the replication of viral ribonucleic acid by the interfering virus was required to establish interference. A mechanism of interference based on a competition for replication sites or substrates is compared with other possible explanations.

The practical and theoretical implications of viral interference have stimulated considerable interest in this research area. Many reports have been published since the discovery of interference in plant viruses (14) and in animal viruses (11). Those papers that appeared prior to the discovery of interferon (13) have been reviewed extensively by Henle (10) and Schlesinger (18). The role of interferon as an important factor in the development of nonspecific resistance of the host cells to superinfection with a second related or unrelated virus is now firmly established (6). It is presently difficult to determine which of the early reports on viral interference were concerned with interferon production by the host or by other factors. It is apparent, however, that there are several types of viral interference that do not involve the mediation of interferon (5, 7, 12, 15–17).

This report describes a noninterferon-mediated interference among different arboviruses; this interference requires that the interfering virus be able to replicate viral ribonucleic acid (RNA) in the host cell before it can interfere with the growth of the challenge virus. Further, interference of the challenge virus appears to occur after its uncoating but before synthesis of infectious viral RNA.

MATERIALS AND METHODS

Virus strains. In most of the experiments reported here, the virus used to induce the interference was either the Trinidad strain of Venezuelan equine encephalitis (VEE) virus or strain T, which is a small-plaque, temperature-sensitive variant of this virus. In a few experiments, the interfering viruses were: a large-plaque revertant of T, designated V5, whose maximum growth temperature was unchanged (S. Halle, *personal communication*); an attenuated variant (A) of VEE virus originally described by Berge et al. (1); and a temperature-sensitive mutant (Ets-4) of the Louisiana strain of eastern equine encephalitis virus (EEE). EEE virus served as the challenge virus in most experiments. Properties of these viruses, except for Ets-4 and V5, have been described by Brown (3).

Cell cultures. Cell cultures were prepared from 10-day-old chick embryos. The chick embryo (CE) monolayers were grown, in lactalbumin hydrolysate medium containing 10% calf serum, for 24 hr at 37 C before infection. Details of the preparation of monolayers, medium, and growth conditions were described in an earlier paper (22).

Virus growth. Except where noted, CE cell monolayers in 60-mm plastic petri dishes were infected with a virus seed prepared as a 10% CE seed, or as undiluted tissue culture fluids obtained from infected CE monolayers. The virus was allowed to adsorb for 15 min at room temperature; the cells were washed twice with phosphate-buffered saline (PBS) at *p*H 7.4 to remove excess unattached virus, and were overlayered with 5 ml of lactalbumin hydrolysate medium containing 10% calf serum. The cultures were incubated at 37 C in a 5% CO_2–95% air incubator.

When the interfering and challenge virus were simultaneously added to CE monolayers, the virus seeds were mixed before infection of the cells. When the challenge virus was added at some time after infection of the cells with the interfering virus, the culture medium was removed, the challenge virus was added, and, after a 15-min adsorption period at room temperature, the infected cells were washed twice with PBS and overlayered with the lactalbumin hydrolysate medium. The cultures were reincubated at 37 C and samples of the culture medium were collected at various intervals. In most of the experiments, actinomycin D (1 or 2 μg/ml) was incubated with cells for 30 min before infection and was in the culture medium at the same concentration throughout the experiment.

Virus assay. Virus assays were performed on 24-hr

CE monolayers prepared from 10-day-old chick embryos. The overlay medium was lactalbumin hydrolysate-agar (21). In those studies involving mixed infection with VEE and EEE viruses, titers in the growth medium were determined in the presence of a 1:100 dilution of anti-VEE serum (whose plaque neutralization titer exceeded 1:10,000) added to the agar overlay medium. Plaque formation by VEE virus was inhibited but that of EEE virus was not. This permitted assay of EEE virus growth in the presence of a large excess of VEE virus. When strain T was used, it was not necessary to add antiserum to the overlay because this virus formed very small plaques and was easily distinguishable from EEE virus when assays were made on samples from mixed infections. In reconstruction experiments involving mixtures and controls, it was found that 100- to 200-fold excess of T over EEE did not inhibit plaque formation of EEE, thus justifying the procedure of plaquing and counting EEE in the presence of excess T.

Extraction and assay of infectious RNA (IRNA). Infected CE monolayers were removed with a rubber policeman and suspended in 0.02 M phosphate-0.001 M ethylenediaminetetraacetic acid buffer (*p*H 7.4). The cells were extracted twice with cold phenol (4 C), and the viral RNA was precipitated from the aqueous phase with three volumes of 95% ethyl alcohol containing 2.0% potassium acetate. The precipitate was dissolved in PBS, and the IRNA was assayed on CE monolayers treated with 1.0 M NaCl in a 0.1 M tris-(hydroxymethyl)aminomethane-chloride buffer (*p*H 8.3) by the method of Colon and Idoine (4).

RESULTS

Demonstration of interference. Interference of the challenge virus could be demonstrated in two ways: (i) by infecting cells with VEE virus several hours before superinfecting the cultures with EEE virus at multiplicities equal to those used for VEE virus, and (ii) by infecting the CE cells simultaneously with two viruses at different multiplicities; the interfering virus was added at an input multiplicity of about 10 plaque-forming units (PFU) per cell, whereas the challenge virus was used at a multiplicity of 0.1 PFU per cell.

When equal multiplicities of the two viruses were employed, the degree of interference was dependent upon the time of superinfection with the challenge virus (Table 1). The degree of interference increased with the time that elapsed before superinfection with the second virus. Maximal inhibition of the growth of EEE virus was observed when it was used to superinfect cells 5 to 6 hr after infection with T virus.

The effect of infecting CE cells simultaneously with strain T and EEE virus is shown in Table 2. Strain T was added at a constant multiplicity of infection (MOI) of 10 and EEE virus was added at an input MOI ranging from 1.0 to 0.01. The previous experiment and others showed that simultaneous infection at equal input multiplicities of 10 resulted in no interference of either virus; multiplicities higher than 1 were therefore omitted. The results showed a progressive increase in the degree of interference as the multiplicity of EEE virus decreased. Maximal interference was observed when the lowest multiplicity of challenge virus was used. The effect of multiplicity in this experiment was probably only another manifestation of the effect of time, since, at the lower multiplicities, more than one cycle of challenge virus multiplication would be required to reach the levels obtained by the interfering virus in one

TABLE 1. *Effect of time of superinfection on interference with challenge virus*

Time of superinfection with EEE virus[a]	Growth response of challenge virus (EEE)	
	PFU/ml[b]	Log_{10} inhibition
hr		
0[c]	7.7×10^9	0
1	3.1×10^9	0.4
2	1.9×10^9	0.6
3	4.5×10^8	1.2
4	1.9×10^8	1.6
5	2.5×10^7	2.5
6	9.0×10^6	2.9
7	1.1×10^7	2.9

[a] Cultures were infected with strain T virus at an input MOI of 10. Cultures were then washed two times with PBS and overlayered with culture medium. At the indicated times, the medium was removed and the cultures were superinfected with EEE virus at the same multiplicity; the cultures were then washed twice and overlayered with growth medium.

[b] Cultures were held at 37 C for 24 hr after addition of the challenge virus before virus assays were made.

[c] This 24-hr titer was approximately the same for all the EEE singly infected control cultures.

TABLE 2. *Effect of input multiplicity of the challenge virus on the interference with challenge virus*

Input multiplicity of challenge virus[a]	PFU/ml (24 hr)	Log_{10} inhibition
Control[b]	1.9×10^9	0
1.0	4.0×10^8	0.7
0.1	4.7×10^7	1.6
0.01	5.1×10^5	2.6

[a] Multiplicity of the interfering virus (strain T) was held constant at 10 PFU/cell.

[b] The 24-hr control value was the growth response of EEE virus in the absence of strain T; this was approximately the same for each of the multiplicities tested.

cycle. In the absence of strain T, EEE virus grew normally and to high titer; although the data are not shown, there was no interference with T virus growth in such doubly infected cells.

There was a strong inhibition of the growth of the challenge virus when the interfering virus was given a growth advantage in the CE cells either by being inoculated several hours earlier or at a significantly higher MOI than the challenge virus (Tables 1 and 2). If the interfering virus was treated with specific neutralizing antiserum just before infecting CE cells, interference to superinfection with the challenge virus was not observed. Controls consisting of neutralizing antiserum to EEE virus, or normal serum, when incubated with VEE as interfering virus prior to infection, did not prevent the interference. These results showed that infection by the virus particle was necessary to establish interference and that the interference was probably not due to interferon in the virus suspension. The latter conclusion was supported by the fact that virus that was sedimented and washed twice had the same interfering capacity as crude virus.

Interference induced by different strains of VEE virus. Interference of the growth of EEE virus could also be demonstrated when other strains of VEE virus were used. In addition, EEE virus could be used as the interfering virus and could inhibit the growth of any strain of VEE virus. However, there seemed to be some variation among virus strains in their capacity to serve as interfering viruses (Table 3). Strain A was consistently more effective as an interfering virus than was the Trinidad strain or the others; the Trinidad strain was more effective than either T or V5; there was no significant difference between T and V5. The differences in the degree of inhibition induced by different strains may possibly be explained by differences in the capacity of the different virus genomes to attach to replication sites within the host cell or by differences in their rates or extent of viral RNA replication.

TABLE 3. *Capacity of different strains of VEE virus to interfere with the growth of EEE virus in chick embryo cell culture*

VEE virus strain[a]	Degree of EEE virus interference[b]
A	2.4
Trinidad	1.6
T	1.4
V5	1.1

[a] Input MOI, 100 PFU/cell.

[b] Input MOI of EEE virus, 1 PFU/cell. The degree of interference is expressed as $\log_{10}$ units of EEE virus at 20 hr. Values are averages of four experiments.

TABLE 4. *Effect of actinomycin D on interference with EEE virus multiplication by strain T[a]*

Time	EEE virus (PFU/ml)		
	Strain T + EEE virus[b]	Strain T + EEE virus[b] + actinomycin D[a]	EEE virus alone
hr			
0	1.2×10^5	1.2×10^5	1.2×10^5
6.5	4.1×10^6	6.4×10^6	6.0×10^7
22	4.7×10^7	4.6×10^7	2.4×10^9

[a] Actinomycin D (1.0 μg/ml) added at 2 hr before infection.

[b] Infection by strain T followed by EEE 3 hr later at equal multiplicities (100).

Is the interference of virus growth mediated by interferon? Actinomycin D was used to help obtain evidence on whether interferon was a factor in the interference that was observed. This drug is known to inhibit both the formation and action of interferon in virus-infected cells, yet it does not interfere with the synthesis of many RNA viruses (8, 20). If interferon were involved in the interference observed here, then the challenge virus should be able to multiply normally in the presence of actinomycin D. When actinomycin D (1 μg/ml) was added 2 hr prior to infection, it had no effect on the interference of EEE virus in cells that had been previously infected with a high multiplicity of strain T virus (Table 4). In both the presence and absence of actinomycin D, the growth of the challenge virus was inhibited to the same extent, about 1.6 $\log_{10}$ less than that obtained for the control culture. That the actinomycin D was active was shown by the fact that growth of vaccinia virus was inhibited by more than 99% in CE cells in the same experiment. In addition, the drug abolished the interference resulting from added chick interferon (50 plaque-inhibiting units) in a Sindbis virus-CE cell test system. From these results, it seems likely that the interference observed does not result from the formation or action of interferon by the host cell. Subsequent experiments were carried out in the presence of actinomycin D, and by infecting with equal multiplicities of the viruses 3 to 4 hr apart.

RNA synthesis by the challenge virus. The interference of EEE virus growth that resulted when strain T was inoculated onto CE cells 4 hr earlier is shown in Fig. 1. The EEE virus titer was reduced 2.7 $\log_{10}$ below that observed for the control culture. In doubly infected cells, the synthesis of

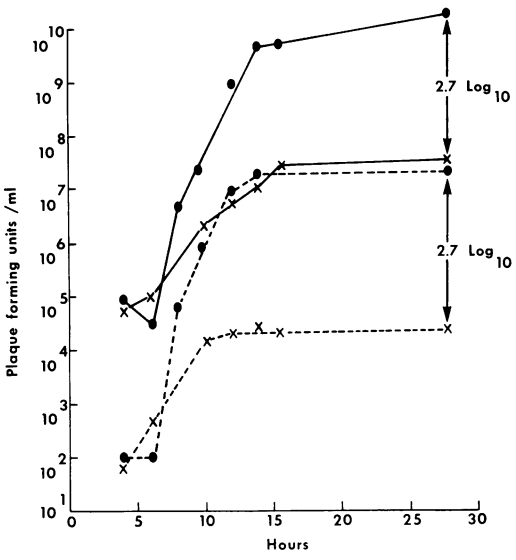

FIG. 1. *Interference of challenge virus (EEE) growth and infectious RNA synthesis by strain T virus. Viruses infected at equal multiplicity (10 PFU/ml). Challenge virus was added to the culture 3 hr after infection with strain T virus. Symbols:* (●—●) *singly infected EEE virus titer;* (×—×) *doubly infected EEE virus titer;* (●- -●) *singly infected EEE IRNA;* (×- -×) *doubly infected EEE IRNA.*

IRNA was reduced to the same proportion as that of the mature virus. These results suggest that the interference phenomenon involves a very early step in the synthesis of the challenge virus, probably before the virus has had the opportunity to synthesize its IRNA. On the basis of our limited data (Fig. 1), there appears to be no obvious preferential interference between IRNA synthesis and viral structural protein synthesis; otherwise, the curves of virus interference and IRNA synthesis would not be expected to parallel each other. However, since no direct measure of viral protein synthesis was made independent of infectious virus, this conclusion is tentative.

Apparently, the early step of the challenge virus infection that was inhibited was not adsorption, penetration, or uncoating of the virus genome, because interference was of the same magnitude when IRNA of the challenge virus was used in the place of infectious virus. This conclusion receives additional support from some incidental evidence obtained in an experiment (described below) with the T strain as interfering virus at 42 C.

In previous studies (22), it was established that the RNA of VEE virus enters the cell and is maintained in a viable state, even for prolonged periods at 44 C, although no new RNA is synthesized. The same proved true for strain T virus at 42 C (Zebovitz and Brown, *unpublished data*). EEE virus, on the other hand, replicated normally at this temperature. It was possible, therefore, to inhibit the growth of strain T selectively by incubating infected CE cultures at 42 C prior to and after the cultures were superinfected with EEE virus.

Figure 2 shows the effect of incubating doubly infected cultures at 42 C. In this experiment, T virus was adsorbed to cells at room temperature washed as usual, and incubated at 42 C for 3 hr before superinfecting with EEE virus and incubating further at 42 C. The interference normally observed was largely abolished; i.e., the maximum titer of EEE virus was not inhibited. These results suggest that, as a minimum condition to establish interference, the interfering virus RNA must be able to replicate in the host cell in order to inhibit the growth of the challenge virus effectively.

The results discussed above support the notion that a competition for replication sites or substrates accounts for the interference observed. To explore this idea further, a recently isolated temperature-sensitive mutant of EEE virus was employed. At 37 C, but not at 30 or 42 C, Ets-4 virus exhibited an unusually high rate and extent of viral RNA synthesis; it induced the formation of approximately three times the amount of viral RNA compared to the parent, but produced 90% less infectious virus and at least 50% less complement-fixing antigen (Zebovitz and Brown, *unpub-*

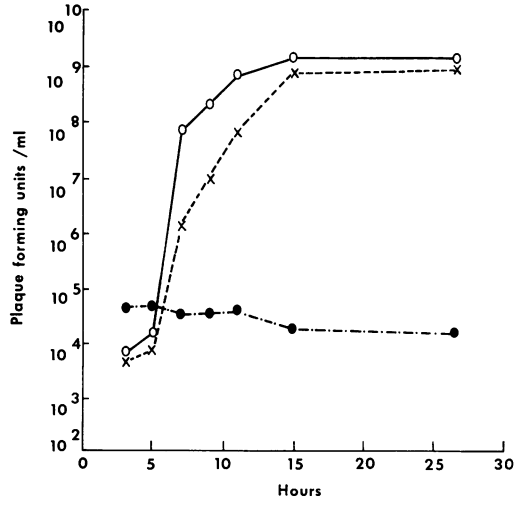

FIG. 2. *Effect of incubation at 42 C on the capacity of strain T virus to interfere with EEE virus growth. Challenge virus (EEE) was added 3 hr after infection of CE cells with strain T. Symbols:* (○) *EEE virus growth in absence of strain T;* (×) *EEE virus growth on CE cells infected with strain T.* (●) *Strain T virus growth in absence of EEE virus.*

TABLE 5. *Comparison of Ets-4 and EEE as interfering virus*

Time after superinfection[a]	Control VEE (PFU/ml)	EEE + VEE (PFU/ml)	Log_{10} inhibition	Ets-4 + VEE (PFU/ml)	Log_{10} inhibition
hr					
0	7.1×10^4	6.0×10^4		5.1×10^4	
5	1.5×10^6	4.0×10^5	0.6	1.0×10^5	1.1
8	2.0×10^7	3.0×10^6	0.8	1.9×10^5	2.0
10	2.0×10^9	7.2×10^7	1.6	3.5×10^5	3.8

[a] Cells were infected with each virus at an MOI of 10, 3 hr apart.

lished data). Because this mutant produces larger amounts of viral RNA than does the parent, one might predict that more replication sites would be occupied and therefore a greater degree of interference should result when it, instead of the parent, is used as the interfering virus. Ets-4 was indeed a much better interfering virus than its parent when VEE was used as a challenge virus (Table 5). From doubly infected cells, VEE virus was counted in the presence of a 1:100 dilution of anti-VEE serum (titer, 1:100,000). The interfering virus in this experiment was incubated at 37 C for 3 hr before challenge virus was added. If the initial 3-hr incubation was carried out at 30 or 42 C, at which the rate and extent of viral RNA synthesis of Ets-4 were depressed to levels closer to those found in the parent virus, the degree of interference was likewise reduced (Table 6). It was not reduced to the level induced by EEE because, when incubation was resumed at 37 C after only 3 hr at either of the other temperatures, Ets-4 still had an advantage in the extent of viral RNA synthesis.

TABLE 6. *Effect of initial temperature of incubation on interference induced by Ets-4 virus*[a]

Time after superinfection	Initial temp of incubation[b]		
	37 C	30 C	42 C
hr			
5	1.4	0.9	1.0
8	2.2	1.2	1.4
20	3.8	2.8	2.9

[a] Expressed as the log_{10} decrease of VEE virus in doubly infected cells compared to controls incubated with VEE virus alone.

[b] Cultures infected with Ets-4 virus were incubated at 37, 30, or 42 C for 3 hr prior to superinfection with VEE virus. Incubation was then resumed at 37 C for all cultures.

DISCUSSION

Several types of interference among viruses have been described (2) that do not involve the mediation of interferon. Except for a few reports (16, 19), all of the interference phenomena described required that the interfering virus be able to initiate certain synthetic processes in the host cell. To demonstrate the interference between arboviruses, it was necessary to give the interfering virus a growth advantage either by previous infection of the host cells or by using high multiplicities of the interfering virus relative to the challenge virus. The greater the growth advantage given to the interfering virus, the greater the degree of interference. The interference, however, did not result from a general deterioration of cell metabolism due to infection by the interfering virus. At least 40 hr of infection (at multiplicities greater than 1) with EEE or VEE viruses was required for the cells to show a cytopathic effect, even though peak titer was attained between 10 and 12 hr. Furthermore, in our hands these viruses cause little or no inhibition of total RNA or protein synthesis in monolayer cultures of CE cells for at least 12 hr after infection.

In contrast to the interference described by Pohjanpelto and Cooper (16), the presence of the virus genome in the cell without accompanying IRNA replication in our system was not sufficient to induce interference of challenge virus growth. When the growth of strain T was prevented by incubation at 42 C, the growth of the challenge virus (EEE) was not inhibited. It is known that the block in the growth of T virus occurs because synthesis of IRNA is inhibited at 42 C. These data therefore indicate that the interfering virus genome must be able to replicate IRNA in the cell in order to prevent the growth of the challenge virus.

The data above appear to support the hypothesis of Cords and Holland (5) that interference of challenge virus occurs because of competition for replication sites or substrate necessary for viral replication. This hypothesis was further strengthened by showing that Ets-4 was a better interfering virus than was EEE at 37 C, a temperature at which it produces three times as much viral RNA as does the parent EEE virus. At initial temperatures of incubation of 30 and 42 C, at which Ets-4 virus begins to produce viral RNA at rates ap-

proaching that of EEE virus, Ets-4 interferes to a lesser extent with VEE virus growth than at 37 C.

Interference of challenge virus was observed in one experiment in which superinfection was carried out with the IRNA of challenge virus instead of the virion, and in a second experiment in which the inhibition of IRNA synthesis of challenge virus after superinfection with the virion was evident. It seems reasonable to conclude, therefore, that interference probably occurred at some point after the entry and uncoating of the viral nucleic acid but before the initiation of IRNA synthesis. This conclusion is similar to that reported by Cords and Holland (5) for enteroviruses.

The evidence on the requirement for viral RNA synthesis to establish interference, and the inhibition of synthesis of IRNA of challenge virus, support the interference mechanism based on a competition for replication sites or metabolites. Interference of this type, although less directly stated or supported, has been described for arboviruses by Henderson and Taylor (9) and by P. T. Allen (*unpublished data*). Recent preliminary experiments indicate that various arboviruses can interfere with Newcastle disease virus and vesicular stomatitus virus, and vice versa, in the presence of actinomycin D. We have not, however, eliminated adsorption, penetration, or uncoating of challenge virus in these combinations, nor have we yet followed viral RNA of challenge virus in these systems. If, in fact, the actinomycin D-resistant interference is as broad as suggested above, the major hypotheses reviewed by Bratt and Rubin (2) as alternatives to the competition hypotheses would be eliminated because of the relative specificity required. Further experiments are needed, not only to determine how broad is the interference described here, but also to provide direct proof where possible of the competition hypotheses.

If the kind of interference described in the present paper is found to extend nonspecifically to unrelated viruses, then its role in vivo as a nonspecific mechanism of resistance to virus disease would have to be evaluated, particularly in relation to interferon-mediated interference. The latter is usually assumed to play a dominant role in most of the in vivo interference that has been described before and after the discovery of interferon.

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