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TECHNICAL MANUSCRIPT 477

INTERFERENCE AMONG GROUP A ARBOVIRUSES

Eugene Zebovitz
Arthur Brown

OCTOBER 1968

DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland

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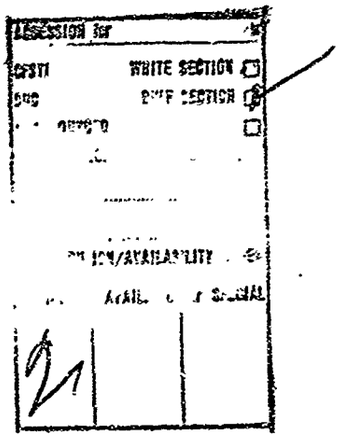

DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland 21701

TECHNICAL MANUSCRIPT 477

INTERFERENCE AMONG GROUP A ARBOVIRUSES

Eugene Zebovitz

Arthur Brown

Virus and Rickettsia Division
BIOLOGICAL SCIENCES LABORATORIES

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ABSTRACT

Interference among group A arboviruses that did not involve the mediation of interferon is described. 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 RNA of the challenge virus was significantly retarded. With temperature-sensitive viruses or mutants, the replication of viral RNA by the interfering virus was required to establish interference. A mechanism of interference based on a competition for replication sites or substrates was compared with other possible explanations.

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I. INTRODUCTION

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[1] and in animal viruses.[2] Those papers that appeared prior to the discovery of interferon[3] have been reviewed extensively by Henle[4] and by Schlesinger.[5] 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 the result of interferon production by the host or of other factors. It is apparent, however, that there are several types of viral interference that do not involve the mediation of interferon.[7-12]

The present report describes an interference not mediated by interferon among different arboviruses that requires that the interfering virus replicate viral RNA in the host cell before it can interfere with the growth of the challenge virus. Further, interference with the challenge virus appears to occur after its uncoating but before synthesis of infectious viral RNA.

II. MATERIALS AND METHODS

A. 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;* an attenuated variant (A) of VEE virus originally described by Berge, Banks, and Tigertt;[13] and a temperature-sensitive mutant (Ets-4) of the Louisiana strain of eastern equine encephalitis virus (EEE).[14,**] 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.[15] The special properties of Ets-4 pertinent to its use are described in Section III of this report.

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* Halle, S., personal communication.

** Unpublished data.

B. CELL CULTURES

Cell cultures were prepared from 10-day-old chick embryos. The chick embryo (CE) monolayers were grown in lactalbumin hydrolyzate medium containing 10% calf serum for 24 hours at 37 C before infection. Details of the preparation of monolayers, medium, and growth conditions were described in an earlier paper.[16]

C. 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 undiluted tissue culture fluids obtained from infected CE monolayers. The virus was allowed to adsorb for 15 minutes at room temperature; the cells were then washed twice with phosphate buffered saline (PBS) at pH 7.4 to remove excess unattached virus and overlayed with 5 ml lactalbumin hydrolyzate medium with 10% calf serum. The cultures were incubated at 37 C in a 5% CO_2 - 95% air incubator.

In those experiments where the interfering and challenge viruses were added simultaneously 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-minute adsorption period at room temperature, the infected cells were washed twice with PBS and overlayed with the lactalbumin hydrolyzate medium. The cultures were reincubated at 37 C and samples of the culture medium were collected at various intervals. In most of the experiments, as indicated in Section III, actinomycin D at 1 or 2 μg/ml was preincubated with cells for 0.5 hour before infection and was held at the same concentration throughout the experiment.

D. VIRUS ASSAY

Virus assays were performed on 24-hour CE monolayers prepared from 10-day-old chick embryos. The overlay medium was lactalbumin hydrolyzate agar described previously.[17] In those studies involving mixed infection with VEE and EEE viruses, titers in the supernatant 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, 100- to 200-fold excesses 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.

E. EXTRACTION AND ASSAY OF INFECTIOUS RIBONUCLEIC ACID

Infected CE monolayers were removed with a rubber policeman and were suspended in 0.02 M phosphate - 0.001 M ethylenediaminetetraacetic acid (EDTA) buffer, pH 7.4. These suspensions were extracted twice with cold (4 C) phenol, 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 infectious ribonucleic acid (IRNA) was assayed on CE monolayers treated with 1.0 M NaCl in a 0.1 M Tris-HCl buffer at pH 8.3 according to the method described by Colon and Idoine.[18]

III. RESULTS

A. DEMONSTRATION OF INTERFERENCE

Interference with 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 per cell, while the challenge virus was used at a 100-fold lower multiplicity.

In the case where equal multiplicities of the two viruses were employed, the degree of the 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. Maximum inhibition of the growth of EEE virus was observed when it was used to superinfect cells 5 to 6 hours after infection with T virus.

TABLE 1. EFFECT OF TIME OF SUPERINFECTION ON INTERFERENCE WITH CHALLENGE VIRUS

Time of Superinfection[a] with EEE Virus, hours	Growth Response of Challenge Virus (EEE)	
	24-Hour Virus Titers, pfu/ml[b]	Log_{10} Inhibition
0 (Control)[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 overlayed with culture medium. At the indicated time the medium was removed and superinfected with EEE virus at the same multiplicity; the cultures were then washed twice and overlayed with growth medium.

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

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

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 input MOI ranging from 1.0 to 0.01. The degree of interference increased progressively as the multiplicity of EEE virus decreased. Maximum interference was observed when the lowest multiplicity of challenge virus was used. In the absence of strain T, EEE virus grew normally and to high titer, and, although the data are not shown, there was no interference with T virus growth in such doubly infected cells.

TABLE 2. EFFECT OF MULTIPLICITY OF THE CHALLENGE VIRUS ON THE INTERFERENCE WITH CHALLENGE VIRUS

Multiplicity of Challenge Virus (EEE)[a]	Titer (24-Hour)	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-hour 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.

The data in Tables 1 and 2 show that 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 that of the challenge virus. 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.

B. INTERFERENCE INDUCED BY DIFFERENT STRAINS OF VEE VIRUS

Interference with 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 shows the average results of four experiments. The degree of inhibition of EEE virus induced by each virus strain varied. Among

VEE viruses, strain A was the most effective interfering virus, followed by the Trinidad strain and finally strains 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 virus genomes to attach to replication sites within the host cell and/or to differences in their rates or extent of viral RNA replication (see later discussion concerning Ets-4 virus).

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 Interference in Log_{10} Units of EEE Virus Titer at 20 Hours[b]
A	2.4[c]
Trinidad	1.6
T	1.4
V5	1.1

a. VEE virus strains at input MOI of 100 pfu/cell.
b. EEE virus used at input MOI of 1 pfu/cell.
c. Values are averages of four experiments.

C. 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.[19,20] If interferon were involved in the interference observed here, the challenge virus should be able to multiply normally in the presence of actinomycin D. Table 4 shows that actinomycin D (1 μg/ml), when added 2 hours prior to infection, had no effect upon the interference with EEE virus in cells that had been previously infected with a high multiplicity of strain T virus. 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 inhibition of growth of vaccinia virus 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 hours apart.

TABLE 4. EFFECT OF ACTINOMYCIN D UPON INTERFERENCE WITH EEE VIRUS MULTIPLICATION BY STRAIN T[a/]

Time, hours	Plaque-Forming Units/ml of EEE Virus		
	Strain T + EEE Virus	Strain T + EEE Virus + Actinomycin D[a/]	EEE Virus Alone
0	1.2×10^{5}[b/]	1.2×10^{5}[b/]	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 added at 1.0 μg/ml 2 hours before infection.
B. Infection by strain T followed by EEE 3 hours later at equal multiplicities (100).

D. IRNA SYNTHESIS BY THE CHALLENGE VIRUS

The interference with EEE virus growth that resulted when strain T was inoculated onto CE cells 4 hours earlier is shown in Figure 1. The EEE virus titer was reduced 2.7 $\log_{10}$ below that observed for the control culture. Superimposed on this curve are two curves for the synthesis of IRNA of the challenge virus. In doubly infected cells the synthesis of 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 the opportunity to synthesize its IRNA. There appears to be no obvious preferential interference between IRNA synthesis and viral structural protein synthesis; otherwise, the degrees of the two inhibitions would be expected to vary more significantly than was found.

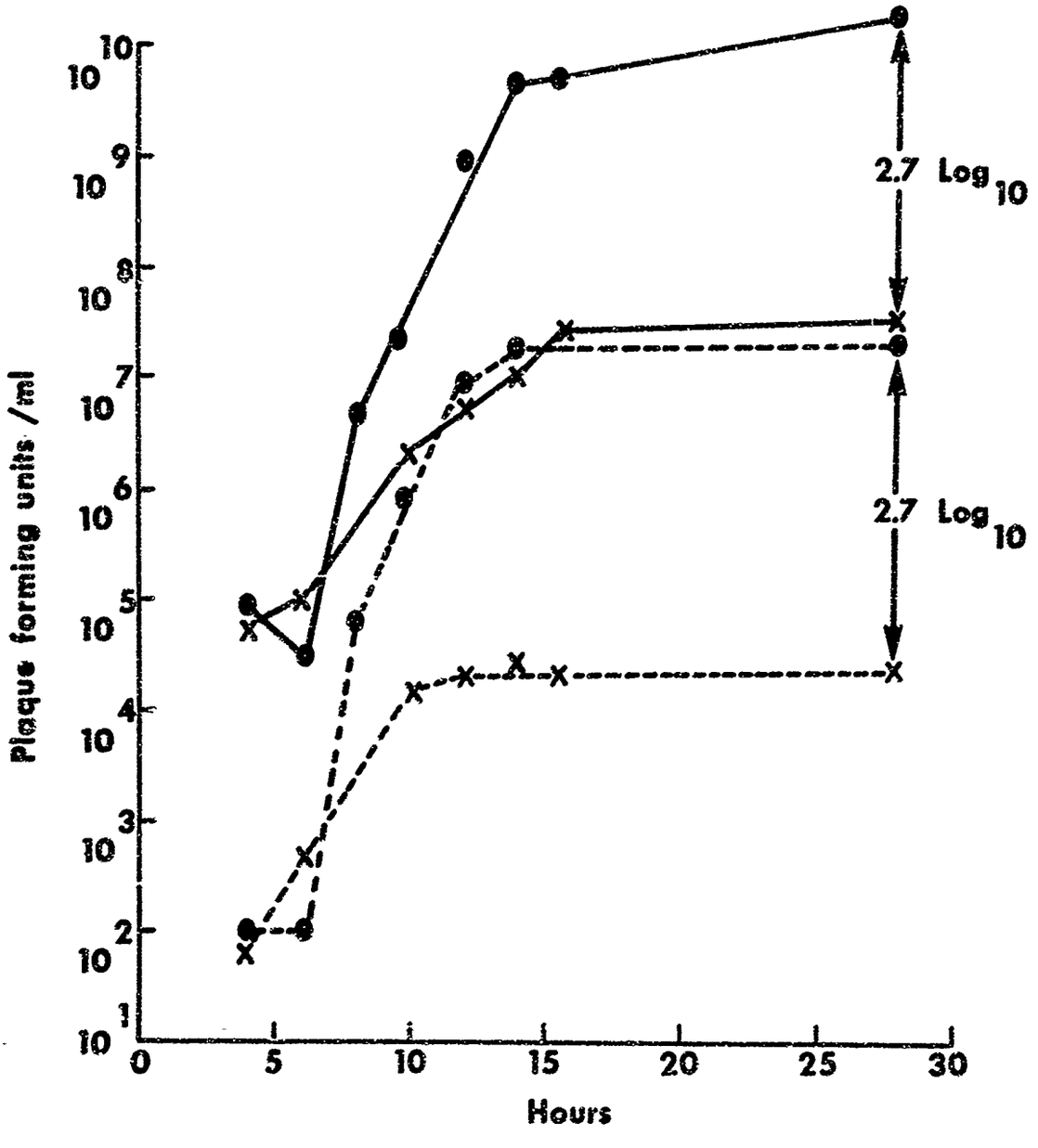

Figure 1. Interference with Challenge Virus (EEE) Growth and IRNA Synthesis by Strain T Virus. Viruses infected at equal MOI (10 pfu/ml). Challenge virus was added to culture 3 hours after infection with strain T virus. Symbols: ●—●, singly infected EEE virus titer; X—X, doubly infected EEE virus titer; ●--●, singly infected EEE IRNA; X--X, doubly infected EEE IRNA.

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 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 using the T strain as interfering virus at 42 C.

In previous studies,[16] the RNA of VEE virus entered the cell and was maintained in a viable state even for prolonged periods at 44 C, although no new RNA was synthesized. The same proved true for T virus at 42 C.* 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 hours before superinfecting with EEE virus and incubating further at 42 C. Thus, 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 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 C or 42 C, Ets-4 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 with the parent, but produced 90% less infectious virus and at least 50% less complement-fixing antigen.[14],** Because this mutant produces larger amounts of viral RNA than 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. The results presented in Table 5 show that Ets-4 is indeed a much better interfering virus than its parent when VEE is used as a challenge virus. From doubly infected cells, VEE virus was counted in the presence of a 1:100 dilution of anti-EEE serum (titer was 1:100,000). The interfering virus in this experiment was incubated at 37 C for 3 hours before challenge virus was added. If the initial 3-hour incubation was carried out at 30 C or 42 C, where the rate and extent of viral RNA synthesis of Ets-4 was depressed to levels closer to those found in the parent virus, the degree of interference was likewise reduced (Table 6).

* Zebovitz, E.; Brown, A. Unpublished data.
** Unpublished data.

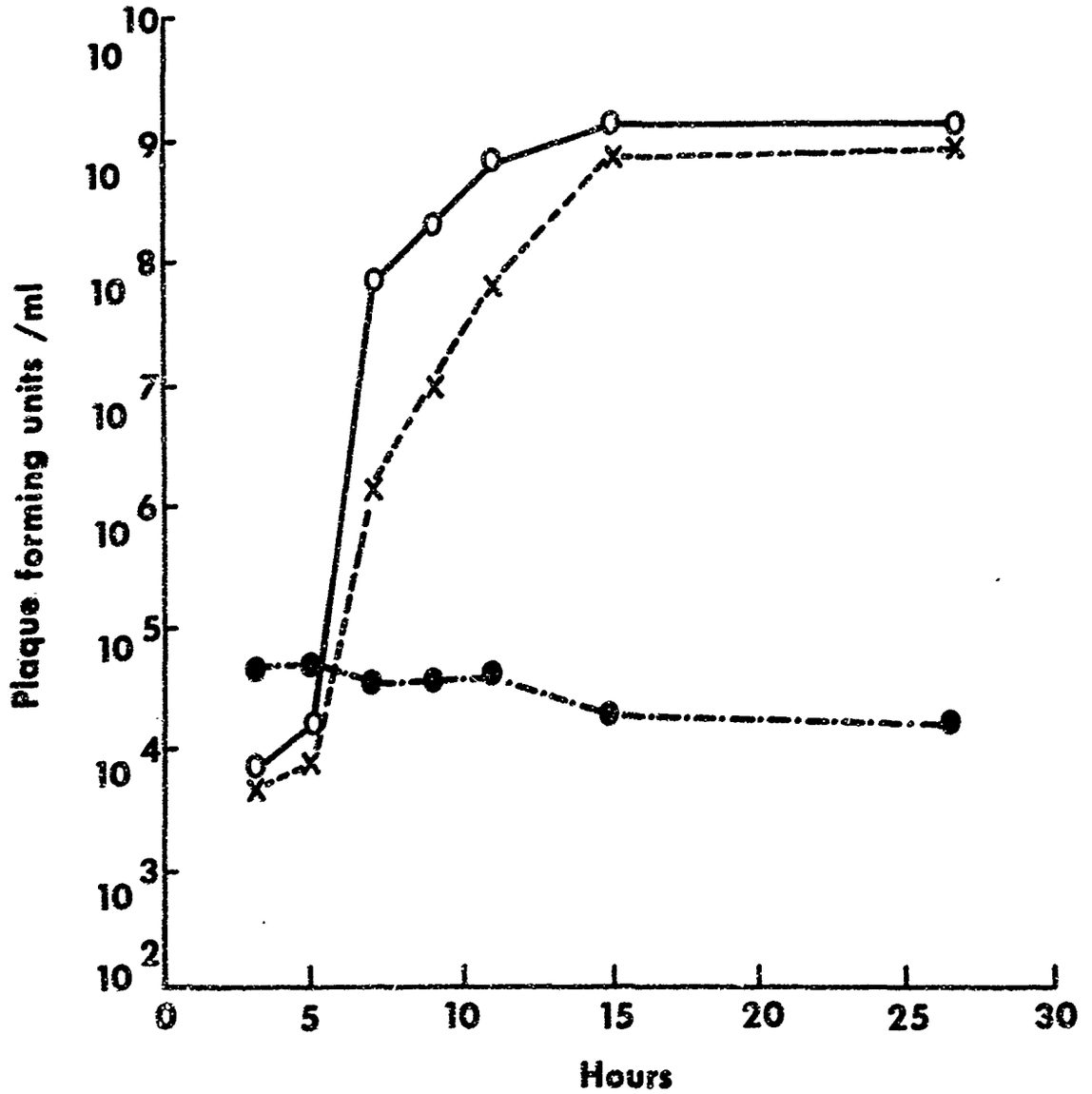

Figure 2. Effect of Incubation at 42 C Upon the Capacity of Strain T Virus to Interfere with EEE Virus Growth. Challenge virus (EEE) added 3 hours after infection of CE cells with Strain T. Symbols: O—O, EEE virus growth in absence of strain T; X--X, EEE virus growth on CE cells infected with strain T. ●--●, strain T virus growth in absence of EEE virus.

It was not reduced to the level induced by EEE because, when incubation was resumed at 37 C after only 3 hours at either of the other temperatures, Ets-4 still had an advantage in the extent of viral RNA synthesis.

TABLE 5. COMPARISON OF Ets-4 AND EEE AS INTERFERING VIRUS

Hours After Superinfection[a]	Control VEE, pfu/ml	EEE + VEE		Ets-4 + VEE	
		pfu/ml	Log_{10} Inhibition	pfu/ml	Log_{10} Inhibition
0	7.1×10^4	6.0×0^4		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 hours apart.

TABLE 6. EFFECT OF INITIAL TEMPERATURE OF INCUBATION ON INTERFERENCE INDUCED BY Ets-4 VIRUS

Hours After Superinfection	Degree of Interference with VEE[a] / Initial Temperature of Incubation[b]		
	37 C	30 C	42 C
5	1.4	0.9	1.0
8	2.2	1.2	1.4
20	3.8	2.8	2.9

a. Log_{10} decrease of VEE virus titer in doubly infected cells compared with controls incubated with VEE virus alone.

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

IV. DISCUSSION

Several types of interference among viruses have been described (reviewed by Bratt and Rubin[21]) that do not involve the mediation of interferon. Except for a few reports (e.g., Pohjanpelto and Cooper,[11] Steck and Rubin[22]) 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 those of the challenge virus. The greater the growth advantage given to the interfering virus, the greater was 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 hours of infection (at multiplicities greater than 1) with EEE or VEE viruses were required for the cells to show a cytopathic effect, even though peak titer was attained between 10 and 12 hours. Furthermore, in our laboratory these viruses cause little or no inhibition of host-cell RNA or protein synthesis in monolayer cultures of CE cells for at least 12 hours after infection.

In contrast to the interference described by Pohjanpelto and Cooper,[11] the presence of the virus genome in the cell without accompanying IRNA replication was not sufficient to induce interference of challenge virus growth in our system. 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[10] 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 EEE at 37 C, a temperature at which it produces three times as much viral RNA as the parent EEE virus. At initial temperatures of incubation of 30 C and 42 C, where Ets-4 virus begins to produce viral RNA at rates approaching that of EEE virus,[14,*] Ets-4 interferes to a lesser extent with VEE virus than at 37 C.

* Unpublished data.

Interference of challenge virus was observed in one experiment where superinfection was carried out with the IRNA of challenge virus instead of the virion, and in a second experiment where 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[10] for enteroviruses.

The evidence on the requirement for viral RNA synthesis to establish interference on one hand, and the inhibition of synthesis of IRNA of challenge virus on the other, supports the explanation that the interference mechanism is 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[23] and by Allen.[24] Recent preliminary experiments indicate that various arboviruses interfere with Newcastle disease virus and vesicular stomatitis 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 broad as suggested above, the major hypotheses reviewed by Bratt and Rubin[21] 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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	Department of the Army Fort Detrick, Frederick, Maryland, 21701

13. ABSTRACT

Interference among group A arboviruses that did not involve the mediation of interferon is described. 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 RNA of the challenge virus was significantly retarded. With temperature-sensitive viruses or mutants, the replication of viral RNA by the interfering virus was required to establish interference. A mechanism of interference based on a competition for replication sites or substrates was compared with other possible explanations. ( )

14. Key Words

Arboviruses, group A
Venezuelan equine encephalitis virus
Eastern equine encephalitis virus
Interference
Ribonucleic acid

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