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Induction of Interferon by Nonreplicating Single-Stranded RNA Virus[1] (37824)

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The event(s) during viral infection which is the stimulus for cellular production of interferon has not been determined finally. Also undetermined is whether the viral stimulus or inducer is the same for all viral infections. Isaacs *et al.* (1, 2) presented evidence that viral or nonviral nucleic acids could be a stimulus. The finding by Field *et al.* (3) that double-stranded ribonucleic acids (RNA) were the most effective among the isolated nucleic-acid inducers raised the possibility that double-stranded viral RNA is the common stimulating factor (4). On the other hand, isolated single-stranded nucleic acid can be stimulatory (5, 6), and it may be one of the viral stimulatory substances. Additional stimulating and controlling mechanisms are likely since the production of intracellular viral nucleic acids is sometimes inadequate to act as a stimulus (7).

In a previous study (8), mouse L cells stimulated with the single-stranded RNA virus, Newcastle disease virus (NDV), produced messenger RNA for interferon during inhibition of protein synthesis and in the absence of formation of detectable viral double-stranded RNA. Since there was no detectable synthesis of viral RNAs, it was suggested that a component of the input NDV was the stimulus for interferon production. However, the subsequent demonstration of a small quantity of RNA transcriptase associated with NDV virions (9) raised the possibility that undetectable amounts of double-stranded NDV RNA could have been produced by the input virion and thus have stimulated interferon. To eliminate this possibility, we repeated the study using a stimulating virus (chikungunya virus, group A arbovirus) which does not contain a virion-associated RNA transcriptase (10). The findings again indicate that some fraction of the input virus can stimulate interferon production.

Materials and Methods. Cycloheximide was used at a final concentration of 100 μg/ml and DL-*p*-fluorophenylalanine (FPA) at 5 μg/ml. These drugs were purchased from Sigma Chemical Co. Their activity and/or reversibility was controlled by measuring their effect on the incorporation of labeled precursors. Actinomycin D was used at a concentration of 10 μg/ml.

Chikungunya virus, grown in the brains of newborn mice or in BHK-21 cells, was used as the inducer of interferon at an input multiplicity of 50 ID_{50}/cell unless otherwise specified. This multiplicity of infection was obtained by diluting the 10% mouse brain pool of virus 1 to 3. Primary or passaged cultures of rat embryo (RE) cells were prepared by standard methods. Culture medium for experiments was Eagle's medium plus 2% fetal bovine serum. Interferon was assayed by the single-cycle Sindbis virus yield reduction assay as previously described (11). Interferon samples were maintained at pH 2 for 24–48 hr before assay to inactivate virus. In every experiment, an additional aliquot of the virus which was used as inducer was maintained at pH 2 and tested to ensure

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its loss of interfering activity after acidification. The absence of interference eliminated the possible presence of residual interfering virus, interferon activity, or extraneous interferon-inducing nucleic acids in the preparation of chikungunya virus.

Purification of chikungunya virus was performed as previously described (12) for the experiments in which viral RNA was extracted. Chikungunya virus was titrated by the CPE endpoint method in BHK-21 cells and the titers expressed as $TCID_{50}$.

Results. Induction of mRNA for interferon by chikungunya virus during inhibition of protein synthesis. The experimental design was to permit production of mRNA for interferon in rat embryo cells induced with chikungunya virus during inhibition of protein synthesis (8). In this way it could be determined whether interferon mRNA could be induced during the time that viral replication and synthesis of viral components were blocked by the inhibition of formation of viral specific proteins such as RNA polymerase. Rat embryo cells were pretreated with cycloheximide for 30 min and then stimulated with chikungunya virus. The block to protein synthesis was maintained by the presence of cycloheximide and FPA. After 4.5 hr at 37°, actinomycin D was added to inhibit any further transcription of mRNA for interferon. After incubation for another hour, the cultures were washed 4 times (to reverse the inhibition of protein synthesis by cycloheximide and FPA but not the inhibition of transcription of RNA by actinomycin D) and refed with inhibitor-free medium. After overnight incubation, the culture fluids were harvested and assayed for interferon content.

As was previously found in mouse cells (8), protein synthesis (as measured by incorporation of ^{14}C-proline) in the rat cells was inhibited $> 98\%$ but RNA synthesis was unaffected (as measured by incorporation of ^{3}H-uridine) during the 5.5 hr of treatment with cycloheximide and FPA. After removal of cycloheximide and FPA, protein synthesis resumed fully within 0.5 hr, indicating the reversibility of the inhibition of protein synthesis. In comparison, transcription was irreversibly blocked (96% decreased) by the added actinomycin D. Additional controls for inhibitor effectiveness demonstrated that rat cell cultures which were stimulated with chikungunya virus in the continued presence of cycloheximide and FPA did not produce the interferon protein when tested at 5.5 hr or 20 hr. Cultures treated with actinomycin D for 1 hr and then washed before stimulation with chikungunya virus did not produce any interferon, indicating the irreversibility of this inhibition of transcription.

Table I and Fig. 1 show the yield of interferon in rat embryo cultures which were stimulated with chikungunya virus in the presence or absence of the metabolic inhibitors. It may be seen that full interferon production occurs under the conditions of sequential metabolic inhibition. This finding indicates that the mRNA for interferon is stimulated by chikungunya virus during inhibition of protein synthesis since the mRNA could not have been transcribed after the later addition of actinomycin D.

To determine whether a lower multiplicity of infection would give a similar result, the experiments were repeated using an input multiplicity of 5. Again, interferon production occurred (150 U/ml) under the conditions of the metabolic inhibition, but it was somewhat reduced as compared with an input multiplicity of 50.

Examination for possible replication of chikungunya virus or its RNA during inhibition of protein synthesis. Experiments were done to determine if synthesis of virus

TABLE I. Interferon Production by Rat Embryo Cells Stimulated with Chikungunya Virus During Inhibition of Protein Synthesis.

Treatment of cultures before addition of actinomycin D at 4.5 hr	Interferon production (U/ml)		
	Experiment		
	1	2	3
Chikungunya virus[a]	320	320	1000
Cycloheximide + Chikungunya virus + FPA[b]	320	320	1000

[a] Multiplicity of infection = 50.

[b] See text for details of sequential treatment.

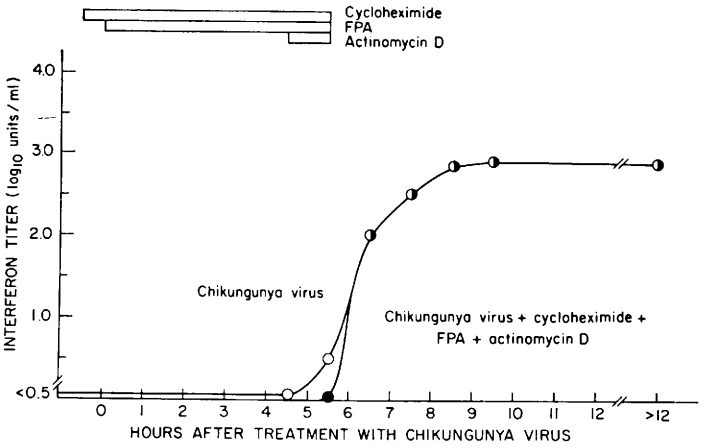

FIG. 1 Kinetics of interferon production in rat embryo cells induced with chikungunya virus in the presence or absence of inhibition of protein synthesis.

or double-stranded viral RNA had occurred in the inhibitor-treated cultures. Infectivity titrations demonstrated absence of virus replication in the presence of cycloheximide and FPA for 5.5 hr or 20 hr. Experiments were performed to determine whether partial replication of the viral RNA occurred under the experimental inhibitory conditions. Monolayers of rat embryo cells (10^8 cells/culture) were treated with 10 μg/ml actinomycin D for 1 hr to reduce the background of cellular RNA synthesis. The cultures were then infected with an input multiplicity of 50 PFU of chikungunya virus in the presence or absence of cycloheximide plus FPA. After 30 min of incubation, 20 μC/ml of tritiated uridine and 20 μC/ml of tritiated adenine (Amersham) were added. Five hours later, the medium was decanted and the cultures washed with cold phosphate-buffered saline containing cycloheximide. Then the cells were suspended with a rubber policeman, and the RNA was extracted through the use of phenol (13).

The amount of radioactivity incorporated into RNA from infected cells treated

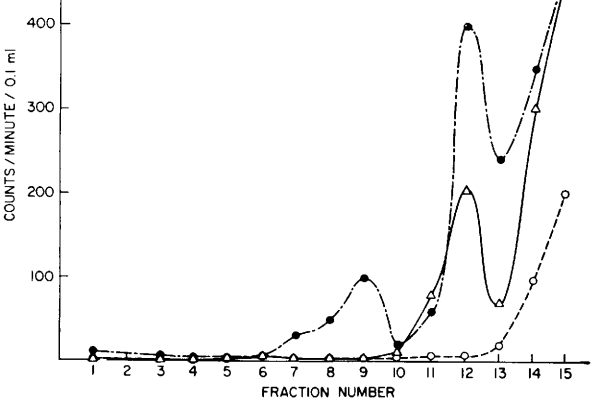

FIG. 2. Sucrose density gradient (15–30%) centrifugation of ^{3}H-labeled RNA extracted from (a) rat embryo cells pretreated with actinomycin D and infected with chikungunya virus in the presence (○- - -○) or absence (●- - -●) of inhibition of protein synthesis and (b) purified chikungunya virus (△- - -△).

with cycloheximide and FPA was less than 1/10 of that from infected cells not treated with these inhibitors. Analysis of both RNA preparations in a 15–30% sucrose gradient for 90 min (12) (Fig. 2) showed that labeled RNA from the infected cells treated with actinomycin D had two peaks as previously reported (12). In comparison, the radioactivity from infected cells treated with cycloheximide and FPA in addition to actinomycin D was found only at the top of the gradient. This finding confirms the prediction that inhibition of protein synthesis prevents detectable synthesis of chikungunya virus RNA.

Biological assay for the presence of double-stranded RNA. To test further the possibility that interferon was stimulated by amounts of double-stranded RNA which were undetectable by isotopic techniques, the extracted RNA from (a) infected cells treated with cycloheximide and FPA, (b) infected control cells, and (c) purified virions were tested for their ability to induce interferon. This experiment is based on the previous findings that isolated double-stranded RNA is a potent inducer of interferon (3) and isolated single-stranded RNA is a weak inducer (5). To digest single-stranded RNA but preserve double-stranded RNA, half of each RNA preparation (RNA extracted from 10^8 cells or from 10^{11} $TCID_{50}$ of virus) was treated with 2 μg/ml of purified RNase (Worthington) for 20 min at 37° in 0.15 *M* buffered saline. Two-tenths of a milliliter was inoculated into each of 2 tube cultures of rat embryo cells containing medium and 200 μg/ml DEAE-dextran to enhance the action of any active RNA (14). Twelve hours later, the culture fluids were harvested and assayed for interferon. The results are shown in Table II. It may be seen that neither the RNA from cells infected in the presence of cycloheximide and FPA nor the RNA from purified virions stimulated the production of interferon. This finding indicates that neither preparation contained sufficient quantities of double-stranded RNA to induce interferon.

TABLE II. Interferon-Inducing Activity of RNA Extracted from Cells Infected with Chikungunya Virus or from Purified Virions.[a]

Source of RNA	Treatment	Interferon production (U/ml)
Cells infected in the presence of inhibitors	None	<3
	RNase	<3
Cells infected in the absence of inhibitors	None	32
	Rnase	32
Purified virions	None	<3
	RNase	<3

[a] Cells were treated with 200 μg/ml of DEAE-dextran.

In comparison, the RNA from productively infected cells did stimulate the production of interferon (Table II). The expected finding of RNase-resistant (double-stranded) RNA in cells infected in the absence of inhibitors of protein synthesis (Table II) indicates that chikungunya virus does produce double-stranded RNA during its normal replicative cycle, and it validates the usefulness of this method to detect double-stranded RNA under the present experimental conditions.

Discussion. The present results indicate that even when the syntheses of detectable viral components are blocked by cycloheximide and FPA, full yields of interferon may be produced. It is reasonable to conclude that the input virion or some component of it can be an effective interferon inducer. If the effective component of the virus is RNA, the present data suggest that single-stranded RNA can be the effective inducer during some viral infections. Although single-stranded viral RNA may have some double-stranded regions or regions of homology with cellular nucleic acids, they would not be expected to stimulate as much interferon as the replicative form of RNA. The finding that nonreplicating virus can induce as much interferon as replicating virus argues against this possibility. Thus, it is possible that, in some cases, the input virion-associated single-stranded viral RNA may be as effective as replicated double-stranded RNA. The reason that isolated single-stranded RNA is a less effective stimulator than is isolated

double-stranded RNA may be its greater sensitivity to degradation by intracellular and extracellular RNases.

A recent study showing enhanced interferon production by chicken cells pretreated with interferon and then stimulated with chikungunya virus also indicated that nonreplicating chikungunya virus can induce interferon (15).

Although viral nucleic acid is the most likely virion component responsible for induction of interferon, other substances associated with the input virion could induce interferon. The small fraction of double-stranded RNA detected in some viral preparations (16) is not likely to have been the stimulus for interferon under the present conditions because (a) purified chikungunya virions contained no detectable double-stranded RNA when tested chemically and biologically, and (b) only concentrated preparations of virus have been reported to contain detectable quantities of double-stranded RNA (16). The present experiments employed as little as 5 PFU/cell (1 to 30 dilution) to obtain consistent yields of interferon—not nearly enough to contain sufficient double-stranded RNA to stimulate the cells.

The virion lipids and proteins are other possible stimuli under the present conditions. Lipid or protein is less likely than nucleic to be the inducing substance since those few lipids or proteins which stimulate interferon production *in vivo* are ineffective in the type of cell culture used in the present study (17, 18). There is not enough information available to assess the possible stimulating roles of viral attachment, penetration, uncoating events, or cellular enzymatic factions.

Summary. A study was undertaken to help determine whether the input virions of a nonreplicating single-stranded RNA virus could stimulate interferon production. Rat embryo cells were treated with inhibitors of protein synthesis during infection with chikungunya virus. After 4.5 hr incubation, RNA synthesis was inhibited with actinomycin D and 1 hr later the inhibition of protein synthesis was reversed by washing. Thereafter, the culture fluids were tested for production of interferon. Since not even partial replication of chikungunya virus or its components was demonstrable under the conditions of inhibition of protein synthesis, the finding of full yields of interferon indicated that a component of the input virion stimulated production of the interferon. The results of control experiments make it unlikely that contaminating double-stranded RNA was the stimulus for interferon production. Since viral and other nucleic acids are the most general inducers of interferon, and since the input chikungunya virion contains single-stranded RNA, it seems probable that at least certain single-stranded viral RNAs can stimulate interferon production.

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