Replication of Vesicular Stomatitis Virus: the Effect of Purified Interfering Component

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SUMMARY

High concentrations of interfering component added to BHK cells infected with the Indiana strain of vesicular stomatitis virus (VSV) inhibited the synthesis of virus and interfering component. The inhibition was at an early stage in virus replication. Lower concentrations of interfering component led to the formation of interfering component and depression of virus synthesis. When virus replication was depressed with interfering component prepared from a second strain of VSV (Brazil strain) the RNA of the Brazil interfering component was also replicated at the expense of the Indiana virus. The proteins of the progeny interfering component particles were of the Indiana virus type. It is proposed that interference can take place at different stages during infection, depending upon the amount of interfering component present.

INTRODUCTION

Cells infected at a high multiplicity with unfractionated vesicular stomatitis virus (VSV) produce low yields of infectious virus (Cooper & Bellett, 1959). Under these conditions, an excess of smaller non-infectious particles is synthesized which has been shown to inhibit the synthesis of infectious virus (Crick, Cartwright & Brown, 1966; Huang & Wagner, 1966; Hackett, Schaffer & Madin, 1967). The purified interfering component contains an RNA which is smaller than the virus RNA (Huang & Wagner, 1966; Brown et al. 1967) but has the same polypeptides (Kang & Prevec, 1969; Wagner, Schnaitman & Snyder, 1969) and is antigenically indistinguishable from the virus (Huang, Greenawalt & Wagner, 1966).

Huang & Baltimore (1970) have postulated that interfering component arises from a deletion mutant in the parent virus. Replication of this mutant would give rise to a smaller piece of nucleic acid which would be capable of combining with virus structural proteins to form a defective or interfering component. Once formed, the interfering component is incapable of self-replication (Crick, Cartwright & Brown, 1969). The problem arises therefore whether all the particles are synthesized via the replication of deletion mutants or the infecting interfering particles can use preformed enzymes for multiplication. Stampfer, Baltimore & Huang (1969) have suggested that the interfering component RNA can be replicated by the virus-induced polymerase. In such a system the interfering component RNA would be replicated at the expense of virus RNA, reducing the yields of infectious virus. In the present study, evidence is presented to show that interfering component RNA can replicate by such a mechanism.
METHODS

Viruses, interfering component and cells. The methods used for growth and titration of the Indiana strain of VSV in BHK cells have been described previously (Wild, 1971). The Indiana interfering component was prepared by infecting BHK cells with undiluted harvests of virus for three serial passages. The harvest from the third passage was mixed with an equal vol. of glycerol and kept at −20 °C as a stock. Passage of this stock virus at a multiplicity of approximately 1/100 gave a high yield of interfering component. The Brazil strain of the virus (Federer, Burrows & Brooksby, 1967) was passaged in BHK cells at low input multiplicity (0.01 p.f.u./cell) for virus production and for interfering component synthesis it was passaged once at an input multiplicity of approximately 50 p.f.u./cell.

Interfering component preparations were purified as follows. After an initial clarification the harvests were concentrated by precipitation with an equal volume of saturated ammonium sulphate and resuspended in 0.04 M-phosphate, pH 7.6. After a further clarification the suspensions were pelleted in the MSE angle-rotor no. 59595 at 20000 rev/min for 60 min. The pellets were resuspended in the minimum vol. of 0.04 M-phosphate, pH 7.6, and 2 ml samples centrifuged on 26 ml of 15 to 45% sucrose gradients in 0.04 M-phosphate, pH 7.6, at 20000 rev/min for 2 h in an SW 25.1 Spinco rotor. The fractions containing the interfering activity were diluted in 0.04 M-phosphate, pH 7.6, and recycled on a second sucrose gradient to reduce the amount of contaminating virus to less than 10^6 p.f.u./ml. The recycled material was then used in the experiments.

Measurement of interfering activity. Monolayers of BHK cells in 4 oz prescription bottles (approximately 10^7 cells/bottle) were co-infected with virus (input multiplicity 10 p.f.u./cell) and tenfold dilutions of the interfering component preparation. After 30 min at 37 °C the cells were washed with Eagle's medium and incubated for 6 h in 10 ml of the same medium. The yield of infectious virus was measured by the plaque assay method.

Examination of RNA. RNA was extracted from the various fractions by adjusting them to 0.1% SDS and extracting with water-saturated phenol. The RNA was precipitated from the aqueous phase with 2 vol. of ethanol at −20 °C. Samples of 1 ml of the resuspended RNA in 0.15 M-NaCl, 0.001 M-EDTA and 0.01 M-tris, pH 7.6, were centrifuged on 13 ml of a 5 to 25% sucrose gradient in 0.1 M-NaCl at 22000 rev/min for 16 h in the MSE swing-out rotor, no. 59108, at 4 °C. Gradients were fractionated into 0.5 ml or 0.75 ml fractions and acid-insoluble radioactivity determined by precipitation with an equal vol. of 10% TCA in the presence of carrier bovine serum albumin.

Density determinations. Samples of 0.2 ml were centrifuged on 4 ml potassium tartrate gradients (1.1 to 1.28 g/ml) at 30000 rev/min for 2 h in the SW 39 Spinco rotor. Density was determined by refractometry.

Complement-fixing activity. Quantitative estimations of complement-fixing activity were carried out by estimating the 50% haemolytic end-point at one level of complement, using the antigen dilution method. The results are expressed as the reciprocal of the dilution giving 50% haemolysis. Hyperimmune antisera against the Indiana and Brazil strains of VSV were prepared in guinea-pigs.

RESULTS

Effect of high concentrations of interfering component on virus synthesis

BHK cell monolayer cultures in 4 oz prescription bottles were infected at a multiplicity of 10 p.f.u./cell for 10 min at 37 °C with the Indiana strain of the virus. The cells were then incubated in the presence of 1 µg/ml actinomycin D and 2 µCi/ml [3H]-uridine. Purified
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Ten ml of purified interfering component at 10⁻² dilution were added to each 4 oz prescription bottle (approximately 10⁶ cells/bottle).

interfering component of the same strain of virus was added to replicate bottles of infected cells at hourly intervals. Six hours after infection, the cells and medium were separated and estimated for infectivity and TCA-insoluble radioactivity. Addition of this level of interfering component to cells in the first hour after infection inhibits virus synthesis by 98% or more and incorporation of radioactivity into the cell and supernatant fractions was reduced by at least 80% (Table 1). Addition of interfering component 3 h or more after infection did not reduce the yield of cell-associated infectious virus or incorporation of radioactivity into the cell and only slightly reduced the release of TCA-insoluble radioactivity into the medium.

**Effect of low concentration of interfering component on virus synthesis**

It was noticed that, when lower levels of interfering component were used to inhibit virus synthesis, the release of radioactively labelled material into the medium was as great as in controls in which no interfering component was added, but the yields of infectious virus were reduced. To investigate this phenomenon, BHK cells were infected with virus (input multiplicity 10 p.f.u./cell) and dilutions of purified interfering component, then incubated in 1 μg/ml actinomycin D and 4 μCi/ml [³H]-uridine. The events following the use of levels of interfering component which inhibited synthesis of infectious virus by less than 90% were investigated. Six hours after infection the medium and cells were separated and samples of the medium centrifuged on sucrose gradients. In the control sample (virus-infected only) 95% of the incorporated radioactivity was associated with the virus peak, whereas in the partially inhibited samples a second peak in the position of the interfering component was found (Fig. 1). That this fraction was interfering component and not ribonucleoprotein was confirmed by centrifuging in a potassium tartrate gradient (Fig. 2). It had a density of 1·135 g/ml compared with a density of 1·225 g/ml expected for ribonucleoprotein (Crick et al. 1966).

Although the total amount of [³H]-uridine incorporated into both virus and interfering component released from the cell is constant for the uninhibited and inhibited cultures, the distribution of radioactivity between the two fractions varies with the amount of interfering component used for co-infection of the cells. The synthesis of progeny interfering component is inversely proportional to the synthesis of virus. These experiments suggest that the interfering component and virus are competing for a common replication site such as a RNA polymerase.

### Table 1. Effect of time of addition of purified interfering component on yield of infective virus and incorporation of [³H]-uridine into TCA-insoluble material

<table>
<thead>
<tr>
<th>Time of addition of interfering component (h)</th>
<th>Infectivity at 6 h (p.f.u./ml)</th>
<th>Incorporation of [³H]-uridine into TCA precipitate (ct/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cells Medium</td>
</tr>
<tr>
<td>0</td>
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<td>2159 0</td>
</tr>
<tr>
<td>1</td>
<td>4·7 × 10⁶</td>
<td>2887 960</td>
</tr>
<tr>
<td>2</td>
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<td>9536 4305</td>
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</tr>
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<td>16563 4310</td>
</tr>
<tr>
<td>5</td>
<td>2·5 × 10⁶</td>
<td>15941 6000</td>
</tr>
<tr>
<td>No interfering component</td>
<td>2·4 × 10⁶</td>
<td>16047 6100</td>
</tr>
</tbody>
</table>
Fig. 1. Sucrose-gradient analysis of VSV (Indiana strain) grown with different concentrations of the same strain of interfering component and labelled with [3H]-uridine in the presence of 1 µg/ml actinomycin D. (a) No added interfering component; (b) 10^{-8} dilution of interfering component; (c) 5 × 10^{-4} dilution of interfering component; (d) 2 × 10^{-4} dilution of interfering component. Two ml samples of the medium taken 6 h after infection were centrifuged on 15 to 45% sucrose gradients at 20,000 rev/min for 2 h in the SW 25.1 Spinco rotor.

Fig. 2. Potassium tartrate density-gradient analysis of interfering component preparations isolated from Fig. 1. Fractions 15 +16 isolated from Fig. 1(b) and (c) were combined and centrifuged on a preformed potassium tartrate gradient (1.1 to 1.28 g/ml) at 30,000 rev/min for 2 h in the SW 39 Spinco rotor. The density is the continuous line.
Characterization of the interfering component of the Brazil strain of virus

In order to test the foregoing hypothesis, it is necessary to show that the ingoing strand of interfering component RNA is acting as a template for the synthesis of new RNAs and that the observed synthesis of interfering component (RNA) does not arise from the secondary effects, such as selective degradation of virus RNA. A method of testing this would be to inhibit the replication of the virus with an interfering component from another source and to examine the products. Interfering component from the Brazil strain of VSV inhibits the replication of the Indiana virus to the same extent as the homotype and has been found to...
Fig. 5. Sucrose-gradient analysis of VSV (Indiana) grown in the presence of different concentrations of Brazil interfering component and labelled with [3H]-uridine in the presence of 1 μg/ml actinomycin D. (a) No interfering component; (b) 10⁻¹ dilution of Brazil interfering component; (c) 2·5 × 10⁻² dilution of Brazil interfering component; (d) 6·25 × 10⁻³ dilution of Brazil interfering component; (e) 2·5 × 10⁻⁴ dilution of Brazil interfering component (not superinfected with virus); (f) 6·25 × 10⁻³ dilution of interfering component (not superinfected with virus). One ml samples of the medium removed 6 h after infection were added to purified [3P]-Indiana interfering component and centrifuged on 15 to 45% sucrose gradients at 22000 rev/min for 2 h in the MSE swing-out rotor, no. 59108. Fractions (0·75 ml) were analysed. ○——○, [3H]-products of heterotypic interference.

have an RNA with a different sedimentation coefficient (23 S) from the Indiana interfering component RNA (19 S; F. Brown, J. F. E. Newman & J. Crick, personal communication).

BHK cell monolayer cultures were infected at an input multiplicity of 50 p.f.u./cell with the Brazil strain of virus and incubated with 5 μCi/ml [3H]-uridine. The harvest was concentrated with ammonium sulphate and centrifuged on a sucrose gradient (see Methods). Three peaks could be distinguished in the sucrose gradient (Fig. 3). The fastest sedimenting peak (A) co-sedimented with Indiana virus particles. Examination in the electron microscope showed that the second peak (B) contained mainly truncated-type particles, whereas peak (C) contained mainly ribonucleoprotein. Assays on peaks B and C for interfering activity against the Indiana virus showed that it was confined to peak B. Fig. 3 also shows a parallel experiment with the Indiana strain of virus passed at high multiplicity and processed similarly. The interfering component of the Brazil strain sedimented faster than the Indiana strain.
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Fig. 6. Sucrose-gradient analysis of RNA extracted from the products of heterotypic interference. The medium was removed from BHK cells co-infected with the Indiana strain of virus and (a) the $2.5 \times 10^{-2}$ dilution of Brazil interfering component and (b) the $6.25 \times 10^{-2}$ dilution of Brazil interfering component concentrated by ultracentrifugation. The RNA was extracted from the pellets, mixed with $[\alpha^2P]$-RNA extracted from Indiana virus passaged at high multiplicity (processed similarly) and centrifuged on 5 to 25% sucrose gradients at 22000 rev/min for 16 h in the MSE swing-out rotor, no. 59108. •—•, $[\beta^H]$-RNA; ○—○, $[\alpha^2P]$-Indiana interfering component RNA.

The RNA was extracted from both strains of interfering component with 0.1% SDS mixed with $[\beta^P]$-BHK cell RNA and centrifuged in a sucrose gradient. The Indiana RNA had a sedimentation value of 19 S compared with 28 S and 18 S for BHK ribosomal RNAs (Brown et al. 1967), whereas the RNA from the Brazil interfering component sedimented at 23 S with a minor peak at 14 S (Fig. 4). The relationship between the two RNAs is not yet understood. The analysis of the Brazil interfering component showed it to be sufficiently different from the corresponding Indiana particle to be suitable to test the hypothesis.

Superinfection of Indiana virus infected cells with Brazil interfering component

BHK cells in monolayer culture in 20 oz prescription bottles were infected with the Indiana strain of virus (input multiplicity 10 p.f.u./cell) and fourfold dilutions of a purified Brazil interfering component preparation. The cells were then incubated in medium containing 1 $\mu$g/ml actinomycin D and 2.5 $\mu$Ci/ml $[\beta^H]$-uridine. Six hours after infection the
medium was removed and 1 ml samples together with a marker of $[^{32}\text{P}]$-Indiana interfering component were analysed on sucrose gradients (Fig. 5). At the highest concentration of interfering component the yield of infectious virus was 1% of the control and there was no detectable radioactive product. This agreed with the results obtained with the homotypic interference studies. Diluting the interfering component 4- and 16-fold reduced the infectious virus yield to 20% and 40%, respectively, but permitted synthesis of radioactively labelled material to the levels of 30% and 107% of the uninhibited controls. In both these preparations two peaks were separated on sucrose gradients, one corresponding to the virus and the other sedimenting in the position of the Brazil interfering component. The amount of $[^{3}\text{H}]$-interfering component synthesized was proportional to the concentration of Brazil interfering component used for inoculating the cells.

The media from the harvests containing interfering component (Fig. 5c, d) were centrifuged at 20000 rev/min for 60 min in the MSE angle rotor, no. 59595. The pelleted material was resuspended in 0.15 M-NaCl, 0.001 M-EDTA and 0.01 M-tris, pH 7.6, and the RNA extracted with 0.1% SDS phenol. $[^{32}\text{P}]$-RNA extracted from the Indiana strain passaged at high multiplicity and processed in a similar manner was added as marker and the mixtures were centrifuged on sucrose gradients (Fig. 6). The RNA extracted from both interference samples sedimented at 23 S, compared with 19 S for the Indiana RNA marker. The results show that superinfection of Indiana virus infected cells with Brazil interfering component leads to the replication of the Brazil interfering component RNA.
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Identification of the interfering component structural proteins formed in heterotypic interference

Interfering component has been shown to have little or no transcriptase activity (Bishop & Roy, 1971), therefore its genetic information would be expected to be redundant. Genetic studies on VSV (Pringle, 1970) would support this point of view. If this is the case, then the 23 S RNA found in the truncated particles in the above experiment would be expected to have Indiana coat proteins.

To investigate this possibility the previous experiment was repeated, using cells grown in Roux bottles (approximately 10⁸ cells/bottle). The supernatant fluids harvested 6 h after infection were concentrated by centrifuging at 20000 rev/min for 1 h in the MSE angle rotor, no. 59595. The pellets were each resuspended in 2 ml of 0.04 M-phosphate, pH 7.6, and centrifuged on sucrose gradients. Gradients were analysed for complement-fixing activity against Indiana and Brazil antisera which were type-specific in this test (Cartwright & Brown, 1972). Fig. 7 shows the result of one of the gradients in which interfering component and virus synthesis occurred. There was no complement-fixing activity with Brazil antiserum, using the highest concentration of antigen (0.2 ml/tube) permissible, but the interfering component fraction was as antigenic as the virus when the Indiana antiserum was used. It can be concluded, therefore, that the 23 S Brazil RNA was encapsulated by proteins coded for by the Indiana virus.

DISCUSSION

Concentrations of interfering component which suppressed infectious virus synthesis by 99% or more also inhibited the synthesis of interfering component to a similar extent. Addition of this level of interfering component at intervals after infection confirmed Huang & Wagner's (1966) observation that the interference was primarily concerned with an early event in the replicative cycle. Under these conditions there was also a reduction in the poly-somes formed (Wild, unpublished observation) which in turn would reflect in the amount of protein and polymerase available.

Under the conditions described, the absence of interfering component synthesis posed the question of the origin of interfering component and its mode of replication. Virus which had been plaque-purified free of interfering component still gave rise to interfering component particles on serial passage at high multiplicity (Crick et al. 1969; Stampfer, Baltimore & Huang, 1971). Although the possibility of contamination of the original clones with interfering particles cannot be completely eliminated, it was strong evidence that virus can give rise directly to interfering particles.

Crick et al. (1969) showed that purified interfering component particles were unable to self-replicate. Therefore, in the present study, this problem has been approached by co-infecting cells with virus and interfering component at sufficient levels to allow a limited synthesis of virus.

Addition of interfering component in amounts which reduced infectivity yields less than 90% directed the synthesis of progeny particles from that of virus to interfering type. Prevec & Kang (1970) came to a similar conclusion when superinfecting cells with 'long' and 'short' interfering components. This would suggest that the interference was acting either by causing selective degradation of the particles or by competing with the virus for the replicative apparatus. To distinguish between these two possibilities it was necessary to use an interfering component which could be readily differentiated from the homotype. When Brazil interfering particle, which could be distinguished from the Indiana strain on the
basis of its size and the sedimentation coefficient of its RNA, was used to inhibit the replication of the Indiana virus, the progeny particles contained the RNA of the Brazil strain and the coat proteins of the Indiana strain. Therefore, the present experiments suggested that the RNA of the interfering particles was competing with virus RNA for replicative sites, presumably the polymerase. Such a mechanism has been proposed by Stampfer et al. (1969).

It was not surprising that the Brazil RNA from these experiments was encapsulated in Indiana proteins. Interfering component preparations contained little or no transcriptase activity (Bishop & Roy, 1971) and so any genetic information contained in the particles would be redundant. This latter observation was confirmed by the inability of interfering component to complement ts mutants of the virus (Pringle, 1970).

It appeared that interference of VSV virus replication could take place at two levels according to the number of interfering particles present. If a sufficient quantity of the particles was available, it could act on an early event. If the concentration of interfering component was sufficient to allow a limited amount of virus replication and hence synthesis of virus proteins and enzymes, it could compete with the virus RNA for these entities.

In multicyle infections, such as will occur in VSV-infected animals, initially small amounts of interfering component can be replicated at the expense of virus RNA. Eventually the level of interfering component would reach a concentration which inhibited an early event.

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REFERENCES


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