Effect of Interferon on the Replication of Sendai Virus

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SUMMARY

The incorporation of [3H]uridine into the four species of RNA specified by Sendai virus in monolayers of chick embryo fibroblasts was inhibited to an equal degree by addition of purified chick interferon to cultures before infection with virus. The inhibition of RNA synthesis was dose-dependent. Similarly, [3H]uridine incorporation into RNA of virus nucleocapsid and polyribosomes was completely inhibited by pretreatment with interferon. When interferon was added 2 hr after infection, there were only small effects on the synthesis of total virus-specific RNA and significantly greater reduction was observed in [3H]uridine incorporation into the RNA of nucleocapsid and polyribosomes. Although Sendai infection does not interfere with host-cell protein or RNA synthesis, interferon added 6 hr or later after infection did not affect any Sendai replicative functions.

INTRODUCTION

Interferon inhibits the synthesis both of infectious virus RNA (Wagner, 1961; De Somer et al. 1962; Lockart, Sreevalsan & Horn, 1962; Gordon et al. 1966; Mécs et al. 1967) and of species of virus-specific RNA detected by labelling with radioactive RNA precursors (Friedman & Sonnabend, 1965; Gordon et al. 1966; Mécs et al. 1967) in cells infected with several small RNA viruses. It also prevents the appearance not only of the virus RNA polymerase in cells infected with Mengo virus (Miner, Ray & Simon, 1966) or Semliki Forest virus (Sonnabend et al. 1967) but also other virus-specific proteins in Semliki Forest virus infection (Friedman, 1968). DNA polymerase production and virus DNA production in cells infected with vaccinia virus, a large DNA virus, are also inhibited by interferon (Joklik & Merigan, 1966; Levine et al. 1967).

Interferon thus probably blocks virus replication at a step between virus penetration of cells and synthesis of virus-specified proteins. Attempts to delineate further the specific action of interferon have yielded contradictory results. Marcus & Salb (1966) and Levy & Carter (1968) reported evidence for a functional defect in the ribosomes of interferon-treated cells which prevented specific translation of virus messenger RNA. More recently, however, Kerr, Sonnabend & Martin (1970) were unable to show any alteration in protein synthetic activity of ribosomes from interferon treated cells and several investigators have failed to find structural changes in ribosomes from interferon-treated cells (personal communications from G. Bodo and E. M. Martin & J. A. Sonnabend).

Because the primary action of interferon remains unclear, we undertook this study of the effects of a purified preparation of chick interferon on the replication of Sendai virus (Parainfluenza I), a large RNA virus which continues to replicate in chick embryo cells for
many hours without greatly inhibiting host protein and RNA synthesis (Blair & Robinson, 1968, personal communication). The long replicative cycle of Sendai virus with minimum interruption of host-cell function permitted us to test the effect of interferon, added after virus replication had begun, on several specific steps in virus replication.

**METHODS**

*Materials.* 5-[³H]uridine (25·8 c/mmole) was obtained from Schwarz BioResearch, Inc., Orangeberg, New York and crude yeast RNA was from Sigma Chemical Co., St Louis, Missouri. Pharmaceutical Actinomycin D (Lyovac® Cosmegan®–Merck, Sharpe and Dohme) was employed.

*Buffers.* Isotonic buffer contained 0·15 m-NaCl, 0·01 m-tris (pH 7·5), 0·001 m-MgCl₂. Hypotonic buffer contained 0·01 m-NaCl, 0·01 m-tris (pH 7·5), 0·001 m-MgCl₂. RNA buffer contained 0·01 m-NaCl, 0·01 m-tris (pH 7·5), 0·001 m-EDTA.

*Virus.* The HARRIS strain of Sendai virus was grown in chick embryos, as previously described (Blair & Robinson, 1968). The virus stock gave a positive haemagglutination test at a dilution of 1/400 (Blair & Robinson, 1970).

*Cell culture and media.* Medium 199 + tryptose phosphate broth + calf serum (85:10:5) was employed as culture medium throughout these studies. Chick embryo fibroblast secondary cultures were started with 6 × 10⁶ cells/100 mm. plastic Petri dish. After 1 day, when monolayers were confluent, they were infected with 3 ml. of a 1/5 dilution of virus stock in culture medium and after incubation at 37 ° for 2 hr, the virus inoculum was replaced with 6 ml. of medium. Eighteen hr after infection, the incubation medium was frozen for virus assay.

*Interferon treatment of cells.* Monolayers were exposed to the specified concentration of chick interferon in 6 ml. of medium 199 for 12 hr.

*Sendai virus RNA labelling, preparation and fractionation.* Eighteen hr after infection, 6 ml. of medium containing 10 µg./ml. actinomycin D were incubated with the cells for 30 min. and were replaced with 4 ml. of medium containing 0·1 mc ³H]uridine. Twenty hr after infection, three monolayers were washed three times with cold isotonic buffer, detached by swirling in 4 ml. 0·1 m-Na acetate with 0·5 % sodium dodecyl sulphate (pH 5·1), and extracted three times with equal volumes cold washed phenol. The aqueous phase was twice precipitated with three volumes absolute ethanol and redissolved in 0·3 ml. RNA buffer. One half of this material was layered on a 13 ml. 5 to 20 % (w/v) linear sucrose density gradient and centrifuged in a SW 4 ° rotor for 250 min. at 40,000 rev./min. at 4 °. Fractions were collected from the bottom of the tube, their extinction measured in microcuvettes in a Zeiss spectrophotometer at 260 mm. and then precipitated and their radioactivity counted. The E₂₆₀ values for all gradients were virtually the same.

*Sendai polyribosome and nucleocapsid preparation and fractionation.* For each gradient, four chick embryo fibroblast monolayers in 10 cm. Petri dishes were used. At the indicated time after infection, 5 ml. of medium containing 5 µg./ml. actinomycin D were placed on the cultures for 20 min. and then replaced with 4 ml. of medium containing 100 µc. [³H]uridine for 90 min. The monolayers were then washed once with isotonic buffer, covered for 4 min. with cold hypotonic buffer, scraped off with an amber rubber stopper and disrupted in a tight-fitting Dounce homogenizer with 12 strokes. The material was centrifuged in an International centrifuge at 4 ° for 6 min. at 2000 rev./min. The supernatant fluid was removed and divided into two equal parts. One was placed on a 5 to 20 % (w/v) linear sucrose density gradient in isotonic buffer. To the other, EDTA was added to a final
Interferon and Sendai virus replication

concentration of 0.01 M. This material was placed on a 5 to 20% sucrose density gradient in RNA buffer. The gradients were centrifuged in an SW40 rotor at 40,000 rev./min. at 4°C for 45 min. They were collected, precipitated, and counted as for Sendai RNA.

Assay of virus titre. Egg infectivity and haemagglutination assay were performed as described by Blair & Robinson (1968).

RNA precipitation and scintillation counting. Radioactivity precipitable by trichloracetic acid was measured as previously described (Blair & Robinson, 1968).

Interferon. Purified chick interferon was prepared by pooling 28 l. of chorioallantoic fluid obtained 96 hr after infecting 9- to 10-day-old chick embryos with the ws strain of influenza. This pool was acidified to pH 4.0, chromatographed on an XE-64 column, dialysed against distilled water and 0.1 M-NaPO₄ (pH 5.1), and chromatographed on a CM-Sephadex column (Merigan, Winget & Dixon, 1965). A pool of peak fractions eluted between pH 6.00 and 6.90 had an interferon titre of 7000 units/ml when assayed on chick embryo fibroblasts with vesicular stomatitis virus (Merigan et al. 1965) and the $E_{290}$ was 1.0. Thus, the potency of the purified material was 10 units/μg protein. Bovine serum albumin 0.5 μg/ml was added to stabilize the interferon (Lampson et al. 1963) during storage at −20°C.

RESULTS

Effect of preinfection interferon treatment on Sendai RNA synthesis

Blair & Robinson (1968, 1970) reported that Sendai virus infection of chick embryo fibroblast cells resulted in the appearance of four distinct RNA species, a 57S single-stranded RNA indistinguishable from virus particle RNA and three other RNAs sedimenting at 35S, 22S and 18S. The incorporation of 5H]uridine into all four RNAs between 20 and 22 hr after infection was diminished in cells pretreated with interferon (Fig. 1). Actinomycin D was used to inhibit DNA-dependent RNA synthesis (Blair & Robinson, 1968). Inhibition in pretreated cells was proportional to the interferon dose. Each of the four RNA species was inhibited to the same degree by any given concentration of interferon. Interferon also reduced the virus cytopathic effect in proportion to the dosage. The effects of interferon on virus RNA synthesis and cytopathic effect were correlated with a reduction of infectious virus production (Table 1).

Effect of the time of interferon treatment on Sendai virus RNA synthesis

When interferon treatment was started 6 hr before infection, there was marked inhibition of virus RNA synthesis (Fig. 2a). When interferon treatment was started 2 hr after infection, there was no significant reduction in virus-specific RNA synthesis (Fig. 2b).

Interferon added after infection had a variable effect in different experiments with as much as 20% inhibition of RNA synthesis in experiments when interferon treatment was started between 2 and 14 hr after infection. However, in an experiment using a tenfold smaller virus inoculum, interferon treatment of cells 2 hr after infection also failed to reduce RNA synthesis directed by Sendai virus significantly.

Effects of interferon on Sendai virus nucleocapsid and polyribosome formation

Eight, 14 and 20 hr after infection, monolayers were incubated with actinomycin D for 30 min., [3H]uridine was added and incubation continued for 90 min. The cells were then disrupted and cell extracts sedimented in sucrose gradients. Only a small amount of radioactive RNA in polyribosomes was present 8 hr after infection, and no 200S nucleocapsid was detected. By 14 hr, labelled RNA was present in polyribosomes and nucleocapsid,
Table 1. Effect of interferon concentration on RNA from Sendai-infected cells correlated with cytopathic effects on monolayers and virus production

<table>
<thead>
<tr>
<th>RNA species</th>
<th>Radioactive incorporation (counts/min.)</th>
<th>Interferon concentrations (units/ml.)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>control</td>
<td>2</td>
</tr>
<tr>
<td>18 s Total</td>
<td>33,792</td>
<td>22,718</td>
</tr>
<tr>
<td>%</td>
<td>100</td>
<td>67</td>
</tr>
<tr>
<td>22 s Total</td>
<td>15,408</td>
<td>10,509</td>
</tr>
<tr>
<td>%</td>
<td>100</td>
<td>69</td>
</tr>
<tr>
<td>35 s Total</td>
<td>13,830</td>
<td>8,147</td>
</tr>
<tr>
<td>%</td>
<td>100</td>
<td>59</td>
</tr>
<tr>
<td>57 s Total</td>
<td>22,630</td>
<td>13,030</td>
</tr>
<tr>
<td>%</td>
<td>100</td>
<td>58</td>
</tr>
<tr>
<td>CPE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Egg infective dose 50</td>
<td>10^{-9.5}</td>
<td>10^{-9.5}</td>
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</tbody>
</table>

Gradients were prepared as described in Fig. 1. The summation of the counts in the following fractions were taken to represent the incorporation in each of the peaks: 18 s (fractions 24 to 31); 22 s (fractions 19 to 24); 35 s (fractions 13 to 19); 58 s (fractions 1 to 8). Cytopathic effect was determined as follows:

* Rounding of some cells.

† About 50% of cells revealed marked changes in cell morphology with cell rounding, cell fusion and cytoplasmic vacuolation.

‡ Above changes in the majority of the cells.

Fig. 1. Effect of chick interferon on Sendai virus RNA synthesis. Sucrose density gradient analysis of Sendai RNA labelled with [3H]uridine. Monolayers were exposed to interferon during the 12 hr preceding infection. The 18 s and 28 s arrows mark the peaks of the E260 measurements and represent cellular RNA. No interferon, ○—○; interferon 10 units/ml. ●—●.
but less than there was 20 hr after infection (Fig. 3a, b). The time of maximum [\textsuperscript{3}H]uridine-incorporation, 20 hr after infection, was used in subsequent experiments on the effects of interferon.

Fig. 2. Effect of time of interferon treatment of Sendai virus RNA synthesis. Sucrose density gradient analysis of [\textsuperscript{3}H]uridine-labelled Sendai RNA showing the effect of interferon treatment between 6 hr before to 6 hr after infection (a) and from 2 to 14 hr after infection (b). Control, \textcircled{O}; interferon, \textbullet\textbullet\textbullet.

Treatment of the monolayers with 100 units/ml. of interferon 6 hr before infection almost completely prevented the formation of virus nucleocapsid and polyribosomes as measured by [\textsuperscript{3}H]uridine incorporation (Fig. 4). When interferon treatment was initiated 2 hr after infection, [\textsuperscript{3}H]uridine incorporation into virus nucleocapsid and polyribosomes was significantly reduced, and in several experiments the incorporation of [\textsuperscript{3}H]uridine into RNA of these structures was reduced significantly more than incorporation into total virus-specific RNA (see Fig. 3) when interferon was added for 12 hr, starting 2 hr after infection. Interferon added 6 hr after infection had no effect on the formation of these virus components. Interferon treatment in other experiments initiated 10, 14 and 18 hr after infection also failed to alter the incorporation of [\textsuperscript{3}H]uridine into these virus structures.
DISCUSSION

Our results clearly show that replication of Sendai virus in chick embryo fibroblasts is inhibited by interferon. The sensitivity of this virus to interferon appears to be similar to that of vesicular stomatitis virus in chick embryo fibroblasts (10 units/ml. of interferon reduces Sendai virus yield by 90%). Our results also show that treatment of cells with interferon before infection equally reduces the synthesis of all four virus-specific RNA species.
in infected cells and the inhibition is dose-dependent. The effect of interferon on virus RNA synthesis is correlated with reduction in cytopathic effects and infectious virus production. Similarly to our findings with Sendai virus, Gordon et al. (1966) observed a parallel depression of all Mengo virus RNA species found in infected L cells when high concentrations of interferon were added before infection. Similarly, Mécs et al. (1967) and Friedman et al. (1967) demonstrated that synthesis of all three RNA species specific to Semliki Forest
virus-infected cells was inhibited by interferon added before infection of chick embryo fibroblasts, although two species, the double-stranded replicative intermediate and the 'inter- jacent' RNA, were considered to be less sensitive than the 45S infectious RNA species (Mécs et al. 1967) Sendai virus and Mengo virus infection. Significant effects of interferon on virus RNA synthesis resulted solely when interferon was added to cells before infection and only small effects, if any, were observed when interferon was added after virus infection. Wagner (1961) using Eastern equine encephalomyelitis virus, and De Somer et al. (1962) and Lockart et al. (1962) using Western equine encephalomyelitis virus found only partial inhibition of synthesis of infectious virus RNA when high concentrations of crude interferon were added as late as 2 to 6 hr after infection. The apparently greater effect of interferon added after infection with these arboviruses compared with the effect with Sendai virus could have been due to intrinsic differences in the virus cell system, differences in experimental method (measuring infectious RNA compared with radioactive labelling), or to the very high concentrations of crude interferon which were used in the arbovirus experiments.

Our experiments also show that interferon inhibits [3H]uridine incorporation into the RNA of virus nucleocapsid and polyribosomes. As with virus RNA synthesis, the effect on nucleocapsid and polyribosome formation was greatest when interferon was added before infection. The failure to observe [3H]uridine labelling of polyribosomes and nucleocapsid when interferon was added before virus infection could have been due completely to the inhibition of virus RNA synthesis by interferon. However, in several experiments in which interferon was added 2 hr after infection, a significantly greater reduction in [3H]uridine incorporation into the RNA was observed, suggesting that interferon may exert an effect on polyribosome and nucleocapsid formation which is independent of inhibition of RNA synthesis. Such an effect is compatible with a primary action of interferon on polyribosomes and virus-specific protein synthesis. Such a mechanism would account for the complete inhibition of virus RNA synthesis in cells treated with high concentrations of interferon before infection because virus RNA synthesis is dependent on protein synthesis. It would also account for a greater effect on polyribosome and nucleocapsid formation than on virus RNA synthesis when interferon was added after infection. Proof that this mechanism is interferon's primary action will require more direct evidence than is provided by our experiments or any others reported so far. As indicated in the introduction, experiments by many investigators have produced no reproducible or conclusive evidence for an alteration in ribosomes or cell-free protein synthesizing systems from interferon-treated cells. No effects have been reported of interferon added to cell-free systems.

Two aspects of interferon's action may in part account for the failure so far to demonstrate its primary site of action: (1) the long time (at least 6 to 8 hr) required to observe an inhibitory effect, and (2) failure of interferon to block or interrupt virus replication, or to do so only minimally when added after infection. The finding that interferon's action on cells appears to depend on the integrity of cellular RNA and protein synthesis (Taylor, 1964; Levine, 1964; Lockart, 1964; Friedman & Sonnabend, 1964) may also contribute both to the delay in its action on cells and to the difficulty of demonstrating a primary site of action.

Sendai virus continues to replicate in chick embryo fibroblasts for many hours after infection without greatly inhibiting host cell RNA or protein synthesis (Blair & Robinson, 1968, personal communication). With this virus we have shown that interferon does not interrupt any of the steps in virus replication which we examined when interferon was added 8 hr or more after virus infection after virus production had begun, even though high concentrations of interferon were used and were incubated with infected cells for 12 to 14 hr before the cells were examined for an interferon effect. Such a result suggests that interferon
blocks a very early step in virus replication and once completed the step is no longer necessary for continued virus production, or in some other way, the interferon-sensitive process in the cell becomes refractory to interferon action after the cell is infected. In contrast to interferon, inhibitors of protein synthesis like cycloheximide stop the synthesis of Sendai components at the time of their addition even well after infection has begun (unpublished data of W. S. Robinson).

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REFERENCES


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