Interferon Production by Double-stranded RNA: A Comparison of Induction by Reovirus to that by a Synthetic Double-stranded Polynucleotide

By W. F. LONG and D. C. BURKE*
Department of Biochemistry, Marischal College, University of Aberdeen, Scotland

(Accepted 1 March 1971)

SUMMARY

Treatment of chick cells with reovirus resulted in the production of an inhibitor of virus replication which was characterized as an interferon. No infectious virus was released, nor could any virus-specific RNA synthesis be detected in cells receiving virus. The ability of reovirus to induce interferon formation in chick cells was less sensitive to irradiation with ultraviolet light than was its ability to produce infectious virus in L cells. These data suggest that the interferon was induced by the double-stranded RNA of the inoculum virus. Comparison of this system with that induced by a synthetic double-stranded polynucleotide showed that the latter was considerably less sensitive to the effects of metabolic inhibitors, suggesting that the two inducers do not stimulate interferon formation by the same mechanism. The effect of addition of two metabolic inhibitors simultaneously to cells previously treated with a synthetic polynucleotide suggests that their effect is not a direct one on the formation of an interferon messenger RNA and its translation.

INTRODUCTION

The mechanism of interferon production by reoviruses which contain double-stranded RNA within the virus particle (Gomatos & Tamm, 1963), is of particular interest in view of the suggestion that formation of double-stranded RNA is a necessary step in interferon production by viruses containing single-stranded RNA (Field et al. 1967). The suggestion was put forward because several natural and synthetic double-stranded, but not single-stranded, polynucleotides are potent inducers of interferon. The mechanism by which interferon is produced in response to synthetic double-stranded polynucleotides differs, however, in some respects from that which follows infection with viruses containing single-stranded RNA. In some tissue culture cells interferon appears more quickly in the former system, and its production is less sensitive to inhibition by actinomycin (Finkelstein, Bausek & Merigan, 1968). Moreover, interferon production in response to synthetic inducers in vivo and in some tissue culture cells is not suppressed by inhibitors of protein synthesis (Finkelstein et al. 1968; Youngner & Hallum, 1968). Indeed, in rabbit kidney cells such inhibitors enhance interferon production, and it has been suggested that in these cells an endogenous inhibitor of interferon production is normally synthesized soon after administration of the synthetic inducer (Vilcek, 1970).

* Present address: Division of Biological Sciences, University of Warwick, Coventry, Warwickshire.
It seemed important to examine whether reovirus-induced interferon production shared any characteristics with that stimulated by a synthetic double-stranded polyribonucleotide, and this paper reports a comparison of the two inducers in chick embryo cells.

**METHODS**

**Materials.** Polyriboinosinic (In) (control number 39726) and polyribocytidylic (Cn) (control number 29725) acids were obtained from Miles Laboratories Ltd, Elkhart, Indiana, U.S.A. DEAE-dextran (mol. wt. $2 \times 10^6$) was obtained from Pharmacia Ltd, Uppsala, Sweden. Pepsin was obtained from Worthington Biochemical Corporation, Freehold, New Jersey, U.S.A., and pancreatic ribonuclease (RNase) from Calbiochem Ltd, London. ‘Arkline’ P was a gift of Imperial Chemical Industries Ltd, Mond Division, Run-corn, Cheshire. Noble agar was obtained from Difco Laboratories, Detroit, U.S.A. DL-p-fluorophenylalanine was obtained from Koch-Light Laboratories Ltd, Colnbrook, Buckinghamshire, and cycloheximide from Sigma Chemical Company Ltd, London. Actinomycin D was a gift of Merck, Sharp and Dohme, Inc, New Jersey, U.S.A. [5-$\text{H}$]-uridine (30 c/m-mole), [2-$\text{H}$]uridine (42.3 mc/m-mole), [3$\text{H}$]DL-valine (480 mc/m-mole) were obtained from the Radioactive Centre, Amersham, Buckinghamshire. Heat-inactivated antiserum, prepared in rabbits against reovirus type 3 DEARING strain, was given by Dr C. R. Pringle, then of Department of Bacteriology, University of Aberdeen.

**Media and cells.** Chick embryo cells were prepared and cultivated as described by Walters, Burke & Skehel (1967) using medium 199 (Glaxo Laboratories Ltd, Greenford, Middlesex) supplemented with calf serum (Flow Laboratories Ltd, Irvine, Ayrshire). Interferon and interference assays were also carried out using this medium. Mouse fibroblast L cells were cultured as monolayers in Eagle’s minimal essential medium (Wellcome Reagents Ltd, Beckenham, Kent) supplemented to 5% with foetal bovine serum (Flow Laboratories). All other experimental work was carried out using this medium. Cultures were prepared in Roux bottles for virus propagation, on glass Petri dishes (6 cm. diameter) for radioactive experiments or on plastic Petri dishes (5 cm. diameter) for plaque assay of virus.

**Virus.** Reovirus type 3 was obtained from the Central Public Health Laboratory, London, as 15th monkey kidney passage of the DEARING strain, and subsequently passaged in L cells before cloning by three consecutive isolations from single plaques and storage at $-70^\circ$. The infectious titre of the virus was estimated by plaque assay on L cells by a modification of the procedure described by Gomatos et al. (1962). The overlay medium used consisted of Eagle’s minimal essential medium supplemented with foetal bovine serum (to 5%) and agar (to 0.9%). For virus propagation, L cell cultures were incubated for 2 hr at 37° with 5 ml. of virus (m.o.i. 5 to 10). Fifty ml. of medium were then added to the cultures, which were reincubated for 48 hr. Cultures were then frozen and thawed, and suspensions clarified by low-speed centrifugation. The supernatant, which contained $1 \times 10^9$ p.f.u./ml., was used, after a tenfold dilution in medium, as inoculum for most of the experiments described. Such material was partially purified before ultraviolet irradiation by centrifugation at 180,000g for 1 hr. The pellet was resuspended in phosphate-buffered saline (PBS: 0.15 M-sodium chloride, 0.006 M-sodium phosphate, pH 7.0) using a Dounce homogenizer, and an equal volume of the fluorocarbon ‘Arkline’ P added. The two phases were mixed in the homogenizer, and then separated by low-speed centrifugation. The aqueous layer containing virus was diluted with PBS for use. Ultraviolet irradiation of virus was carried out as described by Long & Burke (1970). The virus (initial titre $10^{6.7}$ p.f.u./ml.) received 12 hits/4 min. irradiation as measured by reduction in infectivity.
Interferon production by double-stranded RNA

Preparation of In, Cn. Homoribopolymers were dissolved in PBS and solutions adjusted to known molarities spectrophotometrically using the following molar extinction coefficients derived from phosphate analyses by Sigler, Davies & Miles (1962):

\[
\begin{align*}
\text{Cn} & \quad \epsilon_{298} = 6.3 \times 10^3, \\
\text{In} & \quad \epsilon_{248} = 10.4 \times 10^3.
\end{align*}
\]

Molar concentrations of polyribonucleotides are expressed in terms of the molarity of the total nucleotide content. Stock solutions of polymers (2 \times 10^{-4} M) were stored at -20°C. Immediately before use, equimolar solutions of In and Cn were mixed. Formation of the In-Cn complex, as determined by hypochromic effect (32% at 235 nm.), occurred within a few min. at room temperature. The ultraviolet spectrum of the complex showed a sharp thermal transition at 63°C.

Inoculation of chick cells or L cells with reovirus or In, Cn. Cultures were incubated for 1 hr at 37°C with 0.5 ml. of reovirus (normally at an m.o.i. of 5) or In, Cn (plus DEAE-dextran as indicated in the text). Fluid was then removed from the cultures, the monolayers washed twice with medium before addition of 2 ml. of medium/culture, and reincubation at 37°C.

Assay of interference with virus growth and of interferon. For assay of interference, medium was removed from chick cell cultures 18 to 24 hr after cells received In, Cn and replaced by 0.25 ml. of a suspension of Semliki Forest virus containing sufficient virus to give 55 to 80 plaques/culture. After an adsorption period of 1 hr, the virus suspension was replaced by 5 ml. of an overlay medium which consisted of medium 199 supplemented with calf serum (to 2%) and agar (to 0.9%). Cultures were reincubated at 37°C and plaques counted after 36 hr. Chick cell interferon was assayed as described by Gandhi & Burke (1970).

Incorporation of radioactive precursors and estimation of radioactivity. These were carried out as described by Long & Burke (1970).

Extraction and fractionation of radioactive RNA. RNA was extracted from L cells and from chick cells using sodium dodecyl sulphate and phenol and was fractionated on polyacrylamide gels as described by Cartwright & Burke (1970).

RESULTS

The reovirus-chick cell system

The kinetics of release of infectious virus from L cells was as shown in Fig. 1. Under similar conditions, virus adsorbed to chick cells but no infectious virus was released. The medium removed from chick cell cultures 24 hr after treatment with reovirus contained an inhibitor of virus multiplication with many of the properties of interferon. Activity was not decreased by prolonged dialysis at pH 2, treatment with reovirus antiserum, by heating at 65°C for 1 hr, or by centrifugation at 190,000g for 1 hr. Activity was destroyed by treatment with pepsin (0.01% at 37°C and pH 2 for 1 hr) but not by treatment with ribonuclease (100 μg./ml. with 1 mM ethylene diamine tetraacetate (EDTA) for 1 hr in medium). In all subsequent experiments the fluids were heated at 65°C for 1 hr before assay. This procedure decreased the infectivity of reovirus by a factor of 10⁶ without any effect on the interferon titre, and was adopted in order to remove any possible contribution of unadsorbed reovirus.

The yield of interferon increased with increasing multiplicity of exposure over the range tested (Fig. 2). The linear relationship between the yield of interferon and the log of multiplicity suggests that the yield of interferon was a function of the amount of input virus adsorbed. No infectious virus was produced at any of the multiplicities examined.
Inoculation of chick cells with reovirus irradiated with ultraviolet light

Partially purified virus was irradiated with differing doses of ultraviolet light and tested for its ability to yield infectious virus in L cells and to produce interferon in chick cells. Purification of the virus had no effect on its capacity to induce interferon. Interferon

Fig. 1. Kinetics of reovirus release from L cells (○—○) and chick cells (●—●).

Fig. 2. Effect of virus multiplicity on the yield of interferon produced in chick cells.
Interferon production by double-stranded RNA production was much less sensitive to irradiation than was the ability to produce infectious virus (Fig. 3), suggesting that virus multiplication was not necessary for interferon production. The loss of inducing ability might result from a failure to remove the virus protein coat, or from a failure to dissociate the nucleic acid from the inner core of the virus, as is the case in a similar situation with heavily irradiated Newcastle disease virus (A. Meager and D. C. Burke, unpublished results). Alternatively, interferon production might require the expression of part of, but not all, the virus genome, and the following experiment was designed to test whether any virus RNA synthesis could be detected in chick cells receiving reovirus.

![Graph](image.png)

Fig. 3. Effect of u.v. irradiation of reovirus on the yield of infectious virus released from L cells (○—○) and on the yield of interferon produced by chick cells (●—●), both shown as a percentage of the figures obtained with unirradiated virus (5 × 10⁸ p.f.u. and 125 PDD₅₀/culture respectively).

Virus RNA synthesis in infected L and chick cells

Virus RNA synthesis could readily be detected in infected L cells which had been treated with a low concentration of actinomycin sufficient to depress cellular RNA synthesis (Fig. 4). This dose of actinomycin had no effect on virus yield and infection had no effect on the rate at which [³H]uridine entered the acid soluble intracellular pool. A comparison of the kinetics of RNA synthesis to that of virus release (Fig. 1) suggests that the stimulation represented synthesis of progeny virus RNA. No such stimulation was found in chick cells after treatment with actinomycin and reovirus, suggesting that virus RNA synthesis did not occur in these cells between 11 and 14 hr after infection. This interpretation was strengthened by an experiment in which RNA synthesised in infected cells was examined by electrophoresis on polyacrylamide gels. No RNA synthesis was detected in actinomycin-treated L cells, while three major and some minor RNA components were detected in similarly treated cells after inoculation with reovirus (Fig. 5a). (Compare, for example, Banerjee & Shatkin, 1970.) No RNA components were detected in actinomycin-treated chick cells, whether or not the cultures received reovirus (Fig. 5b).

The possibility that virus RNA synthesis in chick cells was more sensitive to inhibition by actinomycin than was virus RNA synthesis in L cells was considered. Fig. 6 shows the fractionation of RNA synthesised in the absence of actinomycin in chick and L cells after
Fig. 4. Rate of incorporation of $^{3}H$uridine into the trichloroacetic acid-insoluble fractions of reovirus-inoculated L cells (O—O) and chick cells (●—●) as a percentage of the corresponding control receiving no virus. All cultures received actinomycin (0.5 μg./ml.; 2 ml./culture) at the end of the adsorption period. Control values for L and chick cells were $3.11 \times 10^{5}$ and $4.92 \times 10^{5}$ counts/min./culture respectively at 4 hr.

Fig. 5. Polyacrylamide gel electrophoresis of RNA extracted from (a) L cells, (b) chick cells. At the end of the adsorption period all cultures received actinomycin (0.5 μg./ml./culture; 0.5 ml./culture). At 11 hr after inoculation $^{3}H$uridine was added to cultures receiving virus (100 μc/ml.; 0.5 ml./culture) (O—O) and $^{14}$C]uridine added to control cultures (10 μc/ml.; 0.5 ml./culture) (●—●). After reincubation for 3 hr, cells were harvested and the appropriate virus-receiving and control cultures combined before extraction and electrophoresis of the RNA. Migration was from left to right.
Interferon production by double-stranded RNA

inoculation with reovirus. RNA synthesized in infected L cells was resolved into a number of species, some of which were not detected in uninfected cells, and corresponded in electrophoretic migration to those RNA species detected in infected actinomycin-treated cells (Fig. 6a). No RNA species other than those detected in uninfected cells were found in reovirus-inoculated chick cells (Fig. 6b).

![Fig. 6. Polyacrylamide gel electrophoresis of RNA extracted from (a) L cells, (b) chick cells. Details as in Fig. 4 except that cultures did not receive actinomycin. [3H]uridine was added to cultures receiving virus (25 μc/ml; 0.5 ml/culture) (○——○) and [14C]uridine added to control cultures (2.5 μc/ml; 0.5 ml/cultures) (●——●).](image)

The In. Cn chick cell system

Colby & Chamberlin (1969) showed that treatment of chick cells with In. Cn interfered with subsequent virus infection, and that the effect was enhanced by DEAE-dextran. This was confirmed by showing that treatment of chick cells with 20 μM In. Cn eliminated subsequent Semliki Forest virus plaque formation, while 2 μM In. Cn did not reduce plaque numbers significantly, but did so if DEAE-dextran was included in the inoculum (Table 1). Treatment with In or Cn with or without DEAE-dextran did not affect plaque formation.

Before In. Cn could be tested as an interferon inducer, it was necessary to destroy the interfering ability of the polynucleotide still present in the medium. In. Cn could be degraded by treatment with ribonuclease, even in the presence of DEAE-dextran, as shown by the hyperchromic effect at 250 nm. Treatment of In. Cn and DEAE-dextran with ribonuclease (100 μg/ml with 1 mM-EDTA at 37° for 1 hr) before application to cultures prevented the inhibition of plaque formation except at high concentrations of DEAE-dextran (Table 2). When the fluids obtained from cell cultures which had been exposed to In. Cn and DEAE-dextran were treated in this way, a virus inhibitory effect was readily detected (Table 3).
Activity was unaffected by treatment with ribonuclease, with ribonuclease followed by prolonged dialysis against a pH 2 buffer, or with ribonuclease followed by heating at 65°C for 1 hr, but was lost after treatment with ribonuclease and pepsin (0.01% at 37°C and pH 2 for 1 hr). The inhibitor therefore had the properties of an interferon.

Table 1. *In.* Cn-induced interference with Semliki Forest virus growth

<table>
<thead>
<tr>
<th>Additions to cultures</th>
<th>In. Cn (M × 10⁻⁴)</th>
<th>DEAE-dextran (µg/ml.)</th>
<th>Plaques/culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
<td>79</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>72</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>100</td>
<td>79</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>200</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>500</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>500</td>
<td>75</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Effect of RNase on the ability of *In.* Cn/DEAE-dextran to cause interference with Semliki Forest virus growth

<table>
<thead>
<tr>
<th>Concentration of polymers before 1 in 4 dilution*</th>
<th>Plaques/culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>In. Cn (M × 10⁻⁴)</td>
<td>DEAE-dextran (µg./ml.)</td>
</tr>
<tr>
<td>2</td>
<td>800</td>
</tr>
<tr>
<td>2</td>
<td>500</td>
</tr>
<tr>
<td>2</td>
<td>200</td>
</tr>
<tr>
<td>2</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>50</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Samples were diluted fourfold in order to allow for the dilution of the polynucleotide solution used to treat the cells (0.5 ml.) to the final volume harvested (2 ml.).

Table 3. Interferon released from chick cells treated with various concentrations of *In.* Cn and DEAE-dextran

<table>
<thead>
<tr>
<th>DEAE-dextran (µg./ml.)</th>
<th>Interferon titre (PDD 50) <em>In.</em> Cn (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 × 10⁻⁴</td>
</tr>
<tr>
<td>800</td>
<td>45</td>
</tr>
<tr>
<td>400</td>
<td>45</td>
</tr>
<tr>
<td>200</td>
<td>27</td>
</tr>
<tr>
<td>100</td>
<td>29</td>
</tr>
<tr>
<td>50</td>
<td>20</td>
</tr>
<tr>
<td>0</td>
<td>16</td>
</tr>
</tbody>
</table>

Medium collected 24 hr after incubation of cells with *In.* Cn and DEAE-dextran was treated with RNase as described in the text before assay of interferon activity.

Comparison of the reovirus- and *In.* Cn-chick cell systems: kinetics of interferon formation

Interferon production in reovirus-treated cultures began between 12 and 16 hr after inoculation, and was complete by about 20 hr. Interferon was first detected in *In.* Cn-treated cultures at 8 hr and its production was complete by 20 hr.
Interferon production by double-stranded RNA

Effect on interferon production of inhibitors of protein and RNA synthesis

In order to examine the effect of inhibitors on interferon production, cultures were incubated for 1 hr with reovirus or In. Cn (200 μM, containing 500 μg./ml. DEAE-dextran), the fluids removed, cultures washed, and medium containing p-fluorophenylalanine, actinomycin or cycloheximide added. Medium collected at 24 hr was dialysed for 6 days at 5°C against 6 x 10^4 vol. of Earle's balanced salt solution to remove the metabolic inhibitor. The medium from reovirus-receiving cultures was heated and the medium from In. Cn-receiving cultures with RNase, and assayed for interferon activity. The results (Fig. 7) showed that interferon production in the In. Cn chick cell system was less readily inhibited by the compounds than was interferon production in the reovirus-chick cell system. This difference was not due to an effect of the DEAE-dextran treatment on the inhibitory actions of the compounds used, since incubation of chick cells for 1 hr with In. Cn and DEAE-dextran before incubation with cycloheximide or actinomycin did not alter the effect of the inhibitors on the rates of cellular RNA and protein synthesis. The nature of the In. Cn-stimulated interferon resistant to the inhibitors was examined. Table 4 lists the kinetics of interferon production from cultures in the presence of two concentrations of cycloheximide, one of which inhibited interferon production by reovirus but did not affect the In. Cn system, the other of which partially inhibited interferon production by In. Cn. Interferon was released with similar kinetics from cultures in the presence or absence of the inhibitor. A second experiment
Table 4. Kinetics of In. Cn-induced interferon production in the presence of cycloheximide

<table>
<thead>
<tr>
<th>Time after inoculation (hr)</th>
<th>Interferon titre (% 24 hr yield)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control 0.05 µg./ml. 0.5 µg./ml.</td>
</tr>
<tr>
<td>2</td>
<td>0       0               0</td>
</tr>
<tr>
<td>4</td>
<td>0       0               0</td>
</tr>
<tr>
<td>8</td>
<td>12      9               20</td>
</tr>
<tr>
<td>12</td>
<td>48      49              40</td>
</tr>
<tr>
<td>16</td>
<td>93      77              93</td>
</tr>
<tr>
<td>20</td>
<td>106     94              100</td>
</tr>
<tr>
<td>24</td>
<td>100     100             100</td>
</tr>
</tbody>
</table>

After incubation with In. Cn (200 µM, containing 500 µg./ml. DEAE-dextran), chick cell cultures were washed with medium and reincubated for 24 hr with medium (control) or medium containing cycloheximide. Collected fluids were dialysed as described in Fig. 6 and treated with RNase as described in the text before assay for interferon activity. Interferon yields at 24 hr were (a) 80, (b) 85, (c) 40 PDD 50/culture.

Table 5. In. Cn-induced interferon production in the presence of cycloheximide plus actinomycin

<table>
<thead>
<tr>
<th>Additions to cultures</th>
<th>Interferon titre (PDD 50)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cycloheximide (µg./ml.)</td>
<td>Actinomycin (µg./ml.)</td>
</tr>
<tr>
<td>0.5</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>0.1</td>
</tr>
<tr>
<td>0.5</td>
<td>0.2</td>
</tr>
<tr>
<td>0</td>
<td>0.1</td>
</tr>
<tr>
<td>0.5</td>
<td>0.2</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Details as in Table 4 except that cultures received cycloheximide and actinomycin.

DISCUSSION

Several lines of evidence indicate that reovirus does not multiply in chick cells and that interferon production is due to the input virus. First, no yield of infectious virus could be detected over a range of multiplicities of exposure. Second, no progeny virus RNA synthesis could be detected in virus-receiving chick cells, although it was readily detected in infected L cells. Finally, the yield of interferon was directly related to the multiplicity of exposure and interferon production was very much less sensitive to ultraviolet irradiation than was the ability to produce infectious virus. Thus it appears that interferon production in this system does not require virus multiplication, and it is very likely that the inducer is the virus double-stranded RNA. It is not known why reovirus does not multiply or synthesize virus RNA in chick cells, especially as an RNA polymerase is present within the virus particle (Borsa & Graham, 1968; Skehel & Joklik, 1969; Banerjee & Shatkin, 1970; Levin et al. 1970). It may be that the process which leads to the removal of outer capsid coat and exposure of the poly-
merase to nucleoside triphosphates does not occur, although this would imply that the inducer of interferon formation could not be the virus nucleic acid. Alternatively, the central core of the virus may be degraded in chick cells with loss of polymerase function and exposure of the nucleic acid as inducer. These possibilities are being further investigated.

Whatever the mechanism, it is clear that the reovirus-induced system is as sensitive to the effects of metabolic inhibitors as are other virus-induced systems (Burke, 1966) and that the In.Cn-induced system is much less sensitive. This difference has been reported previously (Finkelstein et al. 1968), but since single-stranded RNA viruses were used as inducers, the sensitive stage could have been the formation of a double-stranded RNA molecule. In this report, a difference has been shown in the same cells using double-stranded inducers in both cases, and the difference must reflect a different mechanism of interferon synthesis. The independence of the effects of actinomycin and cycloheximide was unexpected and suggests that the inhibitors are affecting the production of interferon by some process other than their normal effects on RNA and protein synthesis.

We thank the Medical Research Council and the Cancer Research Campaign for grants which supported this work and Mr C. Gibb for technical assistance.

REFERENCES


(Received 12 January 1971)