The Effects of some Different Metabolic Inhibitors on Interferon Superinduction

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SUMMARY

Three different inhibitors of RNA synthesis, actinomycin, α-amanitin and camptothecin, and five different inhibitors of protein synthesis were able to superinduce interferon production in human diploid fibroblasts treated with poly(rI).poly(rC).

Camptothecin was shown to be a reversible inhibitor of virus induced interferon formation. It also substantially reduced the interferon yield from human diploid fibroblasts which had been superinduced with actinomycin D and cycloheximide. This suggests that the previously reported failure of camptothecin to inhibit interferon production in human diploid cells after induction with poly(rI).poly(rC) is the result of two mutually opposing effects: a marked inhibition of interferon messenger RNA synthesis, but a stimulation of the activity of the interferon messenger RNA that is formed.

INTRODUCTION

Interferon production is generally regarded as involving derepression of a cellular gene followed by transcription of the interferon messenger RNA and translation. This scheme was originally based on results obtained with a limited number of metabolic inhibitors, notably actinomycin D and cycloheximide, although recently much more direct evidence has been obtained for the production of a new interferon messenger RNA after treatment of cells with an interferon inducer (De Maeyer-Guignard et al. 1972; Kronenberg & Friedman, 1975; Pestka et al. 1975; Reynolds et al. 1975; Thang et al. 1975). A second gene system, which represses the interferon gene, has also been invoked from the results of experiments in which actinomycin D and cycloheximide were added after interferon production had started. This treatment, known as superinduction, increases the final yield of interferon by prolonging the period during which interferon is produced (Tan et al. 1970; Vilecek & Ng, 1971; Havell & Vilecek, 1972; Vilecek & Havell, 1973; Sehgal et al. 1975, 1976a, b, c); it is thought to involve the inhibition by actinomycin D of the production of a messenger RNA for a regulatory protein exerting negative control at the transcriptional level (Sehgal & Tamm, 1976; Cavalieri et al. 1977; Raj & Pitha, 1977; Sehgal et al. 1977).

Interpretation of experiments with metabolic inhibitors such as actinomycin D depends upon their specificity of action. In addition to its well known effect on transcription (Reich et al. 1962), actinomycin D causes some inhibition of DNA synthesis (Baserga et al. 1965), inhibits transfer of RNA from nucleus to cytoplasm (Sidebottom & Harris, 1969) and

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causes breakdown of messenger RNA (Rovera et al. 1970; Singer & Penman, 1973). The necessity for caution over the ways in which actinomycin D acts is well exemplified by its enhancing effect on the induction of tyrosine aminotransferase in hepatic cells treated with hydrocortisone (Garren et al. 1964), the process for which the term ‘superinduction’ was originally coined. The interpretation of its effect in this system is complex (see discussion by Shields, 1975; Steinberg et al. 1975), and is not simplified by the fact that other inhibitors of RNA synthesis do not cause superinduction (Levinson et al. 1971; Bushnell et al. 1974).

We have therefore examined the effect of two other inhibitors of RNA synthesis, α-amanitin and camptothecin, on interferon induction and superinduction, and of the effect of inhibitors of protein synthesis other than cycloheximide upon superinduction. α-Amanitin specifically inhibits form II DNA-dependent RNA polymerase which is responsible for messenger RNA synthesis (Mahy et al. 1972), although it also has some effect on ribosomal RNA (rRNA) synthesis, and therefore on form I DNA-dependent RNA polymerase at later times (Tata et al. 1972). Camptothecin has its primary effect on rRNA synthesis, but still has some effect on messenger RNA synthesis. For example, 30% heterogeneous RNA synthesis remained under conditions where rRNA precursor synthesis was inhibited by more than 95% (Wu et al. 1971).

METHODS

Materials. Actinomycin D was a gift from Merck, Sharp and Dohme Research Laboratories, Rahway, N.J., U.S.A. Camptothecin (NSC 94600) was a gift from the Drug Development Branch of the National Cancer Institute, Bethesda, Md., U.S.A. The camptothecin obtained was in the δ-lactone form. All experiments reported here were performed with the sodium salt of camptothecin because of its greater solubility. The conversion has been described in detail elsewhere (Atherton & Burke, 1975).

Trichodermin was a gift from Dr W. O. Godtfredsen, Leo Phamaceutical Products, Ballerup, Denmark. Cycloheximide, oxytetracycline and p-fluorophenylalanine were obtained from Sigma Chemical Co. Ltd, London.

The poly(rI).poly(rC) used (PL Biochemicals, Milwaukee, Wisc., U.S.A.—lot 647121) had an $s_{20, \text{w}}$ value of greater than 12, and showed 44% hypochromicity at 248 nm, when heated at pH 7.0.

Media and cells. Two strains of human diploid fibroblast cultures (HEF9 and HFF) and primary chick embryo cells were grown as described by Atherton & Burke (1975). HEF9 were derived from a normal human embryo and were used for all experiments except where indicated; HFF were derived from normal human foreskin.

Interferon inducers and interferon induction. Induction of chick and human cells with either poly(rI).poly(rC) or u.v. inactivated Newcastle disease virus (UV-NDV) has been previously described (Atherton & Burke, 1975).

Interferon superinduction. Human fibroblast cells were seeded in 60 mm Petri dishes at 1 to $2 \times 10^6$ cells/dish. These were confluent in 3 to 4 days and were then maintained for a further 3 to 4 days prior to induction. The medium was removed and the cell monolayers washed twice with medium without serum. Fifty μg poly(rI).poly(rC) was added in 1 ml of serum-free medium and the cells were incubated at 37 °C for 1 h. The inducer was removed, and the cells were washed twice with medium containing 2% foetal calf serum (maintenance medium) before addition of 2 ml of the same medium containing the appropriate protein synthesis inhibitor. The cells were again incubated at 37 °C for a further 4 h and the inhibitor of RNA synthesis was added. After a further 1 h at 37 °C, the inhibition of protein synthesis was reversed by washing twice with maintenance medium and adding 2.0 ml of inhibitor-
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free medium. The culture fluids were collected after a further 18 h incubation at 37 °C and dialysed against phosphate buffered saline before being assayed for interferon.

Interferon assays. A modified version of the inhibition of nucleic acid synthesis (INAS) method described by McWilliam et al. (1971) was used as previously described (Atherton & Burke, 1975). All titres are reported in reference research units.

RNA and protein synthesis. This was determined as previously described (Atherton & Burke, 1975).

RESULTS

The effect of inhibition of RNA synthesis upon virus-induced interferon formation

The effect of actinomycin D, α-amanitin and camptothecin on virus-induced interferon formation was first investigated in order to see if all three inhibitors inhibit transcription of the interferon gene in a similar fashion. Since superinduction of interferon formation is not seen in this or in most other virus-induced systems, this avoided the complications of measuring the effects of a metabolic inhibitor on two systems – transcription of the interferon gene and of the putative repressor gene.

α-Amanitin was a potent inhibitor of interferon production in chick cells treated with UV-NDV (Table I). Other experiments showed that its effect was, like that of actinomycin D, irreversible (data not shown). However, camptothecin is a reversible inhibitor of RNA synthesis (Wu et al. 1971), and it was also found to be a reversible inhibitor of interferon formation (Table 2). When cells were induced with UV-NDV in the presence of 100 μg/ml camptothecin with the drug present for varying periods after induction, it was found that the inhibition of both interferon production and RNA synthesis was reversed upon renewal of the drug.

We also showed that all three inhibitors blocked an early stage in interferon formation, presumably the transcription of the interferon gene, by measuring the effect of the final yield of interferon of adding the inhibitors at increasingly later periods after induction. The results obtained when either α-amanitin or actinomycin D were used were very similar; both indicating that there is continuous production of interferon messenger RNA from 2.5 to 10 h after induction (Fig. 1). The results obtained with camptothecin show that the camptothecin-sensitive process occurs between 1 and 8 h after induction, somewhat earlier than the event inhibited by α-amanitin and actinomycin D. Addition of cycloheximide at different times shows that translation of the interferon messenger RNA occurs between 4.5 and 12 h after induction.

Thus we conclude that all three inhibitions of RNA synthesis inhibit an event early in the production of interferon, and that cycloheximide inhibits a later event.

The use of different metabolic inhibitors for interferon superinduction in human fibroblasts treated with poly(rI).poly(rC)

Superinduction may be accomplished by addition of cycloheximide and either actinomycin D or 5,6-dichloro-1β-D-ribofuranosylbenzimidazole (DRB) to human fibroblasts for the appropriate periods after induction with poly(rI).poly(rC) (Vilcek & Ng, 1971; Sehgal et al. 1976b). It is not known whether other metabolic inhibitors are equally effective, and since superinduction of tyrosine aminotransferase is shown by actinomycin D (Steinberg et al. 1975) and 2-mercapto-l(β-4-pyridethyl) benzimidazole (MPB; Levinson et al. 1971) but not by camptothecin (Bushnel et al. 1974), it was of interest to determine whether this was true for interferon superinduction also. Table 3 shows that two other inhibitors of RNA synthesis, camptothecin and α-amanitin, also caused superinduction in human
Table 1. The effect of α-amanitin on interferon production in chick cells treated with UV-NDV

<table>
<thead>
<tr>
<th>Concentration (μg/ml)</th>
<th>Interferon yield at 24 h (log₁₀ units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.60</td>
</tr>
<tr>
<td>0.1</td>
<td>2.35</td>
</tr>
<tr>
<td>1</td>
<td>1.90</td>
</tr>
<tr>
<td>2.5</td>
<td>1.50</td>
</tr>
<tr>
<td>5</td>
<td>0.60</td>
</tr>
<tr>
<td>10</td>
<td>0.60</td>
</tr>
</tbody>
</table>

* Chick cells were treated with UV-NDV for 1 h at 37 °C, and then drained and re-fed with maintenance medium containing the inhibitor. After overnight incubation, the medium was harvested and dialysed extensively before assay for its interferon content.

Table 2. Reversal of the inhibitory effect of camptothecin (100 μg/ml) on interferon production and RNA synthesis in chick cells treated with UV-NDV

<table>
<thead>
<tr>
<th>Treatment with camptothecin</th>
<th>RNA synthesis as % control</th>
<th>Interferon yield at 24 h (log₁₀ units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>Not done</td>
<td>2.85</td>
</tr>
<tr>
<td>0 h–2 h</td>
<td>Before reversal 11.8*</td>
<td>2.65</td>
</tr>
<tr>
<td>0 h–4 h</td>
<td>After reversal 55.7</td>
<td>2.75</td>
</tr>
</tbody>
</table>

* Cells were incubated with camptothecin for 2 h. Inhibition was then reversed by washing, and the cells were pulse labelled for 30 min with ³H-uridine either immediately (before reversal) or after a 2 h recovery period (after reversal).

fibroblasts treated with poly(rI).poly(rC), while Table 4 shows that four other inhibitors of protein synthesis, chosen for their different modes of action, also caused superinduction. In all cases, the inhibition of protein synthesis was reversible (data not shown).

The effect of camptothecin on interferon production in human fibroblasts treated with poly(rI).poly(rC)

Since camptothecin, together with cycloheximide, can cause superinduction, it must inhibit the transcription of the repressor gene (if the repressor system explanation for superinduction is correct) and such an effect is consistent with its inhibitory effect on cellular RNA synthesis. But camptothecin appears to have no effect on interferon production induced by poly(rI).poly(rC) in the absence of superinduction (Atherton & Burke, 1975), suggesting that interferon transcription is not inhibited by camptothecin. How can camptothecin inhibit the repressor system but not the induction system? A similar situation occurs with DRB, and in this case, Sehgal et al. (1976c) showed that the drug had two opposing effects, an inhibitory effect on the transcription of the interferon gene and a counter-balancing effect resulting from superinduction of the interferon messenger RNA that was formed, through the inhibition of the repressor gene. The net result was no inhibition of interferon formation. A similar explanation for the effect of camptothecin on interferon production was tested according to the method of Sehgal et al. (1976c) in which the drug in question is added along with the poly(rI).poly(rC), and is present throughout subsequent superinduction schedule involving cycloheximide and actinomycin. Since the
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Fig. 1. The effect on the 24 h yield of interferon of adding various inhibitors to chick embryo cells at different times after induction with UV-NDV. Each inhibitor was added to the cells at the indicated times after induction. Interferon yields are expressed as the log₁₀(%) of the yield in control cultures with no inhibitor added. ■—■, Camptothecin (100 μg/ml); ●—●, actinomycin D (0.5 μg/ml); ○—○, α-amanitin (10 μg/ml); △—△, cycloheximide (25 μg/ml). The maximum yield of interferon in the medium was normally detected by 16 h after induction. Each point on the graph represents an average of at least five separate experimental determinations.

Table 3. Superinduction of interferon in HEF9 human fibroblasts after induction with poly(rI).poly(rC) by the use of different inhibitors of RNA synthesis

<table>
<thead>
<tr>
<th>Superinduction conditions</th>
<th>Drug</th>
<th>Conc. (μg/ml)</th>
<th>Time (h)</th>
<th>IF titre at 24 h (log₁₀ units)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
<td></td>
<td></td>
<td>1.80</td>
</tr>
<tr>
<td></td>
<td>Cycloheximide</td>
<td>10</td>
<td>1-6</td>
<td>3.90</td>
</tr>
<tr>
<td></td>
<td>Actinomycin D</td>
<td>1</td>
<td>5-6</td>
<td>3.30</td>
</tr>
<tr>
<td></td>
<td>Cycloheximide</td>
<td>10</td>
<td>1-6</td>
<td>3.30</td>
</tr>
<tr>
<td></td>
<td>Actinomycin D</td>
<td>0.05</td>
<td>5-6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cycloheximide</td>
<td>10</td>
<td>1-6</td>
<td>3.60</td>
</tr>
<tr>
<td></td>
<td>Camptothecin</td>
<td>100</td>
<td>5-24*</td>
<td>3.40</td>
</tr>
<tr>
<td></td>
<td>Cycloheximide</td>
<td>10</td>
<td>1-6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>α-amanitin</td>
<td>10</td>
<td>5-6</td>
<td></td>
</tr>
</tbody>
</table>

* Since the effect of camptothecin is reversible, it was maintained at a concentration of 100 μg/ml in the medium until the interferon was harvested at 24 h.

cells are already maximally superinduced by the cycloheximide and actinomycin, any inhibitory effect of another drug that is present on the final yield of interferon must be due to an inhibitory effect on the transcription of the interferon mRNA. When two different strains of human fibroblasts were induced with poly(rI).poly(rC) in the presence of camptothecin and cycloheximide, and then subsequently treated with actinomycin as described by Sehgal et al. (1976c), camptothecin did cause substantial inhibition of interferon production.
Table 4. Superinduction of interferon in HEF9 human fibroblasts after induction with poly(rI).poly(rC) by the use of different inhibitors of protein synthesis

<table>
<thead>
<tr>
<th>Superinduction conditions*</th>
<th>Conc. (μg/ml)</th>
<th>Time (h)</th>
<th>Inhibition of protein synthesis (%)†</th>
<th>IF titre at 24 h (log$_{10}$ units)</th>
<th>Proposed site of action of protein synthesis inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>—</td>
<td>—</td>
<td>0</td>
<td>2.00</td>
<td>tRNA analogue (Pestka, 1971)</td>
</tr>
<tr>
<td>Puromycin</td>
<td>10</td>
<td>1-6</td>
<td>95</td>
<td>3.40</td>
<td>Elongation and/or termination inhibitor (Cundliffe et al. 1974)</td>
</tr>
<tr>
<td>Trichodermin</td>
<td>1.36‡</td>
<td>1-6</td>
<td>99</td>
<td>4.10</td>
<td>Elongation inhibitor (Pestka, 1971)</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>10</td>
<td>1-6</td>
<td>95</td>
<td>3.95</td>
<td>Peptidyl transferase (Pestka, 1971)</td>
</tr>
<tr>
<td>Oxytetracycline</td>
<td>200</td>
<td>1-6</td>
<td>50</td>
<td>3.00</td>
<td>Analogue of phenylalanine (Pestka, 1971)</td>
</tr>
<tr>
<td>p-Fluorophenylalanine</td>
<td>25</td>
<td>1-6</td>
<td>15</td>
<td>3.30</td>
<td></td>
</tr>
</tbody>
</table>

* Actinomycin D (1 μg/ml) was present from 5 to 6 h in all the superinduction experiments.
† As measured by the incorporation of ³H-leucine for 30 min into trichloroacetic acid precipitable material.
‡ 1.36 μg/ml equivalent to 5 μM.
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Table 5. Effect of camptothecin (100 μg/ml) on interferon production in human fibroblasts in the presence of cycloheximide and actinomycin*

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Not present</th>
<th>Present</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEF9</td>
<td>3.7</td>
<td>2.4</td>
</tr>
<tr>
<td>HFF</td>
<td>3.7</td>
<td>1.85</td>
</tr>
</tbody>
</table>

* According to the procedure of Sehgal et al. (1976c) except that camptothecin (100 μg/ml) was present throughout.

(Table 5). We therefore conclude that camptothecin can, like DRB, inhibit the synthesis of interferon messenger RNA, but that this inhibitory effect is masked by its superinducing effect on the messenger RNA that is formed.

DISCUSSION

Interferon production in chick cells treated with UV-NDV is sensitive to inhibition by actinomycin D, α-amanitin and camptothecin. All three inhibitors have their primary effect on RNA synthesis and α-amanitin is known to be a specific inhibitor of mRNA synthesis by inhibiting the action of form II DNA-dependent RNA polymerase (Mahy et al. 1972). These results therefore support the view that interferon induction requires the derepression of a cellular gene (Burke, 1973) and indicate that the gene is transcribed by form II RNA polymerase. Furthermore, inhibition by camptothecin can, like that by DRB, be reversed. The results of the experiments in which the RNA inhibitors were added at increasingly later periods in interferon production show that all act at an early stage in production, and indicate that interferon messenger RNA is synthesized between 2.5 and 10 h after virus adsorption, while the RNA is translated between 4.5 and 12 h. Interferon is found in the extracellular fluid between 8 and 15 h. The lag after translation prior to release from the cells is most likely the period when the interferon undergoes processing stages such as glycosylation (Weil & Dorner, 1973).

The fact that three different inhibitors of RNA synthesis and four different inhibitors of protein synthesis all caused superinduction in human diploid cells clearly shows that both transcription and translation are required for superinduction, and the results obtained with p-fluorophenylaniline show that synthesis of a non-functional protein is as effective as suppression of protein synthesis. The interferon superinduction system differs in this way from the tyrosine aminotransferase system where superinduction is only shown by MPB and actinomycin D, and then at very high doses of the latter compound in relation to its effect on RNA synthesis.

The effect of camptothecin on interferon induction in human fibroblasts treated with poly(rI).poly(rC) is apparently analogous to that of DRB as shown by Sehgal et al. (1976c). It appears that, like DRB, camptothecin can inhibit the synthesis of interferon messenger RNA but that the effect is masked by its superinducing effect on that mRNA. Thus camptothecin inhibits interferon induced by both viruses and polynucleotides, but since it has not proved possible to superinduce virus-induced interferon production in human or chick cells, it can give different results with the two types of inducer (Atherton & Burke, 1975).
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REFERENCES


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