Cellular Mechanisms of Interferon Production

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ABSTRACT Rabbit kidney cell cultures stimulated with either double-stranded polynosinate-polycytidylate (poly I:poly C) or with ultraviolet-irradiated Newcastle disease virus (UV-NDV) produce two types of interferon response, designated "early" and "late," respectively. The early response is suppressed by inhibitors of RNA or protein synthesis and is therefore thought to represent de novo synthesis of interferon. Circumstantial evidence suggested that this interferon response is regulated by a translation control mechanism. Late interferon production with poly I:poly C only took place in the presence of inhibitors of RNA or protein synthesis. The late interferon is therefore likely to be derived by the activation of an interferon precursor. The stimulation of late poly I:poly C-induced interferon production by cycloheximide suggested the existence of a second, posttranslational level of control of interferon production. This posttranslation control seems to be activated by interferon. UV-NDV can probably suppress the synthesis of the posttranslation inhibitory protein, and therefore it stimulates a late interferon response in the absence of inhibitors of RNA or protein synthesis. It is postulated that both the translation and posttranslation inhibitor participate in the development of a cellular refractory state to repeated interferon stimulation. The picture of interferon which emerges from this study is one of a heterogenous class of proteins whose production is controlled by cellular repressors acting at various levels.

INTRODUCTION

Recent years have witnessed an increased awareness of the complexity of factors involved in interferon production. Some of these complexities have been discussed at this Symposium by Lockart (1) and Burke (2). Most of their studies were performed in cultures of chick embryo cells. The choice of this system for most earlier studies on the induction of interferon production was quite fortunate. It is fair to say that the molecular mechanisms of the initiation of interferon production in this system are still not completely understood. But all available data seem to indicate that interferon induction in chick cells is followed by the synthesis of a new messenger RNA, the translation of this messenger into the interferon protein, and finally, the release of interferon.
from the cell. In other words, the production of interferon in chick embryo cells appears to represent *de novo* synthesis of an induced protein.

The fact that the cellular events leading to interferon production are more complex in some other cell systems became apparent from studies in mice (3, 4) and rabbits (5, 6). In essence, these studies showed that inhibitors of RNA and protein synthesis did not prevent the production of interferon following the injection of animals with some nonviral inducers. Youngner and his colleagues found that in mice injected with either endotoxin (3, 4) or the double-stranded polynucleotide poly I:poly C (7), the yield of interferon was actually increased in the presence of the antibiotic cycloheximide (a potent inhibitor of protein synthesis).

These studies have led to the formulation of the concept of "preformed interferon." According to this idea, some inducers either cause a mere release of preexisting interferon from some depository in the animal organism, or else, they bring about the activation of a preformed inactive precursor of interferon. Many aspects of this work were rationalized at this Symposium by Youngner (8).

The studies described in this paper deal with the production of interferon by poly I:poly C and by ultraviolet-inactivated Newcastle disease virus (NDV) in cultures of rabbit kidney cells. This type of cell culture was first used for the demonstration of antiviral activity of double-stranded RNA's by Lampson et al. (9). The methods used for the production and testing of interferon in rabbit kidney cells were described (10, 11).

**EFFECT OF INHIBITORS OF PROTEIN AND RNA SYNTHESIS ON INTERFERON PRODUCTION WITH POLY I: POLY C**

Several laboratories have shown that treatment of rabbit kidney cells with high concentrations of actinomycin D greatly suppressed the production of interferon in response to poly I:poly C (10, 12, 13). This finding had seemed to suggest that in its essential features interferon production in this system is similar to the virus-induced interferon synthesis in chick embryo cells. It therefore did come as a surprise to find that two inhibitors of protein synthesis, puromycin and cycloheximide, failed to inhibit poly I:poly C-induced interferon production in rabbit kidney cells (10, 14).

The kinetics of the release of interferon from poly I:poly C-treated cultures in the presence or absence of 20 μg/ml of cycloheximide is shown in Fig. 1. It is known from previous experiments that the dose of cycloheximide employed inhibited amino acid incorporation into total cellular protein by about 95% (14). The release of interferon from control cultures reached a peak 4 hr after exposure to poly I:poly C. Thereafter, the release of interferon had rapidly ceased. The release of interferon in the presence of cycloheximide
(included in the medium throughout the duration of the experiment) showed a slight initial delay, but then, unlike in control cultures, interferon production continued at a high rate, with maximum production observed between 7 and 22 hr after treatment with poly I:poly C. The total amount of interferon released in the presence of cycloheximide was therefore much higher than that in control cultures. Cycloheximide alone, in the absence of poly I:poly C, did not stimulate the release of interferon from rabbit kidney cells. Therefore, the increased interferon yield observed in the presence of cycloheximide could not be due to the induction of interferon by cycloheximide alone.

A comparison of the effects of cycloheximide and puromycin on early and late interferon release is shown in Table I. Both inhibitors of protein synthesis (present throughout the duration of the experiment) caused a suppression of interferon release in the first 4 hr and an increase thereafter. However, cycloheximide had a much less marked inhibitory effect on early interferon production and caused a much more striking stimulation of the late interferon release than did puromycin.
A similar experiment with different doses of actinomycin D is shown in Table II. To avoid, as best as possible, an effect on the uptake of poly I:poly C, cells were treated with actinomycin D for 30 min following a brief exposure to poly I:poly C. Doses of 0.3 \( \mu \)g/ml and higher suppressed the early yield, but the late interferon yield was increased by treatment with 0.1 or 0.3 \( \mu \)g/ml of actinomycin D.

**Table I**

**EFFECT OF PUROMYCIN AND CYCLOHEXIMIDE ON THE RELEASE OF INTERFERON AT DIFFERENT TIMES AFTER THE ADDITION OF POLY I: POLY C**

| Treatment  | Interferon yield | 1-4 hr | 4-22 hr |
|------------|------------------|--------|---------|
|            |                 | U/ml   | % control | U/ml | % control |
| None       | 32               | 4      | -        | 4    | -        |
| Puromycin  | 4                | 12.5   | 32       | 800  |
| Cycloheximide | 16           | 50.0   | 512      | 12,800|

Cells were treated with 40 \( \mu \)g/ml of poly I:poly C for 1 hr as described in Fig. 1. Fluids were collected 4 hr after exposure to poly I:poly C, the cultures were washed and replenished with fresh medium. Fluids were again collected 22 hr after poly I:poly C treatment. Puromycin and cycloheximide (10 \( \mu \)g/ml) were present throughout the duration of the experiment and were removed by dialysis before titrating the interferon content of the collected fluids.

**Table II**

**EFFECT OF ACTINOMYCIN D ON URIDINE-3H INCORPORATION AND THE RELEASE OF INTERFERON AT DIFFERENT TIMES AFTER THE ADDITION OF POLY I: POLY C**

| Actinomycin D | Uridine-3H Incorporation | Interferon yield | 1-5 hr | 5-22 hr |
|---------------|--------------------------|------------------|--------|---------|
| Average       | % inhibition          | U/ml | % control | U/ml | % control |
| \( \mu \)g/ml|                          | control |        |        |
| None          |                          | 662    | 128     | 4     | 128     |
| 0.1           | 306                      | 54     | 100     | 32    | 800     |
| 0.3           | 218                      | 67     | 12.5    | 32    | 800     |
| 1.0           | 22                       | 96     | <2      | <1.5  | 4       |
| 3.0           | 14                       | 97     | <2      | <1.5  | <2 <50  |

Duplicate dish-cultures, each containing cells grown on three cover slips, were treated with the indicated concentrations of actinomycin D for 30 min and were washed and pulsed for 1 hr with 2 \( \mu \)Ci of uridine-3H (Nuclear Chicago Corporation, Des Plaines, Ill.; 2730 mCi/mM) in the presence of excess cold thymidine. The methods of extraction and counting were the same as described earlier (14).

A separate set of cultures was treated with 40 \( \mu \)g/ml of poly I:poly C for 30 min, then thoroughly washed and treated with the indicated doses of actinomycin D for 30 min. Thereafter, the cultures were washed free of actinomycin and replenished with fresh medium. Culture fluids were collected at 5 and 22 hr as described in Table I.
PARADOXICAL EFFECTS EXPLAINED BY THE SUPPRESSION OF ENDOGENOUS INHIBITOR OF INTERFERON PRODUCTION

We have postulated earlier that the paradoxical effects of inhibitors of RNA and protein synthesis might be explained by their suppression of an endogenous cellular inhibitor of interferon production. This still hypothetical inhibitor appeared to be responsible for the cessation of interferon production in control rabbit kidney cells 4–5 hr after exposure to poly I:poly C. The

![Graph showing the effect of cycloheximide and puromycin added at different times before or after poly I:poly C on subsequent interferon yield. Cultures were treated with 40 μg/ml of poly I:poly C (added at time 0) for 1 hr, then thoroughly washed and replenished with medium. At the indicated times, the cultures were washed and received medium containing cycloheximide (10 μg/ml), puromycin (10 μg/ml), or control medium; they were then incubated with the respective medium throughout the rest of the experiment. (The −1 hr group was treated with the antibiotics for 1 hr before the addition of poly I:poly C as well as immediately after the removal of poly I:poly C.) All fluids were collected at 21 hr after treatment with poly I:poly C and dialyzed as described in Fig. 1. Results represent the amount of interferon made from the time of addition of antibiotic or control medium until 21 hr after poly I:poly C treatment. (In the −1 hr group results represent total interferon yield from the end of poly I:poly C treatment.) Numbers at individual points in the graph designate actual interferon titers in units per milliliter.]

Figure 2. Effect of cycloheximide and puromycin added at different times before or after poly I:poly C on subsequent interferon yield. Cultures were treated with 40 μg/ml of poly I:poly C (added at time 0) for 1 hr, then thoroughly washed and replenished with medium. At the indicated times, the cultures were washed and received medium containing cycloheximide (10 μg/ml), puromycin (10 μg/ml), or control medium; they were then incubated with the respective medium throughout the rest of the experiment. (The −1 hr group was treated with the antibiotics for 1 hr before the addition of poly I:poly C as well as immediately after the removal of poly I:poly C.) All fluids were collected at 21 hr after treatment with poly I:poly C and dialyzed as described in Fig. 1. Results represent the amount of interferon made from the time of addition of antibiotic or control medium until 21 hr after poly I:poly C treatment. (In the −1 hr group results represent total interferon yield from the end of poly I:poly C treatment.) Numbers at individual points in the graph designate actual interferon titers in units per milliliter.
synthesis of this inhibitor was suppressed by actinomycin D, cycloheximide, or puromycin, and interferon, therefore, continued to be produced under these conditions (11, 14).

To test this hypothesis further, cycloheximide, puromycin, or control medium was added to rabbit kidney cells at different times before or after their exposure to poly I:poly C. Then we measured the yield of interferon from the time of addition of the antibiotic until 21 hr after the treatment with poly I:poly C. Fig. 2 shows that cycloheximide, and to a lesser extent puromycin, increased the interferon yield when added 1 hr before poly I:poly C. Addition of the inhibitors at 2 hr after poly I:poly C caused an even greater stimulation of subsequent interferon production. No increased interferon production was observed when the inhibitors were added as late as 4 hr after exposure to poly I:poly C.

The results of a similar experiment with actinomycin D are shown in Fig. 3. Treatment with a high dose of actinomycin D (2 μg/ml) before the addition

![Graph](image)

**Figure 3.** Effect of actinomycin D added at different times before or after poly I:poly C on subsequent interferon yield. Rabbit kidney cell cultures were treated with poly I:poly C as described under Fig. 2. Actinomycin D (2 μg/ml) or control medium was added to cells at the indicated times for 30 min. The cultures were then thoroughly washed and replenished with actinomycin-free medium. Results represent yield of interferon from the time of removal of actinomycin until 22 hr after poly I:poly C treatment when all fluids were harvested. (In the −0.5 hr group results represent total interferon yield from the end of poly I:poly C treatment.) Numbers at individual points in the graph are actual interferon titers in units per milliliter.
of poly I:poly C greatly reduced the interferon yield. When added 2 hr after poly I:poly C, actinomycin D had no effect on the amount of subsequently produced interferon. The same dose of actinomycin D added at 3.5 hr significantly increased subsequent interferon production; a less marked but still significant increase was observed when actinomycin D was added as late as 5.5 hr after poly I:poly C treatment.

**EFFECT OF CYCLOHEXIMIDE ON INTERFERON PRODUCTION WITH NEWCASTLE DISEASE VIRUS (NDV)**

It was of interest to determine whether some of the observations made with poly I:poly C-stimulated interferon also hold true for virus-induced interferon production in rabbit kidney cells. We have shown earlier that, unlike poly I:poly C-stimulated interferon, the total amount of interferon produced with live NDV was decreased in the presence of puromycin or cycloheximide (14). However, the degree of inhibition of interferon production by cycloheximide was smaller than the degree of inhibition of cellular protein synthesis afforded by this drug. Furthermore, since live NDV was used in this experiment, it was unclear whether the observed inhibition of the interferon yield had resulted from the effect on interferon production per se, or from the inhibition of viral syntheses preceding interferon induction.

The kinetics of interferon production stimulated with UV-inactivated NDV in the presence or absence of cycloheximide are shown in Fig. 4. This experiment suggests that the release of interferon from control cells is biphasic, with an early peak at about 5 hr, and a later peak with a maximum reached sometime between 7 and 22 hr after exposure to UV-NDV. In its time of appearance the early peak seems quite similar to the peak of poly I:poly C-stimulated interferon made in the absence of cycloheximide, while the second peak is more similar to the late interferon produced with poly I:poly C in the presence of cycloheximide (compare data in Fig. 1). Cycloheximide reduced the amount of interferon produced with UV-NDV; however, while it seemed to inhibit more markedly the early peak, the total amount of interferon produced was only reduced by a little over 50%. This relatively low degree of inhibition is not proportionate to the much higher degree of inhibition of amino acid incorporation into total cellular protein. The dose of cycloheximide employed was shown to inhibit amino acid-14C incorporation in rabbit kidney cells by about 95% (14).

The relatively large amount of interferon produced with NDV in the presence of cycloheximide also suggests that viral protein synthesis is not required for interferon induction in this system. This would probably mean that the single-stranded RNA of the input virus can serve as the stimulus for interferon production. This conclusion agrees with the observations of other investigators (2, 15).
TOLERANCE TO REPEATED INDUCTION OF INTERFERON

It has been observed frequently that cells which had earlier been induced to make interferon cannot be restimulated to make a second crop of interferon (16–18). A similar state of tolerance to restimulation with homologous and heterologous inducers was observed in vivo (19, 20). The development of a refractory state was also noted in cells which had been treated with interferon before the exposure to an interferon inducer (21, 22). Chany (23) has recently demonstrated that a line of cells which lost its sensitivity to the antiviral action of interferon could not be shown to develop refractoriness to repeated interferon stimulation. Youngner and Hallum (24) and Paucker and Golgher (25) have recently shown that treatment with interferon suppressed the subsequent induction of interferon with poly I:poly C in L cells. These studies have raised the possibility that the refractory state to repeated interferon induction is mediated by interferon.

It seemed reasonable to assume that a relationship might exist between the development of a refractory state to interferon induction and the postulated shut-off mechanism for interferon production in poly I:poly C-stimulated
rabbit kidney cells. Table III shows that 6 as well as 24 hr after exposure to poly I:poly C, a second stimulation with either poly I:poly C or UV-NDV produced a much reduced interferon yield. Thus the refractory state seems to develop concurrently with the cessation of interferon production in response to the first poly I:poly C treatment. This finding suggested that the shut-off phenomenon and the refractory state to repeated stimulation could indeed be the result of the same cellular events.

Table IV shows the effect of treatment with various doses of interferon on the subsequent early and late interferon production stimulated with either

| Time of second stimulation (hours after first stimulation) | 6 | 24 |
|-----------------------------------------------------------|---|----|
| None | None | <2$ | N.D.§ |
| Poly I:poly C | None | 4 | <2 |
| None | Poly I:poly C | 512 | N.D. |
| Poly I:poly C | Poly I:poly C | 16 | 64 |
| None | UV-NDV | 1024 | N.D. |
| Poly I:poly C | UV-NDV | 64 | 16 |

* Cultures in 60-mm Petri dishes were treated for 1 hr with 50 µg poly I:poly C, or with UV-NDV (100 p.f.u./cell), or with plain medium.
$ Interferon units in fluids collected 24 hr after second stimulation.
§ Not done.

**Table IV**

**EFFECT OF INTERFERON TREATMENT ON THE PRODUCTION OF INTERFERON BY RABBIT KIDNEY CELL CULTURES**

| Interferon inducer | Treatment | Yield of interferon μ/ml |
|--------------------|-----------|--------------------------|
|                    | 1-5 hr | 5-24 hr |
| Poly I:poly C      | None   | 512 | 2 |
|                    | Interferon, 200 U | 512 | 4 |
|                    | Interferon, 40 U | 512 | 4 |
|                    | Interferon, 8 U | 512 | 2 |
| UV-NDV             | None   | 32 | 512 |
|                    | Interferon, 200 U | 16 | <4 |
|                    | Interferon, 40 U | 32 | 8 |
|                    | Interferon, 8 U | 32 | 64 |

* Cultures in 60-mm Petri dishes were treated for 1 hr with 50 µg poly I:poly C or with UV-NDV (100 p.f.u./cell).
‡ For 24 hr before exposure to interferon inducer.
pol y I: poly C or UV-NDV. Interferon treatment did not suppress interferon production with poly I: poly C. (The release of this interferon was virtually completed in the first 5 hr.) Interferon treatment had no significant effect on the early interferon release from UV-NDV-stimulated cells. However, the late UV-NDV interferon response was greatly reduced in cells treated with interferon.

DISCUSSION

Experiments with poly I: poly C and with UV-NDV reported in this study have suggested that two types of interferon response exist in rabbit kidney cells; the two responses have tentatively been named early and late interferon production. When stimulated with poly I: poly C rabbit kidney cells only produce the early response. This early response is depressed by inhibitors of RNA and protein synthesis. Instead, the suppression of the early response gives rise to the late response which only develops in the presence of inhibitors of RNA and protein synthesis. It seems that the two types of responses can be partially superimposed upon each other. This lack of clear separation of the two responses might be the reason for the only partial inhibition of early interferon production by cycloheximide (Fig. 1 and Table I). It seems reasonable to assume that the early response represents \textit{de novo} synthesis of the interferon protein. On the other hand, the late response is more likely to represent the activation of a preformed interferon precursor.

Experiments with NDV have suggested that the virus-induced interferon response in rabbit kidney cells also consists of two components, possibly identical with the early and late poly I: poly C-interferon response. Unlike poly I: poly C, UV-NDV produced both the early and late response in the absence of inhibitors of RNA or protein synthesis. It seems possible, however, that with NDV as an inducer, the virus itself causes an inhibition of host cell biosyntheses which might be necessary for the production of late interferon. The late UV-NDV-interferon is likely to be derived mainly from preformed protein, because it is only slightly suppressed by cycloheximide.

Some experiments suggested the existence of not only one, but two different endogenous cellular inhibitors of poly I: poly C-induced interferon production in rabbit kidney cells. The suppression of one inhibitor probably leads to the “superinduction” phenomenon observed when a high dose of actinomycin was added 3.5 or 5.5 hr after poly I: poly C (Fig. 3). Two observations suggest that the inhibitor involved in this phenomenon is different from the inhibitor blocked in the presence of cycloheximide or puromycin. (a) Unlike actinomycin D, cycloheximide and puromycin only increased the interferon yield if added not later than 3 hr after poly I: poly C (Fig. 2), indicating that the event sensitive to inhibitors of protein synthesis has been completed by this time. (b) We have observed earlier that the superinduction by actinomycin D,
added 3.5 hr after poly I:poly C, is blocked in the presence of puromycin (11). This actinomycin D-sensitive inhibitor is therefore likely to be a translation inhibitor. This probably is the inhibitor responsible for the cessation of the early (newly synthesized) interferon production. The addition of actinomycin D prevents the synthesis of this translation inhibitor and thus allows the translation of the relatively stable interferon messenger RNA already present at the time of treatment with actinomycin D. This type of translation control is known to be operative in the synthesis of several other induced proteins in mammalian cells (26).

The second cellular inhibitor, made early after treatment with poly I:poly C, cannot be a translation inhibitor because its inhibition leads to increased interferon production in the presence of cycloheximide and puromycin—both of which are potent translation inhibitors. The latter cellular inhibitor is, therefore, likely to act at a stage of interferon production following translation. This inhibitor could act by preventing the activation of an interferon precursor, or else, it could be an enzyme causing the degradation of either interferon or a component necessary for the activation of interferon. This second or posttranslation inhibitor is likely to act specifically on the production of the late (probably preformed) interferon.

It is possible that both postulated endogenous inhibitors are involved in the development of the refractory state to repeated interferon induction. The first (translation) inhibitor might be responsible for the refractoriness to a repeated stimulation with poly I:poly C and, possibly, might also suppress the early response to UV-NDV. The second (posttranslation) inhibitor is more likely to produce the refractory state to the late UV-NDV interferon response. A suggestion of such a dual mechanism of tolerance can be found in the data shown in Table III. 6 hr after treatment with poly I:poly C, rabbit kidney cells were more refractory to a second stimulation with poly I:poly C than with UV-NDV, but at 24 hr refractoriness was more marked against UV-NDV than against poly I:poly C.

Experiments on the effect of treatment with interferon on subsequent interferon production (Table IV) supplement the other observations made in this study. It was found that the late interferon production with UV-NDV was greatly suppressed by moderate doses of interferon, while the early response with either UV-NDV or poly I:poly C was resistant to inhibition by the same concentrations of interferon. These findings have suggested that the postulated cellular posttranslation inhibitor might be an interferon-stimulated protein. Only if the synthesis of this protein is suppressed, by either metabolic inhibitors or by the inducing virus itself, can the late interferon response take place. In view of the demonstrated high sensitivity of the late UV-NDV-interferon response to suppression by interferon treatment, it seems that UV-NDV must have a built-in mechanism for counteracting the inhibitory
effect of interferon. Otherwise, the late interferon response could not be produced. It has indeed been known for some time that several paramyxoviruses, including NDV, can render cells refractory to the antiviral action of interferon (27–30). The antiviral action of interferon is also known to require cellular RNA and protein synthesis (31).

It is hardly surprising that the picture of the mechanism of interferon production which now appears to be slowly emerging from our studies is rather complex. The production of interferons is apparently under the control of several repressors which operate at different levels. In addition to the translation and posttranslation levels of control suggested by our studies, transcription control is also likely to be involved. The heterogenous group of viral and nonviral “interferon inducers” can apparently act at various levels, depending on the type of inducer and host cell involved. Binding of the interferon inducer to one or more cellular repressors appears to be the most important primary mechanism of interferon induction. In addition, secondary effects of interferon inducers (viruses, in particular) on cell metabolism are of importance in the regulation of interferon production. In some instances, inhibition of protein synthesis alone, e.g. with cycloheximide, has led to interferon production in the absence of an inducer (3), probably by affecting the posttranslational level of control. Furthermore, the action of interferon feeds back into interferon production.

Along with earlier evidence (32), this study has also suggested that interferons are a heterogenous class of proteins which possess regulatory functions in animal cells. New evidence has been obtained for the regulatory effect of interferon in the control of its own production. This effect is apparently independent of the antiviral action of interferon, however, like the antiviral action, it also seems to require cellular protein synthesis. It seems likely that interferons exert other hitherto unrecognized regulatory effects on animal cells.

Addendum. Bausek and Merigan (personal communication) have recently obtained independent evidence for (a) differences in the nature of the refractory state to repeated stimulation with poly I:poly C and NDV, and (b) a difference in the sensitivity of poly I:poly C-induced and NDV-induced interferon production to inhibition by interferon treatment. They have postulated the existence of two mechanisms of refractoriness to repeated interferon stimulation. I am indebted to Doctors Bausek and Merigan for kindly sending me a copy of their unpublished manuscript.

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