Evidence that IFN-α/β Induces Two Antiviral States Active Against Different Viruses

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SUMMARY

IFN-α/β has been suggested to require only one round of mRNA and protein synthesis to induce an antiviral state. We have examined the mechanism of induction of the antiviral states shown against three types of viruses: mengovirus (plus strand, sense RNA), vesicular stomatitis virus (VSV, minus strand RNA), and vaccinia virus (DNA). Mouse L cells were treated with IFN-α/β and cycloheximide and then with actinomycin D on a schedule which allowed only one round of mRNA and protein synthesis. The cells were challenged with virus under single cycle growth conditions to determine the amount of antiviral activity against the particular challenge virus employed. These studies confirmed that most of the antiviral effect directed against VSV is achieved with one round of macromolecular synthesis. However, most of the antiviral effect directed against mengovirus and vaccinia virus seemed to require more than one round. These results suggest that IFN-α/β induces two different antiviral states: one requiring one round of synthesis which is primarily responsible for the inhibition observed for VSV; and another requiring more than one round of synthesis which is primarily responsible for the inhibition observed for mengovirus and vaccinia virus.

Dianzani et al. (1969, 1976) have shown that murine IFN-α/β and human IFN-β induce the antiviral state directly, i.e. by one round of synthesis involving the induction of mRNA for the antiviral protein, then its translation to form antiviral protein(s), without any intermediary proteins. In contrast, the antiviral state induced by IFN-γ was found to be a slower process and has been suggested to require two rounds of synthesis, that is, induction of mRNA for an intermediary protein, then formation of the intermediary protein before induction of an antiviral protein mRNA and antiviral proteins (Dianzani et al., 1978, 1980). In this paper, we have tested the observation of Dianzani et al. (1969, 1976) that most of the antiviral state induced by IFN-α/β developed in a single round of synthesis, by employing three viruses, vesicular stomatitis virus (VSV, a negative-strand RNA virus), mengovirus (a positive-strand RNA virus) and vaccinia virus (a double-stranded DNA virus).

The experimental design developed to investigate the requirement for macromolecular synthesis in the induction of the antiviral states against the viruses is shown in Fig. 1. Mouse L929 cell monolayers were incubated with IFN-α/β in the presence or absence of cycloheximide (20 μg/ml) for timed intervals beginning at 12 h prior to actinomycin D addition. The addition of cycloheximide with IFN-α/β permitted the transcription of mRNAs for proteins which were directly induced by interferons. At 0 h, actinomycin D (5 μg/ml) was added to the cultures to block further transcription before the translational block established by cycloheximide was released. This procedure allowed the transcription of mRNAs for only those proteins directly induced by IFN-α/β. This procedure prevented transcription of mRNAs induced secondarily by interferon-induced intermediary proteins. With the removal of cycloheximide and IFN-α/β at +1 h, the translational block was removed (Colombo et al., 1965) and the mRNA transcribed...
during the first round of transcription was translated. The cells were evaluated for the development of antiviral protection by viral challenge in a single-cycle virus yield assay 3 h later at +4 h. Supernatant fluids were harvested after 24 h and assayed for progeny virus. The portion of the antiviral state that developed under the conditions of sequential treatment of cells with cycloheximide and actinomycin D was interpreted as resulting from antiviral proteins induced and synthesized with one round of cellular mRNA transcription and translation. The portion of the antiviral state that did not develop was interpreted as being due to a block in the process of induction and synthesis of the antiviral proteins which required more than one round of cellular mRNA transcription and translation.

Fig. 2(a) presents the data of a representative experiment employing VSV as the challenge virus. Maximum protection was reached after about 7 h of IFN-α/β treatment. In this experiment, treatment with 100 U/ml of IFN-α/β in the absence of added antimetabolites induced the development of an antiviral state which reduced virus yield approximately 140-fold. Actinomycin D treatment enhanced the IFN-α/β-induced antiviral state 1-3-fold, as previously reported (Chany et al., 1979). Sequential treatment of control monolayers with the antimetabolites caused a small but significant reduction in VSV replication, probably because VSV replication has been shown to require the constant synthesis of a host factor (Pringle, 1978; Szilagyi & Pringle, 1975). The appropriate control treatments, shown in (a), were performed in all experiments, but have been omitted from (b) and (c) for clarity. Sequential addition of cycloheximide and actinomycin D to IFN-α/β-treated monolayers appeared to slow the development of the antiviral state; however, this treatment did not substantially block the induction of the antiviral state directed against VSV. That is, under experimental conditions permitting one round of synthesis, the results of 13 experiments showed that an average of 66% of the control level of protection developed (Table 1). This must be regarded as a low estimate of the level of protection, since the antimetabolites alone had a significant effect on the replication of VSV. These results confirmed previous reports in the literature (Dianzani et al., 1969, 1978) and suggested that only one round of synthesis was necessary for the induction of most of the antiviral state directed against VSV.

When mengovirus was used as the challenge virus (Fig. 2b), 100 U/ml of IFN-α/β reduced the virus yield approximately 17-fold. Again, actinomycin D treatment enhanced the IFN-α/β-induced antiviral state (2-4-fold, data not shown). Sequential treatment with cycloheximide and actinomycin D had little effect on viral replication; however, unlike the observation with VSV, this treatment significantly blocked the induction of the antiviral state. The results of 19 experiments showed that an average of only 21% of the control level of protection developed with one round of synthesis (Table 1), suggesting that more than one round of macromolecular synthesis was necessary for the development of most of the antiviral state directed against mengovirus.

Fig. 1. Time schedule for basic experimental procedure. Mouse L cell monolayers were incubated with IFN in the presence or absence of cycloheximide (cyclo; Sigma) for timed intervals (shown as h) beginning at 12 h prior to actinomycin D (Act D; Merck, Sharp & Dohme) treatment. The addition of cycloheximide with the IFN allows transcription of one round of host mRNA. At 0 h, actinomycin D was added before the removal of cycloheximide (or control cultures were infected) to inhibit further mRNA transcription. One h later, the IFN and cycloheximide were removed. This reversed the translation inhibition and the mRNA that was transcribed during IFN treatment could then be translated. Cultures were challenged with virus 3 h later. Virus yields were harvested 24 h later and measured by plaque assay as previously described (Fleischmann & Simon, 1974).
Fig. 2. Kinetics of development of the antiviral state. Mouse L cell monolayers were incubated for timed intervals with 100 units/ml mouse IFN-α/β in the presence or absence of 20 μg/ml cycloheximide (or control medium). At 0 h, 5 μg/ml actinomycin D was added before removal of cycloheximide (or controls infected) to inhibit further mRNA synthesis. One h later IFN + cycloheximide was removed. Cultures were then challenged 3 h later with (a) VSV (Indiana serotype), (b) mengovirus (Franklin isolate) or (c) vaccinia virus at a multiplicity of infection of 10. Virus yields were harvested 24 h later and measured by plaque assay.

Because the mengovirus and VSV results conflicted, it was necessary to determine if the observation with mengovirus was unique. Vaccinia virus was next employed as the challenge virus. As shown in Fig. 2(c), 100 U/ml of IFN-α/β reduced virus yield by approximately 34-fold. Again, actinomycin D treatment enhanced the IFN-α/β-induced antiviral state (1.2-fold, data not shown). Sequential treatment with the antimetabolites had little effect on viral replication. However, this treatment significantly blocked the induction of the antiviral state. The results of eight experiments showed that an average of 20% of the control level of protection developed with one round of synthesis (Table 1). These results were similar to those with mengovirus, and suggested that the bulk of the antiviral state active against vaccinia virus also required more than one round of mRNA and protein synthesis. The data suggest that the proteins synthesized under conditions permitting only one round of macromolecular synthesis had only a minimal effect on vaccinia virus and mengovirus replication. Before this hypothesis was accepted, various components of the experimental design were examined to determine whether there were any trivial explanations for the observations.

Even though the effects of actinomycin D have been reported to be irreversible (Reich et al., 1961), it was necessary to ensure that the 1 h treatment with 5 μg/ml of actinomycin D in our experimental procedure was sufficient to establish an irreversible block of mRNA synthesis. We therefore modified our basic experimental design to evaluate the effectiveness of the time of
Table 1. Effect of antimetabolites on the IFN-induced antiviral state

<table>
<thead>
<tr>
<th>Virus</th>
<th>No. of experiments</th>
<th>Fold virus inhibition† induced by IFN plus Antimetabolites</th>
<th>Percent protection of antimitabolites‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>VSV</td>
<td>12</td>
<td>165 ± 45</td>
<td>19.5 ± 3.9</td>
</tr>
<tr>
<td>Mengo</td>
<td>18</td>
<td>21 ± 3</td>
<td>1.9 ± 0.2</td>
</tr>
<tr>
<td>Vaccinia</td>
<td>6</td>
<td>33 ± 8</td>
<td>2.6 ± 0.6</td>
</tr>
</tbody>
</table>

* Single-cycle virus growth experiments were performed with mouse L cells incubated 12 h with 100 units/ml IFN-αβ in the presence or absence of antimetabolites before virus challenge.
† Fold inhibition was calculated by dividing yield of medium-treated cells (virus control) by yield of IFN-treated cells. S.E.M., the standard error of the mean, is shown.
‡ Percent protection is the level of protection developed with IFN plus antimetabolites (see Fig. 1) relative to the level developed with IFN alone and was calculated by comparing virus yields of cells with various treatments using the formula \[\log_{10} (\text{cyclo + Act D}) - \log_{10} (\text{IFN + cyclo + Act D})/\log_{10} (\text{virus control}) - \log_{10} (\text{IFN})]\.

actinomycin D treatment and the concentration of actinomycin D. It was found that increasing the actinomycin D treatment from 1 to 3 h had no effect on the virus yields of cells pretreated with IFN-αβ plus cycloheximide (data not shown). Also, comparable virus yields were obtained with either a high (20 μg/ml) or a low (5 μg/ml) concentration of actinomycin D (data not shown). Finally, we tested the effectiveness of actinomycin D treatment by measuring the incorporation of $^3$H]uridine. Treatment of cell monolayers with 5 μg/ml or 20 μg/ml of actinomycin D for 1 h followed by replacement with growth medium inhibited $^3$H]uridine incorporation by 93.8% ± 0.8% and 94.5% ± 1.8%, respectively. Thus, these results taken together indicate that the time of treatment (1 h) and the concentration (5 μg/ml) of actinomycin D used in our studies were sufficient to block the synthesis of mRNA for IFN-αβ-induced proteins.

Next, the concentration of cycloheximide was examined. Comparable virus yields were obtained when either 20 μg/ml or 100 μg/ml of cycloheximide was used (data not shown). Further, we measured the effectiveness of cycloheximide treatment by measuring the incorporation of $^3$H]leucine. Treatment of cell monolayers with 20 μg/ml or 100 μg/ml of cycloheximide inhibited $^3$H]leucine incorporation by 95.7% ± 0.8% and 97.5% ± 0.1%, respectively. Thus, these results taken together indicate that the concentration of cycloheximide used in our studies was sufficient to block the synthesis of IFN-αβ-induced proteins.

Another factor examined was the time allowed for translation after removal of the cycloheximide block and before virus infection. During this time, the mRNAs synthesized during the IFN-αβ plus cycloheximide pretreatment were translated into proteins. Time periods from 1 to 7 h were allowed for translation of the IFN-αβ-induced proteins. There was no significant increase in the establishment of the antiviral state with increased translational time for VSV, vaccinia virus, or mengovirus (data not shown). Thus, the 3 h period allowed for translation was apparently sufficient to allow essentially complete translation of the existing message.

Another possible explanation for the observed differences in kinetics of development of the antiviral state among the viruses might have been differences in the relative sensitivities of the three viruses to IFN-αβ. To address this possibility, IFN-αβ concentrations that caused the development of the same relative amounts of protection (100-fold) against the three viruses were employed (100 units for VSV, 400 units for mengovirus and 250 units for vaccinia virus). Increasing the concentration of IFN-αβ to provide equivalent protection against mengovirus and vaccinia virus did not affect the results (data not shown).

Finally, it might be argued that cells do not need a second round of macromolecular synthesis to synthesize another protein in order to establish an antiviral state against vaccinia virus and mengovirus, but rather that the cell must continually synthesize a previously existing protein in order to offset viral degradation of that protein. Indeed, several investigators (Whitaker-Dowling & Youngner, 1983; Rice & Kerr, 1984; Paez & Esteban, 1984a, b) have shown that
vaccinia virus infection blocks the action of the interferon-induced protein kinase and 2'5'-oligoadenylate synthetase systems. However, this is unlikely to be the explanation for our data, since cells treated with IFN-α/β and actinomycin D in the absence of cycloheximide develop their full antiviral state against vaccinia virus and mengovirus despite the shutdown of further mRNA synthesis.

In addition to the evidence presented here for IFN-α/β induction of two different antiviral states, others have suggested that interferons may induce different antiviral mechanisms. Nilsen et al. (1980) found that the replication of encephalomyocarditis virus (EMC) but not VSV was inhibited in interferon-treated embryonal carcinoma cells. Czarniecki et al. (1981) found that replication of EMC and murine leukemia virus in mouse 3T3 cells was affected by interferon through different mechanisms. Samuel & Knutson (1983) demonstrated that recombinant DNA-derived IFN-αA induced different antiviral states in human cells, because reovirus was resistant and VSV was sensitive to the interferon treatment. Hallum et al. (1970) also provided evidence for different interferon-induced antiviral states in chick embryo cells when they showed that the antiviral state against VSV decayed more slowly than that against Newcastle disease virus.

The evidence presented in this report confirms in a different system the previous observations that cells respond to interferon treatment by producing different antiviral states. Further, it suggests that there are two antiviral states induced by IFN-α/β which have different kinetics and different mechanisms of development. Our evidence for the induction of at least two antiviral states with different induction kinetics may help provide an explanation for the differential sensitivities of viruses to interferon.

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REFERENCES


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