The Refractory State after Induction of Interferon with Double-stranded RNA

By A. BILLIAU*

Rega Institute for Medical Research, University of Leuven, Belgium

(Accepted 6 February 1970)

SUMMARY

After a first exposure to single-stranded polyriboinosinic and polyribocytidylic acids, which initiates interferon production, rabbit kidney cultures become refractory for about 3 days to further stimulation with polyriboinosinic and polyribocytidylic acids. However, when polyriboinosinic and polyribocytidylic acids are left in contact with the cells for several days, the interferon response is not restored to normal, as appears from a gradually decreasing production rate and a decreasing responsiveness to a higher dose. This is interpreted as indicating that refractoriness is not merely due to exhaustion of interferon precursors, but also to a blocking of de novo synthesis of interferon or interferon precursors. Small doses of polyriboinosinic and polyribocytidylic acids which did not induce measurable interferon, but which did stimulate resistance to virus, caused a hyper-reactivity to a second exposure to a high dose of polyriboinosinic and polyribocytidylic acids. It was concluded that in rabbit kidney cells, refractoriness is not correlated with the antiviral state resulting from stimulation of the interferon mechanism.

INTRODUCTION

In order to clarify the factors which control interferon production in tissue culture, several authors have studied the effect of stimulation of the interferon mechanism on subsequent induction of interferon by live or inactivated viruses (Lindenmann, Burke & Isaacs, 1957; Burke & Isaacs, 1958; Isaacs & Burke, 1958; Vilcek, 1962; Vilcek & Rada, 1962; Lockart, 1963; Cantell & Paucker, 1963; Burke & Buchan, 1965; Friedmann, 1966; Levy, Buckler & Baron, 1966; Paucker & Boxaca, 1967). Depending on the experimental system, either increased or decreased interferon yields may result from prior treatment of the cells with inactivated virus or with interferon itself. The mechanism of these modifications is not clear. Inhibition of interferon induction by viruses in cells which have been pretreated with interferon or interferon inducers, does not necessarily imply a reduction in the capacity of cells to respond. It may also be the result of a different processing of the interferon inducer. Even in the case of infection of cells with inactivated virus (Huppert, Hillova & Gresland, 1969), or with conditionally lethal mutants which induce interferon at the non-permissive temperature (Skchel & Burke, 1968), limited synthesis of virus RNA can occur. This RNA, which may be double-stranded, and whose synthesis is inhibited by prior induction of, or exposure to, interferon, may well be the real triggering substance for interferon release. Hence, refractoriness to a second viral inducer could merely be the result of inhibition of the formation of such triggering RNA.

The mechanisms of refractoriness have been re-investigated by use of the potent synthetic double-stranded RNA inducers which recently became available (Field et al. 1967).

METHODS

Primary rabbit kidney cultures were grown in plastic Petri dishes or on the bottom of 12 x 120 mm. glass tubes, using a modified Eagle's medium containing lactalbumin hydrolysate as amino acid source and 10% calf serum. RK13 cells were grown in 12 x 120 mm. culture tubes using Eagle's basal medium with 10% calf serum. Interferon induction experiments and interferon titrations were made using Eagle’s basal medium with 2% calf serum.

Single-stranded polyriboinosinic (In) and polyribocytidylic (Cn) acids (PL-Laboratories Inc., Milwaukee, Wisconsin, U.S.A.) were dissolved in Dulbecco’s phosphate buffered saline at 1 mg./ml. They were annealed by combination of equal volumes, heating at 90° and slowly cooling to room temperature. Hypochromicity at 245 nm. was more than 40%. Yeast RNA (highly polymerized) was obtained from Calbiochem, Los Angeles, California, U.S.A.

Interferon titrations were made by inhibition of cytopathic effect on RK13 cells. Duplicate cultures were incubated for 24 hr with serial 0.5 log. dilutions of interferon. Vesicular stomatitis virus (VSV) was added after 24 hr at a multiplicity of 1/1000 and the cytopathic effect was read 4 days later. RK13 cells are relatively resistant to induction by In. Cn and a concentration of 10 μg./ml. was needed to cause inhibition of cytopathic effect. This permitted the assay of interferon in the presence of In. Cn.

Resistance to VSV infection induced by In. Cn in primary rabbit kidney cultures was determined as follows. The cultures in glass tubes were incubated for 24 hr with serial 0.5 log. dilutions of In. Cn. They were drained and challenged with VSV at a multiplicity of 20. Excess virus was removed by washing the cultures three times. The cultures were then refed and harvested after 24 hr of incubation. The VSV infectivity was determined by plaque assay on L cells; a reduction of 0.5 log. in VSV yield is significant at the P < 0.05 level.

RESULTS

Optimal conditions for induction of interferon by In. Cn in rabbit kidney cells

Interferon can be induced readily in primary rabbit kidney cultures by exposure to In. Cn. To avoid difficulties in the titration of interferon in the presence of interfering quantities of In. Cn, the cultures were exposed for a limited time to the inducer, then thoroughly washed, refed and further incubated. Optimal conditions for induction of interferon under these circumstances were determined by incubating different cultures with In. Cn (50 μg./ml.) for 1, 2 and 3 hr. The In. Cn was removed and the cultures were re-incubated for 6 hr, at which time fresh medium was again put on the cells. A final interferon harvest was taken at

Table 1. Optimum timing conditions for interferon production by In. Cn in rabbit kidney cultures

<table>
<thead>
<tr>
<th>Treatment with 50 μg./ml. In. Cn (hr)</th>
<th>Interferon activity (units/ml.) in supernatant fluid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0-6 hr</td>
</tr>
<tr>
<td>Expt 1</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>650</td>
</tr>
<tr>
<td>3</td>
<td>3000</td>
</tr>
<tr>
<td>Expt 2</td>
<td>3000</td>
</tr>
<tr>
<td>3</td>
<td>2000</td>
</tr>
</tbody>
</table>
Interferon induction with synthetic RNA

24 hr. Representative results are shown in Table 1. Maximal induction of interferon was achieved by exposure to In.Cn for 2 to 3 hr, and the bulk of interferon was released within 6 hr after removal of In.Cn. Between 6 and 24 hr only a further 1% was produced. The following schedule was adopted therefore for interferon induction studies: the cultures were incubated with In.Cn for 3 hr, washed 3 times and refed. Interferon was harvested after incubation for 6 hr or overnight, according to convenience.

Refractoriness to induction of interferon in cells pretreated with In.Cn

Termination of interferon production in tissue cultures exposed for a limited time to In.Cn could be due either to the waning of the stimulatory effect or to the loss of the capacity to produce more interferon. The following experiment was made to distinguish between these possibilities. Rabbit kidney cultures were pretreated with In.Cn (10 μg./ml.) for 3 or 24 hr. An untreated culture was included. The cells were washed free of inducer and further incubated for 24 hr. At this time the supernatant fluid was harvested for interferon determination and the cultures were rechallenged with In.Cn (50 μg./ml.) for 3 hr. They were again washed free of inducer and re-incubated for 24 hr, at which time the second interferon yield was collected (Table 2). The culture exposed to In.Cn for 24 hr did not produce interferon during the subsequent 24 hr, while that left in contact with In.Cn for 3 hr produced 1000 units/ml. When the cultures were exposed a second time, less than 10% of the normal amount of interferon was released. This shows that termination of interferon production is not only due to discontinuation of the stimulus, but also to the development of a refractory state.

Two possible explanations for such refractoriness can be proposed. Interferon precursor ('preformed' interferon) may be exhausted, or, interferon synthesis (either complete de novo synthesis or conversion of inactive to active interferon) may be interrupted by some feed-back mechanism. Such interruption might be associated with resistance to virus resulting from interferon induction. In order to distinguish between the proposed mechanisms, we determined whether refractoriness would develop after exposure to small doses of In.Cn which do not induce measurable interferon. At the same time we tested the relationship to resistance to virus. Primary rabbit kidney cultures were pretreated for 24 hr with In.Cn at concentrations from 0-0005 to 50 μg./ml. They were washed, refed and re-incubated for 24 hr, and exposed to In.Cn (50 μg./ml.) for 3 hr. Interferon production was measured during the next 24 hr. A separate set of cultures was exposed to different concentrations of In.Cn for 3 hr, washed, refed and incubated for 24 hr to measure interferon production after the first exposure. Finally, a third set of cultures was treated with different concentrations of In.Cn for 24 hr and then challenged with VSV to test resistance to virus (Fig. 1). Unexpectedly, it was found that cells pretreated with small doses of In.Cn which did not induce measurable interferon but did induce resistance to virus, resulted in increased res-
pensiveness to a second induction with In. Cn. It can be concluded that refractoriness is not linked to antiviral resistance. On the other hand, there was a quantitative relationship between the amount of interferon induced after the first exposure to In. Cn and the degree of refractoriness which developed subsequently. This suggests that refractoriness was caused by some form of exhaustion.

![Graph](image-url)

**Fig. 1.** Interferon production, resistance to virus, and refractoriness to a second stimulus in rabbit kidney cultures pretreated with different concentrations of In. Cn. Rabbit kidney cultures were exposed to In. Cn at different concentrations for 3 hr and interferon production measured during the subsequent 24 hr (○—○). A second set was exposed to In. Cn for 24 hr, washed, refed and incubated for 24 hr at which time they were given In. Cn (50 μg./ml.) for 3 hr; interferon production was measured during the next 24 hr (●—●). A third set of cultures was exposed to In. Cn for 24 hr, washed and challenged with VSV to measure resistance to virus (△—△).

**Table 3. Duration of refractory state in rabbit kidney cultures pretreated with In. Cn**

<table>
<thead>
<tr>
<th>Dose of In. Cn at first exposure (μg.)</th>
<th>Yield of interferon in 24 hr (units/ml.) after a second contact on*</th>
<th>Day†</th>
<th>Day 2</th>
<th>Day 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>650</td>
<td>300</td>
<td>300</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>&lt; 30</td>
<td>65</td>
<td>160</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>&lt; 30</td>
<td>40</td>
<td>300</td>
<td></td>
</tr>
</tbody>
</table>

* In. Cn 50 μg./ml. for 3 hr.
† Days after discontinuation of first exposure to In. Cn.

Since In. Cn does not destroy cells, it was probable that refractory cultures would regain their capacity to respond to In. Cn. To test this, three series of cultures were incubated for 24 hr with 0, 10 or 50 μg./ml. of In. Cn. They were then washed free of inducer and reincubated at 37° for 24, 48 or 72 hr. At each of these times a group of cultures was challenged with 50 μg./ml. of In. Cn for 3 hr, washed and refed. Interferon was harvested 24 hr later. Refractoriness to a second induction lasted for 48 hr (Table 3), and the response was again normal at 72 hr. This period can be interpreted as the time necessary to restore the interferon precursor to its normal pool size, or it may also be the time during which *de novo*
synthesis of interferon is repressed. In the first mechanism, if restoration of the pool of interferon precursor is independent of the presence of In.Cn, reactivity should also be restored in the continued presence of In.Cn. That this was not so was demonstrated in the following experiment.

Fig. 2. Interferon production and refractory state in rabbit kidney cultures after prolonged exposure to In.Cn. Rabbit kidney cultures were incubated with In.Cn (10 µg./ml.) for 0, 1, 2, 3, 4 or 5 days. At each day one set of cultures was drained, washed and reincubated during 24 hr. Interferon production during these 24 hr was measured (○—○). Then the cultures were restimulated with In.Cn (50 µg./ml.) for 3 hr and interferon production measured during the subsequent 6 hr (●—●). * Production in first 24 hr in presence of In.Cn.

Rabbit kidney cells were incubated for 0, 1, 2, 3, 4 or 5 days with In.Cn (10 µg./ml.). Each day the cultures were drained and refed with fresh In.Cn solution. At the end of incubation with In.Cn the cells were washed three times and refed with medium. The supernatant fluids were again harvested after 24 hr of incubation. Then the cultures were rechallenged with 50 µg./ml. of In.Cn for 3 hr, washed, refed and harvested again after 6 hr of incubation (Fig. 2). Reactivity to a second exposure to In.Cn was reduced after one day of contact with In.Cn. It continued to decrease as the cells were left in contact with the inducer for 5 consecutive days, instead of returning to normal as was the case when the contact with In.Cn was discontinued after the first day. Such a decrease in reactivity could not be explained by further exhaustion of interferon precursors by actual production of interferon during exposure to In.Cn. Indeed, the amounts which were released during
contact with In.Cn, or immediately after its removal, were minute compared to the initial or total production capacity.

In order to exclude the unlikely possibility that refractoriness is due to a direct effect of nucleic acids, cultures were incubated with high concentrations of single-stranded In.Cn or yeast RNA. Control cultures received either double-stranded In.Cn or plain medium. After 24 hr the medium was removed, the cells were washed, refed and reincubated for 24 hr. At this time all the cultures received In.Cn (50 μg./ml.) for 3 hr. They were washed again, refed, incubated for 24 hr and harvested. Table 3 shows that refractoriness was present after contact with double-stranded In.Cn but not after exposure to the single-stranded RNAs. This again indicated that the development of refractoriness was closely linked to the induction of interferon.

Table 4. Influence of pretreatment with single-stranded RNA on subsequent induction of interferon by In.Cn

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Yield of interferon in 24 hr (units/ml.) after challenge with In.Cn*</th>
</tr>
</thead>
<tbody>
<tr>
<td>In (50 μg./ml., 24 hr)</td>
<td>10,000</td>
</tr>
<tr>
<td>Yeast RNA (300 μg./ml., 24 hr)</td>
<td>3300</td>
</tr>
<tr>
<td>In.Cn (10 μg./ml., 24 hr)</td>
<td>330</td>
</tr>
<tr>
<td>Control</td>
<td>6000</td>
</tr>
</tbody>
</table>

* Induction with In.Cn (50 μg./ml., 3 hr), 24 hr after removal of first inducer.

**DISCUSSION**

When primary rabbit kidney cultures were incubated with In.Cn for 3 hr, interferon was produced during the subsequent 6 hr. When the cultures were exposed a second time to the inducer, significantly less interferon was released. This refractory state only developed when the cultures were exposed to a dose of In.Cn which was high enough to induce measurable interferon, and there was a good correlation between the amount of interferon produced and the degree of refractoriness to a second dose. This suggests the existence of some exhaustion mechanism, but does not exclude the possibility of a direct feed-back to the synthesis of interferon. It was also found that refractoriness did not occur when the cultures were incubated with single-stranded, non-inducing RNA, rather than with double-stranded In.Cn, again suggesting that the development of refractoriness is closely linked to the induction of interferon itself.

When the contact with In.Cn was discontinued, refractoriness lasted for 3 days, at which time responsiveness was restored to normal. This 3-day interval may be interpreted as the time necessary for interferon precursor ('preformed' interferon) to be restored to its initial pool size. Alternatively, it may mean that during 3 days after a first exposure to In.Cn, *de novo* synthesis of interferon is blocked. If refractoriness is merely due to exhaustion of interferon precursor, and if such precursor can be restored within 3 days, the rate of production of interferon in the continued presence of In.Cn should after a few days equilibrate to roughly 1/3 of the initial production rate. However, it appeared that in the continued presence of In.Cn, the rate of production of interferon rapidly decreased to a negligible level. Re-exposure to a higher dose also resulted in a response decreasing progressively to almost zero. This indicated that during refractoriness either the inducing effect of In.Cn is neutralized or that the synthesis of interferon or its precursors is interrupted.
Interferon induction with synthetic RNA

The mechanism of such interruption is not clear. One possibility is that it is due to a feedback mechanism operating through interferon and the antiviral protein. The following observations rule out this possibility. Exposure of rabbit cells to small doses of In.Cn which induced strong resistance to virus without production of measurable interferon, resulted in an enhanced responsiveness to a second dose. The same conclusion can be reached from a study by Desmyter (personal communication) in which pretreatment of rabbit kidney cells with interferon increased their reactivity to In.Cn. However, in L cells the situation may be different since Youngner & Hallum (1969) reported inhibition rather than priming of the interferon response to In.Cn after pretreatment with mouse interferon.

Vilcek (1969) reported that cycloheximide enhanced the induction of interferon by In.Cn in rabbit kidney cultures. As a possible explanation, it was suggested that cycloheximide prevents the synthesis of a protein which inhibits interferon production. Such a protein could also be responsible for refractoriness. Attempts to demonstrate directly the presence of an inhibitor of interferon production in culture fluids after exposure to In.Cn, or to prevent refractoriness by cycloheximide were unsuccessful in our laboratory.

It has been reported that small doses of In.Cn stimulate and that large doses inhibit the cellular synthesis of RNA (Margolis & Levy, 1969). The increased interferon response to In.Cn after pretreatment with small doses, and refractoriness after preincubation with higher doses may be related closely to these effects on cellular RNA synthesis. It can be proposed alternatively that small doses of In.Cn stimulate the accumulation of interferon precursor which is released after exposure to a high dose.

The author gratefully acknowledges the technical assistance of Miss F. Cornette, Miss C. Dillen and Mr R. Conings.

REFERENCES


(Received 10 December 1969)