Refractory State of Cells to Interferon Induction

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SUMMARY

The effect of interferon on interferon induction either by NDV or poly I:C was studied. A priming effect of NDV-induced interferon was obtained after pre-treatment of the cells for 24 h with low concentrations of homologous interferon. After pre-treatment with higher doses, interferon production was diminished. Purified homologous interferon preparations had a biological effect similar to that of unpurified preparations, while purified heterologous interferon had no effect. The destruction of the interferon molecule or of ‘associated proteins’ by trypsin abolished the blocking effect. Similar results were obtained when poly I:C was used, instead of NDV, as an inducer. In somatic monkey-mouse hybrid cells, mouse or monkey interferon was effective in blocking mouse or monkey interferon synthesis. In clones weakly sensitive to primate interferon, no significant effect was observed.

INTRODUCTION

When cells are challenged with suitable virus or non-virus interferon inducers, they often become refractory to new stimulation. When cells are subjected to interferon treatment prior to induction, the interferon production is either increased or, on the contrary, diminished (Vilcek, 1962; Vilcek & Rada, 1962; Lockart, 1963; Burke & Buchan, 1965; Levy, Buckler & Baron, 1966; Friedman, 1966; Paucker & Boxaca, 1967; Youngner & Hallum, 1969). However, there are cell populations which did not exhibit such a refractory state; one of these is, in addition, insensitive to interferon action (Chany & Vignal, 1968; Stewart, Gosser & Lockart, 1971a; Margolis, Oie & Levy, 1972). Different hypotheses have been proposed to explain the mechanism(s) of the refractory state: an inhibitor, acting as a repressor of interferon production could appear simultaneously with interferon (Paucker & Boxaca, 1967; Chany & Vinal, 1968; Vilcek, 1970; Tan et al. 1970; Borden & Murphy, 1971); or, the antiviral state could feed-back on interferon synthesis through an interferon-induced protein (Youngner & Hallum, 1969; Bausek & Merigan, 1970; Chany & Vignal, 1970; Rousset, Fournier & Chany, 1970). This study reports data supporting the involvement of the second mechanism, which, however, does not exclude the first.

METHODS

The cells were cultivated in a modified Eagle’s medium (GRM o111, Eurobio), supplemented for cell growth with 5 to 10 % heat-inactivated calf serum.

Cells. L cells (strain 929, poly I:C sensitive strain from Flow Laboratories) and BSC cells were routinely carried in the laboratory.

MSV IF+ and MSV IF° cells were isolated by Chany & Vignal (1968) from Balb/c
mouse cells infected with mouse sarcoma virus (Moloney strain). MSV IF+ cells were propagated in medium supplemented with 50 units/ml of mouse interferon, for more than 200 passages. MSV IFo cells were MSV IF+ cells cultivated without interferon in the medium for less than 10 subsequent passages.

The different somatic monkey-mouse hybrid cells employed were clonal populations selected from the original hybrid MKCV III cells. These hybrid clones were clone 4 (Cassingena et al. 1971), and subclone 33 of clone 4 (Suarez et al. 1972). Hybrid cells were grown in the presence of HATG 1 % (10^{-4} M-hypoxanthine, 10^{-5} M-aminopterin, 4 \times 10^{-5} M-thymidine and 10^{-5} M-glycine) as described by Cassingena et al. (1971).

Viruses. Newcastle disease virus (NDV), L. Kansas strain, and Hertfordshire strain were routinely propagated in the chorio-allantoic cavity of 9-day-old embryonated Leghorn eggs. Infectivities were assayed using the egg ID_{50} method.

Vesicular stomatitis virus (VSV), Indiana strain, was grown in L cells and assayed with the routine plaque technique in L 929 cells.

West Nile virus was propagated by intracerebral inoculation of adult Swiss mice. Infectious titres were evaluated using the 50 % lethal doses.

Preparation of crude and partially purified interferons

Sources. Tissue culture interferons were obtained from MSV IF+ or MSV IFo cells (Chany & Vignal, 1968) induced by NDV. Mouse interferon was also obtained from Swiss mice after intracerebral inoculation with West Nile virus (Finter, 1964). Human interferon was prepared on human leucocytes suspensions as previously described (Gresser, 1961; Falcoff et al. 1966).

Interferon induction. Induction by NDV. Cell cultures in 30 ml plastic Falcon flasks were infected at an input multiplicity of 200 ID_{50}/cell. After 1.5 h incubation at 37 °C, non-adsorbed virus was removed. The cells were washed once with phosphate-buffered saline and 5 ml of fresh medium supplemented with 2 % calf serum were added for a further incubation period of 24 h at 37 °C. Supernatant fluids were then collected. Residual NDV was inactivated by lowering the pH of these preparations to pH = 2 with 1/N-HCl for 5 days at +4 °C; pH was then readjusted to pH = 7.4 with 1/N-NaOH.

Induction by poly I:C. L cells grown in 30 ml plastic Falcon flasks were incubated for 1 h at 37 °C with 5 to 10 µg of poly I:C (Microbiological Associates) and 200 µg of DEAE-dextran in 2 ml of phosphate-buffered saline. After that time the inducer was removed, the cultures were washed twice with phosphate-buffered saline and 5 ml of fresh medium supplemented with 2 % calf serum were added. Supernatant fluids were collected after a further incubation period of 24 h at 37 °C.

Interferon purification. In some experiments crude interferon preparations were concentrated tenfold by pressure dialysis and centrifuged at 80000 g for 4 h. Concentrated interferons were then purified by filtration on a Sephadex G 75 column (Andrews, 1964). The specific activity of partially purified mouse interferon varied from 3.4 \times 10^{8} units/mg of protein to 5 \times 10^{4} units/mg of protein.

Interferon assay. The antiviral activity of the different interferon preparations was assayed either by 50 % inhibition of the c.p.e. of VSV (input multiplicity 0.1 p.f.u./cell) or by inhibition of the virus yield in suitable cell systems.*

Chemicals. Three times crystallized, salt-free, lyophilized trypsin was purchased from Worthington Biochemical Corporation. The trypsin inhibitor used was Iniprol (Choay Laboratory).

* One unit is equivalent to 4 reference research units.
RESULTS

Refractory state of cells to induction of interferon by NDV

Effect of the pre-treatment of the cells with crude interferon

The effect of interferon treatment on subsequent interferon production in L cells was studied.

L cells were treated, for 20 to 24 h at 37 °C, with increasing concentrations of mouse interferon. Cultures were then washed and induced by NDV as described in Methods. The amount of interferon produced and diffused into the tissue culture medium was evaluated using the 50 % inhibition dose of VSV c.p.e. (Fig. 1).

When interferon doses < 20 units/ml were used for pre-treatment, the amount of interferon produced was about four times greater than in the control. On the contrary, for interferon doses > 20 units/ml, interferon production decreased with the increasing quantities of interferon used for pre-treatment.

A similar inhibition was obtained with BSC cells treated with increasing doses of human leucocyte interferon (Table 1).

In further experiments we explored the possibility that the blocking effect of interferon was not due to interferon itself but to an impurity present in the preparations.
Table 1. Effect of human interferon pre-treatment on interferon production by monkey BSC cells

<table>
<thead>
<tr>
<th>Human interferon used for pre-treatment (units/ml)*</th>
<th>Interferon released in 24 h (units/ml)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1024–2048</td>
<td>1st expt.</td>
</tr>
<tr>
<td>256–512</td>
<td>≤ 8</td>
</tr>
<tr>
<td>64–128</td>
<td>2nd expt.</td>
</tr>
<tr>
<td>No interferon treatment</td>
<td>≤ 8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>NDV input multiplicity = 200 EID&lt;sub&gt;50&lt;/sub&gt;/cell</th>
<th>64–128 64–128</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>256–512 256–512</td>
</tr>
</tbody>
</table>

* Units/ml expressed as the reciprocal of the dilution which inhibits 50 % VSV-c.p.e.

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**Fig. 2.** Effect of chromatographic fractions of mouse interferon on interferon production in mouse L cells. ■, control production, no interferon pre-treatment; ◊, interferon present in chromatographic fractions; □, interferon production of interferon-treated cells.

**Pre-treatment with partially purified interferon**

Different experiments were carried out by treating L cells, prior to induction, with chromatographic fractions of mouse interferon obtained after gel filtration. The different fractions were incubated for 24 h at 37 °C with the cells. The cells were then washed and induced by NDV. Supernatant fluids were collected after an incubation period of 24 h at 37 °C. The amounts of interferon produced were assayed using the 50 % VSV-c.p.e. inhibition test.

The results obtained are summarized in Fig. 2. No blocking effect on interferon production was observed in L cells treated with fractions which did not contain interferon in detectable amounts. On the contrary, inhibition was regularly obtained when fractions containing interferon were employed. The blocking effect increased with interferon concentrations and was maximal at the peak of interferon activity.

Parallel results were obtained with BSC cells treated with chromatographic fractions of human leucocyte interferon prior to induction.
Interferon induction by NDV or poly I:C

Table 2. Effect of purified mouse interferon pre-treatment on interferon production of monkey BSC cells

<table>
<thead>
<tr>
<th>Mouse interferon used for pre-treatment (units/ml)*</th>
<th>Induction</th>
<th>Monkey interferon released in 24 h (units/ml)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>50,000</td>
<td>NDV input</td>
<td>64</td>
</tr>
<tr>
<td>5,000</td>
<td>multiplicity = 100 EID₅₀/cell</td>
<td>128</td>
</tr>
<tr>
<td>500</td>
<td></td>
<td>128</td>
</tr>
<tr>
<td>50</td>
<td></td>
<td>128</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>128</td>
</tr>
<tr>
<td>No interferon treatment</td>
<td></td>
<td>128</td>
</tr>
</tbody>
</table>

* Units/ml expressed as the reciprocal of the dilution which inhibits 50 % VSV-c.p.e.

Table 3. Action of trypsin on the blocking effect of interferon on interferon production in L cells

<table>
<thead>
<tr>
<th>Pre-treatment</th>
<th>Units/ml*</th>
<th>Interferon released (units/ml)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interferon + medium</td>
<td>&lt; 8</td>
<td>256-512</td>
</tr>
<tr>
<td>Interferon + trypsin</td>
<td>&lt; 8</td>
<td>256-512</td>
</tr>
<tr>
<td>Medium</td>
<td>—</td>
<td>256-512</td>
</tr>
<tr>
<td>Medium + trypsin</td>
<td>—</td>
<td>128-256</td>
</tr>
</tbody>
</table>

* Units/ml expressed as the reciprocal of the dilution which inhibits 50 % VSV-c.p.e.

These data are in agreement with those reported by Stewart et al. (1971 a).

In addition, to check that hyporeactivity was not related to a factor present in interferon preparations but not specifically related to the cell species, monkey BSC cells were treated with different amounts of partially purified mouse interferon prior to induction. No detectable effect on interferon production was observed (Table 2), and, as expected, no antiviral state was induced (unpublished experiments).

Nature of the interferon blocking factor present in interferon preparations

Partially purified mouse interferon was treated with 250 units/ml of trypsin for 45 min at 37 °C. The proteolytic action of the enzyme was then stopped by addition of a trypsin inhibitor (Iniprol, 8000 units/ml) acting during a new incubation period of 20 h at 4 °C. The trypsin completely destroyed the blocking effect of interferon preparations (Table 3).

Refractory state of L cells to poly I:C induction

Repeated inductions of interferon by poly I:C

L cell cultures were twice induced by poly I:C as described in Methods, after either a 24 h or a 48 h incubation period between the two inductions. Supernatant fluids were collected 24 h after each induction and the cells replenished with fresh medium. As controls for re-induction, primary induced L cells were employed. The antiviral activity present in the supernatant fluids was evaluated by 50 % c.p.e. inhibition in L cells, using VSV as a challenge virus. As shown in Table 4, after the first induction, re-induction did not produce newly released interferon, since the amount of interferon present in the supernatant fluid was not greater than the amount liberated from the cells after primary induction.
Table 4. Refractory state in L cells re-induced with poly I: C

<table>
<thead>
<tr>
<th>Poly I: C inductions</th>
<th>Interferon released (units/ml)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1st day</td>
</tr>
<tr>
<td>1st induction</td>
<td>1024</td>
</tr>
<tr>
<td>2nd induction (24 h)</td>
<td>32–64</td>
</tr>
<tr>
<td>(256–512)†</td>
<td></td>
</tr>
<tr>
<td>2nd induction (48 h)</td>
<td></td>
</tr>
<tr>
<td>(512–1024)†</td>
<td></td>
</tr>
</tbody>
</table>

* Units/ml expressed as the reciprocal of the dilution which inhibits 50 % VSV-c.p.e.
† Primary induced controls.

Effect of mouse interferon treatment on subsequent interferon induction by poly I: C

L cells were treated with increasing concentrations of partially purified mouse interferon for 24 h at 37 °C. After elimination of interferon the cells were washed and induced for interferon synthesis by poly I: C (as described in Methods). Supernatant fluids were collected after a 24 h incubation period at 37 °C. Antiviral activity of these preparations was evaluated by 50 % VSV c.p.e. inhibition. The dose–response relationship is shown in Fig. 3. No ‘priming’, but an inhibition of interferon production was observed. These results confirm those of Youngner & Hallum (1969) and those of Stewart et al. (1971a).

When instead of mouse interferon, human leucocyte interferon was employed in the same L cell system, no such effect on poly I: C-induced interferon yield was observed.

All the data here presented seem to indicate that the protein responsible for the blocking effect of interferon on interferon induction, either by virus or poly I: C, cannot be distinguished from interferon itself. In addition, these data show that interferon from heterologous cell species induced by the same virus has no such effect. We therefore postulated that the antiviral state might be at least partially responsible for the observed blocking effect. The available somatic monkey-mouse hybrid cells appeared to represent a suitable system to check this hypothesis.

Effect of pre-treatment of monkey-mouse hybrid cells with mouse or human interferon on subsequent interferon production

The somatic monkey–mouse hybrid MKCV III clone 4 or subclone 33 of clone 4 were used for these experiments. These hybrid cells of clone 4 contain about 19 bi-armed monkey chromosomes, and about 68 telocentric mouse chromosomes (Cassingena et al. 1971). These two clones produce both mouse and monkey interferon. Clone 4 is sensitive to both types of interferon, but subclone 33, although sensitive to mouse interferon, is only weakly sensitive to primate interferon.

Effect of pre-treatment of clone 4 with mouse or human interferon on mouse interferon production

Hybrid cells of clone 4 were treated, as in the previous experiments, with increasing concentrations of either mouse or human interferon for 24 h and were subsequently induced by NDV. Antiviral activity of the supernatant fluids collected 24 h later was evaluated in mouse L cells by 50 % VSV-c.p.e. inhibition. Each interferon affected the production of mouse interferon by the hybrid cells (Fig. 4) which were sensitive to both types of interferon (Table 5).
Effect of pre-treatment of subclone 33 with mouse or human interferon on mouse or monkey interferon production

Subclone 33 cells were treated as described above with increasing concentrations of mouse or human interferon and subsequently induced by NDV. Antiviral activity of the supernatant fluids collected was evaluated both in mouse L cells and in monkey BSC cells by 50% VSV-c.p.e. inhibition.

It is of interest that in the case of subclone 33, which is weakly sensitive to human interferon (although the cells produce monkey interferon) the pre-treatment with primate interferon had no significant effect either on mouse or on monkey interferon production (Figs. 5 and 6).

In parallel experiments, the antiviral state in this hybrid subclone treated either with mouse or human interferon was explored. As shown in Table 6, the antiviral state induced in this clone by mouse interferon was significant, while that induced by human interferon was hardly detectable.
Fig. 4. Mouse interferon produced in hybrid clone 4 after pre-treatment with mouse or human interferon. ■, control production, no interferon pre-treatment; □, mouse interferon production of cells treated with mouse interferon; □□, mouse interferon production of cells treated with human interferon.

Table 5. Antiviral state induced by mouse or human interferon in hybrid cells of clone 4

<table>
<thead>
<tr>
<th>Mouse interferon used for treatment (units/0.2 ml)</th>
<th>VSV p.f.u./0.5 ml</th>
<th>Human interferon used for treatment (units/0.2 ml)</th>
<th>VSV p.f.u./0.5 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1024</td>
<td>5.6 × 10^4</td>
<td>256</td>
<td>N.D.†</td>
</tr>
<tr>
<td>128</td>
<td>3.3 × 10^3</td>
<td>64–128</td>
<td>8.6 × 10^3</td>
</tr>
<tr>
<td>16</td>
<td>3.1 × 10^2</td>
<td>16</td>
<td>5.4 × 10^3</td>
</tr>
<tr>
<td>0</td>
<td>9.5 × 10^6</td>
<td>0</td>
<td>1.3 × 10^7</td>
</tr>
</tbody>
</table>

* Units/0.2 ml expressed as the reciprocal of the dilution which inhibits 50% VSV-c.p.e.
† N.D. = not done.
Interferon induction by NDV or poly I:C

Fig. 5. Mouse interferon produced in hybrid subclone 33 of clone 4 after pre-treatment with mouse or human interferon. ■, control production, no interferon pre-treatment; □, mouse interferon production of cells treated with mouse interferon; □□, mouse interferon production of cells treated with human interferon.

Table 6. Antiviral state induced by mouse or human interferon in hybrid cells of subclone 33

<table>
<thead>
<tr>
<th>Mouse interferon used for treatment (units/0.2 ml)*</th>
<th>VSV p.f.u./0.5 ml</th>
<th>Human interferon used for treatment (units/0.2 ml)*</th>
<th>VSV p.f.u./0.5 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>128–256</td>
<td>1.1 × 10^3</td>
<td>256</td>
<td>1.2 × 10^4</td>
</tr>
<tr>
<td>32–64</td>
<td>4.2 × 10^3</td>
<td>32–64</td>
<td>2.1 × 10^3</td>
</tr>
<tr>
<td>8</td>
<td>7.9 × 10^4</td>
<td>16</td>
<td>4.7 × 10^3</td>
</tr>
<tr>
<td>0</td>
<td>5.9 × 10^6</td>
<td>0</td>
<td>7.4 × 10^6</td>
</tr>
</tbody>
</table>

* Units/0.2 ml expressed as the reciprocal of the dilution which inhibits 50% VSV-c.p.e.
Fig. 6. Monkey interferon produced in hybrid subclone 33 of clone 4 after pre-treatment with mouse or human interferon. ■, control production, no interferon pre-treatment; □, monkey interferon production of cells treated with mouse interferon; ☐, monkey interferon production of cells treated with human interferon.

DISCUSSION

The effect of interferon on interferon production varied with the concentration of interferon used for pre-treatment. At low interferon concentrations a priming effect was obtained in mouse cells, while at high concentrations (> 20 units/ml) the amount of synthesized interferon decreased with increasing interferon concentrations. Both the priming and blocking effect are probably due to the interferon molecule itself. This is supported by the observations here reported showing that: (1) partially purified interferon preparations had a biological effect similar to that of unpurified preparations; (2) fractions obtained during chromatographic purification had a blocking effect parallel to the interferon peak; (3) partially purified heterologous interferon had no similar effect. Both the priming and blocking effect of interferon preparations were cell species specific, as already described for crude interferon preparations by Paucker & Boxaca (1967); (4) destruction of the interferon molecule or of ‘associated proteins’ (Paucker & Stancek, 1972) (by trypsin) abolished the blocking effect. In addition, in somatic monkey–mouse hybrid cells (clone 4) sensitive to primate and to mouse interferon, both primate and mouse interferon diminished mouse interferon production; however, no detectable priming effect was observed. In a hybrid
subclone of clone 4, resistant to primate interferon but sensitive to mouse interferon, only mouse interferon had a blocking effect. It is of interest that in this subclone able to synthesize both monkey and mouse interferon, the treatment of the cells with mouse interferon blocked the synthesis of both types of interferon.

The mechanisms by which interferon primes or blocks interferon synthesis are probably completely unrelated. The blocking effect of interferon on interferon production could be a consequence of a mechanism involving the action of the antiviral protein on the incoming virus nucleic acid and on interferon synthesis (Youngner & Hallum, 1969; Bausek & Merigan, 1970; Rousset et al. 1970). The refractory state of the cells which appeared after a first induction of interferon synthesis could be the consequence of a repressor (Paucker & Boxaca, 1967; Chany & Vignal, 1970; Tan et al. 1970; Vilcek, 1970; Borden & Murphy, 1971) and/or of a feedback mechanism involving the antiviral protein (or a simultaneously produced factor) (Chany & Vignal, 1968; Rousset et al. 1970). These mechanisms could implicate the function of the cellular interferon genome or of the interferon messenger RNA (Vilcek & Ng, 1971). Contradictory results were published in the case of poly I:C induced interferon. The priming effect is present in most of the cases, however a blocking effect is not always reported (Tan et al. 1970; Vilcek, 1970; Stewart et al. 1971a; Margolis et al. 1972). In our system, as for Youngner & Hallum (1969) and Stewart et al. (1971a), a blocking effect is clearly shown; it is possible that the blockage of interferon synthesis is not due to the same mechanism in the case of a virus induction and in the case of induction by poly I:C (Bausek & Merigan, 1970). It can be postulated, therefore, that priming is unrelated to the antiviral state (Stewart, Gosser & Lockart, 1971b) and could involve some membrane phenomenon (Ng, Berman & Vilcek, 1972). Interferon might change, in some instances, cell permeability: an increased sensitivity to the cytotoxic effect of poly I:C has been observed in cells pre-treated with interferon (Stewart et al. 1972). Further experiments are needed to test all these possibilities.

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