Mass Production of Human Interferon in Diploid Cells Stimulated by Poly-I:C

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SUMMARY

The production of interferon by human diploid cells stimulated by poly-I:C can be increased by pretreatment of the cells with interferon (priming effect). Large amounts of interferon (30,000 units/ml) can be obtained by combining priming with a superinduction schedule adapted from that described for rabbit kidney cultures (Tan et al. 1970; Vilcek & Ng, 1971). Human plasma protein could replace bovine serum albumin in the production medium.

INTRODUCTION

Three methods have in the past been envisaged for mass production of human interferon: (i) infection of suspended human leucocytes with Newcastle disease virus (NDV) or Sendai virus (Gresser, 1961; Falcoff et al. 1966; Strander & Cantell, 1966, 1967; Wheelock, 1966; Hadhazy, Strander & Cantell, 1969; Strander, 1969; Horowitz, Stjernholm & Wheelock, 1970; Valenta et al. 1970; Tovell & Cantell, 1971); (ii) induction of human skin fibroblasts with Newcastle disease virus (Merigan, Gregory & Petralli, 1966) or poly-I:C (Billiau, Joniau & de Somer, 1972a; Ho, Tan & Armstrong, 1972); and (iii) infection of suspended human amniotic membrane with NDV (Fournier, Falcoff & Chany, 1967). From these three methods only the leucocyte system has, until now, been used on a more or less large scale. However, in the event of generalized application in medicine, the method would require a regular and massive supply of fresh human material. Cells of different individuals would have to be pooled for each preparation, and would have to be monitored for the presence of different endogenous virus agents. Further handicaps for the use of leucocyte preparations in humans would be: the presence of residual inducer virus, of cell lysis material and of bovine serum or serum albumin.

In a previous study (Billiau et al. 1972a), we reported the production of relatively large quantities (1000 units/ml) of human interferon by diploid cells, after stimulation with the non-virus inducer polyriboinosinic-polyribocytidylic acid (poly-I:C). Studies reported in the present paper were directed towards further increasing the yields. The experimental approach was based on the observation that rabbit kidney cells produced large quantities of interferon provided the stimulation by poly-I:C was followed by exposure to cycloheximide and actinomycin D (Tan et al. 1970; Vilcek & Ng, 1971). This 'superinduction' phenomenon was explained by assuming that actinomycin D, when added at a suitable time, prevents the synthesis of an endogenous inhibitor, which cuts off interferon synthesis. The role of cycloheximide would be to temporarily suppress translation of the interferon protein until the actinomycin D can be removed. Release from both inhibitors would then result in sudden translation of accumulated messenger and release of large quantities of interferon.
A second approach to increase the yields of human interferon came from the observation that pretreatment of cells with interferon or small amounts of double-stranded RNA can enhance their response to stimulation by a massive dose of poly-I:C (Billiau, 1970; Rosztoczy & Mees, 1970; Stewart, Gosser & Lockart, 1971; Billiau et al. 1972a; Billiau, van den Berghe & de Somer, 1972b). This effect, termed 'priming' was also used in the present study.

**METHODS**

**Cell cultures.** Diploid cells (VGS strain) (Billiau, 1970; Billiau et al. 1972a, b) were grown from a normal human embryo. They were subcultured in Eagle's minimum essential medium (EMEM) with 10% heated foetal bovine serum. Maintenance medium contained 2% heated calf serum. L-929 cells, used for preparation of mengovirus, were grown in the same medium.

**Chemicals.** The preparation of poly-I:C solutions has been described (Billiau et al. 1972b). Cycloheximide was purchased from Sigma Chemical Co., St Louis, Mo., U.S.A., and actinomycin D from Serva Feinbiochemica GmbH & Co., Heidelberg, W. Germany. Human plasma protein was obtained as a 5% pasteurized solution prepared for human use by the National Blood Service (Belgian Red Cross).

**Induction of interferon.** For induction of interferon, VGS-cells were grown on the bottom of unstoppered, disposable glass cups (diameter 27.5 mm) with flat bottom, kept in a CO₂ incubator. When the cups were seeded with approx. 30000 cells, confluent monolayers (100000 cells/cm² or 600000 cells/cup) were reached in 3 to 4 days. The cultures were used 7 to 10 days after confluency. During induction or production, the cups contained 1.25 ml of medium. Each interferon determination was done on pooled medium from four cups.

Alternatively, interferon was produced in roller bottles (height 23 cm, diameter 10 cm). The apparatus used was a RC-41 Rollacell bench model from New Brunswick Co., Inc., New Brunswick, N.J., U.S.A. It was operated at 45 rev/h.

Various induction schedules, as specified in Results, were used. The times of incubation with different substances always refer to the time poly-I:C was added to the cultures as being 0 h.

**Titration of interferon.** Interferon was titrated with a dye-uptake method adapted from that described by Finter (1969). VGS-cells were grown on the bottom of unstoppered disposable glass tubes (diameter 12 mm) with round bottom, kept in a CO₂ incubator. Duplicate monolayers were incubated for 24 h with serial 0.5 log₁₀ dilutions of interferon (1 ml/tube). The tubes were drained and challenged with mengovirus at an input multiplicity of 0.01 p.f.u./cell. After 24 h the medium was decanted and 1 ml of a 0.06 mg/ml neutral red solution in Dulbecco's phosphate buffered saline (PBS) was added. After 3 h incubation in the dark at 37 °C, the tubes were washed with PBS and drained. Neutral red incorporated by the cells was then eluted by adding 2 ml of ethanol buffered with an equal volume of Sörensen citrate buffer at pH 4.2.

Extinctions of elution fluids were read at 542 nm. Titration end-points were determined by graphically calculating the dilution which corresponded to a neutral red uptake of 50% of that of control cells not treated with interferon and not challenged with mengovirus. Two laboratory reference preparations of human interferon were included in each titration. This allowed us to express all values in terms of the 69-19 IAMS-reference preparation (International Symposium on Interferon and Interferon Inducers, 1970).

**Determination of bovine serum albumin.** Trace amounts of bovine serum albumin (BSA) in interferon samples were quantitated by a radio-immunooassay adapted from Catt & Treager (1967). Polystyrene haemolysis tubes (Falcon Plastics, type 2038) were antibody-
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coated with 1 ml volumes of a solution containing 1 μg of a Na₂SO₄-precipitated γ-globulin fraction of a rabbit anti-BSA antiserum. This corresponded to a 10000-fold dilution of the original antiserum. Calibration curves were run using 250 μl samples of a series of BSA solutions with concentrations ranging from 1 to 100 ng/ml, each containing in addition 10 ng/ml of [¹²⁵I]-labelled BSA. All dilutions were made in PBS or EMEM with 10 % normal rabbit serum. The tubes were counted for their [¹²⁵I] content on a crystal scintillation gamma spectrometer (Packard, type 5213). Interferon samples (2 ml) to which 0.2 ml normal rabbit serum and 20 ng [¹²⁵I]-BSA were added, were handled in the same way. BSA-titres were read by interpolation on the calibration curve.

RESULTS

Priming effect of interferon on stimulation of interferon production by poly-I: C in human cells

Optimal conditions for priming of human fibroblasts (vgs-strain) by poly-I:C were described in a previous paper (Billiau et al. 1972a). The following experiment was designed to test whether enhancement of interferon induction could also be obtained by pre-incubating human cells with interferon. In order to evaluate optimal conditions for priming, the cultures were incubated for different periods (3, 6 and 16 h) with different concentrations of interferon (0, 1, 10 and 100 units/ml). After removal of the interferon, the cultures were exposed for 2 h to 50 μg/ml of poly-I: C. Cultures were rinsed and refed. Interferon was determined in the supernatant fluid harvested at 8 h. Fig. 1 shows a time- and dose-dependent priming effect of interferon on the subsequent interferon response to poly-I: C. The highest interferon titre was obtained using a 16 h pre-incubation with 100 units/ml of interferon.

![Graph](image)

Fig. 1. Priming effect of interferon on interferon induction by poly-I: C. Cultures pre-incubated with interferon were stimulated for 2 h with 50 μg/ml of poly-I: C, washed and re-incubated. Interferon was harvested after 6 h.
Table 1. Superinduction of interferon in human cells stimulated by poly-I:C and treated with cycloheximide and actinomycin D

<table>
<thead>
<tr>
<th>Inhibitors added</th>
<th>Amount of interferon (log₁₀ units/ml) harvested from</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 to 6 h</td>
</tr>
<tr>
<td>None</td>
<td>2·6</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>2·4</td>
</tr>
<tr>
<td>Actinomycin D</td>
<td>3·1</td>
</tr>
<tr>
<td>Cycloheximide + actinomycin D</td>
<td>1·0</td>
</tr>
</tbody>
</table>

Cultures were exposed to 50 μg/ml of poly-I:C from time 0 to 2 h. The inducer was washed off and fresh medium containing 0 or 10 μg/ml of cycloheximide was added. At 3·5 h after treatment actinomycin D was added to a final concentration of 1 μg/ml. At 6 h the medium was harvested, cultures were washed and refed fresh medium. A second interferon harvest was taken at 20 h.

Although the priming effect was consistently observed in all our experiments, its magnitude could vary widely. In an experiment similar to that described above, cultures were primed for 24 h with 100 or 3000 units/ml of interferon. One hundred units/ml caused an increase of 0·6 log₁₀ units/ml in production, while 3000 units/ml caused the production to rise by 0·9 log₁₀ units/ml over that in unprimed cultures. Therefore it was concluded that, for production purposes, it is advisable to prime with at least 3000 units/ml for an overnight period.

Superinduction of interferon in human cells, stimulated by poly-I:C and treated with cycloheximide and actinomycin D

The following experiment was designed to test whether addition of cycloheximide and actinomycin D would increase the late yields of interferon in human cells, as was described for rabbit cultures (Tan et al. 1970; Vilcek & Ng, 1971). VGS-cells were induced with 50 μg/ml of poly-I:C for 2 h. After being washed, the cultures were divided in four groups. Two groups received 10 μg/ml of cycloheximide, the others received control medium. At 3·5 h after treatment, actinomycin D (final concentration 1 μg/ml) was added to one group of cycloheximide and one group of control cultures. A first interferon harvest was taken from each of the four sets at 6·5 h. The cultures were rinsed again and refed with plain medium. A second interferon harvest was taken at 24 h.

The results of this experiment are shown in Table 1. It can be seen that cycloheximide caused a shift in the time of interferon production, as described previously (Billiau et al. 1972a; Vilcek & Ng, 1971). Actinomycin D, under the conditions used, caused an increase both in early and late interferon production. Combined treatment with both inhibitors caused a distinct inhibition of early interferon production followed by a strongly increased release of interferon after removal of the inhibitors (superinduction).

The possibility that residual cycloheximide and actinomycin D affected interferon titration was excluded by comparative titration of an interferon sample diluted 1/2 in control medium and in medium from a culture which had received the superinduction schedule without poly-I:C.

Combined application of priming, cycloheximide and actinomycin D in human cultures

The following experiment was designed to test possible additive effects of priming and superinduction procedures. Cultures were exposed to interferon (0 or 1000 units/ml) for
**Interferon stimulation by poly I: C**

Table 2. Production of interferon after combined application of priming, cycloheximide and actinomycin D in human cultures

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cycloheximide from 1 h to time (h)</th>
<th>Actinomycin D added at time (h)</th>
<th>Amount of interferon (log10 units/ml) harvested from</th>
<th>Not primed cultures</th>
<th>Primed cultures</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly-I: C only</td>
<td>—</td>
<td>—</td>
<td>1st harvest 2nd harvest</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Poly-I: C + inhibitors</td>
<td>5.5</td>
<td>2</td>
<td>2.7 2.1</td>
<td>3.3 2.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>2.0 3.6</td>
<td>2.4 4.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>2.2 4.3</td>
<td>2.4 4.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>2.3 4.0</td>
<td>2.6 4.4</td>
<td></td>
</tr>
<tr>
<td>Poly-I: C + inhibitors</td>
<td>5.5</td>
<td>3.5</td>
<td>— —</td>
<td>— 4.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6.5</td>
<td>4.5</td>
<td>— —</td>
<td>— 4.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7.5</td>
<td>5.5</td>
<td>— —</td>
<td>— 3.7</td>
<td></td>
</tr>
</tbody>
</table>

Cultures were primed by adding 10^4 units/ml of interferon at time — 24 h. Poly-I: C (50 μg/ml) was added at 0 h. It was removed at 1 h and replaced by cycloheximide (10 μg/ml). Actinomycin D was added to a final concentration of 1 μg/ml at times indicated. Medium was harvested after removal of inhibitors (1st harvest). Cultures were washed, refed and incubated until 24 h for a second interferon harvest.

24 h. The medium was replaced by 50 μg/ml of poly-I: C for 1 h. Cultures were washed and refed with medium containing 10 μg/ml of cycloheximide. Actinomycin D was added to the cultures at different times as indicated in Table 2. The cultures were harvested and washed at 5.5 h and fresh medium without inhibitors was added. A second harvest of interferon was taken at 24 h.

The time of exposure to poly-I: C (1 h) was kept shorter than in former experiments (2 h). This was done to avoid waste of interferon produced early after induction. It has been described, indeed, that priming shortens the induction period (Stewart et al. 1971). Moreover, from earlier experiments (Billiau et al. 1972a) it appeared that a 1 h contact period with poly-I: C is sufficient for maximal induction of vgs-cells.

The results shown in Table 2 demonstrate that, in the absence of cycloheximide or actinomycin D, the production of interferon was increased by priming with interferon. In cultures which had not been pre-exposed to interferon, the addition of cycloheximide or actinomycin D caused inhibition of interferon production as long as the inhibitors were present. After their removal amounts of interferon were released far in excess over those released by cultures not exposed to the inhibitors. When cultures were pre-exposed to interferon and treated with cycloheximide and actinomycin D, the yields of interferon were slightly higher than when priming was omitted. In an additional experiment, 8 parallel culture sets were used to test this effect; an average difference of 0.24 log10, statistically significant at \( P < 0.001 \), was found.

In the second experiment, shown in Table 2, the total time of incubation with cycloheximide was varied and actinomycin D was added 2 h before its removal. From these data it was concluded that optimal conditions for production of interferon consisted of priming combined with exposure to cycloheximide (from 1 to 6.5 h) and actinomycin D (from 4.5 to 6.5 h).

Finally, the dosage of cycloheximide and actinomycin D was adjusted. For actinomycin D, an optimal dose-response curve was found (optimal dose 1 μg/ml); for cycloheximide dosages ranging from 1 to 100 μg/ml had equal effects.
Comparison of calf serum, human serum albumin and foetal human serum as additives for production of interferon in human cells

Because of their antigenicity, the presence of bovine serum proteins should be avoided in interferon preparations for human use. Attempts to produce interferon by priming and superinduction, in the complete absence of serum or serum components were unsuccessful; yields were about 30 times lower than in the presence of calf serum. The following experiment was done to test the possibility of producing interferon by the priming and superinduction schedule, in medium containing foetal human serum or human plasma protein instead of calf serum. Three groups of 4 culture sets were primed for 24 h with $10^8$ units/ml of interferon, followed by the superinduction schedule described in the preceding paragraph. During the entire operation, the media used for the three groups of cultures were supplemented with 2% calf serum, 2% foetal human serum, or 5% human plasma protein. Yields averaged $10^{4.25}$, $10^{4.15}$ and $10^{4.27}$ units/ml, respectively.

Since the cultures used for interferon production were grown in 10% foetal bovine serum, trace amounts of bovine protein might still be present in preparations harvested from cultures induced in medium with human plasma protein. It was determined by radio-immunoassay that such a preparation contained 0.011 μg/ml of bovine serum albumin.

Production of human interferon by the priming and superinduction method in roller bottles

In order to further increase the yield of interferon in crude preparations, and also to design a system for mass production, a tissue culture roller flask system was used. Confluent monolayers of VGS-cells in roller bottles were primed with 20 ml of interferon at a concentration of $\geq 10^8$ units/ml. After 24 h, the interferon was removed and 10 ml of poly-I:C (50 μg/ml) was added. The inducer was washed off after 1 h and replaced by 10 ml of medium containing 10 μg/ml of cycloheximide. Actinomycin D was added at 4.5 h to a final concentration of 1 μg/ml. At 6.5 h the bottles were washed again and refed with 20 ml of maintenance medium. Interferon was harvested after overnight incubation. With a set of eight roller places serving both cell and interferon production, two bottles a week could be used for interferon production. The yields varied from week to week, probably depending on the condition of the cells. If cell growth was quick (confluency in 3 to 5 days after a 1:2 split) and if the cells were used 10 days after seeding, the yields averaged $10^{4.5}$ units/ml. This corresponded to a 2.5-fold increase over average yields in cups, i.e. well below the expected ninefold increase from the difference in surface/volume ratio between roller bottles and cups. The highest yields obtained in roller bottles were $10^{4.9}$ units/ml.

DISCUSSION

A method is described whereby high yields of human interferon can be produced in diploid skin fibroblasts, stimulated with a non-virus interferon inducer. The method uses two effects which have been observed previously in rabbits, mouse or human cells: (i) the 'priming effect' and (ii) the 'superinduction effect'.

Priming, or enhancement of interferon production in cell cultures by pretreatment with interferon has been described by several authors (Rosztoczy & Mecs, 1970; Stewart et al. 1971; Tovell & Cantell, 1971), either in systems where viruses or non-virus inducers were used. Human cells induced by poly-I:C can effectively be primed by pre-incubation with a small amount of poly-I:C (Billiau et al. 1972a, b). In the present study, priming by interferon in human skin fibroblasts was found to be dose- and time-dependent, maximum
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effects being obtained by overnight incubation with a concentration of interferon greater than 10⁸ units/ml.

Superinduction of interferon has been described in rabbit kidney cultures and in human embryonic fibroblasts stimulated by poly-I:C or u.v.-irradiated Newcastle disease virus (Tan et al. 1970; Vilcek & Ng, 1971; Ho et al. 1972). Addition of cycloheximide for 3 to 4 h after poly-I:C and actinomycin D, 0.5 to 1 h before removal of cycloheximide was found to result in inhibition of interferon production as long as the inhibitors were present, followed by release of large quantities of interferon soon after their removal. A possible explanation for this phenomenon was that actinomycin D, given at a suitable time, prevented the synthesis of a protein which blocked translation of the interferon messenger.

By adapting time and dosage of exposure to cycloheximide and actinomycin D, we could obtain a 50- to 60-fold increase in interferon production in treated cultures over control cells exposed to poly-I:C only. A combination of priming and superinduction procedures produced 1.8 times more interferon than superinduction only. Average yields thus obtained were 10⁴¹ units/ml.

A further increase to 10⁴⁵ units/ml could be obtained by using roller bottles instead of stationary cultures. However, this increase did not correspond to the ninefold higher surface/volume ratio in the roller bottles. Therefore, the available cells in this system were less efficiently used than in stationary cultures. Additional investigation is needed to find a suitable system for mass production.

The highest yields obtained by our method were 10⁴⁹ units/ml. A comparison with yields described in literature is difficult because the sensitivity of assay systems varies between different laboratories, and reference preparations have only recently become available (International Symposium on Interferon and Interferon Inducers, 1970).

If only the recent studies (Tovell & Cantell, 1971; Ho et al. 1972), which deal with the best available production systems, are taken into account, it appears that our yields in roller bottles are four- and tenfold higher than those obtained, respectively, in the mass leukocyte-Sendai virus system (Tovell & Cantell, 1971) and in fibroblasts stimulated by Newcastle disease virus (Ho et al. 1972). Our preparation has also the advantage over virus-induced interferon in containing less cell lysis material and no residual inducer virus or virus antigens. Moreover, in the event of production for administration in humans, preparations made on diploid cells can more easily be monitored for presence of endogenous agents than those prepared in leukocytes. Omission of bovine serum from the medium resulted in a 30-fold decrease of interferon production. No decrease in yield was noted when human foetal serum or human plasma protein were added. However, complete elimination of bovine protein, left over from the growth medium, was not achieved. Traces of bovine serum albumin amounting to 0.011 µg/ml could still be detected by radioimmunossay. For this reason the preparation as such may not be safe for systemic use in humans.

Note added in proof

Results similar to those published here have recently been obtained by Havell & Vilcek (1972).

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