Induction of Mouse Interferon in a Chemically Defined System

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Production of interferon from cultured cells has required the presence of serum in the cell culture medium although it may be omitted during the period of interferon induction. In the system reported here a fully defined medium, free of protein, is employed. This permits the culture of the cells and all phases of interferon induction and release to be studied under completely defined conditions. The final product can easily be separated from its inducers to yield a preparation of high specific activity.

The LS strain of mouse fibroblasts, adapted to grow in suspension, has been under regular culture in this laboratory (Griffiths & Pirt, 1967; Birch & Pirt, 1969, 1970, 1971; Blaker & Pirt, 1970) in the protein-free medium (Birch & Pirt, 1970). The only polymer used in the medium is the uncharged polysaccharide methylcellulose. Interferon produced by cells growing in this medium is assayed according to the method of Finter (1969), in which neutral red is used for an estimation of cytopathic effect due to the challenge by Semliki Forest virus (at 10⁷ p.f.u./ml.) upon mouse L cells grown in monolayer cultures. A calibrated laboratory standard preparation was included in all assays so that the potency values could be expressed in units based on the mouse interferon reference preparation of the National Institute of Health, Bethesda, Maryland, USA.

Polyinosinic-polycytidylic acid (poly rI. poly rC) was purchased in lyophilized form from PL Biochemicals, Milwaukee, Wisconsin, USA, and dissolved as prescribed by Field et al. (1967). Double-stranded RNA (ds-RNA) prepared from a virus of Penicillium chrysogenum (Buck, Chain & Himmelweit, 1971) and dissolved in saline citrate buffer at pH 6-2, was a gift from Sir Ernst Chain, Imperial College, London. Using these inducers, interferon could be detected only in the presence of a suitable polycation. Diethylaminoethyl-dextran (DEAE-dextran; mol. wt about 2 x 10⁶ from Pharmacia (BG) Ltd, London) is suitable for this purpose (Dianzani et al. 1968). Inducers were removed by washing the cells twice. Polyanions have been used to neutralize the potentiating and toxic effects of DEAE-dextran (Tilles, 1970; Dianzani, Gagnoni & Cantagalli, 1970). In our system the addition of heparin (trade mark Pularin, Evans Medical Ltd, Speke, Liverpool; B.P. specification) after onset of induction was found to be an effective means of stopping production of more interferon and for preventing the development of antivirus activity, due to the inducer, in the subsequent assay. Experiments were carried out to determine the appropriate quantities of heparin required to prevent induction of interferon. The ds-RNA, DEAE-dextran and heparin in various combinations were added to the cells simultaneously and left in contact with the cells for 6 hr after which the cells were removed, washed twice, and resuspended in fresh medium. Interferon was harvested at 24 hr and assayed. No heparin was added to the controls. The results are shown in Table 1.

The use of heparin at a concentration equal to that of DEAE-dextran was subsequently adopted for the termination of all inductions employing polyribonucleotide inducers. Inducers, thus neutralized with heparin and mixtures of DEAE-dextran and heparin alone were used as controls in the assays and were shown to be devoid of antivirus activity, nor did they affect the growth rate of the cells. This is in contrast to the effects of RNA or dextran alone or in combination, both of which are toxic to the cells.
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Table 1. *Neutralization by heparin of the potentiating effect of DEAE-dextran on the induction of interferon by a double-stranded myxophage RNA*

<table>
<thead>
<tr>
<th>Heparin (μg./ml.)</th>
<th>DEAE-dextran (μg./ml.)</th>
<th>ds-RNA (μg./ml.)</th>
<th>Interferon titre as a percentage of the control</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>50</td>
<td>1.0</td>
<td>0</td>
</tr>
<tr>
<td>50</td>
<td>50</td>
<td>1.0</td>
<td>0</td>
</tr>
<tr>
<td>25</td>
<td>50</td>
<td>1.0</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>50</td>
<td>1.0</td>
<td>55</td>
</tr>
<tr>
<td>1.0</td>
<td>50</td>
<td>1.0</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 2. *A comparison of the inducers employed, under conditions optimal for each*

<table>
<thead>
<tr>
<th>Inducer concentration</th>
<th>Poly rI.rC (μg./ml.)</th>
<th>ds-RNA (myxophage) (μg./ml.)</th>
<th>NDV u.v. (μg./ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEAE-dextran concentration</td>
<td>10.0</td>
<td>1.0</td>
<td>*</td>
</tr>
<tr>
<td>Time of heparin addition</td>
<td>3.0 hr</td>
<td>1.0 hr</td>
<td>—</td>
</tr>
<tr>
<td>Concentration of LS cells per ml.</td>
<td>1.0 × 10⁶</td>
<td>5.0 × 10⁶</td>
<td>1.0 × 10⁶</td>
</tr>
<tr>
<td>Time of appearance of interferon</td>
<td>2.0 hr</td>
<td>5.0 hr</td>
<td>6.0 hr</td>
</tr>
<tr>
<td>Time of harvest of interferon</td>
<td>12.0 hr</td>
<td>24.0 hr</td>
<td>2.0</td>
</tr>
<tr>
<td>Maximum concentration of interferon in log units</td>
<td>2.7 to 3.0</td>
<td>2.7 to 3.0</td>
<td>2.0</td>
</tr>
</tbody>
</table>

* 12.8 haemagglutination units/ml.

The system also proved susceptible to induction by Newcastle Disease virus (strain H) inactivated by u.v. light according to the method of Youngner *et al.* (1966). Subsequent interfering activity of the virus was destroyed by dialysis of the interferon sample against glycine hydrochloride buffer at pH 2.0 for 5 days, after which it was back dialysed to pH 7.0 prior to assay.

An important feature of our defined system is the absence of serum protein. The levels of protein (Lowry *et al.* 1951; bovine plasma albumin standard) obtained in crude preparations of interferon were in the range 50 to 150 μg./ml. as compared with about 1.5 mg./ml. for a preparation containing 2% serum. Potency is not affected by the presence of 2% serum; therefore, under the optimal conditions described below (Table 2), minimum specific activities of the crude interferons are 300 units/mg. protein and 3000 units/mg. protein respectively in the presence and absence of 2% serum.

Fig. 1 shows a typical time course of the appearance of interferon in response to poly rI.poly rC employing the conditions described in Table 2.

The antivirus activity obtained from these inductions exhibited properties compatible with those of mouse interferon (Lockart, 1966), viz. all activity was rapidly destroyed by trypsin; was insensitive to levels of ribonuclease that degraded double-stranded RNA (hyperchromic effect at 260 nm. and loss of interferon-inducing ability) and was unaffected by deoxyribonuclease. Full activity was recovered from the supernatant fluid after centrifuging for one hr at 100,000 g. It was stable at pH 2.0 for 1 week and was retained by dialysis tubing (Visking) at pH 2.0 and at pH 7.0. At pH 7.5 activity was retained by a collodion membrane, under pressure, with a specified mol. wt ‘cut off’ of 25,000 (Sartorius Membranfilter GmbH, Göttingen, Germany). Approximately 90% of the activity was lost after one hr at 56° and 50% after 12 hr at 37°. No loss in activity was measurable after
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Fig. 1. The appearance of interferon in response to poly rI, poly rC employing the conditions employed in Table 2. The arrow shows the time of addition of heparin.

1 week at 2°. Crude material stored at −20° varied in its stability. On one occasion activity was lost after 3 months but generally it was stable for longer than 6 months. The material conferred no protection from cytopathic effects of Semliki Forest virus on chick embryo fibroblasts nor upon human foetal lung cells under conditions when poly rI, poly rC did so but a partially purified sample was found to protect against the cytopathic effects of vesicular stomatitis virus (Dr I. Gresser, personal communication).

Probit analysis (Finney, 1952) of the dose response curves from the assay of this material together with a preparation of mouse serum interferon, which had been calibrated against the NIH reference preparation by Dr N. B. Finter, showed the two to be directly comparable in a valid assay.

Partial purification of the interferon was achieved using a method similar to that of Falcoff et al. (1966). The crude material was exhaustively dialysed against 0.1M-phosphate buffer, extracted batchwise by CM Sephadex (C50; Pharmacia (GB) Ltd) and subsequently eluted by a rising pH gradient after extensive washing at pH 6.0 (Merigan, Winget & Dixon, 1965). Activity was recovered between pH 6.0 and pH 8.0 and subsequently concentrated by ultrafiltration according to the final titre required. Specific activities in the range 1.0 to \(5.0 \times 10^5\) reference units of mouse interferon per mg. of protein were thus obtained, with an overall recovery of activity at least in excess of 30%. Stored at −20° in the presence of bovine serum albumen (Cryst., Armour Pharmaceutical Co. Ltd, Eastbourne, Sussex) at 5 mg./ml., no loss in activity occurred over a 6-month-period. Washing at pH 6.0 removed polyribonucleotide, DEAE-dextran, heparin and methylcellulose so that the final eluate contained only interferon and associated cell proteins in phosphate buffer.

We gratefully acknowledge the gift of suspensions of Semliki Forest virus and Newcastle Disease virus from Dr C. J. Bradish of the Microbiological Research Establishment, Porton Down, Wilts. We are similarly indebted to Dr N. B. Finter (then of Imperial Chemical Industries, Pharmaceuticals Division, Macclesfield, Cheshire) for the gift of a strain of mouse L cells and for supplies of mouse interferon, calibrated against the NIH reference preparation.

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REFERENCES


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