Mouse Interferons: Production by Ehrlich Ascites Tumour Cells Infected with Newcastle Disease Virus and its Enhancement by Theophylline

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SUMMARY

Conditions are described for the production of 0.3 to 0.7 NIH mouse reference standard units of interferon per cell from Ehrlich ascites tumour cells cultured as monolayers and induced by infection with Newcastle disease virus (NDV). Inclusion of theophylline (6 mM) in the medium increased the interferon yield three to four times. Cells infected with NDV started to lyse at about 15 h p.i., but infected, theophylline-treated cells lysed only 24 h p.i.

Several other methylxanthines (e.g. theobromine, caffeine and isobutylmethylxanthine), when tested at concentrations similar to that of theophylline, did not boost interferon production. Dibutyryl cyclic AMP (10⁻⁶ to 10⁻² M) did not substitute for theophylline in increasing interferon production, and, if used together with theophylline, did not cause further enhancement.

INTRODUCTION

Interferons are glycoproteins produced by a large variety of vertebrate cells upon virus infection or some other stimuli. They are released from the producing cells and bind to other cells in which they induce an 'antiviral' state in which virus replication is impaired. Interferons also affect the immune system in various ways and may impair cell proliferation (Finter, 1973; Stewart, 1979).

We have produced mouse interferons from Ehrlich ascites tumour (EAT) cells grown in monolayer cultures and infected by Newcastle disease virus (NDV) (Gupta et al. 1973; Kawakita et al. 1978). Here we describe the use of theophylline to enhance interferon production. This compound was shown earlier to enhance interferon yields in cultures of L cells (Mecs, 1973) and 1 day-old chick embryo fibroblasts infected with influenza virus (Reizin et al. 1975).

METHODS

Buffers and media. Phosphate-buffered saline (PBS): 137 mM-NaCl, 2.7 mM-KCl, 8.1 mM-Na₂HPO₄, 1.5 mM-KH₂PO₄, (pH 7.2); monolayer growth medium: Eagle's minimal essential medium with Earle's salts (medium 410-1500; Gibco, Grand Island, N.Y., U.S.A.) supplemented with 7% (v/v) foetal calf serum (Gibco), 100 units/ml penicillin, 100 μg/ml streptomycin and 2 mg/ml additional glucose. Interferon induction and production media: Eagle's minimal essential medium with Earle's salts supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mg/ml additional glucose and 0.5% (v/v) foetal calf serum.
**Cells and viruses.** EAT cells (Van Venrooij et al. 1970; Kawakita et al. 1978) were grown in monolayer growth medium in disposable 75 cm² plastic tissue culture flasks at 37 °C in a 5% CO₂ incubator or (with screw caps tight) in a 37 °C constant temperature room. The cells were passaged on the day when they became confluent (approx. 2 × 10⁷ cells/75 cm²). The cells were suspended by treatment with 1 mM-EDTA in PBS at 37 °C for a few minutes, sedimented by low speed centrifugation, resuspended in fresh monolayer growth medium and dispensed into new tissue culture flasks. When passaged at a split ratio of 1:10, the cells formed a confluent monolayer after 3 to 4 days.

**NDV (Beaudette strain)** was passaged in 10 day-old embryonated chicken eggs (Roman & Simon, 1976). A stock of the virus in allantoic fluid was diluted 1:10000 in PBS supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin, and 0.2 ml was injected into the allantoic cavity of each egg. The infected eggs were incubated in a humidified incubator at 38 °C for approx. 44 h and were then chilled at 4 °C for 3 h. The allantoic fluid was collected, clarified by low speed centrifugation, supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin and stored at 4 °C for up to 1 month. The titre of the virus was usually between 1 × 10⁹ and 2 × 10⁹ p.f.u./ml and did not decrease significantly when stored at 4 °C for only a few weeks. Long term storage of the virus was at −50 °C. The virus was assayed by adsorbing appropriate serial dilutions of the virus on to freshly confluent primary chick embryo fibroblasts (CEF). The virus inoculum was then aspirated and monolayer growth medium, supplemented with 0.8% agarose, was added. The dishes were incubated at 37 °C in 5% CO₂ for 48 h or until plaques were visible. Cells were stained with 0.02% neutral red in PBS and the plaques were counted.

Avian reovirus (isolate S1133; Van der Heide et al. 1974) was obtained from Dr L. Van der Heide (University of Connecticut, Storrs, Conn., U.S.A.). It was passaged in primary CEF and its titre was determined by plaque assay on primary CEF cultures (Spandios & Graham, 1976).

Vesicular stomatitis virus (VSV), Indiana strain (Stampfer et al. 1971), was passaged at 0.1 p.f.u./cell in mouse L cells. Aliquots of the clarified supernatant from VSV-infected cells were stored at −50 °C.

**Standard conditions for interferon production in EAT monolayers in stationary flasks.** The cells were induced on the day that they became confluent. The spent culture medium was decanted and 5 ml of induction medium containing the required amount of NDV was added to each 75 cm² flask. Virus was adsorbed to cells at 37 °C, with occasional tilting of the flasks. Two h p.i., the virus solution was decanted and 10 ml of production medium containing any required additives were added. The flasks were returned to the 37 °C incubator. Interferon was harvested 25 h p.i. (unless otherwise indicated) by decanting the culture medium and centrifuging it at 7000 g to pellet the debris. The supernatant containing the crude interferon was processed for interferon assay, or was supplemented with 10 μM-phenylmethylsulphonyl fluoride (PMSF; Sigma, St. Louis, Mo., U.S.A.) and 3.1 mM-NaNO₃ to decrease proteolysis and microbial growth and stored at 4 °C until purified.

**Processing of crude interferon for interferon assay.** A 2 ml amount of the crude interferon was dialysed at 4 °C for 24 h against at least 100 vol. of 50 mm-KCl, 10% glycerol, adjusted to pH 2 with HCl. Thereafter, the pH of each sample was adjusted to neutrality with a few drops of 0.5 M-Na₂HPO₄. The samples were assayed immediately for interferon or were stored in plastic tubes at 4 °C. The plaque reduction assay was performed using L929 cells and VSV as described by Kawakita et al. (1978) and Vassef et al. (1973).

The amounts of interferon are expressed in international reference units in terms of the NIH mouse reference preparation, G002-904-511.
RESULTS AND DISCUSSION

Enhancement of interferon production by theophylline

When mouse interferon is produced in EAT cells as described in Methods the amount of interferon released into the medium by about 25 h p.i. is about 0.08 units/cell. Reports on the enhancement of interferon production (Mecs, 1973; Reizin et al. 1975) by treatment with agents presumed to boost cAMP levels (e.g. theophylline; Robison et al. 1968; Lauzon et al. 1976) prompted us to perform similar experiments. Fig. 1 shows that theophylline at 6 mM, the optimal concentration, increased interferon production 3.5-fold. (In other experiments this increase varied between two- and fourfold and was on average threefold.) It was established in a separate experiment that the addition of 6 mM-theophylline to interferon samples before processing them for the assay had no effect on the outcome of the assay (not shown).

The data in Fig. 2 suggest that theophylline increases the rate of accumulation of interferon in the medium and extends the length of time during which interferon is produced. The optimal m.o.i. with NDV for interferon production in our conditions is about 20 p.f.u./cell (Fig. 3).

A further boost in interferon yield (up to twofold) was obtained when the production medium containing 6 mM-theophylline was replaced by fresh medium with the same theophylline content at any time between 5 and 8 h p.i. Further addition of 6 mM-theophylline (alone) at this time did not substitute for replacement of the medium. Treatment of cells with 6 mM-theophylline for 18 h before infection with NDV and/or the inclusion of theophylline together with NDV during infection did not enhance interferon production (data not shown). Attempts to enhance production further by 'superinduction' (Vilcek et al 1969; Tan et al. 1970, 1971; Sehgal et al. 1975; Wiranowska-Stewart et al. 1977), i.e. by treatment with cycloheximide (20 µg/ml) and actinomycin D (2 µg/ml) in addition to 6 mM-theophylline at various times after infection, were unsuccessful.
Fig. 3. Interferon production in the presence of 6 mM-theophylline: effect of m.o.i. with NDV.

Table 1. Interferon production in NDV-infected EAT cells* is not increased by dibutyryl cAMP or any of several methylxanthines other than theophylline

<table>
<thead>
<tr>
<th>Addition to the production medium</th>
<th>Yield of interferon (units/cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expt. 1 None</td>
<td>0.079</td>
</tr>
<tr>
<td>6 mM-theophylline</td>
<td>0.29</td>
</tr>
<tr>
<td>0.001 mM-dibutyryl cAMP</td>
<td>0.069</td>
</tr>
<tr>
<td>0.1 mM-dibutyryl cAMP</td>
<td>0.085</td>
</tr>
<tr>
<td>1 mM-dibutyryl cAMP</td>
<td>0.085</td>
</tr>
<tr>
<td>10 mM-dibutyryl cAMP</td>
<td>0.091</td>
</tr>
<tr>
<td>6 mM-theophylline plus</td>
<td></td>
</tr>
<tr>
<td>10 mM dibutyryl cAMP</td>
<td>0.18</td>
</tr>
<tr>
<td>Expt. 2 None</td>
<td>0.12</td>
</tr>
<tr>
<td>1 mM-theophylline</td>
<td>0.21</td>
</tr>
<tr>
<td>6 mM-theophylline</td>
<td>0.36</td>
</tr>
<tr>
<td>0.5 mM-theobromine</td>
<td>0.12</td>
</tr>
<tr>
<td>2.5 mM-theobromine</td>
<td>0.050</td>
</tr>
<tr>
<td>1 mM-caffeine</td>
<td>0.11</td>
</tr>
<tr>
<td>5 mM-caffeine</td>
<td>0.053</td>
</tr>
<tr>
<td>1 mM-isobutylmethylxanthine</td>
<td>0.036</td>
</tr>
<tr>
<td>5 mM-isobutylmethylxanthine</td>
<td>&lt;0.007</td>
</tr>
</tbody>
</table>

*EAT cells were infected with NDV at a m.o.i. of 15.

Studies on the basis of the enhancement of interferon production by theophylline

Theophylline inhibits the cleavage of cAMP by phosphodiesterase, thereby causing an increase in cAMP levels in various cells (Robison et al. 1968). Furthermore, dibutyryl cAMP, or agents thought to increase the level of cAMP, have been reported to decrease (Dianzani et al. 1972) or to increase (Mecs, 1973; Reizin et al. 1975) interferon production in various cells. We, therefore, tested the effect of dibutyryl cAMP on interferon production (see Table 1, Expt. 1). If added at concentrations between 0.001 and 10 mM 2 h p.i., this compound had no effect on interferon production. When 10 mM-dibutyryl cAMP was added 2 h p.i., together with 6 mM-theophylline, it caused a 2.2-fold enhancement in interferon production, whereas theophylline added alone caused a 3.5-fold enhancement.

Some other methylxanthines, e.g. theobromine, caffeine or isobutylmethylxanthine, are also thought to stabilize cAMP by blocking its cleavage by phosphodiesterase (Robison
Mouse interferon production

et al. 1968). Nevertheless, at the concentrations tested, these agents did not substitute for theophylline in boosting interferon production (Table 1, Expt. 2).

These results suggest that theophylline may enhance interferon production independently of its effect on cAMP levels. The potentiation of a calcium ion-dependent potassium ion conductance in neurons by theophylline was also reported not to be due to inhibition of phosphodiesterase (Smith et al. 1979).

Further tests established that treatment with 6 mM-theophylline had no effect on the amount of NDV recovered from EAT cells: at 25 h p.i. with NDV at a m.o.i. of 18, 0.7 p.f.u. of NDV was recovered per EAT cell whether or not the cells were treated with theophylline. Moreover, 6 mM-theophylline did not enhance interferon production in an experiment in which avian reovirus at a m.o.i. from 1 to 40 was used as the interferon inducer. In this experiment, the yield of interferon at the highest m.o.i. tested was 0.12 units/cell with or without theophylline (data not shown).

Treatment of EAT cells with 6 mM-theophylline 2 h p.i. with NDV (at a m.o.i. of 18) delays the onset of cell lysis until about 24 h p.i.; the cells not treated with theophylline begin to lyse at about 15 h p.i. (not shown).

The mechanism by which theophylline treatment enhances the production of interferon by EAT cells infected by NDV remains to be elucidated.

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REFERENCES


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