Production and Initial Characterization of Rat Interferon

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SUMMARY

Rat interferon of relatively high specific activity (about $10^8$ units/mg protein) was produced in embryonic rat cells treated with Newcastle disease virus at a high m.o.i. The cells were cultured in serum-free medium and the interferon was precipitated and concentrated with 0.02 M-zinc acetate or with ammonium sulphate at 85% saturation. With both methods the increase in interferon activity was greater than the concentration factor. The rat interferon activity was stable on treatment with 0.15 M-perchloric acid or three cycles of freezing and thawing, but incubation at $37^\circ C$ for 1 h resulted in a 50% loss in activity. It had no cross activity in human or mouse cells. The sensitivity of different types of rat cells for rat interferon differed widely and was dependent on the challenge virus. Human interferons had no detectable antiviral activity on rat cells and did not block the activity of rat interferon.

In the few published studies on rat interferon there are discrepancies in the physico-chemical properties reported and in the activity of rat interferon in cells of other species (Biernacka & Lobodzińska, 1973; Lobodzińska et al. 1973; Illinger et al. 1976). An interesting finding is that human interferons have antiviral activity in rat cells and that human interferon inhibits rat interferon activity (Chany, 1976; Duc-Goiran et al. 1971). However, all these studies have been performed with rather impure interferon preparations. This study was designed to produce rat interferon of high specific activity and to study some of its physico-chemical and biological activities.

The origin, propagation and titration of herpes virus hominis type I, vaccinia and vesicular stomatitis virus (VSV) were as described elsewhere (Stitz & Schellekens, 1980). Newcastle disease virus (NDV), Komorow strain, was grown in the allantoic fluid of 10-day-old chicken embryos. The titre of this virus was expressed in p.f.u, established in primary chicken embryo cells.

Rat embryo cells (REC) were prepared from approx. 15-day-old embryos of WAG/Rij rats. Cells from the first passage were pooled and stored in liquid nitrogen with dimethyl-sulphoxide (DMSO) as a cryoprotective agent and subcultures derived from these were used in our experiments. Normally, REC could be serially passaged up to the 30th passage, but one strain was subcultivated further, and after the 40th passage it transformed, apparently spontaneously. We designated this rat cell line Ratec and these cells have now undergone more than 100 passages in our laboratory without deterioration in growth. XC cells (derived from a Rous sarcoma virus-induced tumour) and RR 1022 cells (from a Schmidt-Ruppin sarcoma in a Rattus norvegicus) were obtained from the American Type Culture Collection. RSC cells, derived from a radiation-induced rat skin carcinoma, RUC II cells from rat urethral carcinomas, RM cells from a spontaneous rat rhabdomyosarcoma and ROS cells from an osteosarcoma were donated by Dr G. W. Barendsen (Radiobiological Institute TNO, Rijswijk, The Netherlands). WIRA rat cells were donated by Dr I. Gresser (Institut de Recherches Scientifiques sur le Cancer, Villejuif, France). RSb cells are Rous sarcoma virus-transformed human cells and were donated by Dr T. Kuwata (Chiba University, Japan), Mouse L929 cells were originally obtained from Flow Laboratories (Irvine, Scotland). Chicken embryo cells were prepared from 10-day-old chicken embryos.
All cells were routinely propagated in Brockway prescription bottles in Dulbecco's modification of Eagle's minimal essential medium (DMEM), supplemented with 10% foetal calf serum (FCS) and antibiotics. When cells were grown in roller bottles, DNEM was supplemented with a pH 7.2 buffer consisting of 0.0075 M-N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid (HEPES), 0.005 M-N-tris-(hydroxymethyl)methyl-2-amino-ethane sulphonic acid (TES) and 0.005 M-morpholinopropane sulphonic acid (MOPS).

To produce rat interferon $2 \times 10^7$ Ratec cells in 100 ml DMEM plus 10% FCS were grown in 1.5 l roller bottles being rotated at 0.5 rev/min. Usually within 4 days confluency was reached and the cells were re-fed with 100 ml DMEM plus 10% newborn calf serum (NCS). The next day the culture medium was discarded and the cells were treated with NDV at a m.o.i. of 30 to 50 p.f.u./cell for 90 min. After challenge the cells were washed twice with PBS and 50 ml DMEM without serum added. After 24 h the medium was harvested and centrifuged for 2 h at 14,000 g to remove the bulk of NDV. The remaining NDV was inactivated by treatment at pH 2.0 for 5 days, or by adding perchloric acid to 0.15 M for 30 min, which had no effect on the antiviral activity. After neutralization with 50 M-NaOH to pH 6.0, the preparation was stored frozen at $-20^\circ$C and designated 'crude interferon'. No residual live NDV could be demonstrated after inoculation of the preparations in embryonated chicken eggs.

Mouse interferon, induced with NDV in L929 cells, had a specific activity of $10^6$ units/mg protein. Human leucocyte interferon, a kind gift from Dr K. Cantell (State Serum Institute, Helsinki, Finland) was prepared as described before (Cantell, 1970); its sp. act. was $10^6$ units/mg protein. Human fibroblast interferon, prepared as described before (Billiau et al. 1973) and obtained by the courtesy of Dr A. Billiau (Rega Institute, University of Leuven, Leuven, Belgium), had a sp. act. of $10^6$ units/mg protein.

Cells were grown in microtitre plates at a concentration of $2 \times 10^4$ cells per well in DMEM supplemented with 10% FCS. When a monolayer was formed, the supernatant was removed and the preparation to be tested was added in twofold dilutions in DMEM with 1% FCS. After overnight incubation, the supernatant was again removed. The cells were washed with PBS (Dulbecco's modification) and infected with VSV at 10 TCID$_{50}$ per well. When the untreated infected control showed > 90% c.p.e., the assay cells were stained with crystal violet. The interferon activity was expressed as the reciprocal of the maximum dilution protecting 50% of the cells as estimated visually or established by the dye elution of Armstrong (1971).

In every assay a new sample of a laboratory rat interferon reference stored at $-70^\circ$C was included as a control for variables between the different tests. The mean result of the first ten tests of this standard was 96 units and so it was considered to contain 100 laboratory reference units of rat interferon. All rat interferon activities in this study were titrated against this standard, which has now been tested on 28 different occasions. When the results of all these tests were normalized by a log transformation, they were shown to be normally distributed with a mean log titre of 1.85 (i.e. 78 units) and a standard deviation of 0.24 (coefficient of variation of 13%). The research reference preparation G-002-904-511 was used in the mouse interferon assay in L$_{929}$ cells and the reference preparation 69/I9 in the human interferon assay in R5b cells. Hence the activities of these interferons could be expressed in international reference units. Poly(rI).poly(rC) was obtained from Boehringer, Mannheim, and its activity as an interferon inducer was established in L$_{929}$ cells.

Interferon production in rat cells increased with increasing challenge doses of NDV. The highest production was obtained with the highest challenge dose tested ($10^4$ p.f.u./cell). Routinely, however, we used a challenge dose of 30 to 50 p.f.u./cell, so that unconcentrated NDV in allantoic fluid could be used.
The interferon-producing capacity of rat embryo, Ratec, WIRA and RR 1022 cells with NDV was tested. The secondary rat embryo cells and Ratec cells were the best producers (10³ to 10⁴ units/ml). WIRA and RR 1022 cells produced only moderate amounts of interferon (10².⁰ and 10².₉ units/ml, respectively). The type (foetal or newborn) and concentration (0 to 10 %) of serum did not influence production.

Poly(rI).poly(rC) can be a potent inducer of interferon in vitro, but when added to embryonic rat cells at a concentration of 100 µg/ml it induced only minimal amounts of interferon. Its inducing capacity was only slightly increased by adding DEAE dextran or after priming. Superinduction, successfully employed in human embryonic cells (Billiau et al. 1973; Wiranowska-Stewart et al. 1977), did not enhance interferon production.

Ammonium sulphate has been widely used to precipitate various interferons and we tested this with rat interferon. Some activity precipitated at 25 % saturation and the precipitation was complete at 85 % saturation, independent of pH in the range of pH 2 to pH 7.5. Rat interferon activity could also be precipitated with zinc acetate at a concentration of 0.02 M (Table 1). No loss in activity was encountered during precipitation, and indeed, unexpectedly, both methods of precipitation resulted in an increase in activity. The preparations used in the present study were concentrated and purified by zinc acetate.

Rat interferon is stable for at least 2 years when stored frozen in the presence of 10 % FCS at −70 °C. At 0 °C no loss in activity was observed during 24 h incubation. At a room temperature of 20 °C, activity started to decrease after 4 h. One hour at 37 °C resulted in a 50 % loss in activity. Heating at 100 °C destroyed all activity after 15 min. Three cycles of freezing and thawing had no discernible effect on activity, but seven resulted in the loss of half of the activity.

The antiviral activity in our preparations met the following criteria for an interferon. It was stable at pH 2-0, but sensitive to trypsin treatment. There was no activity with mouse and human cells, both sensitive to their homologous interferon, but rat cells could be protected against both VSV and vaccinia virus.

In Table 2 the antiviral activity of rat interferon against the c.p.e. of VSV and herpes viruses in different rat cells is compared. With VSV, Ratec cells were the most sensitive and RUC II cells the least. With herpes virus, Ratec cells were the least sensitive and ROS and RR 1022 cells were equally well protected. There was no correlation between the protection against VSV and herpes virus induced by rat interferon in these cells. Neither human

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**Table 1. Flow sheet for partial purification of rat interferon**

<table>
<thead>
<tr>
<th>Ammonium sulphate precipitation method</th>
<th>Zinc acetate precipitation method</th>
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</thead>
<tbody>
<tr>
<td>Initial: 100 ml crude rat interferon.</td>
<td>Total 12000 units. Sp. act. 100 units/mg protein</td>
</tr>
<tr>
<td>Precipitate with (NH₄)₂SO₄ added to 85 % saturation</td>
<td>Precipitate with zinc acetate added to 0.02 M</td>
</tr>
<tr>
<td>Centrifuge 60 min at 10000 rev/min</td>
<td>Centrifuge 60 min at 2000 rev/min</td>
</tr>
<tr>
<td>Dissolve precipitate in 10 ml PBS</td>
<td>Dissolve precipitate in 10 ml 0.2 N-HCl</td>
</tr>
<tr>
<td>Dialyse extensively against PBS</td>
<td>Dialyse first against saline then against PBS</td>
</tr>
<tr>
<td>Centrifuge for 30 min at 10000 rev/min</td>
<td>Centrifuge for 30 min at 10000 rev/min</td>
</tr>
<tr>
<td>Sterilize by Millipore filtration</td>
<td>Sterilize by Millipore filtration</td>
</tr>
</tbody>
</table>

Final: 10 ml purified rat interferon

<table>
<thead>
<tr>
<th>Total 68000 units/ml</th>
<th>Total 68000 units/ml</th>
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<tbody>
<tr>
<td>Sp. act. 250000 units/mg protein</td>
<td>Sp. act. 630000 units/mg protein</td>
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</table>
Table 2. Antiviral activity of rat interferons (10^4.8 units/ml) in rat cells of different origin

<table>
<thead>
<tr>
<th>Challenge virus</th>
<th>Ratec</th>
<th>RUC II</th>
<th>RSC</th>
<th>ROS</th>
<th>RM</th>
<th>XC</th>
<th>RM 1022</th>
<th>WIRA</th>
</tr>
</thead>
<tbody>
<tr>
<td>VSV</td>
<td>10^8</td>
<td>10^8</td>
<td>10^4</td>
<td>10^8</td>
<td></td>
<td></td>
<td>10^4</td>
<td></td>
</tr>
<tr>
<td>Herpes virus</td>
<td>10^8</td>
<td>ns*</td>
<td>ns</td>
<td>10^7</td>
<td>ns</td>
<td>ns</td>
<td>10^4</td>
<td></td>
</tr>
</tbody>
</table>

* ns = Not sensitive (virus does not produce cytopathogenic effects in these cells).

Fibroblast nor leucocyte interferon had any antiviral effect in these rat cells and pre-treatment with 1000 units of either of these interferons did not influence the sensitivity of rat embryonic cells to rat interferon.

This study shows that a high challenge dose of NDV is necessary to induce reasonable amounts of interferon in rat cells. Secondary embryonic rat cells and Ratec cells were the best producers of interferon of all cells studied.

Serum in the culture medium is a major source of protein impurities in interferon preparations produced in vitro. We demonstrated that the production of rat interferon was not affected when serum was omitted from the tissue culture medium. This made it possible to produce rat interferon of a high specific activity with the use of a single purification and concentration step.

In contrast to the properties of our rat interferon, Schonne (1966) found that the activity of his rat interferon preparation only precipitated with ammonium sulphate at 100% saturation and could not always be recovered when precipitated by 0.02 M-zinc acetate. He also reported a 50% loss in interferon activity after treatment with 0.15 M-perchloric acid and complete loss after two cycles of freezing and thawing. In the present study, rat interferon completely precipitated at a concentration of 85% and its activity could easily be recovered from a 0.02 M-zinc acetate precipitate; 0.15 M-perchloric acid could be used to inactivate NDV without any loss in interferon activity and the antiviral activity of rat interferon was not affected by three cycles of freezing and thawing.

There was a considerable increase in the total amount of rat interferon activity when our preparations were concentrated and partially purified. This phenomenon was shown not to be due to interferon induction by residual NDV in our preparations. An antagonist of interferon activity produced in embryonic and other tissues has been described (Fournier et al. 1972). It is possible that during concentration this antagonist of interferon was lost. Alternatively, perhaps interferon associates with itself or other substances upon concentration and this association leads to increased antiviral activity.

Our data for the thermal stability of rat interferon are in good agreement with other studies (Schonne, 1966; Billiau & Buckler, 1970). Activity of rat interferon in cells of other species, especially mouse cells, has been reported by several groups (Biernacka & Lobodzińska, 1973; Lobodzińska et al. 1973; Illinger et al. 1976). In the present study this cross-protection could not be established. Also we found no activity of human interferons in rat cells, in contrast to the reports of Chany (1976) and Duc-Goiran et al. (1971). Probably, the explanation for these discrepancies is the purity of the rat interferon employed here. Also the purity of the human interferons employed in these other studies has not always been explicitly stated, but can be assumed to be comparatively low.

From our results we conclude that the rat interferon does not differ as much from other interferons as might be concluded from earlier reports, except for the increase of activity during precipitation.
Short communications

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Department of Virology and Internal Medicine
Erasmus University Rotterdam
P.O. Box 1738, Rotterdam
The Netherlands

H. SCHELLEKENS
G. A. DE WILDE
W. WEIMAR

REFERENCES


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