Interferon Assay Based on the Inhibition of Double-stranded Reovirus RNA Accumulation in Mouse L Cells

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(Accepted 20 November 1972)

SUMMARY

Treatment of mouse L 929 cells in cell culture with mouse interferon, prior to infection with reovirus type 3, decreased the amount of virus progeny formed. In infected, interferon-treated cells less reovirus specified double-stranded RNA (ds reoRNA) and single-stranded RNA accumulated than in infected control cells and the rate of ds reoRNA accumulation was inversely related to the concentration of interferon with which the cells were treated prior to infection. Since ds reoRNA can be conveniently assayed, this forms the basis of a fast and simple assay for interferons.

INTRODUCTION

Interferons are macromolecules produced in vertebrate cells after virus infection or certain other stimuli. They are excreted, react with other cells and make these inefficient in supporting the replication of a broad range of viruses (De Clercq & Merigan, 1970). The identification of the steps in virus replication inhibited in interferon-treated cells is under study in many laboratories. Prerequisites of such studies are animal viruses whose life-cycle is known in some detail and whose replication is inhibited in interferon-treated cells.

Reovirus, an animal virus with a segmented double-stranded RNA (ds RNA) genome, fulfills these prerequisites (Gomatos & Tamm, 1963; Watanabe, Millward & Graham, 1968; Joklik, 1970). The sensitivity to interferon of the multiplication of reovirus (type 2) in human amnion cells (Oie & Leh, 1968) and of reovirus type 3 in mouse L cells (Gauntt, 1972) was reported. In this communication we provide data indicating that the synthesis of reovirus (type 3) specified single-stranded RNA (ss reoRNA) and double-stranded RNA (ds reoRNA) is inhibited in interferon-treated mouse L cells and the extent of inhibition of ds reoRNA synthesis depends on the concentration of interferon to which the cells were exposed prior to infection. This serves as the basis of a convenient, fast assay for interferons. Several interferon assays have been described which are based on the inhibition of total RNA accumulation in cells treated with actinomycin D (to block RNA synthesis on a DNA template) and infected with a virus with a single-stranded RNA (ss RNA) genome (Allen & Giron, 1970; Miller et al. 1970; McWilliams et al. 1971; A. S. Huang, personal communication). The inhibition of reoRNA synthesis in interferon-treated cells was recently reported by Gauntt (1972).

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METHODS

Cells and viruses. L cells (mouse L 929 fibroblasts) were maintained and propagated as monolayers on growth medium (Eagle’s MEM supplemented with 5% foetal calf serum) (Eagle, 1959) in an atmosphere of 5% CO₂ at 37 °C. The monolayers on 60 mm diameter Petri dishes (Falcon Plastics) were seeded with 1 to 2 x 10⁶ cells and were used 48 to 72 h later after having reached confluency.

Reovirus (type 3, the Dearing strain, kindly provided by Dr A. Graham) (Graziadei & Lengyel, 1972) and EMC (encephalomyocarditis virus donated by Dr A. Burness) (Burness, 1969) were propagated on L cells. VSV (vesicular stomatitis virus, the Indiana serotype kindly provided by Dr R. Goldsby) (Mudd & Summers, 1970) had been propagated earlier in chick embryo cells and was adapted to and propagated in L cells.

Plaque assays. Virus solution (0.5 ml) was added at 37 °C to L cell monolayers which had just reached confluency. The monolayer-containing plates were tilted every 15 min to distribute the virus solution evenly. Thirty minutes after infection with VSV or EMC and 90 min after infection with reovirus, the monolayers were overlaid with 4 ml of growth medium supplemented with 10% foetal calf serum and 0.7% Ionagar (Colab Laboratories, Chicago) and were further incubated. On the following day the EMC or VSV-infected monolayers were stained to make the plaques visible by pouring a second overlay identical with the first one except containing 0.6 mg/ml of neutral red. The reovirus-infected plates received their second 4 ml overlay (identical with the first one) 2 days after infection and a third 4 ml overlay containing neutral red 2 days later. Plaques were counted 12 to 24 h after staining.

Interferon preparation. Mouse interferon was produced as described earlier by infecting L cell monolayers with u.v.-irradiated Newcastle disease virus (Paucker et al. 1970). Crude interferon (specific activity 40000 NIH reference units/mg protein) was obtained by dialysing the medium of cells treated with Newcastle disease virus against a pH 2 buffer (to inactivate the virus) and subsequently against a neutral buffer. Partially purified interferon (specific activity 3 x 10⁷ NIH reference units/mg protein) was prepared by chromatography on sulfoethyl Sephadex followed by disc electrophoresis on polyacrylamide gel (Paucker & Stanček, 1972). The eluates from the gel slices containing most of the interferon were pooled. Bovine plasma albumin (0.5%, w/v) was added to stabilize the interferon activity (Stanček & Paucker, 1970).

Interferon treatment of monolayers. Interferon (in the amounts specified in the legends to the figures) was added in 4 ml growth medium to monolayers of L cells on 60 mm plates at about 48 h after the plates had been seeded. At this time the layers were almost confluent (approximately 5 to 7 x 10⁶ cells/plate). After an 18 h incubation at 37 °C the monolayers were washed twice with warm growth medium and used immediately for interferon assays.

Interferon assays

Single-cycle yield reduction. Interferon treated and control monolayers were infected with virus in 0.5 ml growth medium at an input multiplicity of 0.2 p.f.u./cell and incubated at 37 °C. Sixty minutes later in the case of VSV and 90 min later in that of reovirus the monolayers were washed with growth medium to remove the unadsorbed virus, and supplemented with 4 ml of growth medium. The monolayers were further incubated and were frozen 10 h after infection in the case of VSV and 18 h in that of reovirus. The cells in the monolayers were lysed by freezing and thawing them three times and the lysates were centrifuged at 1500 g for 10 min. The amount of infected virus in the supernatant fluid was determined in the plaque assay.
**Results**

Inhibition of VSV, EMC virus and reovirus replication by interferon

The potency of the interferon preparation used in our studies was determined in the plaque reduction assay with VSV. The amount of interferon causing a 50% decrease in the plaque number is defined as one unit (Fig. 1). The interferon sensitivity of VSV (having as its genome single-stranded RNA complementary in sequence to mRNA) (Baltimore, Huang & Stampfer, 1970) differed only slightly, if at all, from that of EMC virus (having as its genome single-stranded RNA serving as mRNA) (Smith, Marcker & Mathews, 1970) (Fig. 1). The interferon sensitivity of VSV was compared with that of reovirus (having as its genome segmented double-stranded RNA) in the single cycle yield reduction assay. About twice as
much interferon was needed to reduce the yield of reovirus by 50% as for VSV (Fig. 2). We could not use the plaque reduction assay in this comparison involving reovirus since our reovirus stock gave small plaques.

Interferon assay based on the inhibition of ds reoRNA accumulation

Reovirus specific double-stranded RNA can be conveniently determined in a cell extract since (a) in uninfected cells the large majority of RNA is apparently single-stranded (Stern & Friedman, 1971) and (b) single-stranded RNA can be selectively hydrolysed with pancreatic ribonuclease A leaving double-stranded RNA intact (Geiduschek, Moohr & Weiss, 1962). We tested the effect of interferon treatment on ds reoRNA accumulation for two reasons: (1) to find out if the accumulation is inhibited in interferon-treated cells in which reovirus multiplication is inhibited and, if so, (2) to see if the inhibition could serve as a basis for an interferon assay. Synthesis of ds reoRNA at 37 °C was reported to start at about 6 h after infection and to proceed for about 14 h (Shatkin, 1969). We followed the kinetics of ds reoRNA synthesis in control cells and in cells treated with interferon. The curves in Fig. 3 reveal that (a) the amount of accumulated ds reoRNA increases with the input multiplicity; and (b) interferon inhibits ds reoRNA accumulation and the extent of inhibition increases with the amount of interferon. The effect of treating L cells with various concentrations of interferon prior to infection with reovirus on the amount of ds reoRNA accumulated between 7 and 11 h after infection is shown in Fig. 4. It can be seen that the inhibition of ds reoRNA accumulation can serve as the basis of an assay for interferon. This assay has been in use in our laboratory for almost 1 year. The sensitivity of it in its present form is one-sixth of that of the plaque reduction assay with VSV; i.e. in our conditions about 6 (VSV plaque reduction) units of interferon caused 50% inhibition of ds reoRNA accumulation. However, both the speed and the reproducibility of the assay are superior. It can be
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Fig. 3

Fig. 3. Inhibition of ds reoRNA accumulation in L cells by interferon treatment: kinetics. Crude interferon was used. The conditions were as described in the section on 'Inhibition of ds reoRNA accumulation' with the following exceptions: the input multiplicity was as indicated in the Fig.; [5-3H]-uridine (1·2 μCi/ml) was added at 4·5 h (the time when unadsorbed virus was removed was taken as 0 time); and samples for determining ds reoRNA were taken at the times indicated in the Fig. All counts were corrected by subtracting from experimental values of infected samples, those for corresponding uninfected samples. The correction was 10 to 15%. O, control; △, 12·5 units of interferon added; □, 50 units of interferon added; ---, input multiplicity of 20 p.f.u./cell; ----, input multiplicity 5 p.f.u./cell.

Fig. 4

Fig. 4. Inhibition of ds reoRNA accumulation in L cells by interferon treatment: dependence on the interferon concentration. Crude interferon was used. Duplicate points represent data from separate plates. For details see the section on 'Inhibition of ds reoRNA accumulation'. All counts were corrected by subtracting from experimental values of infected samples, those for corresponding uninfected samples. The correction was 3 to 5%.

Table 1. Inhibition of ds reoRNA accumulation in L cells by interferon treatment; results of five assays performed on five different days*

<table>
<thead>
<tr>
<th>Assay no.</th>
<th>Labelling period (h after infection)</th>
<th>Concentration of interferon (units/plate)</th>
<th>Amount of interferon causing 50% inhibition of accumulation of RNase-resistant material (units/plate)†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2·5 RNase-resistant material (% of ct/min in control plate with no interferon)</td>
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<tr>
<td></td>
<td></td>
<td>5·0</td>
<td>7·5</td>
</tr>
<tr>
<td>1‡</td>
<td>8 to 10</td>
<td>82·0</td>
<td>34·6</td>
</tr>
<tr>
<td>2</td>
<td>8 to 11</td>
<td>90·1</td>
<td>44·5</td>
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<td>8 to 11</td>
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</tr>
<tr>
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<td>8 to 11</td>
<td>88·7</td>
<td>39·9</td>
</tr>
<tr>
<td>5</td>
<td>8 to 11·5</td>
<td>70·8</td>
<td>43·2</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>83·1</td>
<td>43·2</td>
</tr>
<tr>
<td>S.D.</td>
<td></td>
<td>± 6·8</td>
<td>± 10·0</td>
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</tbody>
</table>

* The conditions were as described in the section on: 'Inhibition of ds reoRNA accumulation'.
† Determined by interpolation between the two interferon amounts causing the next smaller and next larger inhibition than 50%.
‡ In this assay partially purified interferon was used; in all the others, crude interferon.
completed in 1.5 days. Its reproducibility and precision are shown in Table I. For this table five separate assays of the same interferon preparation were performed on different days. The standard deviation in this series was less than 20%. The age of the cell culture affects the sensitivity of the assay (see also McLaren, 1970). We performed the assay routinely with cells which had just reached confluency at the time when we started the interferon treatment. The sensitivity of the assay is 50% higher when performed on cells 2 days after they have reached confluency. The assay can be run without actinomycin D. However, in this case there is more of host specific, ribonuclease-resistant labelled RNA present (as determined in uninfected cells).

To verify that the ds RNA determined in our assay is indeed reovirus-specific, a sample of the ribonuclease A-resistant material extracted from virus-infected cells not treated with interferon was analysed by polyacrylamide gel electrophoresis. The pattern obtained was indistinguishable from that obtained by analysing the ds RNA extracted from purified reovirus (see, for example, Shatkin, 1969).

**DISCUSSION**

Most of the interferon assays in use are based on the inhibition of a manifestation of virus infection. These include reduction of plaque number, plaque size, virus yield and cytopathic effect (Finter, 1966). Recently a new type of assay was described which is based on the inhibition of virus RNA synthesis. This is usually performed in cells treated with actinomycin D to block host RNA synthesis (i.e. transcription of DNA) and infected with a RNA virus with a single-stranded RNA genome, whose replication is only slightly (if at all) sensitive to actinomycin D (Allen & Giron, 1970; Miller et al. 1970; McWilliams et al. 1971). An assay based on ds reoRNA synthesis has the following merits. A well-defined virus component, ds RNA is determined in it and it could be performed without treating cells with actinomycin D, i.e. under more physiological conditions. The assay described in this communication was found to be relatively fast, convenient, and gave reproducible results.

In the course of our studies we found that the accumulation of ds reoRNA was more inhibited by interferon treatment than that of ss reoRNA. Furthermore, no obvious difference was detected either among the inhibition of the accumulation of the various ss reoRNA species or among that of the various ds reoRNA segments (data not shown).

This study has been supported by a research grant from the National Science Foundation. G. B. was a fellow of the Centre National de la Recherche Scientifique, France. We thank Dr A. S. Huang for sending us a preprint.

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(Received 28 July 1972)