Production of Interferon in Rabbit Cell Cultures by Mouse L Cell-bound Poly(rI).Poly(rC)

(Accepted 14 June 1972)

Studies on the interaction with cells of the double-stranded complex of polyriboinosinic acid and polyriboctydyllic acid [poly(rI).poly(rC)] have shown that the polymer binds rapidly to the cell and that of this only a very small amount penetrates into the cell (Bausek & Merigan, 1969). It has been shown further that the interferon inducing capacity of synthetic polynucleotides is markedly enhanced upon pre-incubation of the polymers at 37° in tissue culture medium in the absence of polycations (De Clercq, Wells & Merigan, 1970; De Clercq & Merigan, 1971) and that the preheated polymers bind more rapidly to the cell and persist for a longer time at the outer cell membrane than do the unheated polymers (De Clercq et al. 1971). Although these studies do not indicate whether the interferon response is triggered by the bulk of cell-associated polymer that resides at the cell surface or by the minute amount that penetrates into the cell (Bausek & Merigan, 1969; De Clercq et al. 1971), they suggest that the cell surface may play an important role in the production of interferon by synthetic polynucleotides.

The present report provides additional suggestive evidence for the role of the outer cell membrane in the interferon response to poly(rI),poly(rC). Mouse L cell-bound poly(rI), poly(rC) was found to stimulate the production of interferon in rabbit kidney cells. The activities of rabbit interferon obtained were significantly higher than those expected from the amounts of poly(rI),poly(rC) released from the L cells into the supernatant fluids. A possible explanation for this is that poly(rI),poly(rC) does not enter the cell in order to elicit the interferon response but triggers the production of interferon from the cell surface.

Interferon was measured by a virus plaque-reduction assay in primary rabbit kidney (PRK) cell cultures in 60 mm. Falcon plastic Petri dishes using vesicular stomatitis virus (VSV) (Indiana type). The cell cultures were exposed to serial dilutions of the interferon sample and were challenged with VSV 24 hr later. The activity of interferon is defined as the reciprocal of the highest dilution of sample which reduced VSV plaque formation by 50%.

The N.I.H. reference standard of rabbit serum interferon, defined as 20,000 units/ml., contained 20,000 units in our assay. Thus, the interferon activities presented in this study may be considered to be expressed in research reference units. Interferon was characterized by its sensitivity to trypsin (0.25 mg./ml., 1 hr, 37°), resistance to pancreatic ribonuclease (p. RNase) (40 µg./ml., 1 hr, 37°, 4 x 10⁻⁵ M-EDTA), and lack of activity in human skin fibroblasts as heterologous cells. The VSV plaque-reduction assay in PRK cell cultures was also used to measure the direct antivirus effect of poly(rI),poly(rC). The homopolymer pair poly(rI), poly(rC) was prepared by annealing the individual homopolymers (both purchased from P-L Biochemicals, Milwaukee, Wisconsin) as described previously (De Clercq & Merigan, 1969).

In a first set of experiments (Table 1), confluent monolayers of mouse L cell in Petri dishes were exposed to either 10 or 100 µg./ml. of poly(rI),poly(rC) in MEM (Minimal Eagle's Medium) for 1 hr at 37°. After extensive washing of the L cell cultures with MEM, a suspension of 2 x 10⁶ PRK cells in 4 ml. MEM was added to each Petri dish. The combined L cell-PRK cell cultures were then incubated at 37° for 16 hr. In the second set of experiments
Table 1. Interferon production in primary rabbit kidney cells by L cell-bound poly(rI),poly(rC)*

<table>
<thead>
<tr>
<th>Amount of poly(rI),poly(rC) added to L cell cultures (μg./ml.)</th>
<th>Expt 1</th>
<th>Expt 2</th>
<th>Expt 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amount of rabbit interferon produced upon addition of rabbit kidney cells to poly(rI),poly(rC)-treated L cells (units)†</td>
<td>3</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Amount of poly(rI),poly(rC) released into the supernatant fluids (μg./ml.)‡</td>
<td>0.005</td>
<td>0.05</td>
<td>0.01</td>
</tr>
<tr>
<td>Amount of rabbit interferon produced by the amounts of poly(rI),poly(rC) which are released into the supernatant fluids (units)§</td>
<td>&lt;=3</td>
<td>&lt;3</td>
<td>&lt;=3</td>
</tr>
</tbody>
</table>

* L cell monolayers were exposed to either 10 or 100 μg./ml. of poly(rI), poly(rC) for 1 hr; the cell layers were then washed 6 times with MEM and further incubated for 16 hr in the presence of a suspension of PRK cells (2 x 10⁶ cells/Petri dish) in MEM.
† The supernatant fluids of combined L cell-PRK cell cultures were harvested at 16 hr after addition of the PRK cell suspension to the L cell monolayers; the samples were treated with p.RNase (40 μg./ml., 1 hr, 37°, 4 x 10⁻⁷ M-EDTA) before interferon titration.
‡ Determined in PRK cell monolayers by comparing the antivirus activity of the supernatant fluids (not treated with p.RNase) of combined L cell-PRK cell cultures, harvested at 16 hr, with the antivirus activities of different concentrations of poly(rI), poly(rC).
§ A suspension of 2 x 10⁶ PRK cells was exposed for 16 hr to poly(rI),poly(rC) at concentrations equivalent to those released into the supernatant fluids of poly(rI),poly(rC)-treated L cell cultures; the samples were treated with p.RNase (40 μg./ml., 1 hr, 37°, 4 x 10⁻⁷ M-EDTA) before interferon titration.

Table 2. Interferon production in primary rabbit kidney cells by L cell-bound poly(rI).poly(rC). L cells primed with poly(rI) and then exposed to poly(rC)*

<table>
<thead>
<tr>
<th>Amount of poly(rI) and poly(rC) added to cultures (μg./ml.)</th>
<th>Expt 1</th>
<th>Expt 2</th>
<th>Expt 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amount of rabbit interferon produced upon addition of rabbit kidney cells to the poly(rI)- and poly(rC)-treated L cells (units)†</td>
<td>24</td>
<td>10</td>
<td>30</td>
</tr>
<tr>
<td>Amount of poly(rI),poly(rC) released into the supernatant fluids (μg./ml.)‡</td>
<td>0.2</td>
<td>0.2</td>
<td>0.1</td>
</tr>
<tr>
<td>Amount of rabbit interferon produced by the amount of poly(rI),poly(rC) released into the supernatant fluids (units)§</td>
<td>3</td>
<td>&lt;3</td>
<td>3</td>
</tr>
</tbody>
</table>

* L cell monolayers were exposed to either 10 or 100 μg./ml. of poly(rI) for 1 hr; the cell layers were then washed 6 times with MEM and further incubated for 16 hr in the presence of a suspension of PRK cells (2 x 10⁶ cells/Petri dish) in MEM. Poly(rC) was added 1 hr after addition of the PRK cells at a final concentration of 10 or 100 μg./ml. respectively.
†, ‡, § As for legend to Table 1.

(Table 2), poly(rI) and poly(rC) were added separately. It has been shown that addition of the homopolymers in the order poly(rI), poly(rC) results in a greater antivirus activity than that due to exposure of the cell cultures to the complex poly(rI).poly(rC) (De Clercq* & De Somer, 1971). L cell monolayers were exposed to either 10 or 100 μg./ml. of poly(rI) in MEM for 1 hr at 37°. After extensive washing of the L cells, each dish received a suspension.
of $2 \times 10^6$ PRK cells in 2 ml. of MEM, and 1 hr later, 2 ml. of a solution of poly(rC) (20 or 200 $\mu g./ml.$). The cell cultures were incubated further at 37$^\circ$ for 16 hr. Serum was omitted from the MEM in all experiments.

In both sets of experiments, the supernatant fluids of the combined L cell-PRK cell cultures were collected at the end of the 16 hr incubation period, centrifuged and tested for antiviral activity in PRK cell monolayers. The antiviral activity of the untreated samples was due to residual poly(rI).poly(rC), released from the L cells into the supernatant fluids. The amounts of poly(rI).poly(rC) released into the supernatant fluids were estimated by comparing the reduction of virus plaques by the supernatant fluids with the virus plaque reduction by known concentrations of poly(rI).poly(rC). The amounts of poly(rI).poly(rC) released in the supernatant fluids of combined L cell-PRK cell cultures were identical to those released into the supernatant fluids of L cell cultures not incubated with PRK cells.

To determine the activity of rabbit interferon in the supernatant fluids of the combined L cell-PRK cell cultures, the samples were first treated with p.RNase and then assayed for antiviral activity. Exposure of the samples to 40 $\mu g./ml.$ of p.RNase in MEM + $4 \times 10^{-6}$ M-EDTA for 1 hr at 37$^\circ$ resulted in complete inactivation of the free poly(rI).poly(rC) in the supernatant fluids. This was verified by exposing a standard preparation of 1 $\mu g./ml.$ of poly(rI).poly(rC) to 40 $\mu g./ml.$ of p.RNase and measuring its residual antiviral activity. The antiviral activity of this standard preparation was destroyed completely.

The antiviral activity of the p.RNase treated supernatant fluids of combined L cell-PRK cell cultures could be ascribed to interferon since the activity was destroyed by treatment with trypsin and was not established in heterologous cells. Relatively high levels of rabbit interferon were obtained in rabbit cell suspensions which had been co-incubated with poly(rI).poly(rC)-treated L cells (Table 1) and even higher interferon activities were found in rabbit cell suspensions co-incubated with L cells primed by poly(rI) and then treated with poly(rC) (Table 2).

The amounts of poly(rI).poly(rC) released by the L cells into the supernatant fluids after incubation for 16 hr (0.005 to 0.1 $\mu g./ml.$: third row, Table 1; 0.1 to 1 $\mu g./ml.$: third row, Table 2) were insufficient to account for the levels of rabbit interferon produced in the combined L cell-PRK cell cultures. When poly(rI).poly(rC) was added at concentrations from 0.005 to 1 $\mu g./ml.$ to control suspension of PRK cells only, there was significantly less interferon produced than with L cell-bound poly(rI).poly(rC) (Tables 1, 2).

The titration of free poly(rI).poly(rC) in the supernatant fluids after 16 hr incubation provides a valid estimation of the total amount of poly(rI).poly(rC) released from the L cells during 16 hr of incubation with the PRK cells; this conclusion is based on two types of experiment: first, when poly(rI).poly(rC) was exposed to PRK cells at different concentrations, it could be recovered quantitatively from the supernatant fluids of these cells 16 hr later. Thus, freely circulating poly(rI).poly(rC) was not markedly degraded during incubation for 16 hr. Secondly, interference assays were performed with the supernatant fluids at different times (2, 4, 8, 12 and 16 hr) after addition of the PRK cells to the poly(rI).poly(rC) treated L cell monolayers. The amounts of poly(rI).poly(rC) released into the supernatant fluids at these different times never exceeded the amount of poly(rI).poly(rC) released after incubation for 16 hr. In a representative experiment, poly(rI).poly(rC) at 100 $\mu g./ml.$ was added to the L cells; the amounts of poly(rI).poly(rC) eluted from the L cells at 2, 4, 8, 12 and 16 hr upon addition of the PRK cells were 0.02, 0.01, 0.05, 0.05 and 0.05 $\mu g./ml$ respectively; the amounts of rabbit interferon produced at 2, 4, 8, 12 and 16 hr upon addition of the PRK cells were < 3, 8, 16, 16 and 16 units, respectively.

To determine whether the presence of L cells in the combined cultures increased the pro-
duction of rabbit interferon by poly(rI).poly(rC), PRK cell suspensions incubated in the presence and absence of L cell monolayers, were exposed to 0.2 and 2 μg/ml of poly(rI).poly(rC). Rabbit interferon activity measured in the supernatant fluids of the PRK cell suspensions 16 hr after addition of poly(rI).poly(rC) was increased up to twofold in the combined cultures. Although these results indicate that the presence of L cell material increased the interferon response of rabbit kidney cells to poly(rI).poly(rC), they offer no explanation for the interferon activity obtained with L cell-bound poly(rI).poly(rC) in PRK cells (Tables 1, 2). Firstly, the presence of L cells caused only a twofold increase in the interferon inducing capacity of poly(rI).poly(rC), compared to a five-to tenfold increase observed with L cell-bound poly(rI).poly(rC) (Tables 1, 2). Secondly, the values obtained for the amounts of poly(rI).poly(rC) released into the supernatant fluids of combined L cell-PRK cell cultures (Tables 1, 2, third row) must have been slightly overestimated, due to the activating effect of L cells on the antivirus activity of poly(rI).poly(rC) in PRK cells. Nevertheless, the amounts of rabbit interferon produced by this freely circulating poly(rI).poly(rC) were significantly lower than the amounts of interferon found in combined L cell-PRK cell cultures (Tables 1, 2).

To show that poly(rI).poly(rC) itself and not an intermediate factor (elicited by poly(rI).poly(rC) in the L cells) was responsible for the production of rabbit interferon in the combined L cell-PRK cell cultures, L cell monolayers were exposed to 200 μg/ml of poly(rI).poly(rC) for 1 hr, treated with p.RNase (200 μg/ml., 2 hr, 37°C, 10⁻³M-EDTA), and then incubated with PRK cells. The additional treatment with p.RNase caused a significant reduction in the activity of rabbit interferon produced during the subsequent 16 hr incubation period (3 units instead of 4 units).

Our results suggest that poly(rI).poly(rC) bound to L cells initiates interferon production in rabbit kidney cells, presumably by a direct contact of the L cell-bound polymer with the surface of the rabbit kidney cells. The rabbit interferon activities obtained in the combined L cell-PRK cell cultures cannot be explained by free poly(rI).poly(rC) released from the L cells into the supernatant fluids. Nor can they be explained by an activation of poly(rI).poly(rC), due to the presence of L cell material.

We suggest two alternative mechanisms for this initiation by L cell-bound poly(rI).poly(rC) of the production of rabbit interferon: (1) L cell-bound poly(rI).poly(rC) does not enter the rabbit cell but triggers the interferon response from the outer membrane of this cell; or (2), poly(rI).poly(rC) is transferred directly on contact of the L cell surface with the PRK cell surface and eventually enters the rabbit kidney cell. No evidence is available to distinguish these possibilities.

In a recent report, Wagner, Bugianesi & Shen (1971) stated that poly(rI).poly(rC) covalently bound to an insoluble matrix (sepharose) retained its in vitro biological activity. Provided that the polynucleotide does not elute from the matrix during its incubation with the cells, the use of sepharose-bound poly(rI).poly(rC) may indicate whether poly(rI).poly(rC) triggers the interferon response from the outer cell membrane.

This work was supported by a grant from the Belgian FGWO (Fonds voor Geneeskindig Wetenschappelijk Onderzoek). E. D. C. is ‘Aangesteld Navorser’ of the Belgian NFWO (Nationaal Fonds voor Wetenschappelijk Onderzoek). The excellent technical assistance of Mrs Anita Van Lierde is acknowledged.

Rega Institute for Medical Research
University of Leuven
Leuven, Belgium
REFERENCES


(Received 8 March 1972)