Integrity of Cell-bound Poly(I).Poly(C)

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SUMMARY

The acid-insoluble radioactivity associated with the cells following incubation of the cells with [3H]-labelled poly(I).poly(C) [poly(I).poly(C)*] for 1 h has been analysed by sucrose gradient velocity ultracentrifuging. Intact (poly(I).poly(C)* was recovered from primary rabbit kidney (PRK) cells, partially degraded poly(I). poly(C)* was recovered from mouse L 929 cells and completely degraded material was recovered from HeLa and VERO cells. Priming of the cells with homologous interferon did not alter the sedimentation profile of cell-associated poly(I).poly(C)* in PRK, L 929 or HeLa cells.

INTRODUCTION

Differences in the antiviral activity and interferon inducing capacity among double-stranded polyribo- and polydeoxyribonucleotide complexes cannot be accounted for by differences in the rates of polymer-cell binding or in the amounts of polymer bound to the cells (De Clercq, Wells & Merigan, 1972). Nor do differences in the antiviral activity of poly(I).poly(C), one of the most active and most extensively studied polyribonucleotide inducers of interferon, in different cell cultures correlate with the polymer-cell binding rates or amounts of polymer bound to these cells (De Clercq & De Somer, 1973). However, poly(I).poly(C) bound to cells that are more sensitive to the antiviral activity of the polynucleotide appeared to be more accessible to ribonuclease treatment than poly(I).poly(C) bound to cells that are less sensitive to the activity of the polymer (De Clercq & De Somer, 1973), suggesting that in the more sensitive cell cultures, poly(I).poly(C) is preferentially located at a superficial cell site.

Studies have now been undertaken to evaluate whether the higher antiviral activity of poly(I).poly(C) in the more sensitive cell cultures is related to the maintenance of the poly(I).poly(C) duplex in its intact state. Therefore, the molecular size of cell-bound poly(I).poly(C) was determined in a number of cell cultures varying considerably in their sensitivity to the antiviral activity of the polynucleotide (primary rabbit kidney cells, mouse L 929 cells, Hela and VERO cells). The molecular size of cell-associated poly(I).poly(C) was analysed by sucrose gradient velocity sedimentation and was assessed in both normal and interferon-primed cells. Priming of the cells with interferon has been shown previously to enhance the cells' responsiveness to subsequent interferon induction by poly(I).poly(C) (at least in mouse L cells and rabbit kidney cells: Stewart, Gosser & Lockart, 1971, 1972; De Clercq, Stewart & De Somer, 1973).

* Poly(I).poly(C)* refers to poly(I) complexed with [3H]labelled poly(C).
METHODS

Cell cultures. Primary rabbit kidney (PRK) cells, mouse L 929 cells, HeLa and VERO (African green monkey kidney) cells were grown to confluency in 50 mm Nunc or Falcon plastic Petri dishes in Eagle's minimal essential medium (MEM) supplemented with 10% calf serum.

Poly(I) .poly(C)*. Poly(I) .poly(C)* was prepared by annealing equal amounts of poly(I) and [3H]-labelled poly(C) [both dissolved in Dulbecco's phosphate-buffered saline (PBS) at 50 µg/ml] at 45 °C for 1 h. Poly(I) was purchased from P-L Biochemicals, Milwaukee, Wisconsin and [3H]-labelled poly(C) (44.8 mCi/mmol P, 8.95 µg/µCi, dispensed in 50% ethanol) was purchased from Miles Laboratories, Elkhart, Indiana; poly(C)* was first lyophilized and then dissolved in PBS at 50 µg/ml. The specific radioactivity of the complex poly(I).poly(C)* fell in the range of 30,000 to 40,000 ct/min/µg. The polymer was stored in samples at −20 °C.

Exposure of cell cultures to poly(I).poly(C)*. Confluent cell cultures were first incubated for 16 h at 37 °C with either control medium (MEM + 3% calf serum; 2 ml/Petri dish) or homologous interferon (100 units/ml in MEM + 3% calf serum; 2 ml/Petri dish), washed (3 times) with MEM, and then exposed to poly(I).poly(C)* (10 µg/ml in MEM; 1 ml/Petri dish) for 1 h at 37 °C.

Preparation of cell homogenates. After a series of three washings of the poly(I).poly(C)*-treated cell cultures with cold PBS (o °C), 0.5 ml of cold PBS (0 °C) was added per Petri dish. The cells were scraped off from the Petri dishes with a rubber policeman into glass tubes and disrupted by treatment for 20 s in a 100 W ultrasonic disintegrator (MSE) operating at a nominal frequency of 20 KHz and at a maximum output (8 µm peak-to-peak on the amplitude meter). The samples were kept in an ice bath during these manipulations. Upon sonication the cell homogenates were immediately processed for sucrose gradient velocity ultracentrifuging.

Sucrose gradient velocity sedimentation. The cell homogenates prepared from poly(I).poly(C)*-treated cell cultures, were layered in a vol. of 0.2 ml on to 4.6 ml of a 5 to 40% (w/v) linear sucrose gradient and centrifuged for 20 h at 4 °C in a SW 56.1 rotor (MSE) at 40,000 rev/min. The sucrose solutions were prepared in TNE buffer [0.01 M-tris-HCl (pH 7.5), 0.1 M-NaCl, 0.001 M-EDTA]. After sedimentation, 0.2 ml fractions were collected from the top of the tube (with a model 640 ISCO density gradient fractionator) and analysed for acid-insoluble radioactivity.

Determination of acid-insoluble radioactivity. To 0.2 ml samples were added (at 0 °C) 0.2 ml yeast RNA (0.1%) and 1.0 ml TCA (5%). The precipitates were collected by filtration on Gelman type A glass-fibre discs (2.5 cm) and treated with NCS (Nuclear Chicago Solubilizer) reagent (NCS diluted 1/3 with toluene scintillation fluid, 0.5 ml/filter) for 30 min at room temperature, before addition of 5 ml toluene-PPO scintillation fluid. Radioactivity was determined in a Tri-Carb Packard liquid scintillation spectrometer.

RESULTS

Since all cell homogenates of poly(I).poly(C)*-treated cell cultures were prepared by ultrasonic disintegration for 20 s, it was first explored whether such manipulation altered the sedimentation behaviour of poly(I).poly(C)* in the sucrose gradient. As shown in Fig. 1, control poly(I).poly(C)* sedimented at 1.10 g/ml, poly(I).poly(C)* sonicated for 20 s sedimented at 1.09 g/ml, and poly(I).poly(C)* sonicated for 2 min banded at 1.08 g/ml.
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Fig. 1. Sucrose velocity gradient analysis of poly(I), poly(C)*. Poly(I), poly(C)* prepared in PBS at 10 μg/ml was first submitted to ultrasonic disintegration, then diluted to 1 μg/ml and layered in a 0.2 ml vol. on to 4.6 ml of the linear sucrose gradient (see Methods). A—A, poly(I), poly(C)* sonicated for 2 min; O—O, poly(I), poly(C)* sonicated for 20 s; ●—●, poly(I), poly(C)* not sonicated.

Table 1. Acid-insoluble radioactivity associated with the cells after 1 h contact with poly(I),poly(C)* at 10 μg (400 000 ct/min)/ml/Petri dish

<table>
<thead>
<tr>
<th>Acid-insoluble cell-associated radioactivity*</th>
<th>% (compared to input)</th>
</tr>
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<tbody>
<tr>
<td>PRK cells Control</td>
<td>21 040</td>
</tr>
<tr>
<td>Primed</td>
<td>22 660</td>
</tr>
<tr>
<td>L929 cells Control</td>
<td>26 430</td>
</tr>
<tr>
<td>Primed</td>
<td>19 185</td>
</tr>
<tr>
<td>HeLa cells Control</td>
<td>14 120</td>
</tr>
<tr>
<td>Primed</td>
<td>12 580</td>
</tr>
<tr>
<td>VERO cells Control</td>
<td>12 655</td>
</tr>
</tbody>
</table>

* Measured in confluent cell monolayers. Approximate number of cells per Petri dish: 1.1 × 10⁶ for PRK cells, 2.3 × 10⁶ for L929 cells, 1.6 × 10⁶ for HeLa cells and 2.1 × 10⁶ for VERO cells.
Thus, ultrasonic treatment for 20 s caused a slight shift in the sedimentation behaviour of poly(I).poly(C)* to the lower densities. In the experiments to be described, control poly(I).poly(C)* preparations were always submitted to 20 s ultrasonication, to permit a direct comparison between the molecular size of native poly(I).poly(C)* and cell-bound poly(I).poly(C)*.

PRK, L 929, HeLa and VERO cells differ considerably in sensitivity to the antiviral
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Fig. 3. Sucrose velocity gradient analysis of L 929 cell-associated poly(I).poly(C)*. Upper part: unprimed L 929 cells. Lower part: interferon-primed L 929 cells. ●—●, cell-associated poly(I).poly(C)*; ○---○, control poly(I).poly(C)*.

activity of poly(I).poly(C) (Table 1: De Clercq & De Somer, 1973), yet they do not show appreciable differences in the amounts of cell-associated radioactivity after 1 h contact with the radioactively labelled polynucleotide (Table 1). Likewise, priming of the cells with homologous interferon does not markedly affect the amounts of radioactivity taken up by the cell (Table 1), although the interferon inducing capacity of poly(I).poly(C)* is significantly enhanced in primed PRK and L 929 cells (fig. 1: De Clercq et al. 1973).
Fig. 4. Sucrose velocity gradient analysis of HeLa cell-associated poly(I).poly(C)*. Upper part: unprimed HeLa cells. Lower part: interferon-primed HeLa cells. ●●, cell-associated poly(I).poly(C)*; ○○, control poly(I).poly(C).*
Sucrose gradient velocity analysis of the cell-associated radioactivity following a 1 h contact of the cells with poly(I).poly(C)* revealed considerable differences from one cell type to another. Nearly intact poly(I).poly(C)* could be recovered from PRK cell cultures (Fig. 2); the sedimentation profile of PRK cell-associated poly(I).poly(C)* coincided closely with the profile of control poly(I).poly(C)*, in both unprimed and interferon-primed cells.

Compared to that seen in poly(I).poly(C)*-treated PRK cells, the sucrose density gradient profile obtained in poly(I).poly(C)*-treated L 929 cell cultures was lowered, flattened and shifted to the top of the gradient (Fig. 3). Again, identical profiles were obtained in unprimed and interferon-primed L 929 cells.

Poly(I).poly(C)*-treated HeLa and VERO cells processed in exactly the same conditions as PRK and L 929 cells did not show the sucrose density gradient profile characteristic of intact poly(I).poly(C)*. Instead, they exhibited a low profile of heterogenous material, distributed over the whole gradient (Fig. 4 and 5). Priming of the HeLa cells with human interferon did not affect this sedimentation pattern (Fig. 4).

It should be pointed out that the state of cell-associated poly(I).poly(C)* was evaluated after 1 h contact of the cells with the polymer because a 1 h contact period has proven sufficient for full expression of the antiviral activity and interferon inducing capacity of double-stranded RNAs in cell culture (De Clercq et al. 1971, 1973; Pitha, Marshall & Carter, 1972).
DISCUSSION

It has been shown in a number of reports that synthetic polynucleotides, whether single- or double-stranded polycyano- or polydeoxyribonucleotides [poly(I).poly(C), poly(dI). poly(dC), poly(I), poly(C), ...], are bound to or taken up by the cells (Bausek & Merigan, 1969; Colby & Chamberlin, 1969; Black et al. 1972; De Clercq & De Somer, 1972, 1973; De Clercq et al. 1972, 1973; Field et al. 1972). However, it has never been ascertained in what form poly(I).poly(C) is actually associated with the cell, and, more recently, it has even been contended whether it is taken up as such (Kelly & Levy, 1973).

The results reported herein clearly establish that the state (molecular size) of cell-bound poly(I).poly(C) differs markedly from one cell type to another. From PRK cells largely intact poly(I).poly(C) could be recovered following 1h contact of poly(I).poly(C) with the cells. Partially degraded polymer was detected in mouse L 929 cells and little, if any, intact material could be demonstrated in HeLa and VERO cells.

It should be recalled that PRK cells are among the most sensitive cells to the antiviral activity of poly(I).poly(C), that mouse L 929 cells show an intermediary sensitivity, and that HeLa and VERO cells are among the least sensitive cell lines (De Clercq & De Somer, 1973). Poly(I).poly(C) may owe its activity in PRK cells to the fact that is not readily degraded by the nucleases of these cells. It might, at least partially, resist degradation in L 929: these cells are intrinsically sensitive to the antiviral activity of poly(I).poly(C), although full activity requires priming with interferon (Stewart et al. 1972; De Clercq et al. 1973). In HeLa and VERO cells, however, poly(I).poly(C) may be attacked by cellular nucleases before it can transmit the necessary signal for interferon production. These cells are intrinsically refractory to the antiviral activity of poly(I).poly(C), since previous treatment with interferon does not increase their responsiveness to interferon inducers (Stewart et al. 1972).

Priming of the cells with interferon did not alter the sedimentation profile of cell-associated poly(I).poly(C), even in the cell cultures which do respond to interferon priming with increased interferon production (PRK and L 929 cells). However, this finding is not unexpected in view of the hypothesis proffered before (De Clercq et al. 1973), that interferon priming acts by a specific adjustment of the cellular receptor sites to the polynucleotide structure.

From the data presented in this and a previous report (De Clercq & De Somer, 1973), it appears as though the high antiviral activity of poly(I).poly(C) in PRK cells somehow depends on the persistence of cell-bound polymer in: (1) an intact state (protected against degradation by cellular nucleases) and (2) at a superficial site (accessible to extraneous ribonuclease treatment).

Our data reinforce the assumption that resistance to degradation by nucleases is an important determinant in the antiviral activity of double-stranded RNAs such as poly(I).poly(C). This assumption was originally derived from our previous studies with alternating copolymers [e.g. poly(A-U).poly(A-U)] which exhibited a parallel increase in antiviral activity and resistance to nucleases upon substitution of thiophosphate for phosphate or activation by heating at 37 °C in MEM (De Clercq et al. 1970, 1971). As pointed out before (Black et al. 1973), resistance of the polynucleotide to degradation by nucleases should not necessarily be complete. A threshold resistance may be sufficient, e.g. to overcome premature degradation by cellular enzymes.

Although our data point to a correlation between the inability of HeLa and VERO cells to respond to the antiviral activity of poly(I).poly(C) and the failure to recover intact
polymer from these cells, they do not allow the conclusion that this correlation is a causal one. Rapid degradation of poly(I).poly(C) may only be one of the parameters reflecting the defective character of these cells. In fact, HeLa and VERO cells might be defective for more than one reason (e.g. high nuclease activity, fundamental inaptitude of the receptor sites, deficient transmission of message from receptor site to the gene(s) for interferon formation, etc).

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