Thermal Activation of the Antiviral Activity of Synthetic Polyribonucleotides: Influence of DEAE-dextran in Various Cell Cultures

By E. DE CLERCQ* AND T. C. MERIGAN

Division of Infectious Diseases, Department of Medicine, Stanford University School of Medicine, Stanford, California 94305, U.S.A.

(Accepted 22 September 1970)

SUMMARY

After heating at 37 ° in minimal Eagle’s medium, the alternating polyriboadenylic-polyribouridylic acid [poly r(A–U)] became much more active in producing cellular resistance to virus infection and interferon production in four different cell lines (human skin fibroblasts, mouse embryo fibroblasts, mouse L 929 cells and rabbit kidney (RK 13) cells). The thermal activating effect was neutralized by pre-treatment of the cells with DEAE-dextran, which increased the antiviral activity of unheated and decreased the activity of heated poly r(A–U) to the same level. Under all conditions tested, the degree of cellular resistance to virus infection closely paralleled the amounts of interferon produced suggesting that interferon production is responsible for the antiviral resistance produced by poly r(A–U).

INTRODUCTION

We have recently shown that incubation at 37 ° in minimal Eagle’s medium (MEM) increased the activity of several synthetic polynucleotides by several orders of magnitude in producing cellular resistance to vesicular stomatitis virus (VSV) plaque formation in human skin fibroblasts (HSF) (De Clercq, Wells & Merigan, 1970a). The thermal activating effect was observed for both alternating copolymers and homopolymer pairs but was most marked with the alternating riboadenylic acid-ribouridylic acid copolymer [poly r(A–U)]. Poly r(A–U) reduced VSV plaque formation in HSF at ≥ 1 µg./ml. when pre-incubated at 0 ° and at 0·000004 µg./ml. when pre-incubated at 37 °. Although incubation at 37 ° had no measurable effect on the viscosity, ultraviolet spectrum, CsSO₄ buoyant density and analytical sedimentation velocity of poly r(A–U), its sensitivity to degradation by endonucleases was markedly decreased (De Clercq et al. 1970b). The heat-activated poly r(A–U) was more rapidly bound to the cell and persisted for a longer time at the outer cell membrane than poly r(A–U) which had not been heated.

We now report that (1) the thermal activation of the antiviral activity of poly r(A–U) is not only demonstrable in human skin cells but can also be observed in several other cell cultures, that (2) the effect is markedly suppressed if the cells are pretreated with DEAE-dextran and that (3) under all conditions tested (different cell cultures, with or without DEAE-dextran; poly r(A–U), preheated or not) there is a consistent relationship between the concentrations of polymer required for interferon production and those concentrations causing cellular resistance to virus infection.

* Present address: Rega Institute for Medical Research, Minderbroedersstraat 10, Leuven, Belgium.
METHODS

**Cells.** Four different cell lines were employed: human skin fibroblasts (HSF) and mouse embryo fibroblasts (MEF), both primary diploid cell lines; mouse L 929 cells and RK 13 (rabbit kidney) cells, both continuous heteroploid cell lines. Cells were grown to confluency in 50 mm. Falcon plastic Petri dishes. Calf serum (10%) was added to the medium (MEM) during cell growth but serum was omitted during exposure of the cells to the polymer.

**Virus.** Bovine vesicular stomatitis virus (VSV) (INDIANA strain) which had been prepared in chick embryo fibroblasts was used as the challenge virus.

**Chemicals.** Poly r(A–U) was purchased from Biopolymers Inc., Pinebrook, New Jersey; as it was shipped in tris-HCl buffer (0.01 M, pH 7.9) which interferes with the thermal activation (De Clercq et al. 1970b), poly r(A–U) was dialysed against a 300-fold excess of 0.15 M-NaCl at 4° for 2 days, then further diluted in normal saline and stored at −20° at 20 µg./ml. DEAE-dextran (mol. wt = 2 × 10^6) was obtained from Pharmacia, Uppsala, Sweden (lot no. 5229). A stock solution of 2 mg./ml. was made in MEM and stored at 4°.

**Induction of cellular resistance to VSV infection** was measured by the plaque-reduction assay as described previously (De Clercq & Merigan, 1969; De Clercq et al. 1970a). Serial (1 to 10) dilutions of poly r(A–U) were made in MEM, pre-incubated for 2 hr at either 0 or 37° and then immediately applied to the cell monolayers (4 ml./Petri dish). The cells were incubated for 20 hr at 37°; the supernatant fluid containing polymer was removed and the cells challenged with VSV.

**Interferon production.** Cell monolayers were exposed to serial (1 to 10) dilutions of pre-heated (37°) or pre-cooled (0°) poly r(A–U) in 1 ml. MEM for 3 hr (De Clercq & Merigan, 1969; De Clercq et al. 1970a). After removal of the polymer the cells were washed exhaustively with MEM and further incubated with 4 ml. MEM (containing 3% calf serum)/Petri dish for 5 hr. The culture fluid was harvested at the end of this period and assayed for interferon by exposing new, homologous cell cultures to serial dilutions of the supernatant fluid. The cell cultures were challenged with VSV 24 hr later. The titre of interferon corresponded to the reciprocal of the highest dilution of sample which reduced VSV plaque formation by 50%. Interferon was characterized by its sensitivity to trypsin (0.25 mg./ml. 1 hr, 37°), resistance to pancreatic ribonuclease (40 µg./ml. 1 hr, 37°, 0.001 M-EDTA) and lack of activity in heterologous cells.

**DEAE-dextran treatment of cells.** Cultures were incubated with 100 µg./ml. of DEAE-dextran for 1 hr at 37° (4 ml./Petri dish). The DEAE-dextran was removed, the cells washed with MEM and then immediately exposed to the given concentrations of poly r(A–U).

RESULTS

Pre-incubation at 37° rendered poly r(A–U) markedly more active in reducing vesicular stomatitis virus (VSV) plaque formation in either human skin fibroblasts (HSF), mouse embryo fibroblasts (MEF), RK 13 or L cells (Fig. 1 to 4): the minimal inhibitory concentration of polymer concentration reducing VSV plaque formation by 50% was decreased to 10^4 to 10^5-fold in HSF, 10^2 to 10^3-fold in RK 13, more than 10^6-fold in MEF and more than 10^8-fold in L cells if the polymer was pre-heated at 37° instead of being maintained at 0°. Although poly r(A–U) invariably caused 100% plaque reduction in HSF and RK 13 at concentrations exceeding the minimal inhibitory concentration, complete plaque inhibition
DEAE-dextran, thermal activation and antiviral activity

could not be obtained in MEF or L cells, even if polymer concentrations exceeded the minimal inhibitory concentration by 100 to 1000 times. In fact, the antiviral activity of pre-heated poly r(A–U) in MEF and L cells decreased when the concentration was increased from 0.04 to 0.4 μg./ml.

Polybasic substances such as DEAE-dextran have been reported to enhance the antiviral

---

Fig. 1 to 4. Induction of cellular resistance to virus infection and interferon production by poly r(A–U) in human skin fibroblasts (Fig. 1), RK 13 (Fig. 2), mouse embryo fibroblasts (Fig. 3) and L cells (Fig. 4). ●, virus plaque reduction in control cell cultures; ○—○, virus plaque reduction in DEAE-dextran-treated cell cultures; ■, interferon production in control cell cultures; □, interferon production in DEAE-dextran-treated cell cultures. Polymer concentrations indicated were pre-incubated in MEM at 0 or 37°C for 2 hr. DEAE-dextran-treated cell cultures were exposed to 100 μg./ml. of DEAE-dextran for 1 hr at 37°C, washed and then exposed to poly r(A–U).
activity of synthetic polynucleotides in a variety of cell cultures [L cells (Dianzani et al. 1968; Bausek & Merigan, 1969; Billiau et al. 1969), primary rabbit kidney cells (Vílek et al. 1968; Lampson et al. 1969; Billiau et al. 1969), primary chick embryo cells (Colby & Chamberlin, 1969; Billiau et al. 1969), human leukocytes and human amniotic membrane (Falcoff & Perez-Bercoff, 1969) and human muscle-skin fibroblasts (Tilles, 1970)]. Similarly, DEAE-dextran enhanced the capacity of pre-cooled (0°) poly r(A-U) to induce cellular resistance to VSV plaque formation in all cell lines studied (Fig. 1 to 4). However, DEAE-dextran inhibited the resistance-inducing capacity of heated (37°) poly r(A-U), so that in DEAE-dextran-treated cell cultures pre-heated and pre-cooled poly r(A-U) did not markedly differ in antiviral activity. DEAE-dextran itself did not affect virus plaque numbers in MEF, slightly increased the plaque number in L cells (by 20 to 40%), and increased the plaque numbers in HSF by 100 to 200%, while causing a tenfold reduction of the plaquing efficiency in RK 13. The drop in activity characteristically noted with heated poly r(A-U) in MEF and L cells at 0.4 μg./ml. was not observed if the cell cultures were pre-treated with DEAE-dextran.

With synthetic polynucleotides, e.g. (poly rI) (poly rC) or polyinosinic acid–polycytidylic acid homopolymer pair, cellular resistance to virus infection has been demonstrated at much smaller amounts than required to stimulate detectable interferon production (Field et al. 1968; De Clercq & Merigan, 1969; Colby & Chamberlin, 1969). This raises the question whether interferon production by polynucleotides and induction of cellular resistance to virus infection are related phenomena and whether polynucleotides might cause resistance to virus infection without concomitant interferon production. Interferon production was measured with different concentrations of pre-heated (37°) and pre-cooled (0°) poly r(A-U) in DEAE-dextran-treated and untreated cell cultures. In HSF, MEF or RK 13, interferon could be detected in the supernatant fluid with polymer concentrations which were approximately 100-fold higher than the concentrations required to inhibit the virus-plaquing efficiency by 50%, regardless of the cell type employed, whether or not the cells were treated with DEAE-dextran and whether poly r(A-U) was pre-incubated at either 0 or 37° (Fig. 1 to 4). However, with the concentrations of poly r(A-U) tested, no interferon production could be demonstrated in L cells. Parallel to its effect on cellular resistance to virus infection, DEAE-dextran enhanced the interferon-inducing capacity of pre-cooled poly r(A-U) but suppressed interferon production by pre-heated poly r(A-U). Thus, heat-activated poly r(A-U) stimulated significantly higher interferon titres in HSF, MEF or RK 13 than inactivated poly r(A-U) when added to untreated cells, but failed to do so when added to DEAE-dextran-treated cells. As noted before with cellular resistance to virus infection, interferon production levelled off (HSF) or decreased (RK 13, MEF) at the highest concentration of heated poly r(A-U) tested (4 μg./ml.). This paradoxical effect was not seen in cell cultures treated with DEAE-dextran.

**DISCUSSION**

The antiviral activity of synthetic polynucleotides is significantly increased upon incubation at 37°. This thermal activating effect has been demonstrated with both polyribo- and polydeoxyribonucleotides (De Clercq et al. 1970a) of different sources in several cell cultures (human skin fibroblasts, RK 13, mouse embryo fibroblasts and L cells: Fig. 1 to 4). The polynucleotides tested were either alternating copolymers or homopolymer pairs, but were all capable of forming a double-stranded structure. It is likely that heating to 37° increased their antiviral activity by stabilizing their double-stranded structure (De Clercq et al. 1970a, b). Single homopolyribonucleotides which form single-stranded structures e.g. polyriboadenylic acid, polyribothymidylic acid) or three- or four-stranded structures (e.g.
polyriboinosinic acid, polyriboxanthlyc acid) in the conditions we used (De Clercq & Merigan, 1969), failed to show any increase in antiviral activity after heating to 37°.

DEAE-dextran markedly enhances the production of interferon by (poly rI). (poly rC) in a variety of cell cultures (Dianzani et al. 1968; Vilcek et al. 1968; Falcoff & Perez-Bercoff, 1969; Colby & Chamberlin, 1969; Bausek & Merigan, 1969; Billiau et al. 1969; Tilles, 1970). It increased the antiviral activity of precooled but decreased the activity of pre-heated poly r(A–U) so that no significant difference was observed between the actions of pre-cooled and pre-heated poly r(A–U) in DEAE-dextran-treated cells. The enhancing effect of DEAE-dextran on the antiviral activity of polynucleotides has been ascribed to an increased uptake of the polynucleotide by the cell (Colby & Chamberlin, 1969; Bausek & Merigan, 1969), although increased resistance of the polynucleotide to cellular nucleases could not be excluded (Tilles, 1970). It is conceivable that, no matter where DEAE-dextran acts in the cell, it alters the cell reactivity to poly r(A–U) in a way that favours the action of pre-cooled but hampers the action of pre-heated poly r(A–U). Alternatively, DEAE-dextran might combine directly with the polynucleotide and the configuration resulting from the interaction of DEAE-dextran with the loose, branched helical structure of precooled poly r(A–U) might be similar to that resulting from its interaction with the rigid, unbranched helical structure of preheated poly r(A–U).

It has been mentioned in previous reports that tenfold (Finkelstein, Bausek & Merigan, 1968) or 10,000-fold (Ho & Ke, 1970) higher concentrations of (poly rI). (poly rC) are required for stimulation of interferon production than for production of cellular resistance to virus infection. In our studies the concentration of poly r(A–U) needed for interferon production was consistently 100 times higher than the concentration needed for interference. Under the widely varying conditions tested, interferon production closely paralleled the degree of cellular resistance to virus infection. These findings suggest that even at polymer concentrations which did not stimulate detectable interferon production in the supernatant fluid, cellular resistance to virus infection may be accounted for by interferon production. Conclusions similar to the latter were reached by several groups of investigators. Burke & Isaacs (1960) related interference and interferon production in their studies with ultraviolet-inactivated influenza virus. Friedman (1964) found that actinomycin D had a parallel effect on interference and interferon production by heated Chikungunya virus, and Schäfer & Lockart (1970) found a close relationship between interference and interferon production by (poly rI). (poly rC) in different monkey cell cultures.

Interferon production and cellular resistance to virus infection levelled off or even decreased at the highest concentrations of heated poly r(A–U) (0.4 to 4 µg/ml.) in all cell lines. This paradoxical effect was not observed in DEAE-dextran-treated cells at the same polynucleotide concentrations but might have been apparent at higher concentrations. A possible explanation for the low activity of heated poly r(A–U) at higher concentrations may lie in certain conformational features of the heated polymer preparation which are only evident at higher concentrations and which counteract the antiviral activity of poly r(A–U). This might occur through stimulation of factors which antagonize the activity or production of interferon [such as blocker, enhancer, stimulon or antagonist (De Clercq & Merigan, 1970)].

This work was supported by United States Public Health Service Grant AI-05629. E. de Clercq is a fellow of the Damon Runyon Fund for Cancer Research and ‘Aangesteld Navorser’ of the Belgian N.F.W.O. (Nationaal Fonds voor Wetenschappelijk Onderzoek).
REFERENCES


(Received 19 August 1970)