Interference with Cell Viability and Poliovirus Multiplication by Polyinosinic–Polycytidylic Acid

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Summary

The double-stranded complex of polyinosinic and polycytidylic acids (poly I: C) inhibits the replication of poliovirus in Detroit cells. By a plaque reduction assay a linear relationship was found between the logarithm of the plaque reduction % and the logarithm of the poly I: C concentration, with about 50 % inhibition by 0.1 μg./ml. of poly I: C.

The maximum hypochromic effect at 233 nm. on complexing of the poly-nucleotides occurred with 6 volumes of poly I and 4 volumes of poly C from equimolar solutions of the two homopolynucleotides. Gel-filtration of such a complex on Sepharose 4 B separated double and single strands. Of the added poly I 7 % remained as single strands at the conditions of maximum hypochromic effect.

The poly C was found to have a chain length several times larger than that of poly I.

Cell multiplication proceeded at normal rate for 48 hr in a spinner culture with 5 μg./ml. of poly I: C. With 25 μg./ml. no cell multiplication took place within 3 days, and 65 % of the cells were dead, whereas in a control culture the cell density doubled and only 15 % of cells were dead.

Isolation of RNA from the cells 3 hr after addition of the inducer showed about 20 % higher incorporation of [14C]uridine-2 into the treated cells. The increase of incorporation was evenly distributed in all RNA bands except for the 32 s RNA band which contained about 60 % more 14C than the 32 s RNA of the control.

Introduction

Following the interest in double and multistranded complexes of synthetic homopolynucleotides as inducers of interferon (Field et al. 1967; Vilcek et al. 1968), some observations on the effect of these compounds on cell-virus systems have recently been made.

The antiviral activity of poly I: C is shown to be due to de novo synthesis of interferon (De Clercq & Merigan, 1969). According to Margolis & Levy (1969) the RNA synthesis in mouse L cells is increased several fold in the presence of poly I: C at a concentration of 10 to 50 μg./ml. Most of the excess incorporation into RNA was found in the heterogeneous nuclear RNA.

Reports on the toxicity of interferon inducers are few and scanty. Kawade & Ujihara (1969) found 0.1 to 1 μg./ml. of poly I: C to be toxic to chick embryo cells. Margolis & Levy (1969) working with 10 to 50 μg./ml. on mouse L cells did not report such toxic effects. In the present experiments toxic concentrations of poly I: C are used to study the effect on RNA synthesis; cell number and vital staining are used as indicators of cell damage.
METHODS

Cells and Virus. Detroit-6 cells obtained from the American Type Culture Collection were cultured continuously either in suspension using Eagle's (1959) medium, or as monolayers in Roux bottles. Only monolayer cells were used for virus titration.

Poliovirus, originally isolated from a patient and referred to as polio 3 clone 3, was concentrated and partially purified as follows. Spinner cultures of one l. were infected with

![Graph image]

Fig. 1. Determination of maximal hypochromic effect at 233 nm. in three separate experiments. Numbers on the abcissa indicate applied volumes of equimolar solutions of poly I and poly C in 6 mm-sodium phosphate + 0·15 M-NaCl, pH 7·0. 10 min. after mixing at room temperature the mixtures were transferred to an icebath and left for 1 hr before spectrophotometric observation.

![Graph image]

Fig. 2. Fractionation of the poly I:C complex on Sepharose 4 B. The column of length 27 cm. and diameter 1 cm. was equilibrated with 6 mm-sodium phosphate + 0·15 M-NaCl, pH 7·0 and the poly I:C complex (6:4 ratio; Fig. 1) was eluted with the same buffer at about 1 ml./hr.
Plaque inhibition by poly I:C

10 p.f.u./cell. After 20 hr the cell debris was collected and resuspended in 50 ml. of water for every $2 \times 10^6$ cells. The pH was adjusted to 2.0 with 0.5 M-HCl and the precipitate deposited by centrifugation at 3000 g for 3 min. was discarded. The pH was adjusted to 9.0 with 0.5 M-KOH and the supernatant fluid, after centrifugation, finally adjusted to pH 7.0. The infectivity of such preparations was about $10^9$ p.f.u./ml.

Plaque reduction assay. Infectivity titrations were made on 10 cm. Petri glass dishes. For measurement of plaque reduction by polynucleotides a standard poliovirus preparation giving about 100 plaques per dish was used. Serial dilutions of the interferon inducer were added with virus to the cell monolayers. Each inducer concentration was used in three parallel experiments.

Preparation of the poly I:C complex. Polynosinic acid (poly I) and polycytidylic acid (poly C) (Mile's Laboratories Inc., Indiana) were dissolved in 0.006 M-sodium phosphate and 0.15 M-NaCl, pH 7.0, at a concentration of $4 \mu$moles/ml. The maximum hypochromic effect on complexing (Michelson, Massoulie & Guschlbauer, 1967) was obtained with 60% poly I and 40% poly C (Fig. 1) and this ratio was used in all experiments. With poly I purchased more recently from the same company the maximum hypochromic effect was found with 40% poly I and 60% poly C. This batch of poly I was less soluble than the first, probably due to larger chain length.

Gel filtration of the poly I:C complex on Sepharose 4 B showed that about 7% of the poly I remained as single strands. A small amount of poly C was also present, evident from the spectral characteristics of fractions 13 to 17 (Fig. 2), which could not be estimated in the presence of complex of similar size.

The poly I:C complex was prepared at room temperature and after 10 min. it was left for 1 hr on an icebath before immediate use. During this hour there was no change in extinction at 233 nm.

RNA extraction. Whole cells were extracted by a combination of methods (Scherrer & Darnell, 1962; Penman, 1966; Wagner, Katz & Penman, 1967) which produced pure RNA with no material remaining on the top of the gels after electrophoresis. Extracted RNA was dissolved in electrophoresis buffer (about 1 mg./ml. of RNA) and stored at $-20^\circ$.

Polyacrylamide gel electrophoresis. Gels of 2.2% were used according to Loening (1967), on which the 4 s RNA moved about 4 cm. in 1½ hr at a current of 5 mA/gel.

Gels were prerun for 1 hr before the application of the RNA samples, which were made 5% in respect to sucrose.

After electrophoresis the gels were transferred to a quartz cuvette and u.v.-scanned in a Joyce-Loebl Chromoscan instrument equipped with Ni-Co and p-dimethylamino-benzaldehyde liquid filters.

A Mickle chopper (H. Mickle, Gomshall, Surrey, England) was used for chopping the frozen gels transversely into 1 mm. slabs which were dried on filter paper and assayed for radioactivity.

RESULTS

Preliminary experiments with the poly I:C complex in Detroit cells showed that about 0.1 $\mu$g./ml. resulted in a 50% inhibition of poliovirus plaques. Single strands alone had no effect on plaque formation when tested at up to 10 $\mu$g./ml.

De Clercq & Merigan (1969) reported some, but not all, single strands to be effective at 40 $\mu$g./ml. Kawade & Ujihara (1969) stated that the presence of poly I, poly C or of r-RNA of different origins depressed the ability of poly I:C complexes to induce interferon synthesis. A concentration of 100 $\mu$g./ml. of single strands was required for this effect.
In Fig. 3 the effect of poly I:C on poliovirus plaque formation is shown as a linear relationship between the logarithm of the inducer concentration and the logarithm of plaque reduction %, except above 1 µg./ml. or 80 % plaque reduction.

The toxicity of poly I:C complexes for cells (Kawade & Ujihara, 1969) and animals (M. R. Hilleman, personal communication) and the variation of the degree of toxicity with the type of cell or with the degree of cellular differentiation (Levy, Law & Rabson, 1969) demanded control of the toxicity of poly I:C for Detroit cells. At 5 µg./ml. poly I:C was without effect on cells in spinner culture for 48 hr. The cell density increased from $8 \times 10^6$ to $2.2 \times 10^7$ cells/ml. in this time. At 25 µg./ml. poly I:C caused a small but barely significant increase in cell number, followed by massive cell death. Three days after the addition of the inducer 65 % of the cells were dead, whereas in a control culture only 15 % of dead cells were found by vital staining.

The plaque reducing effect of poly I:C was improved when the inducer was pretreated for 30 min. at 37°C with 0.25 µg./ml. RNase. In some experiments such RNase-treated inducer was ten times as effective as untreated poly I:C. The significance of this effect is not clear. The removal of the ends of single strands from the poly I:C complex may change the secondary structure (De Clercq & Merigan, 1969) of importance to the inducer capacity. Incorporation of [14C]uridine-2 was regularly about 20 % higher in the RNA of cells treated with toxic concentrations of poly I:C.

Analysis of the RNA by polyacrylamide gel electrophoresis showed an even distribution of the excess RNA on all RNA bands, except for the 32 s RNA, in which the incorporation was increased by 60 % (Fig. 4).

Incubation of the cells for 20 hr in the presence of inducer and isotope showed that there was no block in the normal processing of RNA, as the excess isotope measured in the 32 s RNA after incubation for 3 hr was found to accumulate in the ribosomal RNA fractions.
after 20 hr (Fig. 5). The high specific radioactivity of the 32 s RNA band at 3 hr of incubation therefore probably reflects a radioactive pulse passing through the 32 s RNA in response to increased RNA synthesis induced by poly I:C.

Fig. 4. Polyacrylamide gel electrophoresis of RNA from normal Detroit cells (a) and Detroit cells treated with poly I:C (b). The cells were incubated for 3 hr in the presence of 0.05 µg/ml of [3H]uridine-2 and 10 µg/ml of poly I:C (b). The RNA was extracted from whole cells according to the procedure described in the text. 2.2 cm gels were run for 1 hr before application of 30 µg/gel of RNA. Electrophoresis was carried out for 1½ hr at 5 mA/gel. Electrophoresis buffer was: 36 mM tris, 30 mM-sodium dihydrogen-phosphate, 1 mM-EDTA, pH 7.6-7.8 and 0.2 % sodium dodecyl-sulphate. The u.v.-scanning was performed shortly after the electrophoresis was completed. The gels were frozen and sliced and the 1 mm slabs were dried on 1½ X 1½ cm. filter paper bits and transferred to the liquid scintillation vials. ---, Extinction at 265 nm.; -----, 14C (counts/min).

Fig. 5. Polyacrylamide gel electrophoresis for 3½ hr of RNA from normal cells (a) and poly I:C treated cells (b). The cells were incubated with isotope for 20 hr. RNA extraction and electrophoresis were as in Fig. 1. ---, Extinction at 265 nm.; -----, 14C (counts/min).

**DISCUSSION**

The mechanism of action of interferon inducers, and of their toxicity to cells at high concentrations is not known. The results of Margolis & Levy (1969) may indicate a derepressor function in response to which interferon is synthesized (De Clercq & Merigan, 1969).
On the other hand, the amount of inducer used is probably above the toxic level for the cells, and the reported stimulation of RNA synthesis may be part of a general set of mortal reactions induced by the high concentration of inducer. In contrast to previous claims, Bausek & Merigan (1969) found the antiviral activity of poly I: C to be insensitive to actinomycin, making the proposed derepressor function of poly I: C doubtful.

The use of complexes of synthetic double-stranded homopolynucleotides as therapeutic agents in virus infections depends, of course, on toxicity revealed in short- and long-term experiments on cells and on intact animals. The conditions for Detroit cells are promising since 0.01 μg./ml. results in about 20% plaque inhibition, whereas 5 μg./ml. seems to be without toxic effect over 48 hr.

It is worth noticing that previous experiments by some workers with poliovirus and interferon have indicated that this virus responds only poorly to the action of interferon. The present results show that poliovirus multiplication is sensitive to the action of poly I: C at about the same concentration as reported for the viruses of vesicular stomatitis, Semliki Forest and Sindbis.

The increased incorporation of [14C]uridine-2 into the RNA of cells incubated with high concentrations of poly I: C does not necessarily reflect an increased RNA synthesis in these cells. Poly I: C has been reported to increase cell membrane permeability, which could result in a more rapid diffusion of isotope into the cells, although equilibrium with uridine is normally obtained rapidly. More important is the enhancing effect of poly I: C on phosphorylation of uridine, yielding more labelled nucleoside triphosphate available for RNA synthesis.

The reason for the 6:4 optimum ratio in poly I: C is believed to be due to a higher content of short strands in the batch of poly I originally purchased from Mile's Laboratories. A batch purchased 6 months later was far less soluble and showed an optimum ratio of 4:6. It is therefore necessary to maintain careful control of complexing conditions when working with poly I: C.

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REFERENCES


Plaque inhibition by poly I: C


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