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Induction of Interferon Synthesis by Synthetic Double-stranded Polynucleotides

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It has been shown recently that double-stranded ribonucleic acid (RNA) from different sources stimulates the release of interferon into the circulatory system when injected intravenously into rabbits. The same RNA preparations protected mice from viral infections and prevented the viral cytopathic effect in cultures of rabbit kidney cells (A. K. Field et al., Proc. Natl. Acad. Sci. U.S. **58**:2102, 1967). One of the RNA preparations shown to possess such activity was a double-stranded structure produced from homopolymers of polyriboinosinic acid (rI) and polyribocytidylic acid (rC) (A. K. Field et al., Proc. Natl. Acad. Sci. U.S. **58**:1004, 1967). The main purpose of the present study was to find out whether a double-stranded deoxyribonucleic acid (DNA) polymer formed from polydeoxyinosinic acid (dI) and polydeoxycytidylic acid (dC), as well as a double-stranded RNA-DNA homopolymer hybrid formed with rI and dC, could also induce interference and interferon synthesis in a cell culture.

All experiments were carried out in cultures of rabbit kidney cells (RKC) prepared by trypsinization of minced kidney tissue from about 1-month-old rabbits. Cells were grown in Eagle's minimal essential medium (MEM) with 10% heated fetal calf serum and were used in experiments after one or two passages. Cultures grown to confluency in 60-mm plastic petri dishes were used in all experiments. MEM with 2% γ-globulin-free fetal calf serum served as the maintenance medium.

Solutions of rI (molecular weight 1.2×10^5) and rC (molecular weight 4×10^5) (Mann Research Laboratories, New York, N.Y.) were freshly prepared for each experiment in phosphate-buffered saline (PBS; 0.13 M NaCl, 7 mM phosphate, 0.9 mM $CaCl_2$, and 0.5 mM $MgCl_2 \cdot 6H_2O$). Equimolar concentrations of rI and rC were mixed at room temperature. The RNA homopolymers instantly polymerized to form the double-stranded form rI:rC. Polymerization was accompanied by the development of hypochromicity, which was tested with a Cary spectrophotometer (A. K. Field et al., Proc. Natl. Acad. Sci. U.S. **58**:1004, 1967).

Different concentrations of rI, rC, and rI:rC in PBS were added to RKC cultures which were then incubated for 1 hr at 37 C. Thereafter, the cells were washed three times with PBS, fed with 5 ml of maintenance medium, and further incubated at 37 C. Twenty hours later, the media were withdrawn and saved for interferon titrations. The cells were washed twice with PBS and were inoculated with 50 to 80 plaque-forming units (PFU) of bovine vesicular stomatitis virus (VSV). The virus was adsorbed on the cells for 1 hr. Thereafter, cells were overlaid with agar medium without neutral red (J. Vilček and J. H. Freer, J. Bacteriol. **92**:1716, 1966). A second overlay, containing neutral red (1:20,000), was added 2 to 3 days after inoculation and the plaques were counted.

Treatment with 20 to 40 μg of rI:rC per culture completely inhibited the appearance of VSV plaques. Ten or 100 times lower concentrations of rI:rC also caused significant protection. On the other hand, comparable concentrations of rI or rC alone caused no significant decrease in the number of VSV plaques in RKC. These results are in agreement with the data of A. K. Field et al. (Proc. Natl. Acad. Sci. U.S. **58**:1004, 1967).

The presence of interferon in the fluids collected from RKC exposed to polynucleotides was determined by testing their inhibitory effect on VSV plaque formation in RKC cultures. Cells were treated with twofold dilutions of the tested fluids in MEM for 4 hr at 37 C, and were washed and inoculated with 50 to 80 PFU of VSV. Plaques were counted 2 to 3 days after inoculation. Titers of interferon are expressed as reciprocals of the highest dilution of the tested fluid causing an inhibition of at least 50% of the control number of plaques.

In a number of experiments, interferon was

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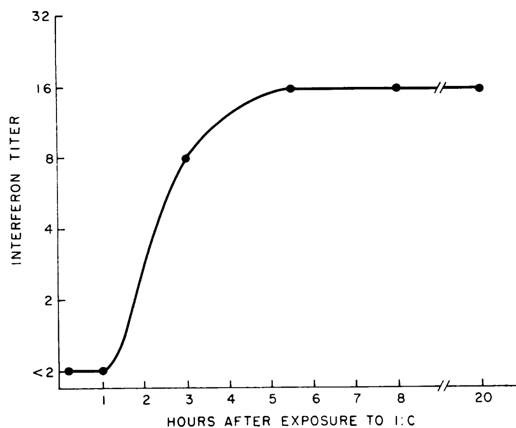

FIG. 1. *Release of interferon into the medium of rabbit kidney cell cultures after a 1-hr exposure to 32 μg of rI:rC.*

always detectable in fluids from cultures treated with 20 to 40 μg of rI:rC. The titers ranged from 2 to 32. Ten or 100 times lower doses of rI:rC induced less or no detectable interferon synthesis, although cultures directly exposed to these concentrations of the double-stranded RNA regularly showed significant protection. Therefore, it can be concluded that direct induction of resistance to viral inoculation is a more sensitive index for the demonstration of polynucleotide activity in this system than is the release of detectable quantities of interferon into the medium. No interferon was found in media of RKC cultures exposed to as much as 50 μg of either rI or rC alone.

The kinetics of the release of interferon into the medium of RKC cultures after a 1-hr exposure to rI:rC are shown in Fig. 1. The first release of interferon was detected between 1 and 3 hr after exposure and the maximal yield was reached within 5.5 hr.

The enzymatically prepared double-stranded polydeoxynucleotide dI:dC (molecular weight between 10^6 and 10^7) and the single-stranded deoxynucleotide homopolymer dC (molecular weight between 10^6 and 5×10^6) were purchased from Biopolymers, Inc., Pinebrook, N.J. They were shipped frozen in 0.01 M tris(hydroxymethyl)aminomethane buffer (*p*H 7.9) and were diluted before use in PBS. The double-stranded RNA-DNA hybrid rI:dC was prepared by mixing equimolar concentrations of rI and dC at room temperature. The double-stranded nature of the dI:dC and rI:dC complexes was confirmed by a sharp increase in the optical density (260 mμ) of the polymers on heating (M. J. Chamberlin and D. L. Patterson, J. Mol. Biol. **12**:410, 1965).

When the dI:dC and rI:dC polymers were

TABLE 1. *Induction of viral resistance and interferon by polynucleotide homopolymers of inosinic and cytidylic acid in rabbit kidney cell cultures*

Polynucleotide (μg/culture)[a]	VSV plaque count[b]		Interferon titer[c]	
	No DEAE-dextran	100 μg of DEAE-dextran per ml	No DEAE-dextran	100 μg of DEAE-dextran per ml
rI (15.0)	71	39.5[d]	<2	<2
rC (30.0)	63.5	49.5[d]	<2	<2
rI:rC (22.5)	0	0	4	32
rI:rC (22.5) + actinomycin D[e]	ND[f]	ND	<2	<2
dC (12.5)	66	30[d]	<2	<2
rI:dC (12.0)	68	0	<2	<2
dI:dC (20.0)	61	43[d]	<2	<2
Control	69.5	38[d]	<2	<2

[a] Cells treated for 1 hr with respective compound in 0.5 ml of PBS or in PBS with DEAE-dextran. I = homopolymer of inosinic acid; C = homopolymer of cytidylic acid; r = ribonucleotide homopolymer; d = deoxyribonucleotide homopolymer; colon = double-stranded homopolymer pair.

[b] In cultures inoculated 20 hr after the end of treatment with polynucleotide.

[c] In culture fluids collected 20 hr after the end of treatment with polynucleotide.

[d] Approximately twofold increase in plaque diameter.

[e] Treated with 1 μg of actinomycin D per ml for 1 hr prior to exposure to rI:rC.

[f] Not done.

tested at concentrations as high as 20 μg per RKC culture, under the same conditions used for the rI:rC, no direct inhibition of VSV plaque formation and no release of interferon were detected. F. Dianzani et al. (*personal communication*) have recently found that the addition of diethylaminoethyl (DEAE)-dextran considerably increased interferon production and the antiviral effect of rI:rC in L-cell cultures. We have therefore tested the activity of rI:dC, dI:dC, and rI:rC, and of the single-stranded homopolymers in RKC in the presence and absence of DEAE-dextran (molecular weight around 2×10^6; purchased from Pharmacia, Uppsala, Sweden). The results of this experiment are shown in Table 1. Pretreatment with DEAE-dextran alone somewhat decreased the VSV plaque count but significantly increased the plaque size. The rI:rC complex conferred complete protection both in the absence and presence of DEAE-dextran; the amount of interferon produced was increased eightfold by DEAE-dextran. Pretreatment with actinomycin D completely inhibited interferon synthesis with rI:rC, both in the presence and in the absence of DEAE-dextran. The RNA-DNA

hybrid rI:dC showed no activity in the absence of DEAE-dextran, but completely inhibited VSV plaque formation when applied in the presence of DEAE-dextran. However, no interferon was detected in the culture fluids of either group of cultures treated with rI:dC. It is felt that the lack of detectable interferon in cultures treated with rI:dC in the presence of DEAE-dextran can be explained by the fact that lower degrees of activity can be more easily shown through direct protection than by demonstration of interferon synthesis. The dI:dC complex and the single-stranded homopolymers were completely inactive under the same conditions.

The following statements can be made about the ability of polynucleotides formed with inosinic and cytidylic acid. (i) Only some double-stranded forms appear to be active. (ii) The rI:rC complex is more active than the RNA-DNA hybrid rI:dC; the deoxyribo-analogue dI:dC appears to be inactive. (iii) Cellular DNA-dependent RNA synthesis is required for the induction of interferon synthesis by rI:rC in RKC cultures because actinomycin D inhibited interferon production.

The differences in the activities of rI:rC, rI:dC, and dI:dC are intriguing, and one might feel tempted to speculate about the possible implications of these findings for the mechanism of induction of interferon synthesis by viruses. However, M. J. Chamberlin and D. L. Patterson (J. Mol. Biol. **12**:410, 1965) have shown that the T_m values and stabilities of the three double-stranded polymers decrease in the same order as their respective activities observed in this study, namely rI:rC > rI:dC > dI:dC. Therefore, we cannot rule out the possibility that the relative activities of the three complexes merely reflect the efficiency with which they can be taken up by cells in a double-stranded form, and, perhaps, their resistance to the action of cellular nucleases.

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