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Interferon Induction by Single-stranded Polynucleotides Modified with Polybases

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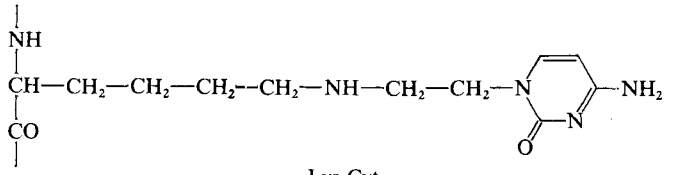
SUMMARY

Single-stranded polynucleotides (poly I and poly C), which do not generally have antiviral activity, became effective inducers of interferon when aggregated with different polycations. Poly I was consistently more active than poly C. The biological activity of the complexes depended more on the nature of the polycationic component than on the presence of the complementary base residue: introduction of covalently bound cytosine residue into the poly I–polybase complex did not have any significant effect.

An inducer with a double helical structure has been found to be necessary for efficient induction of interferon by polynucleotide (Colby, 1971). There are few exceptions to this generalization. One is the inducing activity of the complex of poly I and poly(1-vinylcytosine), (poly IvC, Pitha & Pitha, 1971). The complex lacks a helical conformation and the activity of poly IvC is difficult to understand on the basis of the inducing activity of its components – vinyl analogs alone do not induce interferon (P. M. Pitha, unpublished observations) and poly I shows rather low and variable antiviral activity by itself (Baron *et al.* 1969). However, in human fibroblasts, a higher antiviral effect of poly I, in comparison to poly C, in the absence of polyanions has previously been observed (DeClercq & Merigan, 1969). This suggests that the mechanism of protection by poly IvC may be based on a stimulation of the antiviral properties of poly I, analogous to the stimulation of the inducing efficiency of poly C achieved by pre-treatment of the cell monolayer with DEAE-dextran (Billiau *et al.* 1969). In this work, we have prepared complexes between single-stranded polynucleotides and different polycations, and studied their antiviral effects. Furthermore, we have chemically bound cytosine residue to the polybases or proteins (Fig. 1) and studied whether this additional structural feature would increase the antiviral activity of polyinosinic acid.



Lys-Cyt

Fig. 1. Lys-Cyt.

Primary human fibroblast cells and mouse L cells were used for testing the antiviral activity of the compounds and for the interferon assays. The cells were grown in Eagle's minimal medium supplemented with 5 % of foetal bovine serum. Interferon was assayed colorimetrically (Finter, 1969) using bovine vesicular stomatitis virus (New Jersey serotype) as a challenging virus. The international reference interferon assayed under identical conditions had 80 % of its given activity. Resistance to virus and virus yield assays which were used have been described recently (Pitha & Pitha, 1973).

Table 1. *Conversion of Lys residues to Lys-Cyt*

1-(2,2-diethoxy-ethyl)cytosine (mg)	Protein component	$NaBH_4$ (mg)	Degree of substitution
250	Poly-L-lysine HBr, 100 mg	300	25%, cf. residues substituted
150	Poly-L-(Lys, Lys-Cyt), 43 mg	150	Partly precipitated; in supernatant fluid 42% of residues substituted
150	Poly-D-lysine HBr, 100 mg	150	24% of residues substituted
150	Poly-DL-lysine HBr, 100 mg	150	26% of residues substituted
103	BSA, 288 mg	150	8 Cyt/BSA molecule

Polynucleotides having mol. wt. higher than 2×10^5 were used (purchased from Miles Laboratories, Kankakee, Ill.) and were extensively dialysed against buffer (10 mM-sodium phosphate, 150 mM-sodium chloride, pH 7·0) before use. Solutions in buffer at a concentration of 10 mg/ml were kept frozen at −10 °C; the same preparations were used for all the experiments. DEAE-dextran was purchased from Pharmacia, A.B., Uppsala, Sweden, mol. wt. 2×10^6; poly D- and L-lysine were obtained from Pilot Chemicals, Inc., Watertown, Mass. (mol. wt. 111 000 and 92 000, respectively).

Lysine residues were converted to ε-*N*-(2-(4-amino-2-hydroxypyrimidine-1-yl)ethyl)-lysine residue (Lys-Cyt, Fig. 1) by the following method. An aqueous solution of the aldehyde (0·2 M) was prepared from 1-(2,2-diethoxyethyl)cytosine according to Doel, Jones & Taylor (1969); this was added directly to the appropriate protein (in 10 ml of water or salt solution; pH 9·5). After 50 min at 24 °C, an aqueous solution of $NaBH_4$ was added and the mixture was incubated overnight at 4 °C. The reduced material was then dialysed at 4 °C against buffer (150 mM-sodium chloride, 10 mM-sodium phosphate, pH 7). The absence of degradation of the macromolecule was tested by chromatography of the final clear solution on G-50 Sephadex. In all cases, a single u.v. light absorbing band was eluted at the void vol. of the column. The degree of substitution in polyaminoacids was measured after hydrolysis; the sample was dissolved in 6 N-hydrochloric acid and kept under nitrogen at 100 °C overnight. Completion of hydrolysis was checked by thin layer chromatography on fluorescent silica gel. Elution by the solvent system, ammonia:*n*-propanol, (1:2, w/w) separates lysine from Lys-Cyt. This new amino acid forms a spot identifiable by both u.v. extinction and ninhydrin reaction. In a separate sample of hydrolysate, the number of cytosine residues was measured by u.v. spectrophotometry; the amount of lysine was measured by an amino acid analyser and from this data, the degree of substitution (Table 1) was calculated.

Reactions of some polynucleotides with polybases give, depending on the ratio of components, a number of products which differ in solubility, as well as in antiviral activity (Pitha & Pitha, 1971; Carter *et al.* 1972). To eliminate the variability introduced by a different physical state, we studied conditions which would lead to maximum aggregation of the complex. Solutions containing different ratios of polynucleotide and DEAE-dextran were equilibrated at room temperature and the amount of aggregation measured (Fig. 2). The antiviral protection conveyed by the mixtures with high aggregation was then measured (Table 2). Both polynucleotides were without any significant antiviral activity, when applied alone. When aggregated with DEAE-dextran, poly I induced both antiviral protection and interferon, while the amount of extracellular interferon induced by DEAE-dextran aggregated with poly C was negligible.

Similar conditions were used to test the antiviral protection conveyed by the combination of poly I with both poly-D-lysine and poly-L-lysine. Maximum aggregation for these combinations was observed at a 1:1 ratio (Fig. 2); and results on antiviral activities are in Table 3.

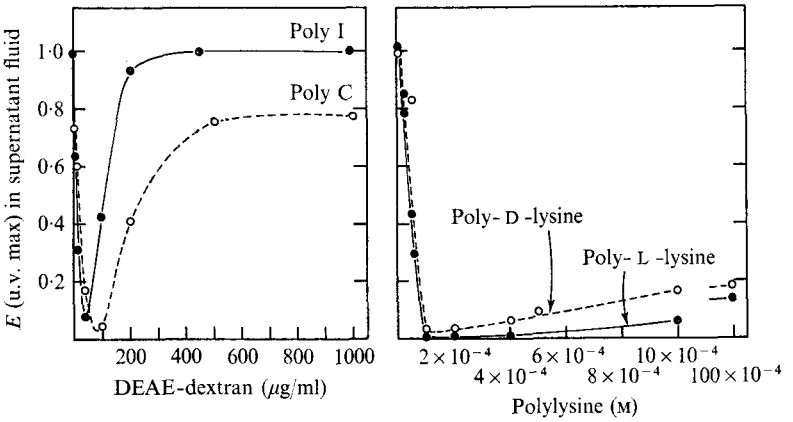


Fig. 2. The mixing curves between a constant amount of polynucleotide (10^{-4} M) and a varying amount of polycations. Solutions were left to equilibrate overnight, centrifuged at 10000 rev/min for 10 min and u.v. spectrum of supernatant fluid measured. All operations were at room temperature. The solvent was 0·15 M-NaCl, 0·01 M-phosphate, pH 7. Experiments with DEAE-dextran were repeated using tris-buffered solutions when no qualitative changes were noted.

Table 2. *The interferon inducing activity of the complexes formed between single-stranded polynucleotides and DEAE-dextran*

Polynucleotide	μg/ml	DEAE-dextran (μg/ml)	% of cells resistant to virus infection *	% of cells resistant to virus infection †	Virus yield (p.f.u./ml) *	Interferon (reference research units) †
Poly I	300	300	100	100	$2{\cdot}1\times10^{4}$	540
	30	30	100	100	$2{\cdot}1\times10^{4}$	240
	3	3	10	14	$1{\cdot}8\times10^{7}$	< 5
	300	—	10	11	$9{\cdot}0\times10^{7}$	—
Poly C	300	1000	100	80	$3{\cdot}3\times10^{4}$	< 5
	30	100	30	14	$3{\cdot}0\times10^{7}$	< 5
	3	10	10	16	$3{\cdot}3\times10^{7}$	< 5
	300	—	10	10	$3{\cdot}4\times10^{7}$	—
	—	—	10	10	$6{\cdot}0\times10^{7}$	—
Poly I.Poly C	30	30	100	100	$1{\cdot}5\times10^{4}$	600
	30	—	100	70	$3{\cdot}1\times10^{5}$	< 5

* Human fibroblast cells.
† Mouse L cells.

The most effective combinations found were the aggregated complexes between poly I and poly-D-lysine; the effect of the complex of poly-L-lysine was significantly lower. Similar experiments were also made for the combinations of poly C with poly-D-lysine and poly-L-lysine. Here again, poly-D-lysine combination was more effective. The treatment of human and mouse cells with both poly-D-lysine and poly-L-lysine alone showed some late toxicity effect (as measured by dye exclusion test, Phillips & Terryberry, 1957). While at 24 h and 48 h after treatment, the percentage of dead cells was only 2 to 5 % in both cell systems, at 72 h, 14 % of the mouse and 20 % of the human cells were dead. The effect was much less pronounced when polylysine-polynucleotide complexes were used. However, neither poly-L-lysine nor poly-D-lysine pre-treatment affected the virus replication under the condition of our experiments (Table 3).

Table 3. *Interferon inducing activity of the complex formed between polynucleotides and polylysines*

Polynucleotide	(M)	Enhancer	(M)	Virus yield (p.f.u./ml) *	Interferon (reference research units) †
Poly I	10^{-4}	Poly-D-lysine	10^{-4}	$4{\cdot}3 \times 10^{5}$	24
	10^{-4}	Poly-D-lysine	2×10^{-4}	$1{\cdot}3 \times 10^{6}$	12
	10^{-5}	Poly-D-lysine	10^{-5}	$2{\cdot}0 \times 10^{8}$	< 2
	10^{-5}	Poly-D-lysine	2×10^{-5}	$3{\cdot}6 \times 10^{8}$	< 2
Poly I	10^{-4}	Poly-L-lysine	10^{-4}	$3{\cdot}2 \times 10^{8}$	< 2
	10^{-4}	Poly-L-lysine	2×10^{-4}	$2{\cdot}8 \times 10^{9}$	—
	10^{-5}	Poly-L-lysine	10^{-5}	$3{\cdot}7 \times 10^{8}$	—
	10^{-5}	Poly-L-lysine	2×10^{-5}	$1{\cdot}3 \times 10^{9}$	—
Poly I	10^{-4}	Poly-L-lysine	—	$1{\cdot}0 \times 10^{9}$	—
Poly C	10^{-4}	Poly-D-lysine	10^{-4}	$3{\cdot}15 \times 10^{7}$	4
	10^{-4}	Poly-D-lysine	2×10^{-4}	$2{\cdot}3 \times 10^{8}$	< 2
	10^{-5}	Poly-D-lysine	10^{-5}	$2{\cdot}3 \times 10^{8}$	< 2
	10^{-5}	Poly-D-lysine	2×10^{-5}	$2{\cdot}7 \times 10^{8}$	—
Poly C	10^{-4}	Poly-L-lysine	10^{-4}	$6{\cdot}0 \times 10^{7}$	< 2
	10^{-4}	Poly-L-lysine	2×10^{-4}	$1{\cdot}5 \times 10^{8}$	< 2
	10^{-5}	Poly-L-lysine	10^{-5}	$2{\cdot}7 \times 10^{9}$	—
	10^{-5}	Poly-L-lysine	2×10^{-5}	$1{\cdot}0 \times 10^{9}$	—
Poly C	10^{-4}	—	—	$1{\cdot}0 \times 10^{9}$	
—	—	—	—	$9{\cdot}0 \times 10^{9}$	
—	—	—	—	$1{\cdot}0 \times 10^{9}$	
—	—	Polylysine‡	10^{-4}	$9{\cdot}0 \times 10^{9}$	
—	—	—	2×10^{-4}	$8{\cdot}0 \times 10^{8}$	

* † See footnote to Table 2.
‡ The difference between D and L series was not significant.

The enhancement of the antiviral activity of poly I and poly C, after complexing with polylysines, cannot be due to the lower susceptibility of these polymers to nucleases. Complex formation with polylysines (both L and D forms) protected polynucleotides from nucleases to a similar extent (Carter *et al.* 1972). Hence, the data indicates that the biological activities of both poly I and poly C complexes with poly-D-lysine were significantly higher than after complexing with poly-L-lysine. Whether this difference can be directly related to the structural requirement of receptors responsible for the triggering mechanism in interferon induction remains to be seen.

To examine whether the antiviral activity of poly I could be potentiated by the presence of cytosine residues, various polylysines were converted to polybases carrying approx. 25% cytosine residues (Table 1 and Fig. 1) in the molecule. The resulting macromolecules formed complexes with polynucleotides with the maximum amount of aggregation again at a 1:1 stoichiometry. This indicates that the observed interaction is through the neutralization of mutual electric charges rather than through base pairing. The antiviral protection induced by these complexes is shown in Table 4. Substitution by cytosine residues increased the antiviral activity of the complex between poly-D-lysine and poly I, but has no effect when poly-L-lysine was combined with poly I. A similar situation was found for the non-complementary complexes between poly C and substituted polylysines: in the poly-D-lysine complex, substitution increases the antiviral activity; in the -L- series, the change is negligible. No differential stimulation was observed by inclusion of cytosine residues into the polynucleotide–

Table 4. *Interferon inducing activity of the complexes formed between polynucleotides and cytosine residue substituted polylysines*

Polynucleotide	(M)	Enhancer‡	(M)	Virus yield (p.f.u./ml) *	Interferon (reference research units) †
Poly I	10^{-4}	Poly-D-lysine cytosine substituted	10^{-4}	$1{\cdot}0 \times 10^{5}$	120
	10^{-4}	Poly-D-lysine cytosine substituted	2×10^{-4}	$2{\cdot}1 \times 10^{6}$	12
	10^{-5}	Poly-D-lysine cytosine substituted	10^{-5}	$2{\cdot}0 \times 10^{8}$	< 2
	10^{-5}	Poly-D-lysine cytosine substituted	2×10^{-5}	$4{\cdot}0 \times 10^{8}$	< 2
Poly I	10^{-4}	Poly-L-lysine cytosine substituted	10^{-4}	$3{\cdot}4 \times 10^{8}$	< 2
	10^{-4}	Poly-L-lysine cytosine substituted	2×10^{-4}	$7{\cdot}8 \times 10^{8}$	—
	10^{-5}	Poly-L-lysine cytosine substituted	10^{-5}	$8{\cdot}3 \times 10^{8}$	—
	10^{-5}	Poly-L-lysine cytosine substituted	2×10^{-5}	$1{\cdot}1 \times 10^{9}$	—
	10^{-4}	—	—	$9{\cdot}0 \times 10^{8}$	—
Poly C	10^{-4}	Poly-D-lysine cytosine substituted	10^{-4}	$2{\cdot}5 \times 10^{6}$	12
	10^{-4}	Poly-D-lysine cytosine substituted	2×10^{-4}	$2{\cdot}2 \times 10^{7}$	5
	10^{-5}	Poly-D-lysine cytosine substituted	10^{-5}	$1{\cdot}2 \times 10^{8}$	—
	10^{-5}	Poly-D-lysine cytosine substituted	2×10^{-5}	$2{\cdot}4 \times 10^{8}$	—
Poly C	10^{-4}	Poly-L-lysine cytosine substituted	10^{-4}	$1{\cdot}0 \times 10^{7}$	6
	10^{-4}	Poly-L-lysine cytosine substituted	2×10^{-4}	$4{\cdot}0 \times 10^{7}$	< 2
	10^{-5}	Poly-L-lysine cytosine substituted	10^{-5}	$8{\cdot}7 \times 10^{8}$	—
	10^{-5}	Poly-L-lysine cytosine substituted	2×10^{-5}	$1{\cdot}5 \times 10^{9}$	—
—	—	Polylysine cytosine substituted	10^{-4}	$8{\cdot}0 \times 10^{8}$	
—	—	Polylysine cytosine substituted	2×10^{-4}	$1{\cdot}0 \times 10^{9}$	

* † See footnote to Table 2.
‡ Approx. 25% substitition (Table 1).

polybase complex. In both complementary and non-complementary cases, activity was increased by approximately the same amount, which may be due to the lack of hydrogen bond formation between poly I and cytosine residues on a polybase.

Finally, we prepared bovine serum albumin which had approximately 8 cytosine residues per protein molecule (Table 1). No detectable complex formation between this molecule and poly I was observed, and consequently, the substituted albumin did not increase the antiviral activity of poly I.

In summary, our results indicate that the inactive single-stranded polynucleotides, poly I and poly C, can induce interferon production and the antiviral state both in human and mouse fibroblast cells, when complexed–aggregated with polybasic substances such as DEAE-dextran and polylysines. In all combinations tested, poly I had higher antiviral activity than poly C when complexed with DEAE-dextran or polylysines. Further experiments will have to elucidate whether this is due to the ability of poly I form stable secondary structure, or due to its higher affinity for receptors. The biological activity of the complex depends more on the nature of the polycation component than on the presence of the complementary base residues in it. In the case where no complex formation was detected (cytosine substituted bovine serum albumin and poly I), the antiviral activity of polynucleotide was not enhanced. Thus, the aggregation of polynucleotide with polybasic compound seems to overcome its intrinsic inefficiency as an interferon inducer.

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