Influence of Polyamines on Induction of Interferon and Resistance to Viruses by Synthetic Polynucleotides (34182)

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It has long been known that polyamines occur in living cells and that they may play an important role in the synthesis and function of nucleic acids (1). It has been found, in particular, that certain polyamines may increase the thermal stability of deoxyribonucleic acid (DNA) (1). Reports (2–8) from this laboratory described the induction of interferon and of resistance to viral infections *in vivo* and *in vitro* by double-stranded but not single-stranded ribonucleic acids (RNA) of synthetic and natural origin. Billiau *et al.* (9) reported an enhancement by certain polybasic substances, including neomycin and streptomycin, of induction of the interferon mechanism by synthetic RNA's. The present report describes the effects of polyamines on the biological activities and physicochemical properties of polyriboinosinic:polyribocytidylic acid complex (poly I:C, rI:rC), of polyriboinosinic acid (poly I, rI), of polyribocytidylic acid (poly C, rC), and of polyriboadenylic:polyribouridylic acid complex (poly A:U, rA:rU). In particular, the influence of polyamines on the polynucleotides with respect to induction of interferon and resistance of viral infections, cell-uptake, toxicity, thermal stability, and sensitivity to destruction by pancreatic ribonuclease (RNase) are reported.

Materials and Methods. Polynucleotides. Synthetic poly I (rI) and poly C (rC) were prepared by Drs. Peter Pollak and John Zabriskie of the Merck Process Research Laboratories. Poly A (rA) and poly U (rU) were obtained from Miles Laboratories, Elkhart, Indiana. The poly I:C and poly A:U complexes were prepared by mixing the corresponding components in equimolar ratio in 0.15 *M* NaCl–0.006 *M* sodium phosphate buffer, pH 7.0, and were stored at a concentration of 1 mg/ml.

Polyamines. Kanamycin was obtained from Bristol Myers, Co., New York, N. .Y; spermine and spermidine from Calbiochem, Los Angeles, California; cadaverine and glucosamine from Nutritional Biochemicals Co., Cleveland, Ohio; and neomycin, neamine (a derivative of neomycin), streptomycin, streptidine, and 1,6-diaminohexane from sources within Merck & Co.

Bioassays. The methods for assaying induction of interferon and resistance to viral infections *in vivo* and *in vitro* were described previously (2). The polynucleotides were tested in the presence or absence of added polyamines. Poly I:C uptake was measured in primary rabbit kidney cells (PRK) using tritium-labeled poly C with or without added polyamine. After incubation for the specified time periods, the cells were washed free of residual non-cell-associated radioactivity in the wash fluid. The concentrated cells were suspended in scintillation fluid, and assayed for radioactivity in a Packard Tricarb liquid scintillation spectrometer.

Physicochemical assays. The thermal denaturation curves (determination of thermal transition midpoint, T_m) were determined in a DB-G recording spectrophotometer equipped with a T_m analyzer. Pancreatic ribonuclease obtained from Worthington Biochemicals Co., Freehold, N. J. was dissolved in the above buffered saline solution, and ribonuclease susceptibility was measured in a DB-G recording spectrophotometer. The linear increase in optical density units over a 30-min period at 37° resulting from the degradation of 34 μg/ml (0.1 μmole/ml) of poly I:C by pancreatic ribonuclease (0.4 μg/ml) was taken as a value

TABLE I. Effect of Added Neomycin on Induction of Interferon in Rabbits by Polynucleotides.

Polynucleotide injected intravenously		Added neomycin	Interferon titer in rabbits
Kind	Amount (μg)	(μg)	
Poly I:C	2.50	150	160, 10
	2.50	0	160, 40
	0.60	150	5, 0
	0.60	0	0, 0
Poly I	550	150	0, 0
	550	0	0, 0
Poly C	500	150	0, 0
	500	0	0, 0
Poly A:U	105.0	150	20, 10
	105.0	0	20, 5
	10.5	150	5, 0
	10.5	0	5, 0
Control	0	150	5,[a] 0
	0	0	0, 0

[a] Antiviral activity at 1:5 may be found occasionally in sera of untreated normal animals.

of 100. Polyamines were added in various molar ratios to poly I:C followed by the addition of RNase. The ratio of the increase in optical density of the test sample to that of poly I:C control multiplied by 100 was the value for RNase susceptibility.

Results. Biological activities. Interferon induction. The observations made in tests to measure the effect on induction of interferon by added neomycin given rabbits intravenously are shown in Table I. The complexed double-stranded poly I:C and poly A:U but not the single-stranded poly I and poly C were active. Added neomycin did not affect the interferon-inducing capacity of poly I:C or poly A:U in rabbits and did not activate the single-stranded poly I or poly C.

Induction of resistance to viral infections in vitro. As shown in Table II, added neomycin in concentrations of 30 or 300 μg/ml enhanced the effectiveness of poly I:C 17-fold in suppressing plaque formation by vesicular stomatitis virus when tested in cell cultures of primary rabbit kidney. No such enhancement was found in RK-13 rabbit kidney line cells or in primary grivet monkey kidney cells. In fact, there seemed to be a slight diminution of activity in these cultures. It is evident from the data in Table III that neomycin, neamine, and streptomycin, in decreasing order of activity, all caused enhancement of poly I:C activity against vesicular stomatitis virus. Spermine, which was toxic in concentrations >5 μg/ml, was inactive or decreased the activity against vesicular stomatitis and vaccinia viruses in these cells.

Induction of resistance to viral infections in vivo. Table IV shows that added neomycin did not alter significantly the activity of

TABLE II. Effect of Neomycin on Poly I:C-Induced Interference against Vesicular Stomatitis Virus in Various Cell Cultures.[a]

Neomycin[b] conc (μg/ml of medium)	Kind of cell culture	Minimal effective dose of poly I:C (μg/ml)	Change in activity (fold)
300	Primary rabbit kidney	0.00015	+17
30		0.00015	+17
0		0.00250	—
300	Rabbit kidney line (RK-13)	>10.50	−2
0		5.25	—
300	Primary grivet monkey kidney	26.00	−8
30		6.50	−2
3		3.25	0
0		3.25	—

[a] Assayed by plaque count.

[b] There was no evidence for cell toxicity at 300 μg/ml; slight enhancement of viral plaque count was noted at this concentration.

TABLE III. Effect of Added Polyamines on the Suppression of Virus by Poly I:C in Primary Rabbit Kidney Cell Culture.

Polyamine added (μg/ml)	Virus[a]	Minimum effective dose of poly I:C (μg/ml)	Change in activity (fold)
Neomycin, 100	VS	0.00015	+17
None (control)	VS	0.0025	—
Neamine, 100	VS	0.00125	+8
None (control)	VS	0.01000	—
Streptomycin, 100	VS	0.003	+2
None (control)	VS	0.006	—
Spermine,[b] 5	VS	>0.02	—2 or more
None (control)	VS	0.01	—
Spermine,[b] 5	Vac	>0.1000	—40 or more
None (control)	Vac	0.0025	—

[a] VS = vesicular stomatitis; Vac = vaccinia.
[b] Spermine in concentrations >5 μg/ml was toxic.

poly I:C in suppressing death in mice caused by pneumonia virus of mice when virus and drugs were given intranasally. Similarly, Table V shows that added neomycin did not cause an appreciable change in the degree of protection afforded by poly I:C against Sendai virus in mice given virus and drugs by the nasal route.

Poly I:C Uptake. The increase in antiviral activity of poly I:C in primary rabbit kidney cells by added neomycin was not due to increased uptake of the polynucleotide by the cells. Conversely, as shown in Table VI, added neomycin markedly reduced the amount of radioactive labeled poly I:C which was taken up by the primary rabbit kidney cells.

Influence of neomycin on poly I:C toxicity. Complexing of poly I:C with neomycin did not alter the toxicity significantly as shown in Table VII and afforded no practical benefit in the use of the polynucleotide complex.

Physicochemical properties. Poly I:C has a relatively lower thermal stability and is more susceptible to destruction by pancreatic RNase than the naturally occurring double-stranded RNA's (2, 4, 5). Figure 1 shows a remarkable stabilization of poly I:C against degradation by RNase when neomycin was added in equimolar amount. This protection by neomycin did not occur for the single-stranded poly C (Fig. 2). (Poly I is not susceptible to destruction by pancreatic

TABLE IV. Effect of Added Neomycin on the Induction of Resistance by Poly I:C in Mice to Infection with Pneumonia Virus of Mice.[a]

Treatment		Survival				
		Nos.			Time (days)	
Poly I:C (μg/mouse)	Neomycin (μg/mouse)	No. survived /no. tested	Survived (%)	Excess survival (%)	Mean	Excess compared with controls
1.00	9.0	13/15	87	83	>14	>7
0.25	9.0	6/15	38	34	13	6
0.06	9.0	1/15	7	3	8	1
0.15	9.0	0/16	0	—4	8	1
1.00	0	12/16	75	71	>14	>7
0.25	0	12/16	75	71	>14	>7
0.06	0	1/16	6	3	8	1
0.15	0	0/16	0	—4	8	1
0 (control)[b]	9.0	0/16	0	—4	7	0
0 (control)	0	2/50	4	—	7	—

[a] The mice were of mixed sex and weighed 9–11 g each. Virus in 100 LD_{50} amount was given intranasally 3 hr after drug administered by the same route.
[b] Control mice received phosphate buffered saline solution without poly I:C.

TABLE V. Effect of Added Neomycin on the Induction of Resistance by Poly I:C in Mice to Infection with Parainfluenza Type 1 (Sendai) Virus.[a]

Treatment		Survival				
		Nos.			Time (days)	
Poly I:C (μg/mouse)	Neomycin (μg/mouse)	No. survived /no. tested	Survived (%)	Excess survival (%)	Mean	Excess compared with controls
60	60	14/16	88	74	>14	>6
30	60	11/16	69	55	>14	>6
15	60	10/16	63	49	>14	>6
60	0	12/15	80	66	>14	>6
30	0	15/17	88	74	>14	>6
15	0	15/17	88	74	>14	>6
0 (controls)[b]	60	0/17	0	—14	8	0
0 (controls)	0	7/50	14	—	8	—

[a] The mice were females and weighed 9–11 g each. Virus in 10 LD_{50} amount was given intranasally 3 hr after drug was administered by the same route.

[b] Control mice received phosphate buffered saline solution in place of poly I:C or neomycin.

RNase.) As shown in Table VIII, 5 of 10 polyamines tested, viz., neomycin, neamine, kanamycin, spermine, and spermidine protected poly I:C against pancreatic RNase when used in the concentrations shown. The resistance to destruction by RNase of the active polyamines–poly I:C complexes was accompanied by an increase in thermal stability (T_m increase 4.5–17.5°). Figure 3 illustrates that the increase in heat stability of poly I:C by the various polyamines was definitely related to the ratio of the concentration of polyamine to poly I:C. Maximal increase in thermal stability was obtained when the ratio of polyamine to poly I:C was about 6 to 9:1. The stabilization by polyamines of poly I:C to thermal denaturation was also markedly affected by the ionic strength. Figure 4 shows that maximal stabilization was obtained at the extremes of 0.01 and 1.0 M Na^+. Gradually decreasing followed by increasing stability was seen in the intermediate concentrations. The thermal stability curve for poly I:C alone followed a straight line function ranging from a T_m of 42° for 0.01 *M* to 76° for 1 *M* Na^+.

TABLE VI. Suppression by Neomycin of Uptake of Radioactive-Labeled Poly $I:C^{H^3}$ by Primary Rabbit Kidney Cells.

Substance[a]	Uptake (%), at 35° as a function of time (hr)			
	0	2	6	24
Poly I:C alone	0.03	0.38	2.20	20.8
Poly I:C plus neomycin	0.03	0.17	0.63	8.5

[a] Poly I:C at 1 μg/ml and neomycin at 300 μg/ml.

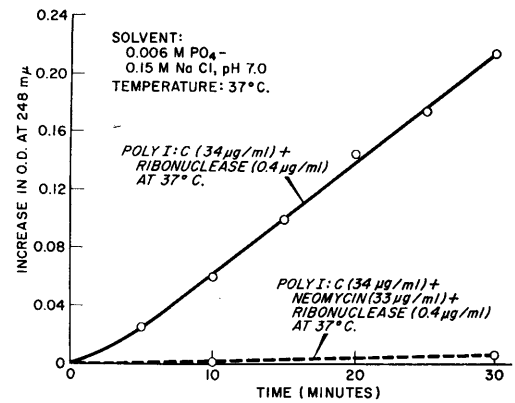

FIG. 1. Inhibition, by neomycin, of pancreatic ribonuclease degradation of poly I:C.

Discussion. It is evident from the present studies that certain basic polyamines of particular molecular structure may complex with poly I:C to cause alterations in physicochemical behavior and in certain biological properties. Altered physicochemical properties of poly I:C by complexing with active polyamines was evidenced by an increase in

TABLE VII. Lack of Effect of Neomycin on Toxicity of Poly I:C for 9–11 g Mice.

Treatment[a]		Result	
Poly I:C (μg/mouse)	Neomycin (μg/mouse)	No. died/no. treated	Death rate (%)
500	250	9/15	60
250	250	2/15	13
125	250	0/15	0
63	250	0/15	0
32	250	0/15	0
500	0	10/15	67
250	0	4/15	28
125	0	1/15	7
63	0	0/15	0
32	0	0/15	0

[a] Drugs administered by the intraperitoneal route.

thermal stability. There was a correlation between elevation in T_m and number and character of basic groups per molecule of polyamine. Hexamino, tetramino, diamino-monoimino, and diguanido (streptomycin) substances were most effective (T_m increase 17.5 to 4.5°) while diamino, monoamino and diguanido (streptidine) compounds were of low activity or were not effective (T_m increase 2.5 to 0°). Molecular ratio was also important in that from about 6–9 molecules of polyamine per molecule of poly I:C were required to achieve maximal stabilization. The marked effect of ionic strength on T_m of poly I:C alone was a straight line function ranging from a T_m of 52° at 0.01 *M* Na^+ to a T_m of 85° at 1 *M* Na^+. The increase of T_m by added polyamines was greatest at 0.01 *M* Na^+ and was progressively diminished to no increase at 1 *M* Na^+. Such thermal stability dependence on ionic strength has been shown (10) for double-stranded DNA. Complexing of poly I:C with polyamines which were effective in thermal stabilization was also effective in prevention of destruction by pancreatic ribonuclease with the exception of streptomycin. Neomycin, however, did not reduce the susceptibility of poly C (single-stranded RNA) degradation by the enzyme.

The alteration in biological activity of the polynucleotides by polyamines was very limited. Neomycin, neamine, and to a small degree streptomycin were capable of potentiating the activity of poly I:C in inducing resistance to vesicular stomatitis virus in primary rabbit kidney cells *in vitro*. In the case of neomycin, this was not due to increased uptake of the poly I:C by the primary rabbit kidney cells. Spermine, by contrast, de-

TABLE VIII. Effect of Polyamines on T_m and Ribonuclease Susceptibility of Poly I:C.[a]

Added polyamine			Finding		
Kind	Basic groups	Concentration (μmole/ml)	T_m (°) Observed	T_m (°) Increase	Relative susceptibility to RNase[b]
None (control)	—	—	60.5	—	100
Neomycin	Hexaamino	0.1	74.0	13.5	0
Neamine	Tetraamino	0.1	65.0	4.5	5
Kanamycin	Tetraamino	0.1	66.0	5.5	5
Spermine	Diamino, monoimino	0.1	78.0	17.5	0
Spermidine	Diamino, monoimino	0.1	65.5	5.0	33
Cadaverine	Diamino	1.0	63.0	2.5	100
Cadaverine	Diamino	0.1	61.0	0.5	100
1,6 Diamino hexane	Diamino	2.5	63.0	2.5	100
Glucosamine	Monoamino	1.2	60.5	0	100
Streptidine	Diguanido	0.1	61.3	0.8	100
Streptomycin	Diguanido	0.1	65.0	4.5	100

[a] The poly I:C concentration was 0.1 μmole/ml in 0.1 *M* Na^+, pH 7.0.

[b] For 30 min, 37°, and at 0.4 μg/ml of enzyme.

creased activity of poly I:C against vesicular stomatitis and vaccinia viruses in these cells.

The increase of activity of poly I:C was highly host-cell specific since such potentiation by neomycin did not occur either in the RK-13 line rabbit kidney line cell or in primary grivet monkey kidney cells; instead, reduction in activity was observed. Added neomycin did not bring about any alteration

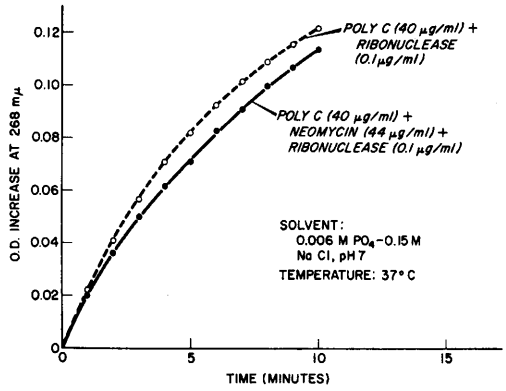

FIG. 2. Failure of neomycin to prevent degradation of poly C by pancreatic ribonuclease.

in the activity of poly I:C in intact animals against pneumonia virus of mice or against parainfluenza 1 virus, and did not decrease the toxicity of the complexed polynucleotides for these animals. The effect of neomycin was entirely limited to *in vitro* activity in one particular kind of cell and showed no potential for practical utilization in human and animal application either in potentiation of poly I:C activity or in reduction of its toxicity. Further, neomycin and other polyamines are highly nephrotoxic in their own right (11, 12).

Added neomycin had no effect on the induction of interferon by poly I:C or poly A:U in rabbits. Further, the single-stranded RNA's remained inactive as inducers of interferon even with added neomycin. Colby and Chamberlin (13) showed that DEAE-dextran was highly active in potentiating induction of resistance in chick embryo cells to Sindbis virus by poly I:C or by poly A:U, but DEAE-dextran did not render single-stranded RNA's (poly I, poly C, poly A, poly U, or poly G) active.

The present data have direct bearing upon

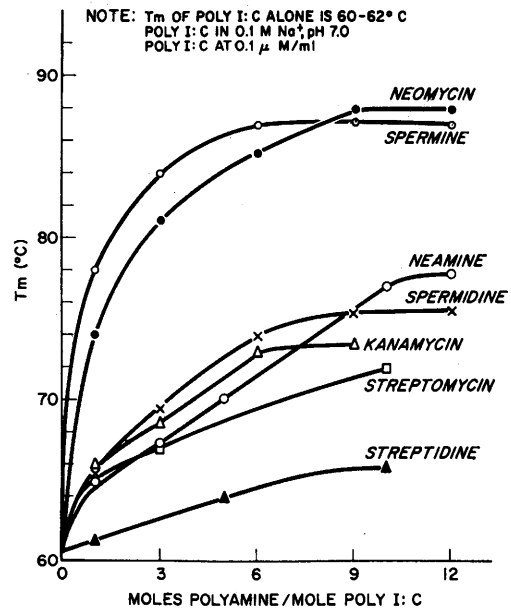

FIG. 3. Increase in heat stability (T_m) of poly I:C by complexing with various polybasic substances.

the question of whether pure single-stranded RNA's are ever capable of inducing interferon or host resistance to viral infections. Billiau *et al.* (9) reported that certain preparations of single-stranded poly I and poly C in relatively high concentration were active *in vivo* and *in vitro* in inducing interferon and resistance to viral infection. Such activity was not found by us (3, 6) or by others (13, 14) using highly purified single-stranded

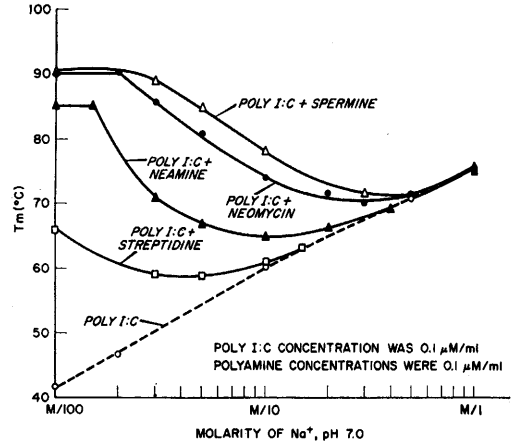

FIG. 4. Influence of ionic strength on heat stabilization of poly I:C by polybasic substances.

polynucleotides. Beyond a reasonable doubt, the capacity of double-stranded RNA to induce interferon or resistance is many times greater (at least 1000-fold) than the alleged capacity of the single-stranded components (3, 6, 13). The burden of proof that interferon can be induced by single-stranded polynucleotide requires rigorous proof of lack of contaminating or inherent double-strandedness (6). Buckler and his co-workers (15) stated that poly I and poly C induce the interferon mechanism in the presence of polybasic substances including neomycin and streptomycin. They stated further, without supporting evidence, that these polybasic substances are known to combine with RNA to protect it against ribonuclease and to increase cellular uptake of RNA. These findings and statements are not supported in the present studies. Thus, neomycin added to poly I or poly C did not render them capable of inducing interferon in rabbits. Added neomycin did not protect poly C from degradation by ribonuclease. Finally, added neomycin suppressed rather than increased the uptake of radioactive labeled poly I:C by primary rabbit kidney cells. Meaningful evidence for induction of interferon or of resistance to viruses by single-stranded RNA's continues to be lacking.

Summary. Certain basic polyamines, such as neomycin, neamine, kanamycin, streptomycin, and spermine, added to poly I:C increased the T_m. The degree of termal stabilization was influenced by the molecular ratio of polyamine to poly I:C and by the ionic strength. Complexing with certain of the polyamines markedly reduced the rate of degradation of poly I:C but not of poly C by pancreatic ribonuclease. Some of the polyamines potentiated induction of resistance by poly I:C of primary rabbit kidney cells but not RK-13 rabbit kidney line or primary grivet kidney cells to infection by vesicular stomatitis virus. Neomycin did not alter the activity of poly I:C in intact animals against pneumonia virus of mice or against parainfluenza 1 (Sendai) virus in mice and did not decrease the toxicity of the complexed polynucleotide for these animals. Added neomycin did not affect the induction by poly I:C of interferon in rabbits. Neomycin did not render the single-stranded RNA's, poly I and poly C, capable of inducing interferon in rabbits and poly C was not made resistant to RNase by neomycin. Neomycin suppressed rather than increased the uptake of poly I:C by primary rabbit kidney cells. The implications of the findings are discussed.

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