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Antiviral Activity and Side Effects of Polyriboinosinic-Cytidylic Acid Complexes as Affected by Molecular Size

(synthetic polynucleotide/interferon/antitumor agent/drug metabolism)

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ABSTRACT **The decrease of the molecular size of poly(I·C) to less than 10^6 decreases its ability to induce interferon, protect mice against virus, or enhance the immune response. Immune adjuvant activity appeared more sensitive to molecular weight than the other protective activities. The composition of the complex—the molecular size of the individual homopolymers when one was large and the other small—did not affect antiviral activity; the activity of a complex made from large poly(I) and small poly(C) was similar to one made from small poly(I) and large poly(C). Molecular size of the complex did not profoundly alter the side effects of poly(I·C). At 2 mg/kg, none of the complexes markedly altered phagocytic function. Only the largest complex sensitized the mouse to endotoxin. However, all of the complexes studied profoundly inhibited drug metabolism by the liver microsomal enzymes between 24 and 72 hr after their inoculation. Decreasing the molecular weight did not alter this inhibition.**

The double-stranded synthetic polynucleotide, polyriboinosinic–polyribocytidylic acid [poly(I·C)], is a potent antiviral agent, interferon inducer (1, 2), and antitumor agent (3, 4; Tarnowski, G. S., Stock, C. C., and Hamilton, L. D., unpublished data). Poly (I·C) also has certain side effects: it is embryotoxic in rabbits and it elevates the concentration of serum transaminases and the prothrombin time in dogs (5) and monkeys (6). The polynucleotide produces effects similar to endotoxin: it is pyrogenic (5, 7, 8), may elicit leukopenia (5, 9), and can provoke the Schwartzman phenomenon (10).

Variations in lethality in mammals of different preparations of poly(I·C) were first noted, and later, variations in antitumor and antiviral activity were observed (11). These observations led to a retrospective correlation of biological potency with $s_{20,w}$ distribution of the complexes (11). A correlation emerged between the increasing $s_{20,w}$ and the median lethel dose (LD_{50}). For the complex with the highest molecular weight distribution, the least amount was required for the LD_{50}; complexes with $s_{20,w}$ less than 20–25 seemed the least toxic. The antiviral and antitumor activity seemed parallel, but although the complexes with the lowest molecular weight distribution were the least effective, the complexes that were more toxic (possibly because of their higher sedimentation distribution) were also less effective against the Ridgeway osteogenic sarcoma and foot-and-mouth disease virus. The data, too few to be conclusive, nevertheless suggested that chain lengths above a certain point may be more toxic and less therapeutically efficient against tumors and viruses (11). To explore this likelihood, we made a series of complexes of poly(I·C) using homopolymers of poly(I) and poly(C) with widely differing $s_{20,w}$ distributions, since we had previously established that the $s_{20,w}$ values in the original homopolymers were important in determining the distribution of $s_{20,w}$ values of the complexes. This paper correlates the molecular weight distribution of poly(I·C) complexes with interferon induction, protection of mice against encephalomyocarditis virus, immuno-enhancing activity, sensitization of mice to endotoxin, and inhibition of drug metabolism *in vivo* and *in vitro*.

Abbreviations: EMC, encephalomyocarditis; LD_{50}, median lethal dose.

MATERIALS AND METHODS

Preparation of Poly(I·C). Synthetic double-stranded complexes of polyriboinosinic and polyribocytidylic homopolymers were prepared and characterized as described (11). The distribution of sedimentation coefficients in polynucleotide samples was determined at 117,000 × *g* in a Spinco model E analytical ultracentrifuge equipped with a photoelectric scanner and UV optics. The polynucleotide sample was made up in 10^{-4} M EDTA–0.1 M phosphate buffer (pH 7) to give an UV absorption of 0.8–1.2 at 260 nm. A 15-μl sample was placed in the well of a Type II double sector, charcoal-filled Epon, band-forming centerpiece; phosphate–EDTA buffer in 50% D_2O was put in both sample and reference sectors. A normalized distribution of sedimentation coefficients, referred to 20° and water, was calculated by a computer program that makes appropriate corrections for density, viscosity, radial dilution, and concentration (Commerford, S. L. and Hamilton, L. D., unpublished data). The distribution of sedimentation values in six samples of polynucleotide complexes is shown in Fig. 1. Two preparations of poly(I·C), prepared by P-L Biochemicals, Milwaukee, Wis., were also compared. The PL complex was preparation CB-1 [poly(I) lot no. 200-14 · poly(C) lot No. 191-14]. The PL-reference complex was lot no. K-4 prepared for the National Institutes of Health, October 1970. All complexes were dissolved or diluted in 0.03 M NaCl at 4° and used within 72 hr of solution.

Interferon Titers were determined on individual mouse sera or serum pools from two mice collected 2 hr after intravenous (i.v.) injection of poly(I·C). Sera were kept at −20° until interferon assays by plaque reduction of vesicular stomatitis virus on L 929 cells were performed. The interferon titer is defined as the reciprocal of the highest dilution of serum that

reduced plaque formation by 50% (PDD_{50}/ml) from control values, and was calculated by the method of least squares. All values were corrected to a simultaneously assayed standard interferon sample that was 945 PDD_{50}/ml ± 89 (SE) for 25 assays.

Antiviral Activity of the complexes was determined by inoculation of adult NYLAR-A Swiss mice i.v. with various doses of polynucleotides or saline, and challenging i.v. 20–24 hr later with 50 LD_{50} of encephalomyocarditis (EMC) virus (ATCC VR129).

Immuno-enhancing Activity was assessed by inoculation of mice i.v. with poly(I·C) or saline and 2 hr later, with about 10^8 sheep erythrocytes i.v. Hemagglutinating or hemolysin antibody titers were determined in individual sera taken 7 days later. Splenic hemolysin plaque-forming cells present in individual spleens, removed 4, 5, and 6 days after immunization, were also determined in some experiments (12).

Phagocytic Activity was assessed by inoculation of mice i.v. with the polynucleotides or saline and, at intervals thereafter, i.v. with ^{51}Cr-labeled sheep erythrocytes. 60 Min after the injection of sheep cells, the percent uptake of labeled erythrocytes into the liver, spleen, and lungs as compared in treated mice with the uptake in control mice.

Sensitization of Mice to Endotoxin was determined by inoculation of mice i.v. with poly(I·C) or saline and 24 hr later, with various doses of *Salmonella typhosa* 0901 lipopolysaccharide (Difco) i.v. Deaths occurring within 72 hr were recorded.

Inhibition of Drug Metabolism In Vivo was assessed by inoculation of mice i.v. with poly(I·C) or saline. At intervals thereafter, mice were inoculated i.v. with hexobarbital (80 mg/kg), and the sleeping time was determined for individual mice (10 mice per group) (13).

Inhibition of Microsomal Mixed Functional Oxidase Enzymes In Vitro was assessed with type I substrate. This procedure evaluates the hydroxylation activity of the liver in the metabolism of foreign compounds. Groups of mice were inoculated with poly(I·C) i.v., and livers were removed from five mice 24 hr later. The 9000 × *g* liver supernatant fractions from individual livers were reacted with aminopyrine and the necessary cofactors, and the conversion of aminopyrine to formaldehyde was determined (14).

Evaluation of Data rested on calculation of the means with standard deviations and standard errors and the use of Student's *t* test for determination of significant differences from control values. For determination of the 50% protective dose of the complexes *in vivo* against EMC virus, the mortality data were analyzed by probit analysis on a CDC 6600 computer, and the 95% confidence limits for the 50% protective dose was calculated.

RESULTS

Relationship of Interferon Induction to Molecular Size. The mode of the sedimentation distribution of the complexes varied from 8S for the smallest complex to 40S for the largest complex (Fig. 1). The ability of these complexes to induce serum interferon decreased with decreasing molecular size, particularly when the comparison was made at a dose of 0.33 mg of complex/kg (Fig. 2).

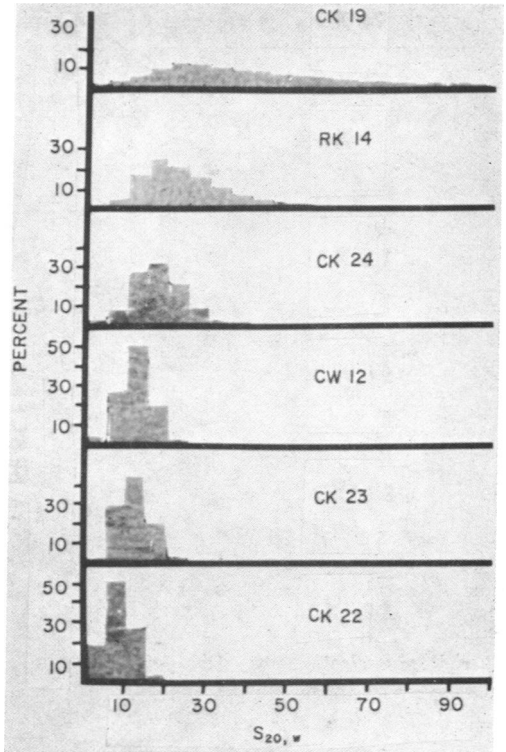

FIG. 1. Distribution of sedimentation values, $S_{20,w}$, in six samples of poly(I·C) complexes. For details, see *Methods*. Modes of sedimentation distribution: CK19 = 40S, RK14 = 25S, CK24 = 18S, CW12 = 11S, CK23 = 11S, and CK22 = 8S.

Relationship of Antiviral Protection to Molecular Size and Composition. The other protective abilities of the complexes also decreased with decreasing size. For the largest 40S complex, the least amount of polynucleotide was required to protect 50% of the mice from death with EMC virus. The smallest (8S) complex was considerably less protective (Table 1).

Composition of the individual homopolymers did not affect antiviral activity. The 18S (CK24) complex was prepared from a large polyribocytidylic acid homopolymer (14S) and a much smaller polyriboinosinic polymer (4S). The 11S (CK23) complex was prepared from a large polyriboinosinic polymer (13S) and a small polycytidylic homopolymer (4S). Both complexes induced equivalent amounts of interferon at a dose of 2 mg/kg (Fig. 2). There was no difference in their induction of antiviral resistance—the 50% protective dose of CK24 was 0.48 (range, 0.29–0.80) mg/kg and that of CK23 was 0.44 (range, 0.28–0.67) mg/kg (Table 1).

Relationship of Immuno-enhancing Activity to Molecular Size and Formulation. The ability of the complexes to enhance the immune response of mice to sheep erythrocytes also decreased with decreasing molecular size. The smallest (8S) complex, at 1–2 mg/kg, did not enhance the immune response as determined either by serum hemagglutinating antibody or splenic hemolysin plaque-forming cells (Table 2). Immunoenhancement may, thus, be more affected by molecular size than interferon induction or antiviral activity. At doses of 1–2 mg/kg, the 8S complex protected mice against EMC and induced significant amounts of interferon. The series of complexes—25S, 12S, and 11S—also showed decreasing

TABLE 1. *Relationship of size of poly(I·C) to antiviral activity*

Poly(I·C)	Dose (mg/kg) i.v. 2	1	0.5	0.33	0.11	0.075	0.019	PD_{50} (mg/kg)	95% Confidence limit (mg/kg)
40S (CK19)	3/19*	6/29	4/11	13/20	9/20	8/10	10/10	0.25	0.15–0.42
11S (CK23)	2/20	10/30	4/10	16/20	13/20	9/10	10/10	0.44	0.29–0.67
18S (CK24)	2/17	10/20	N.D.	9/20	17/20	N.D.	N.D.	0.48	0.29–0.80
8S (CK22)	2/20	17/30	17/20	8/9	7/10	10/10	10/10	0.95	0.63–1.44
PL-Ref.	0/10	5/20	N.D.	10/20	10/10	10/10	10/10	0.41	0.27–0.61

* No. dead/total. Mice were inoculated i.v. with the dose of poly(I·C), and 24 hr later, i.v. with 50 LD_{50} of encephalomyocarditis virus. The 50% protective dose (PD_{50}) was calculated by probit analysis with the 95% confidence limits indicated. The mortality of control animals in these experiments was 83/92 (90%). N.D. = not determined.

immune-adjuvant activity with decreasing molecular size, although all of them significantly increased hemolysin antibody production. The composition of the polynucleotide homopolymers did not markedly affect immuno-enhancement. Both the 11S and 18S complexes significantly increased hemagglutinating antibody response.

Effect of Polynucleotides on Phagocytosis. None of the poly(I·C) complexes markedly affected the reticuloendothelial system when inoculated i.v. at 2 mg/kg, as judged from the weights of organs (liver, spleen, thymus, and lungs) and phagocytosis of sheep erythrocytes.

Sensitization to Endotoxin. At an i.v. dose of 2 mg/kg, the poly(I·C) 40S complex reduced the LD_{50} of *S. typhosa* lipopolysaccharide from 50 mg/kg i.v. to about 18 mg/kg. With challenge doses of endotoxin at 21, 15, and 10 mg/kg, respectively, 4 of 5, 1 of 5, and 0 of 5 mice died. At 2 mg/kg i.v., the PL complex did not cause significant deaths at these doses of endotoxin. With the lower 1 mg/kg i.v. dose of poly(I·C), none of the 40S, 11S, 8S, or PL-reference complexes caused significant deaths at a challenge dose of 20 mg/kg of endotoxin. Thus, only the largest 40S complex sensitized mice to the lethal effects of endotoxin.

Metabolism of Hexobarbital. All the poly(I·C) complexes diminished the metabolism of hexobarbital in mice, prolonging the sleeping time (Table 3). Molecular size was not influential. At 24 hr after administration of complex, the sleeping time after the injection of the largest 40S complex was increased 88%, while sleeping time after that of the smallest 8S complex was increased 167%. At 48 hr after administration of the complexes, they also significantly prolonged sleep. This inhibition of drug metabolism disappeared by day 7 after poly(I·C) administration. For example, the poly(I·C) 12S complex prolonged sleeping time from 36.9 min in controls to only 48.0 min.

Metabolism of Aminopyrine. The polynucleotides also inhibited metabolism of aminopyrine by 9000 × *g* liver supernatant fractions. At 24 hr after polynucleotide administration, the 40S, 8S, and PL-reference complexes inhibited drug metabolism by 33–46% (Table 4). The poly(I·C) 11S complex depressed drug metabolism by only 10%. Thus, the poly(I·C) complexes generally inhibited drug metabolism by liver microsomal enzymes, *in vivo* and *in vitro*. Within the molecular size range studied, there was no decrease in this side-effect with decreasing molecular size.

TABLE 2. *Immunologic enhancement by poly(I·C)*

Complex	Hemolysin titer (log_2) 2 mg poly(I·C)/kg	Hemagglutination titer (log_2) 2 mg poly(I·C)/kg	PFC/10^6 spleen cells 1 mg poly(I·C)/kg
Control	7.9 ± 0.32	9.03 ± 0.26	130 ± 20
Poly(I·C)			
40S (CK19)	N.D.	10.15 ± 0.28*	215.6 ± 30*
25S (RK14)	11.1 ± 0.18*		
12S (TY15)	10.5 ± 0.17*		
11S (CW12)	9.7 ± 0.22*		
11S (CK23)	N.D.	10.60 ± 0.17*	196.9 ± 37
18S (CK24)	N.D.	9.75 ± 0.19*	N.D.
8S (CK22)	N.D.	9.60 ± 0.26	153.2 ± 21

* Values are significantly different from the control at $P < 0.05$. N.D. = not determined; PFC = plaque-forming cells.

DISCUSSION

The estimated molecular weights of the poly(I·C) complexes ranged from 0.11–3.1 × 10^6 for CK22 (8S, equivalent to a molecular weight of 5 × 10^5) to 0.11–366 × 10^6 for CK19 (40S, equivalent to a molecular weight of 38.2 × 10^6) (11). Over this almost 100-fold range of molecular size, all of the protective activities decreased with decreasing molecular size. However, complexes with a sedimentation rate larger than 10S (molecular weight about 0.9 × 10^6) were generally all active antiviral agents, immunologic adjuvants, and interferon inducers. The critical molecular size required for high activity appeared to be about 10^6 for unsubstituted poly(I·C) complexes. The 8S complex with a molecular weight less than 10^6 was markedly less active: its antiviral 50% protective dose was 3.8 times greater than that of the 40S complex. This smallest complex also induced much less interferon, inducing only 7% as much interferon as the 40S complex at the same dosage. The 8S compound was also not an immuno-enhancer, unlike all the other complexes which enhanced the immune response to sheep erythrocyte.

With this series of narrowly characterized poly(I·C) complexes, immune adjuvant activity appears to be the most sensitive to decreases in molecular size. These results agree with those of Niblack and McCreary (15) that there is a continuous relationship between increasing molecular weights of the component homopolymers and biological activities,

TABLE 3. *Prolongation of hexobarbital sleeping time in mice pretreated with poly(I·C)*

Complex	Hexobarbital sleeping time					
	Given after 24 hr			Given after 48 hr		
	Exp. 1*	Exp. 2*	% Increase	Exp. 1*	Exp. 2*	% Increase
0.03 N NaCl	54.9 ± 4.5	36.6 ± 2.0		30.0 ± 2.6	45.1 ± 1.6	
Poly(I·C)						
40S (CK19)	103.3 ± 6.3		88	81.4 ± 2.7		171
25S (RK14)		64.7 ± 6.0	104		N.D.	
12S (TY15)		51.6 ± 3.2	41		98.6 ± 7.8	119
11S (CW12)		N.D.			134.1 ± 15.8	197
11S (CK23)	104.8 ± 6.6		91	42.5 ± 3.9		42
8S (CK22)	146.7 ± 35.6		167	72.3 ± 5.3		141
PL-Ref.	102.6 ± 9.4		87	61.6 ± 8.4		105

* Sleeping time in minutes ±SE after hexobarbital. Mice were inoculated i.v. with 1 mg/kg of poly(I·C), and inoculated i.v. 24 hr and 48 hr later with hexobarbital. All values for mice treated with poly(I·C) are significantly different from those of control mice at $P < 0.05$.

and with the results of Jameson and Grossberg (16). Presumably, the molecular weights of the homopolymers determined the molecular weights of the complexes in their experiments (11). This contrasts with the results of Lampson *et al.* (17), who found interferon induction only reduced at molecular weights less than 1.2×10^5, although they observed reduction in protection of mice against pneumonia virus of mice with lowering of the molecular weight of poly(I·C) complexes from 4.2×10^6 to 1.2×10^6, and then to 4.6×10^5. In their protection experiments against pneumonia virus of mice, protection was dependent on the dose of the polymer complex as well as the size of the complex. Similarly, as reported here, interferon induction as well as antiviral activity depended not only on the molecular size of the complex but also on the dosage. Greater differences in interferon induction were seen at a dose of complexes of 0.33 mg/kg than at a dose of 1–2 mg/kg. The interferon and antiviral results also indicate that at least 100 units of serum interferon are required for significant antiviral activity of poly(I·C) complexes against EMC virus. At 0.33 mg/kg, the 8S complex induced only 67 units and did not protect mice from EMC virus, while the 11S complex induced a mean of 311 units and protected some mice. Our results with poly(A·U) complexes also suggest that 100 units of interferon may be needed for antiviral activity against EMC virus. At doses up to 2 mg/kg i.v., poly(A·U) complexes induced less than 100 units of serum interferon, and did not protect mice against EMC virus (18). The correlation of dose of poly(I·C), serum interferon titer, and ability to protect mice against EMC virus supports the assumption that the poly(I·C) is effective against EMC virus because of its ability to induce interferon; other mechanisms of protection are not excluded. Similarly, the suggestion that at least 100 units of circulating interferon are needed for antiviral activity holds for protection against EMC virus only. Protection against herpes simplex virus in mice has been achieved with both poly(A·U) and poly(I·C), probably by additional protective mechanisms (19).

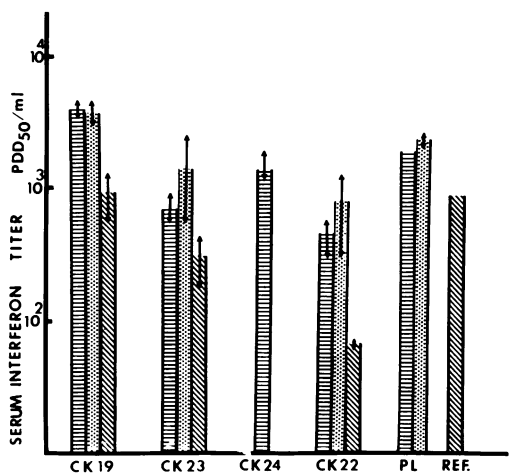

FIG. 2. Serum interferon titers induced by poly(I·C) complexes of decreasing molecular size. *Arrows* indicate the standard error of the mean of the interferon titer for each complex and dose. *Horizontal lines*, 2 mg/kg i.v.; *dots*, 1 mg/kg i.v.; *diagonal lines*, 0.33 mg/kg i.v. A decrease of interferon titer with decreasing molecular size was also observed with the 25S (RK14), 12S (TY15), and 11S (CW12) complexes, which, at 2 mg/kg, induced interferon titers of 1307, 1078, and 888 units/ml, respectively. Modes of sedimentation distribution of complexes are given in Fig. 1.

Composition of the poly(I·C) complexes did not affect antiviral activity, interferon induction, and immunologic adjuvant activity. The 18S complex prepared from large

TABLE 4. *Inhibition of aminopyrine metabolism by poly(I·C)*

Complex	1.0 mg poly(I·C)/kg i.v.	
	μg/mg*	% Inhibition
Control	0.804 ± 0.077	
Poly(I·C)		
40S (CK19)	0.483 ± 0.046†	40.0
11S (CK23)	0.726 ± 0.032	9.7
8S (CK22)	0.537 ± 0.050†	33.2
PL-Ref.	0.435 ± 0.013†	45.9

* μg/mg = μg HCHO/mg protein per 30 min in 9000 × *g* liver supernatant fractions. Aminopyrine metabolism was measured in individual liver supernatant fractions 24 hr after administration of complex.

† Values are significantly different from the control at $P < 0.5$.

poly(C) and small poly(I), and the 11S complex prepared from a large poly(I) and a small poly(C) had similar activities. These findings seem somewhat at variance with those of Tytell *et al.* (20), who found that the ability of poly(I·C) to induce resistance to vesicular stomatitis virus *in vitro* and to pneumonia virus of mice *in vivo* depended more upon maintaining a high molecular weight of poly(I) than of poly(C), but agree with those of Niblack and McCreary (15), who found that the chemical identity of the higher or lower molecular weight components of a poly(I·C) complex seemed insignificant; poly(I) and poly(C) behaved similarly no matter which was the high or low molecular weight homopolymer in the complex. Similarly, Wacker *et al.* (21) found no difference between interferon induction by poly(I)·oligo(C) and poly(C)·oligo(I) complexes (chain lengths of the oligonucleotides ranging from 7–39).

Although the molecular sizes extended over an almost 100-fold range, the complexes behaved similarly in the main in their side effects on the reticuloendothelial system. However, large molecular size appeared necessary for sensitization of mice to endotoxin—the largest (40S) complex reduced the LD_{50} to endotoxin about 2-fold.

At a dose of 3 mg/kg, sufficient for protection but nontoxic, no poly(I·C) complex markedly altered the phagocytic capacity of the reticuloendothelial system. Even the largest (40S) complex did not affect phagocytosis (unpublished results). There was only a slight depression of uptake of erythrocytes into the spleen 24 hr after polymer administration. These results apparently differ from those obtained previously (22, 23). However, in the present experiments, relatively low doses of poly(I·C) were given (25–50 μg/mouse), while in the other experiments, 4–8 times as much complex was given (200 μg/mouse). At the higher levels, the poly(I·C) complexes may indeed depress, or possibly enhance, phagocytic response. However, at the lower antiviral protective levels, phagocytic function was not altered. Thus, alteration of phagocytosis is not a constant side effect of polyribonucleotide administration.

Although the poly(I·C) complexes did not alter phagocytosis, all of the complexes inhibited drug metabolism by liver microsomal enzymes. While phagocytic Kupffer cells may not be affected by polynucleotides, apparently the liver parenchymal cells are affected. This inhibition of drug metabolism occurred both in the intact animal and in liver preparations, indicating that microsomal mixed-function oxidases were inhibited; 24 hr was required for inhibition to be evident (unpublished results). The inhibition might perhaps be related to the ability of poly(I·C) to inhibit RNA and protein synthesis in certain tumors (3). A decrease in the molecular size of the polymers did not markedly affect the inhibition of drug metabolism. Thus, this effect on the host's capacity to metabolize foreign compounds must be taken into account in assessment of the clinical usefulness of the polyribonucleotide complexes. Inhibition of drug metabolism could be a serious side effect of poly(I·C).

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