Influence of Size of Individual Homopolynucleotides on the Physical and Biological Properties of Complexed $rI_n:rC_n$ (Poly I:C) (35170)

ALFRED A. TYTELL, GEORGE P. LAMPSON, A. KIRK FIELD, MARJORIE M. NEMES, AND MAURICE R. HILLEMAN

Division of Virus and Cell Biology Research, Merck Institute for Therapeutic Research, West Point, Pennsylvania 19486

A separate report from these laboratories (1) described the findings in investigations to measure the effect of reduction of molecular weight of $rI_n:rC_n$ (poly I:C) complexes on their physical, chemical, and biological properties. The present report describes the influence of the molecular sizes of the individual homopolymers (rI_n and rC_n) on their ability to form the double-stranded complex ($rI_n:rC_n$) and to induce interferon and resistance to viral infections.

Materials and Methods. Sources of polyribonucleotides. Polyriboinosinic acid (rI_n), and polyribocytidylic acid (rC_n) with sedimentation coefficients of 12.3 and 13.2 respectively, were purchased from the P-L Laboratories, Milwaukee, Wisconsin. The dephosphorylated oligo-inosinic acid trimer $(Ip)_2I$ and the dephosphorylated oligocytidylic acid pentamer $(Cp)_4C$ were obtained from Miles Laboratories, Elkhart, Indiana. All other polynucleotides were prepared by Drs. J. L. Zabriskie and E. E. Harris of the Merck Process Research Group.

Treatment of polyribonucleotides. Sonic radiation of the homopolymers to reduce molecular size was carried out by procedures described earlier (1). Ribonuclease (RNase) degradation of rC_n was carried out as follows: rC_n with $S_{w,20}$ of 9.2 (mol wt $= 1.9 \times 10^5$) was dissolved in 0.15 *M* NaCl–0.006 *M* sodium phosphate buffer solution (PBS), pH 7.0, at a concentration of 1 mg/ml and was incubated with pancreatic ribonuclease A (Sigma Chemical Co., St. Louis, Mo.) at a concentration of 0.01 or 0.1 μg/ml at 23° for varying time intervals. Samples were removed and complexed with rI_n (mol wt $= 1.9 \times 10^5$) in PBS at 1.07 mg/ml concentration. The rapid mixing quenched the RNase activity as evidenced by lack of further increase in optical density (measured at 248 mμ) and by failure to show significant decrease in relative viscosity on standing at 23° for 2 hr or for several days at 5°.

Physical, chemical and biological measurements. Relative viscosity of 1 mg/ml of polynucleotide solutions was measured using an Ostwald viscosimeter having a flow time of 120 sec with water at 25°. Sedimentation coefficients (performed by Dr. E. E. Harris of these laboratories) were determined on dilute solutions of homopolynucleotides in PBS using a Spinco Model E ultracentrifuge equipped with a scanning ultraviolet optical system. Average molecular weights of the homopolynucleotides were calculated directly from the sedimentation coefficients using the empirical equation, mol wt $= 2.82 \times 10^3 \times S^{1.9}_{w,20}$ developed by Maeda (2) for determining molecular weights of single-stranded RNA in phosphate buffered saline solution. *Ribonuclease sensitivities* of $rI_n:rC_n$ were measured as previously described (3). *Hypochromicities* on complexing equimolar solutions of homopolynucleotides were calculated as the percentage decrease in optical density at 248 mμ. Methods for measuring induction of rabbit serum interferon, of resistance of primary rabbit kidney cell cultures to vesicular stomatitis virus (VSV), and of resistance of mice to pneumonia virus of mice (PVM) have been described in an earlier report (4).

Results. Effect of sonic radiation of rC_n on its capacity to complex with rI_n and to induce interferon and resistance to viral infection. Sonic radiation of rC_n ($S_{w,20} = 9.2$)

TABLE I. Effect of Sonic Radiation of rC_n on the Capacity of $rI_n:rC_n$ to Induce Interferon in Rabbits and Resistance Against VSV in Primary Rabbit Kidney Cell Cultures.

		Assay		
		Induction of interferon in rabbits		
Sonic radiation of rC_n,[a] time (min)	Relative viscosity $rI_n:rC_n$	Dose (μg/rabbit)	Interferon titer in individual rabbit sera[b]	Interference in cell cultures; minimal effective dose (μg/ml)
0	3.0	5	20, 320	0.002–0.004
		1	10, 40	—
5	1.7	5	320, 640	0.001–0.002
		1	5, 160	—
15	1.5	5	320, 640	0.002–0.004
		1	160, 640	—
Control (no inducer)		Nil	<5, <5	—

[a] rC_n ($S_{w,20} = 9.2$) prior to sonic treatment.

[b] The sera were collected 2 hr after administration of $rI_n:rC_n$ and were tested for interferon by the tube dilution method in primary rabbit kidney cells using vesicular stomatitis virus for challenge (5).

for 5 or 15 min resulted in the formation of fragments of lower molecular weight. The $S_{w,20}$ of the 15-min sample, *e.g.*, was 4.0. Table I shows that the irradiated rC_n samples which were complexed with rI_n ($S_{w,20} = 9.2$) had relative viscosities of 1.7 or 1.5 while that of the untreated $rI_n:rC_n$ was 3.0. There was no decrease in the capacity of the rC_n to complex with rI_n as evidenced by lack of decrease in hypochromic effect on mixing and by the fact that the ultraviolet absorption spectra of the complexed preparations were identical. It is shown in Table I that sonic radiation of the rC_n did not decrease the ability of the $rI_n:rC_n$ complex to induce interferon in rabbits and interference against VSV in cell cultures.

Effect of ribonuclease degradation of rC_n on its capacity to complex with rI_n and to induce interferon and resistance to viral infection. A 1-mg/ml solution of rC_n ($S_{w,20} = 9.2$ was treated with 0.01 μg/ml of pancreatic ribonuclease for the time periods shown in Table II prior to complexing with untreated rI_n ($S_{w,20} = 9.2$) in equimolar concentration. Exposure of rC_n to enzyme for 2.5 min resulted in little or no decrease in capacity to complex with rI_n and no decrease in capacity to induce resistance against PVM (*in vivo*) and VSV (*in vitro*) infections. There was, however, substantial alteration in the rC_n treated for this time period as evidenced by markedly reduced relative viscosity of the $rI_n:rC_n$ complex (from 4.1 to 1.64). Progressively longer treatment of rC_n with RNase reduced the capacity for complexing with rI_n as evidenced by decrease in hypochromicity on mixing, in further reductions in relative viscosity, and by reduced capacity to protect against viral infections. The decreased capacity of the RNase-treated rC_n to complex with rI_n was reflected also in an increased sensitivity of the $rI_n:rC_n$ complex to degradation by RNase. Figure 1 presents these relationships and shows that the increase in RNase sensitivity of the complexes was proportional to the decrease in viscosity.

Treatment of a 1-mg/ml solution of rC_n with 0.1 μg/ml of RNase rather than 0.01 μg/ml as described above resulted in very rapid degradation of the polynucleotide. As shown in Fig. 2, treatment with RNase for only 4 min totally eliminated its capacity to complex with rI_n as measured by lack of hypochromic effect on mixing.

Effect of altering the molecular weight of rI_n or rC_n on in vitro activity. High molecular weight rI_n or rC_n was complexed in equimolar amount with the corresponding ho-

TABLE II. Effect of Ribonuclease Degradation of rC_n on the Capacity of rI_n:rC_n to Induce Resistance Against PVM in Mice and VSV in Primary Rabbit Kidney Cell Cultures.

Treatment of rC_n (1 mg/ml solution) with 0.01 μg/ml of ribonuclease, time (min)	rI_n:rC_n complex					
	Physical assay			Biological assay		
					VSV in rabbit kidney cells	
	Relative viscosity	$E_{1\%}^{248\,m\mu}$	Hypochromicity on mixing (%)	Minimum protective dose, PVM, mice[a] (μg/ml)	Minimal effective dose (μg/ml)	Relative activity (%)[b]
0	4.1	127	39.7	1	0.001–0.002	—
2.5	1.64	131	38.0	1	0.001–0.002	100
5.0	1.44	136	35.8	4	0.001–0.002	100
7.5	1.31	145	31.5	4	0.002–0.004	50
10.5	1.25	155	26.7	4	0.004–0.008	25
15.0	1.18	167	20.8	>16	0.008–0.015	13
20.0	1.12	179	15.3	—	0.03 –0.06	3–6
30.0	1.08	196	7.1	—	0.13 –0.25	1.5–0.8

[a] Minimum intranasal dose which protected at least 40% of the treated mice against fatal infection with 30 LD_{50} of PVM.

[b] Compared with time 0 sample.

mopolymer of variable sizes as shown in Table III. The various sizes of homopolynucleotides were obtained by sonic treatment and by controlled enzymatic synthesis. The capacities of the complexes to induce resistance against VSV in primary rabbit kidney cell culture were compared with that of rI_n:rC_n prepared by using relatively high molecular weight homopolymers of $S_{w,20} = 9.2$. Using rI_n ($S_{w,20} = 9.2$), there was no loss of activity of the complex until the average molecular weight of rC_n was $<2.3 \times 10^4$ (<70 residues). By contrast, there was marked reduction in activity when the average molecular weight of the rI_n complexed with high molecular weight rC_n ($S_{w,20} = 9.2$) was reduced to 8.4×10^4 (240 residues) or less. It appeared, therefore, that the capacity of rI_n:rC_n complexes to induce resistance *in*

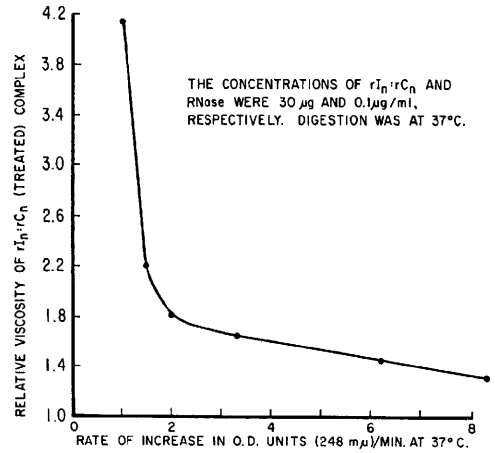

FIG. 1. Increase in RNase Sensitivity of rI_n complexed with rC_n degraded for increasing time periods with RNase.

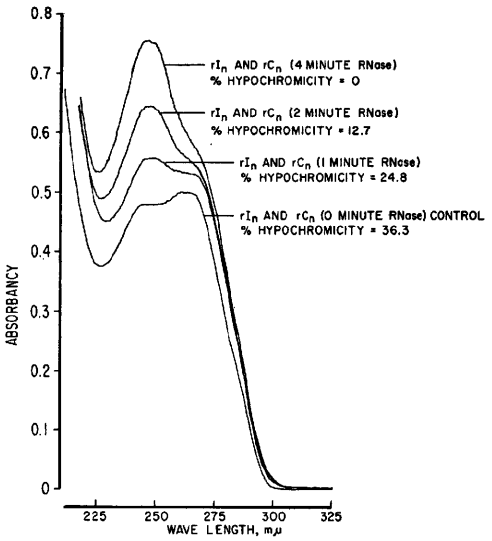

FIG. 2. Effect of 0.1 μg/ml of RNase treatment of rC_n on its capacity to complex with rI_n.

TABLE III. Effect of Altering the Molecular Weight of rI_n or rC_n on the Capacity of rI_n:rC_n to Induce Resistance to VSV in Primary Rabbit Kidney Cell Culture.

Homopolynucleotides					
	Variable				
Constant[a] reagent	Reagent	$S_{w,20}$	Av mol wt	Av no. of nucleotide residues	Relative activity[b] (*in vitro*) (%)
rI_n	rC_n	13.2	3.8×10^5	1160	100
rI_n	rC_n	9.2	1.9×10^5	580	100
rI_n	rC_n	4.0	3.9×10^4	120	100
rI_n	rC_n	3.0	2.3×10^4	70	100
rI_n	rC_n	—	1.6×10^3	5	<0.05
rC_n	rI_n	12.3	3.3×10^5	950	100
rC_n	rI_n	9.2	1.9×10^5	550	100
rC_n	rI_n	6.0	8.4×10^4	240	20–50
rC_n	rI_n	3.0	2.3×10^4	66	5
rC_n	rI_n	—	1.1×10^3	3	0

[a] The $S_{w,20}$ of each homopolynucleotide was 9.2.

[b] Percentage of activity compared with that of rI_n:rC_n in which each component had an $S_{w,20}$ of 9.2.

vitro was more dependent upon maintaining a high molecular weight of rI_n than of rC_n. This finding was true also in *in vivo* experiments in which the mouse PVM assay was used. Thus, Table IV shows that rI_n of high molecular weight was highly active whether complexed with high or low molecular weight rC_n. By contrast, low molecular weight rI_n, was poorly active, whether complexed with high or low molecular weight rC_n.

Discussion. Previous reports (4–9) from these laboratories demonstrated the requirement of double-strandedness of polynucleotides for induction of interferon and of resistance to viral infections. Recently, we reported (1) the influence of molecular weight of rI_n:rC_n on its capacity to induce interferon and resistance to viruses *in vivo* and *in vitro*. Reduction in molecular weight by sonic radiation permitted retention of the complex but caused a decrease in relative viscosity, thermal transition midpoint (*Tm*), resistance to RNase, and hyperchromicity on thermal dissociation. An approximate fiftyfold decrease in average molecular size of the complex was accompanied by a marked reduction in all the biological activities measured.

The relative importance of the molecular size of each of the homopolynucleotides, rI_n and rC_n, was evaluated in the present report. Sonic radiation of rC_n sufficient to diminish the viscosity of complexes formed with high molecular weight rI_n did not inhibit complexing nor did it reduce the activity of these complexes to induce interferon or resistance to viral infections. Greater reduction in the average molecular weight of rC_n brought about by treatment with pancreatic RNase did result in gradually decreased capacity to form complexes with high molecular weight rI_n and this was accompanied by reduced capacity to induce interferon and resistance to viruses. These results reaffirm the initial observations (4–9) by our group that doublestrandedness of polynucleotides is necessary to demonstrate interferon induction and host resistance. Reduction in or loss of capacity to form doublestranded complexes diminished or eliminated the capacity to induce interferon and resistance.

Studies both *in vitro* and *in vivo* in which rI_n or rC_n of variable molecular weight were complexed with the corresponding homopolymer of high molecular weight clearly showed the greater importance of maintaining a high molecular size of rI_n than of rC_n. Maximal activity of rI_n in these experiments depended upon an average molecular weight of $1.9 \times$

TABLE IV. Effect of Molecular Size of rI_n and rC_n on Capacity of Complexed Homopolymers to Protect Mice Against PVM Infection.

$S_{w,20}$ of individual homopolymers		Findings in tests[a] of complexed rI_n:rC_n					
rI_n	rC_n	Dose (μg)	No. survived/total	% Survival	Excess survival (%)	Days survived (mean)	Excess days survived
9.2	9.2	4	19/20	95.0	88.3	>14.0	>8.0
		1	17/20	85.0	78.3	>14.0	>8.0
		0.25	7/20	35.0	28.3	10.0	4.0
9.2	3.0	4	16/19	94.2	87.5	>14.0	>8.0
		1	17/20	85.0	78.3	>14.0	>8.0
		0.25	15/20	75.0	68.3	>14.0	>8.0
3.0	9.2	4	8/20	40.0	33.3	12.0	6.0
		1	5/20	25.0	18.3	8.0	2.0
		0.25	2/20	10.0	3.3	8.0	2.0
3.0	3.0	4	6/20	30.0	23.3	9.0	3.0
		1	5/20	25.0	18.3	8.0	2.0
		0.25	1/20	5.0	−1.7	6.0	—
Control (PBS)	Control (PBS)	—	4/60	6.7	—	6.0	—

[a] Intranasal infection with 30 LD_{50} of PVM virus 3 hr after intranasal instillation of inducer.

10^5 (550 residues) or greater, while full activity of rC_n was still retained when the average molecular weight was reduced to as little as 2.3×10^4 (70 residues).

The effect of alteration of molecular weight of the rI_n:rC_n complex and of the individual homopolymers in the complex on the toxicity for animals will be recorded in a future communication.

Summary. Reduction in the size of rC_n by sonic radiation caused a decrease in viscosity when complexed with rI_n of high molecular weight but did not bring about a decrease in protective activity of the rI_n:rC_n against PVM or VSV viruses. Greater reduction in size of rC_n by treatment with RNase resulted in progressive loss of capacity to complex with rI_n and to protect against viral infections. The capacity of rI_n:rC_n to induce resistance to VSV virus *in vitro* and to PVM virus *in vivo* was more dependent upon maintaining a high molecular weight of rI_n than of rC_n. Activity was markedly diminished when the average molecular weight of rI_n was below 1.9×10^5 and of rC_n below 2.3×10^4.

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