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Interferon Induction in Primates by Stabilized Polyriboinosinic Acid-Polyribocytidylic Acid: Effect of Component Size

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Two series of interferon-inducing complexes containing polyriboinosinic and polyribocytidylic acids, poly-L-lysine, and carboxymethyl cellulose were prepared. One series contained carboxymethyl cellulose, 27,000-molecular-weight poly-L-lysine, and either 4S, 6S, or 9S polyriboinosinic and polyribocytidylic acids. The other series contained carboxymethyl cellulose, 9S polyriboinosinic and polyribocytidylic acids, and poly-L-lysine, whose molecular weights ranged from 2,000 to 27,000. The homogeneity of these double-stranded polynucleotide complexes was confirmed by single-step thermal denaturation profiles and by single peaks in sucrose gradient velocity sedimentation. The complexes have a greater resistance to hydrolysis by ribonuclease than does polyriboinosinic-polyribocytidylic acid. The resistance to ribonuclease increased with the increasing size of polynucleotide homopolymers and poly-L-lysine. In monkeys and, to a lesser extent, in mice, serum interferon levels induced by the different complexes were related to the degree of resistance of the complexes to hydrolysis by ribonuclease. In mice, 4S, 6S, and 9S complexes of polyriboinosinic-polyribocytidylic acid, poly-L-lysine, and carboxymethyl cellulose had a higher level of toxicity than did polyriboinosinic-polyribocytidylic acid as measured by 50% lethal dose. The toxicity was parallel to the ribonuclease resistance of the complexes. It was concluded that an increase in the size of the polynucleotides and the polyamino acids in these complexes leads to higher resistance to hydrolysis by ribonuclease and to greater interferon responses in mice and rhesus monkeys.

The size of the homopolymers from which polyriboinosinic-polyribocytidylic acid [poly(I)·poly(C)] is composed plays a significant role in the magnitude of its interferon-inducing capacity in vitro, with larger polynucleotides being more effective inducers (1). In primates, including humans, even large poly(I)·poly(C) was a poor inducer of interferon (6). It was also found that primate serum contains a relatively high level of enzymatic activity against poly(I)·poly(C), which activity hydrolyzes and inactivates the double-stranded molecule (4). A primate-effective interferon inducer was developed by complexing poly(I)·poly(C) with poly-L-lysine and carboxymethyl cellulose and is called poly(ICLC). This complex is partially resistant to hydrolysis by ribonuclease (RNase) (3). Whereas such an observation is consistent with a correlation between resistance to hydrolysis and effectiveness of this new complex in primates, it does not establish a cause-effect relationship.

In the present study, two series of poly(ICLC) complexes were prepared, one in which the polynucleotide size varied and the poly-L-lysine molecular weight was kept constant (series A); and the other in which the polynucleotide size was kept constant and the molecular weight of the poly-L-lysine varied (series B). A determination of the thermal denaturation (T_m) profile, sedimentation pattern, and resistance to hydrolysis at different concentrations of RNase was made. All compounds were studied for their interferon-inducing capacity in nonhuman primates (rhesus monkeys) and in mice.

This work had its original impetus in the following events. In the original description of the preparation of poly(ICLC) (3), it was stated that the poly-L-lysine used had a molecular weight of 2,000, as given by the manufacturer. When a preparation of poly(ICLC) with poly-L-lysine from another manufacturer, with a molecular weight also given as 2,000, was inactive, a

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series of experiments led to the realization that the original preparation actually had a molecular weight of 22,000, not 2,000, and the bottle had been mislabeled by the manufacturer. The present study was an outgrowth of this problem. The present data indicate a strong correlation, in a given series of complexes, between the resistance to hydrolysis by RNase and the ability to induce interferon in primates.

MATERIALS AND METHODS

Poly(ICLC). Poly(ICLC) was prepared as described previously (3). In the preparation of the series A complexes, homopolymers of approximately 4S, 6S, and 9S were used. The exact values, as determined by the supplier, P-L Biochemicals, Inc., Milwaukee, Wis., in a model E analytic centrifuge were as follows: for the 4S, poly(I) = 3.5, and poly(C) = 4.3; for the 6S, poly(I) = 6.5, and poly(C) = 6.6; and for the 9S, poly(I) = 9.4, and poly(C) = 9.4. Poly-L-lysine (molecular weight, 27,000; Miles-Yeda, Rehovot, Israel) and carboxymethyl cellulose-7H3SF (molecular weight ≅ 700,000; Hercules Powder Co., Wilmington, Del.) were used in the preparation of the series A complexes. Poly(ICLC) preparations made with these homopolymers are referred to as 4S poly(ICLC), 6S poly(ICLC), and 9S poly(ICLC), respectively. In the series B complexes, poly(I) and poly(C) with an $s_{20,w}$ of 9.4S were used (P-L Biochemicals, Inc.). Poly-L-lysine with molecular weights of 2,000, 3,400, 13,000, 17,000, and 72,000 was obtained from Miles-Yeda.

Hydrolysis. For the hydrolysis of poly(I)·poly(C) and poly(ICLC), 5× crystallized, electophoretically pure pancreatic RNase A was used (Sigma Chemical Co., St. Louis, Mo.). Hydrolysis was carried out at room temperature. Polynucleotide complexes were diluted in 0.15 M NaCl–0.01 M PO_4 buffer (pH 7.4) to read an absorbancy at 260 nm (A_{260}) of approximately 0.5. RNase was added to a final concentration of 5 to 50 μg/ml. Hydrolysis was determined by hyperchromic shift and presented as the percent increase in A_{260} after 1 h.

T_m profiles. For T_m determination, the complexes were diluted in 0.1× standard saline citrate to a final A_{260} of approximately 1.0. T_m measurements were made with a Gilford 2400 spectrophotometer.

Relative molecular size determination. The relative molecular sizes of the several complexes were compared by velocity sedimentation in a 10 to 34% (wt/vol) sucrose gradient prepared in 0.15 M NaCl for 16 h at 40,000 rpm with the SW40 rotor in a Beckman L2-65B centrifuge. Double-stranded [^{3}H]deoxyribonucleic acid fragments of known size were used as markers (Bethesda Research Laboratories, Inc., Bethesda, Md.). These markers, of course, being radically different from poly(ICLC) in many physical properties, served only for general standardization of the technique and cannot be used to estimate the weight of the poly(ICLC) complex.

Monkeys. At least three healthy, well-conditioned young adult male or female rhesus monkeys (*Macaca mulata*) weighing 3 to 4 kg were used to study the interferon response to each inducer. The monkeys were housed in individual cages and maintained at constant room temperature (25°C) with a 12-h light cycle. Monkeys were fed four to six commercial monkey chow biscuits per day (Wayne Monkey Diet, Allied Mills, Inc., Chicago, Ill.) and provided water ad libitum.

Mice. Female NIH albino mice weighing 16 to 18 g were used for interferon induction and toxicity studies. Blood samples for interferon assay were obtained by retro-orbital bleeding.

Viruses. Vesicular stomatitis virus (Indiana strain) was used as a challenge in the interferon assay. The virus was propagated in chicken embryo cells and stored at −70°C. The titer of the virus stock was $10^{7.5}$ tissue culture infective doses per ml.

Interferon assay. Monkey interferon was assayed in human diploid fibroblast cultures; mouse interferon was assayed in L-929 cells by methods described previously (3). Assays were standardized against international reference standards (National Institute of Allergy and Infectious Diseases catalog of research reagents, no. G023-901-527 and G002-904-511, respectively).

RESULTS

Molecular characteristics of poly(ICLC) as a function of the component size. (i) T_m. Formation of a stable complex between double-stranded ribonucleic acid and poly-L-lysine, first described by Felsenfeld and Huang (2), is characterized by an increase in T_m over that of the noncomplexed ribonucleic acid (8). In our experiments, formation of 4S, 6S, and 9S poly(ICLC) complexes was also associated with an increase in T_m (Fig. 1). All complexes appeared homogeneous, as judged by the shape of the thermal transition curve, with a single-step sharp increase in A_{260} near the T_m point. There were reproducible increases in T_m of about 2 to 2.5°C, as one went from 4S poly(ICLC) to 6S poly(ICLC) to 9S poly(ICLC), with the latter having a T_m of 88.5°C. In the series B complexes, changing the molecular weight of polylysine had a very slight effect on the T_m. Poly(I)·poly(C) has a T_m of ca. 53°C in the 0.1× standard saline citrate used.

(ii) Size. The sedimentation value of poly(ICLC) in sucrose was found to be a reflection of the size of polynucleotides used in preparation of the complexes, but changing the size of the polylysine had only a very slight effect. 9S poly(ICLC) migrated in the gradient to a position which was approximately equivalent to 10.3S double-stranded deoxyribonucleic acid, whereas poly(ICLC), made with 4S poly(I) and poly(C), had an S value of about 6. Complexes which were collected in sucrose gradient fractions and then dialyzed eight times against 200 volumes of 0.1× standard saline citrate retained their initial T_m.

(iii) Hydrolysis. It was shown previously that 9S poly(ICLC) is more resistant to hydrolysis by RNase than is poly(I)·poly(C) (3). Figure 2 shows the percent increase in optical density of 4S, 6S, and 9S poly(ICLC) upon incubation with different concentrations of RNase. Poly(I)·poly(C) was readily hydrolyzed by low levels of RNase. The complexes with poly-L-lysine and carboxymethyl cellulose were more resistant to hydrolysis. At any given concentration of RNase, the rate of hydrolysis with time was most rapid with poly(I)·poly(C) and was slowest with 9S poly(ICLC) (data not shown). Also, for 9S poly(I)·poly(C), a maximum hydrolysis of 56% was achieved at an RNase concentration of 5 μg/ml. For 9S poly(ICLC), the maximum level of hydrolysis was 15% at 35 μg of RNase per ml. Any further increase in RNase concentration did not result in higher levels of hydrolysis for this complex. A higher level of hydrolysis (33%) at the same concentration of RNase (35 μg/ml) was seen with 6S poly(ICLC). Maximum hydrolysis for 4S poly(ICLC) by 35 μg of RNase per ml was similar to that of 9S poly(I)·poly(C) (56%). Resistance to hydrolysis also increased when the molecular weight of the polylysine increased (Fig. 3).

(iv) Interferon induction by 4S, 6S, and 9S poly(ICLC) complexes. The interferon-inducing activity of 4S, 6S, and 9S poly(ICLC) complexes and 9S poly(I)·poly(C) was studied in mice and in rhesus monkeys.

In studies with mice, 4S, 6S, and 9S poly(ICLC) complexes were injected intravenously (i.v.) at a dose of 3.5 mg/kg. The kinetics of interferon production after administration of the drugs are shown in Fig. 4. A maximum interferon response to poly(I)·poly(C) was found

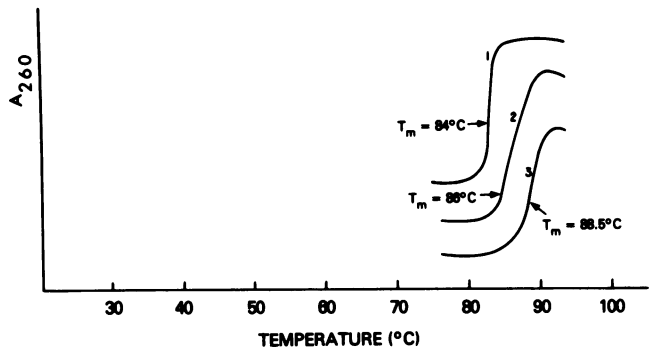

FIG. 1. *T_m profiles of 9S poly(I)·poly(C) and modified poly(ICLC) complexes in 0.1× standard saline citrate. (1) 4S poly(ICLC); (2) 6S poly(ICLC); (3) 9S poly(ICLC). The initial A_{260} was about 1.0.*

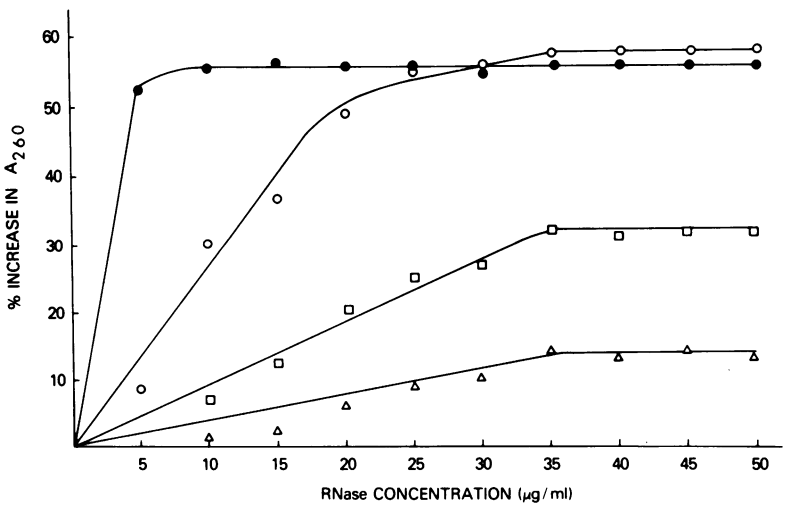

FIG. 2. *Hydrolysis by RNase of poly(I)·poly(C) and poly(ICLC) complexes in 0.01 M PO_4 (pH 7.2)–0.15 M NaCl (percent increase in A_{260} for 1 h at room temperature by the indicated concentration of RNase). Symbols: ●, 9S poly(I)·poly(C); ○, 4S poly(ICLC); □, 6S poly(ICLC); △, 9S poly(ICLC). Poly-L-lysine was 27,000 daltons.*

at 4 to 8 h, with interferon levels reaching approximately 1,000 U/ml. Poly(ICLC) induced peak interferon levels at about 8 h, with titers from 5 to 12.5 times higher than those found with poly(I)·poly(C). At 24 h postinjection, no interferon was detected in mice injected with 9S poly(I)·poly(C). Mice injected with 6S and 9S poly(ICLC) at 24 h after injection still had significant levels of serum interferon, with none detectable at 48 h.

In monkeys, the interferon response to 9S poly(I)·poly(C) and 4S poly(ICLC) was only a few units (<10). Complexes with increased resistance to RNase [6S and 9S poly(ICLC)] injected i.v. at 3 mg/kg produced peak levels of interferon equal to 50 and 1,800 U/ml, respectively. Interferon was found in the sera after injection of 6S and 9S poly(ICLC) for at least 30 h (Fig. 5). In the series B complexes, as the molecular weight of the poly-L-lysine increased, so did the ability to induce interferon in monkeys (Fig. 6).

(v) Toxicity studies in mice. The 50% lethal dose levels of 4S, 6S, and 9S poly(ICLC) and 9S poly(I)·poly(C) were determined. The drugs, diluted in 0.15 M NaCl to the concentrations necessary to obtain doses of 10, 20, 30, 40, and 50 mg/kg, were injected i.v. or intraperitoneally (i.p.). Twenty mice were used for each dose. Animals were observed for 2 weeks. After acute deaths at 24 to 72 h, usually no further deaths were noticed. Three experiments were carried

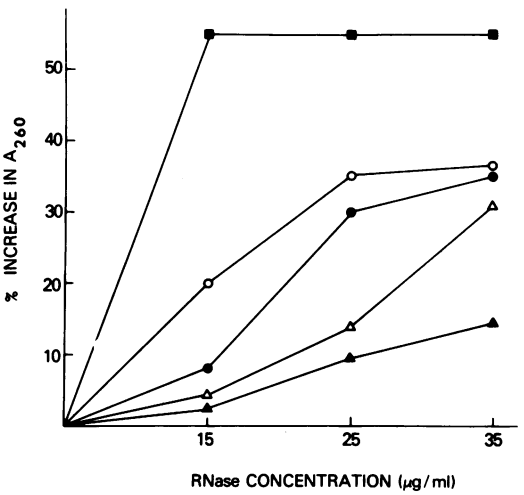

FIG. 3. *Hydrolysis by RNase of poly(I)·poly(C) and poly(ICLC) complexes in 0.01 M PO_4 (pH 7.2)–0.15 M NaCl (percent increase in A_{260} for 1 h at room temperature by the indicated concentration of RNase). Poly-L-lysine molecular weights in the complexes were as follows: ▲, 27,000; △, 13,000; ●, 3,400; ○, 2,000; ■, plain poly(I)·poly(C). Poly(I) and poly(C) were 9S.*

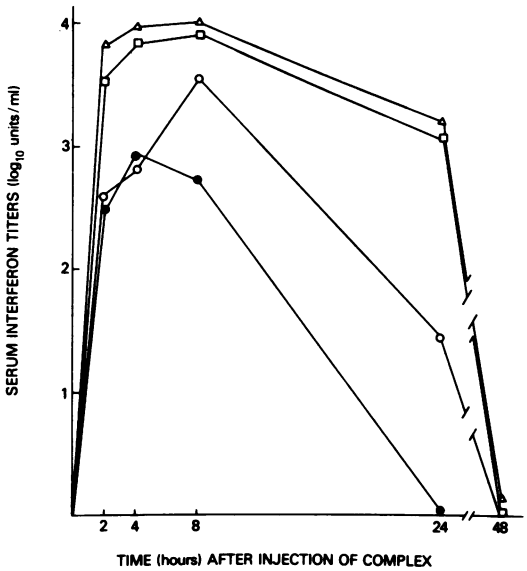

FIG. 4. *Serum interferon response in mice after i.v. administration of poly(I)·poly(C) and poly(ICLC) complexes. Designations are the same as for Fig. 2. There were six mice per group (3.5 mg/kg). Values are mean log titers of individual assays.*

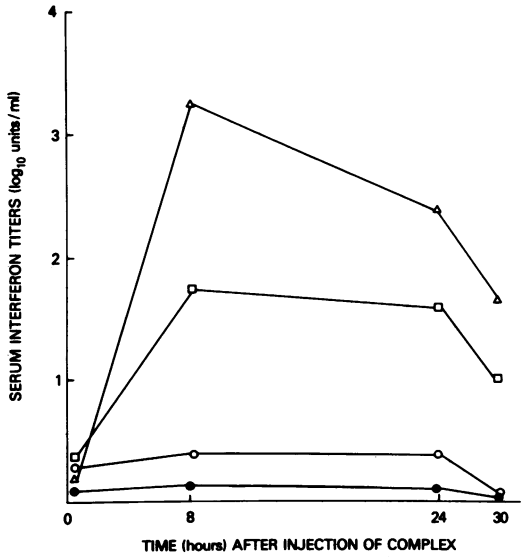

FIG. 5. *Serum interferon levels in rhesus monkeys after single i.v. injection of poly(I)·poly(C) and poly(ICLC) complexes. Designations are the same as for Fig. 2. There were three monkeys per point (3 mg/kg). Values are the mean log titers of individual assays. Levels of significance of comparison were determined by application of the Scheffé multiple-comparison test (7) to the 8-h data, which showed that the mean log interferon titers corresponding to 9S and 6S poly(ICLC) are significantly different at the 1% level. The same is true of the comparison between the mean log titers for 6S and 4S.*

out in this series. 9S poly(ICLC) was more toxic by both routes, i.v. and i.p., than was 9S poly(I)·poly(C) (Table 1). Thus, the 50% lethal dose for 9S poly(ICLC) by the i.v. route was 12.5 mg/kg and by the i.p. route was 13.8 mg/kg as compared with 30 and 45 mg/kg for 9S poly(I)·poly(C) by the i.v. and i.p. routes, respectively. Toxicity in mice for 6S poly(ICLC) was 15.0 mg/kg by the i.v. route and 25.0 mg/kg when given i.p. The least toxic of the poly-L-lysine complexes were those with either the smallest polynucleotide or the smallest polylysine. Their 50% lethal dose values were about 25 mg/kg if given i.v.

Route of injection. (i) Mice. Injection by the i.p. route of 9S poly(ICLC) at 3.5 mg/kg was as effective as the i.v. route for interferon induction in mice (Fig. 7). Drugs injected intramuscularly (i.m.), however, produced a lower and a delayed interferon response, with a peak level at 24 h.

(ii) Monkeys. A comparison of the i.v. route with the i.m. route in monkeys is shown in Fig. 8. The i.m. route in monkeys gave levels of serum interferon slightly lower than those obtained by the i.v. route, but the levels remained elevated longer. This was seen in two additional experiments.

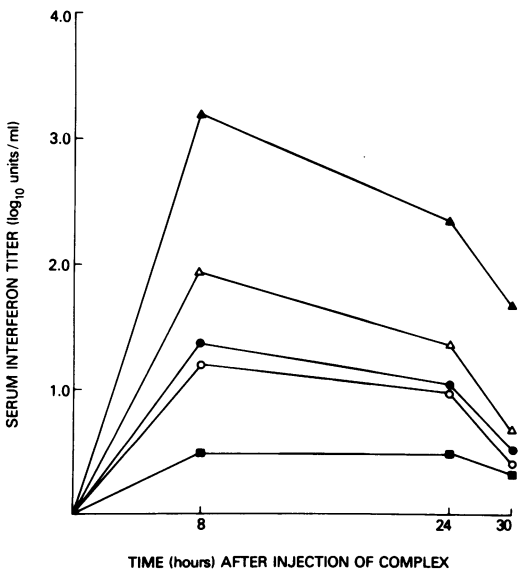

FIG. 6. *Induction of interferon in monkeys by several poly(ICLC) complexes. Groups of three rhesus monkeys (4 to 5 kg each) were injected with the indicated poly(ICLC) and then bled at 8, 24, and 30 h postinjection. Poly-L-lysine molecular weights in the complexes were as follows: ▲, 27,000; △, 13,000; ●, 3,400; ○, 2,000; ■, plain poly(I)·poly(C).*

TABLE 1. *Toxicity in mice of poly(I)·poly(C) and poly(ICLC) complexes administered by different routes*[a]

Drug	LD_{50} (mg/kg)	
	i.v.	i.p.
9S poly(ICLC), 27,000 plL[b]	12.5	13.8
6S poly(ICLC), 27,000 plL	15.0	25.0
4S poly(ICLC), 27,000 plL	25.0	40.0
9S poly(I)·poly(C)	30.0	45.0
9S poly(ICLC), 2,000 plL	26.0	
9S poly(ICLC), 3,400 plL	25.0	
9S poly(ICLC), 13,000 plL	15.0	

[a] Five groups of 20 mice per group were injected with either 10, 20, 30, or 40 mg of each of the complexes per kg. The animals were observed for 2 weeks. Fifty percent lethal dose (LD_{50}) values were calculated by the method of Reed and Muench (5).

[b] Molecular weight of polylysine (plL).

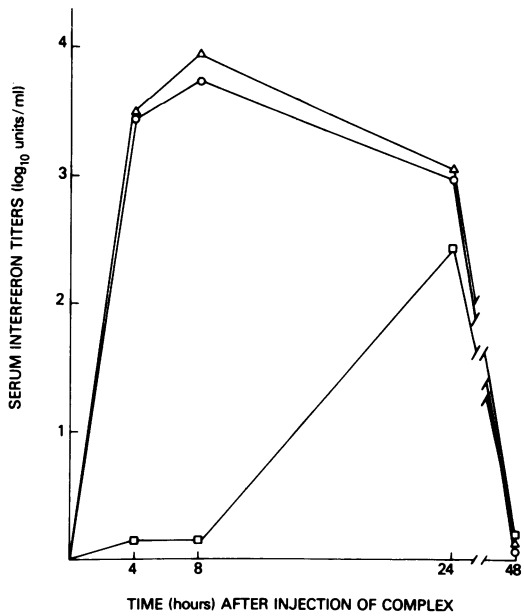

FIG. 7. *Serum interferon levels in mice after single i.v. (△), i.p. (○), or i.m. (□) injection of 9S poly(ICLC) at 3.5 mg/kg. There were six mice per route. Values are mean log titers of individual assays.*

DISCUSSION

The data presented here reveal that when complexes of poly(I)·poly(C) with poly-L-lysine and carboxymethyl cellulose are prepared with different-sized polynucleotides or polylysines, a number of properties are seen to be a function of the component size. Table 2 summarizes these differences.

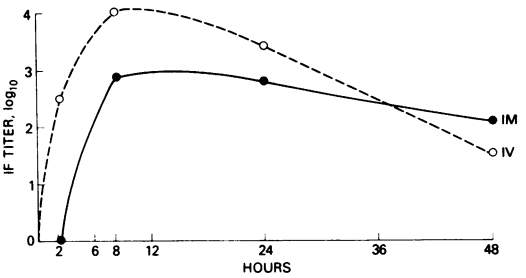

FIG. 8. *Comparison of different routes of injection in monkeys. Two groups of three monkeys (4 to 5 kg each) were injected with poly(ICLC) either i.m. (●) or i.v. (○) at 1 mg/kg body weight. Blood was drawn at the indicated times for serum interferon determinations.*

TABLE 2. *Summary of properties of poly(ICLC) complexes and poly(I)·poly(C)*

Compound	T_m in 0.1× SSC[a]	Maximum rise in OD_{260}[b] on hydrolysis (% rise from original)	Concn of RNase needed for maximum hydrolysis (μg/ml)	Interferon titer induced in monkeys (U/ml of serum)
Poly(I)·poly(C)	53.0	56	5	<
4S poly(ICLC), 27,000 plL[c]	84.0	56	35	<10
6S poly(ICLC), 27,000 plL	86.0	33	35	60
9S poly(ICLC), 27,000 plL	88.5	15	35	1,800
9S poly(ICLC), 3,400 plL	88.0	30		15
9S poly(ICLC), 13,000 plL	88.2	15		90

[a] SSC, Standard saline citrate.
[b] OD_{260}, Optical density at 260 nm.
[c] Molecular weight of polylysine (plL).

Although complexing poly-L-lysine with poly(I)·poly(C) made with any of the homopolymers tested resulted in compounds with a much higher T_m than that of the uncomplexed poly(I)·poly(C), not all of the poly-L-lysine complexes induced interferon in monkeys. The ability to resist hydrolysis by RNase (and presumably primate serum) appears to correspond most closely with the interferon-inducing capacity in primates. This was so whether one measured the final extent of hydrolysis or the rate of hydrolysis.

The larger the polynucleotide or polylysine strand, the smaller the maximum rise in absorbance on hydrolysis. One can only conjecture why this should be so, but it may reflect the possibility that, with the shorter components, there are more unprotected linkages than with the longer strands. This concept would be consistent with the relative T_m values of the complexes, at least for those complexes with different polynucleotides.

The present data lend support to the idea that the degree of resistance to hydrolysis of the complexes is an important determinant in the ability to induce interferon in primates.

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LITERATURE CITED

1. **De Clercq, E., and P. F. Torrence.** 1977. Comparative study of various double-stranded RNAs as inducers of human interferon. J. Gen. Virol. **37**:619–623.
2. **Felsenfeld, G., and S. Huang.** 1959. The interaction of polynucleotides with cations. Biochim. Biophys. Acta **24**:234–242.
3. **Levy, H. B., G. Baer, S. Baron, C. E. Buckler, C. J. Gibbs, M. J. Iadarola, W. T. London, and J. A. Rice.** 1975. A modified polyriboinosinic-polyribocytidylic acid complex that induces interferon in primates. J. Infect. Dis. **132**:434–439.
4. **Nordlund, J. J., S. M. Wolff, and H. B. Levy.** 1970. Inhibition of biologic activity of poly I·poly C by human plasma. Proc. Soc. Exp. Biol. Med. **133**:439–444.
5. **Reed, L. V., and H. Muench.** 1938. A simple method for estimating fifty per cent endpoints. Am. J. Hyg. **27**:493–497.
6. **Robinson, R. A., V. T. DeVita, H. B. Levy, S. Baron, S. P. Holland, and A. S. Levine.** 1976. A phase I-II trial of multiple dose polyriboinosinic-polyribocytidylic acid in patients with leukemia or solid tumors. J. Natl. Cancer Inst. **57**:599–602.
7. **Scheffé, H.** 1959. The analysis of variance, p. 66–67. John Wiley & Sons, Inc., New York.
8. **Tsubio, M., K. Matsuo, and P. O. P. Ts'o.** 1966. Interaction of poly-L-lysine and nucleic acids. J. Mol. Biol. **15**:256–267.