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# Polyriboinosinic-Polyribocytidylic Acid-Poly-L-lysine Complex [Poly(ICL)] without Carboxymethylcellulose (CMC): A New Primate-Effective Interferon Inducer (41329)

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*Abstract.* The complex of polyriboinosinic–polyribocytidylic (poly(I)·poly(C)) with poly-L-lysine in 0.5% carboxymethylcellulose (CMC) [poly(ICLC)] has proven to be an effective interferon inducer in primates, including man. Since no mechanism is known by which the body can degrade CMC, a new complex of lower molecular weight, which contains poly I·poly C complexed with poly-L-lysine [poly(ICL)] but without CMC, was developed. This compound is slightly more resistant than poly(ICLC) to hydrolysis by RNase-A and is also an effective inducer of interferon in nonhuman primates. The new compound without CMC is also less toxic in mice than is poly(ICLC) as indicated by $LD_{50}$.

The synthetic double-stranded RNA polyriboinosinic–polyribocytidylic acid (poly(I)·poly(C)) has been shown to be effective in rabbits and rodents as an interferon inducer (1), as an antitumor agent (2–4), and as an antiviral agent, both therapeutically and prophylactically (5, 6). Unfortunately, however, poly(I)·poly(C) showed little ability to induce interferon in primates, including man (7, 8).

There is present in human serum a high level of enzymes that hydrolyze and inactivate poly(I)·poly(C) (9). Unpublished observations by this laboratory indicated that those species that were easily induced to make interferon had relatively low levels of serum hydrolytic activity.

This laboratory reported the formulation of a new compound, called poly(ICLC), prepared by complexing of poly(I)·poly(C) with poly-L-lysine in 0.5% carboxymethylcellulose (CMC). In the formulation of this compound, all of the compounds were initially dissolved in physiological saline. Without CMC a gummy insoluble precipitate was formed, but the inclusion of CMC allowed for the preparation of a soluble, slightly opalescent solution. This poly(ICLC) compound is partially resistant to hydrolysis by ribonuclease and has been shown to induce significant levels of serum interferon in monkeys, chimpanzees (10), and man (11, 12).

This primate-effective interferon inducer is now being used in clinical trials in humans. Early results have been encouraging with some diseases. Some undesirable side effects, however, have been noted in man (12).

CMC has been used for years as a suspending medium for pharmaceutical preparations used for systemic injections of steroids in humans. There is, however, no known mechanism in mammals for its biodegradation. While the use of poly(ICLC) in clinical trials in humans has not revealed toxicities attributable to the CMC moiety, it would, in principle, be desirable to have a primate-effective interferon inducer which does not contain this large molecule.

The present studies were initiated in order to determine if a nuclease-resistant, interferon-inducing complex could be formulated from poly(I)·poly(C) and poly-L-lysine only. The elimination of CMC from the formulation should result in a complex [poly(ICL)] of significantly lower molecular weight and might eliminate or reduce some

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of the undesirable side effects. The results of these studies are presented here.

Felsenfeld and Huang were the first to describe the formulation of a stable complex between double-stranded RNA and poly-L-lysine (13). The interaction of poly-L-lysine with double-stranded nucleic acids, including poly(I)·poly(C), has since been studied extensively by Tsuboi *et al.* (14). These latter studies were done at very low concentrations of poly(I)·poly(C) (ca. $2.5 \times 10^{-5}$ *M*) and poly-L-lysine (mol wt 60–90,000) ($1.25 \times 10^{-5}$ *M*) and were formed at room temperature. Using the much higher concentrations of poly(I)·poly(C) necessary to induce interferon in primates (ca. 1 mg/ml final) (ca. $3 \times 10^{-3}$ *M*), a soluble complex with poly-L-lysine could not be formed when physiological (0.15 *M*) saline was used as the solvent for the components, even when lower molecular weight poly-L-lysine was used. The technique described under Materials and methods section of this publication makes possible the preparation of poly(ICL) complexes of much higher concentrations than previously obtainable. The results of these studies are presented here.

**Materials and Methods.** Poly-L-lysine (mol wt about 27,000 daltons) is from Miles-Yeda Ltd., Rehovot, Israel. Poly(I) and poly(C) ($S_{20}$ for both = 9.4 S, as determined by the supplier) came from P-L Laboratories (Milwaukee, Wisc.). Sodium CMC 7HSP (approximate mean mol wt = 700,000) is a product of Hercules Powder Company (Wilmington, Del.).

*a. Stock solutions.* Sterile pyrogen-free water (Abbott, Chicago, Ill.) was used as the solvent for all of the chemical components. Equimolar solutions of poly(I) and poly(C) were prepared by dissolving (i) 600 mg poly(I) in 200 ml solvent (3.0 mg/ml) ($8.6 \times 10^{-3}$ *M*) and (ii) 556.8 mg poly(C) in 200 ml solvent (2.784 mg/ml) ($8.6 \times 10^{-3}$ *M*). Poly-L-lysine (630 mg) was dissolved in 200 ml solvent (3.15 mg/ml) ($13.8 \times 10^{-3}$ *M*). All three solutions were filtered through a 0.45-$\mu$m NALGENE filter (Nalge Co., Rochester, N.Y.).

*b. Formulation of poly(I)·poly(C) complexes.* Poly(ICLC) and poly(I)·poly(C) were prepared as described previously (10). The following procedure was used to prepare complexes of poly(I)·poly(C) with poly-L-lysine but without CMC [poly(ICL)]. Just before mixing, the pH of all three solutions was adjusted to 7.8. Because it is known that poly(I) has about 1% double-stranded molecules, this solution was heated to 58–60° and quickly cooled in an ice bath just before mixing. The poly(I) (200 ml) was added (with mixing by a magnetic stirring bar) to poly(C) (200 ml). After 5–10 min of mixing, poly-L-lysine (200 ml) was added, dropwise, with mixing. A cloudy solution was obtained. This mixture was allowed to stand (at room temperature, with slow mixing) for about 24 hr. The solution became nearly clear. NaCl (5 *M*) was then added to a final concentration of 0.15 *M* and the solution was allowed to mix slowly for 1–2 days at room temperature. A uniform, slightly viscous, nearly clear solution was obtained.

The solution described above, which we call FR-3, has a final concentration of poly(I)·poly(C) of ca. $6 \times 10^{-3}$ *M* and a poly-L-lysine final concentration of ca. $4.6 \times 10^{-3}$ *M* if 227 is used as the molecular weight for lysine-HBR. The $PO_4$:$NH_2$ ratio for this preparation is ca. 1:0.77. This concentration of poly-L-lysine was the highest obtainable that would form a stable, clear solution. A complex with the same $6 \times 10^{-3}$ M poly(I)·poly(C) concentration but with a lower final concentration of poly-L-lysine, FR-1 ($3.3 \times 10^{-3}$ *M* lysine), a $PO_4$:$NH_2$ ratio of ca. 1:0.55, was also prepared and will be discussed under Results.

*c. Thermal denaturation profiles* ($T_m$). Each of the complexes were diluted in 0.1× SSC (SSC = 0.15 *M* NaCl, 0.015 *M* sodium citrate) to give a final $A_{260}$ of about 1.0 (about 65 $\mu$g/ml of poly(I)·poly(C)). The $T_m$ was determined using a Gilford 2400 spectrophotometer equipped with a temperature control device.

*d. Hydrolysis by RNase.* Hydrolysis of the poly(I)·poly(C) complexes was carried out using 5× crystallized, electrophoretically pure pancreatic RNase (Sigma Chemical Co., St. Louis, Mo.). Hydrolysis was at room temperature. The complexes were diluted in 0.15 *M* NaCl, 0.01 *M* $PO_4$ buff-

er, pH 7.4 (PBS), to give a final $A_{260}$ of about 0.5. RNase was added to a final concentration of 5–40 μg/ml. Hydrolysis was determined by hyperchromic shift (increase in absorbance) at λ = 260 nm in 1 hr.

*e. Animals.* Husbandry practices conformed to the Guide for the Care and Use of Laboratory Animals (prepared by the Committee on Care and Use of Laboratory Animals, National Research Council, DHEW Publications No. [NIH] 78-23, 1978).

(*i*) *Mice.* NIH female albino mice (weight 18–20 g) were used for toxicity and interferon induction studies. Blood sera samples for interferon assay were obtained by retro-orbital bleeding and kept frozen at −80°.

(*ii*) *Monkeys.* Healthy, conditioned young adult male or female cynomolgous or rhesus monkeys weighing ca. 4 kg were used to study the interferon-inducing capacity of each of the inducers. The monkeys were housed in individual cages at constant room temperature (25°) with a 12-hr light cycle. They were fed a complete commercial non-human primate diet (Purina) and provided with water *ad lib.*

*f. Interferon assay.* Interferon was assayed by a modification of the micro-CPE inhibition method described by Armstrong (15). Mouse interferon was assayed on interferon-sensitive L cells, while monkey interferon was assayed on human foreskin (HFS-30) fibroblasts. Vesicular stomatitis virus was used as the challenge virus in both systems. Reference mouse interferon (G-002-904-511) has a titer of $10^{4.1}$ units/ml, and human reference interferon (G-023-901-527) had a titer of $10^{4.3}$ units/ml in these assays.

**Results.** *a. Thermal denaturation profiles.* The $T_m$ of double-stranded nucleic acids is a measure of the stability of the molecules. The poly(ICL) complex has a $T_m$ which is considerably higher than that of poly(I)·poly(C). Table I shows the comparison of the $T_m$s of poly(I)·poly(C) and poly(ICLC) with the two preparations of poly(ICL) containing different concentrations of poly-L-lysine. All samples were diluted as described under Materials and Methods.

It should be noted that poly(ICL) complexes denature at temperatures which were 29–35° higher than the $T_m$ of poly(I)·poly(C), indicating that the addition of poly-L-lysine increases the stability of the double-stranded RNA molecule. The FR-3 preparation, which contained the highest concentration of poly-L-lysine, had the lowest $T_m$ of the two complexes, but, as will be pointed out later, is the most resistant to hydrolysis by RNase. Poly(ICLC) has a $T_m$ of about 88° under these conditions (0.1 × SSC).

TABLE I. A SUMMARY OF DATA OBTAINED WITH DIFFERENT POLY(I)·POLY(C) COMPLEXES

| Compound[a] | Designation | Approx. $PO_4$:$NH_2$ ratio[b] | Thermal denaturation temperature (Tm)[c] (°) | Hydrolysis by RNase-A[d] | Interferon titers in monkeys[e] (units log/ml serum ± SD) |
|---|---|---|---|---|---|
| P(ICLC) | — | 1:1.1 | 88.0 | 8.7 | 3.34 ± 0.46[f] |
| P(ICL) | FR-3 | 1:0.77 | 82.0 | 2.0 | 2.96 ± 0.33 |
| P(ICL) | FR-1 | 1:0.55 | 88.0 | 41.9 | 2.35 ± 0.06 |
| Poly(I)·poly(C) | — | — | 53.0 | 35.5 | — |

[a] All compounds were prepared from poly(I) (9 S), poly(C) (9 S), and poly-L-lysine (27,000 mol wt).

[b] Since poly-L-lysine is very hygroscopic these ratios of $PO_4$:$NH_2$ are only approximate and probably are too high.

[c] Compounds diluted in 0.015 *M* NaCl–0.0015 *M* sodium citrate (0.1× SSC).

[d] Percentage increase in $A_{260}$ on hydrolysis by 40 μg/ml. RNase-A for 1 hr at room temperature.

[e] Average of two experiments; four monkeys per set for each experiment (3 mg/kg iv).

[f] The mean interferon value of FR-3 was compared with that of poly(ICLC) by Student's *t* test and was significant at the $P = 0.05$ level. FR-1 was compared with FR-3 by Student's *t* test with unequal variance and was significant at the $P = 0.01$ level.

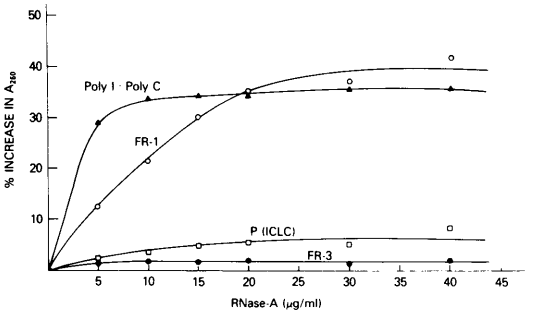


FIG. 1. Hydrolysis by RNase-A of poly(I)·poly(C), poly(ICLC), and poly(ICL) complexes formulated with two different amounts of poly-L-lysine. Ordinate is percentage increase in $A_{260}$ by indicated amount of RNase-A for 1 hr at room temperature.

(*b*) *Hydrolysis by RNase.* One important parameter for the relative ability of a group of poly(I)·poly(C) derivatives to induce interferon in primates is its ability to resist hydrolysis by RNase (Levy, unpublished observations). The rates of hydrolysis of the two poly(ICL) complexes were compared with plain poly(I)·poly(C) and with poly(ICLC) in Fig. 1. Poly(I)·poly(C) was relatively rapidly hydrolyzed by RNase. The complexing of the increased amount of poly-L-lysine to poly(I)·poly(C) caused increased resistance to hydrolysis by RNase. The FR-3 preparation, the poly(ICL) complex which contained the higher amount of poly-L-lysine, was somewhat more resistant to hydrolysis by RNase than was poly(ICLC), the complex which also contains the large CMC molecule. The difference in resistance to hydrolysis by RNase between the two poly(ICL) complexes at low concentration of RNase was small; however, at the highest RNase concentration (40 μg/ml), the FR-3 preparation was much more stable to hydrolysis than the other poly(ICL) preparation.

*c. Interferon induction in mice.* The interferon-inducing capacity of these compounds in mice was compared with that of poly(I)·poly(C) and poly(ICLC).

Six mice per set were injected ip with 100 μg/mouse of each of the compounds. Each set was bled at 2, 7, and 24 hr postinjection. The serum interferon titer of each mouse was separately determined as described, and the arithmetic mean titer for each set

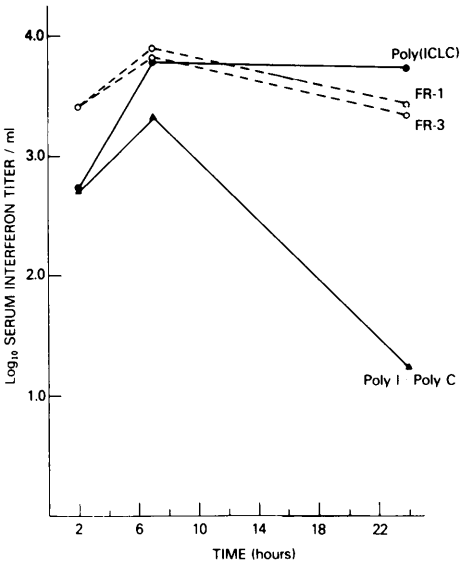


FIG. 2. A comparison of the interferon-inducing capacities of plain poly(I)·poly(C), poly(ICLC), and poly(ICL) complexes in mice.

was calculated. Three independent experiments were performed with essentially the same results.

Poly(ICLC) and the two poly(ICL) complexes were more effective in mice as interferon inducers than was poly(I)·poly(C), as shown in Fig. 2. Serum interferon rose to a higher titer and was present longer. As can be seen, poly(ICL) complexes were comparable to poly(ICLC) as interferon inducers in mice.

*d. Toxicity in mice.* Studies were done in mice to compare the toxicity of poly(ICLC) with that of the poly(ICL) complex which contained the higher poly-L-lysine concentration (FR-3). Twenty mice per set were injected ip with three different dose levels of each of the compounds. The animals were observed daily and the survivors counted. $LD_{50}$ was determined by the Reed and Muench method (16).

In two separate experiments the mean $LD_{50}$ of poly(ICLC) was 12.6 mg/kg while that of poly(ICL) (FR-3) was 25.1 mg/kg. Poly(I)·poly(C) has been shown to have an $LD_{50}$ of about 45 mg/kg. It is clear from these data that the poly(ICL) complex without CMC is less toxic in mice than is poly(ICLC).

*e. Interferon induction in monkeys.* The ability of poly(I)·poly(C) complexes with increasing amounts of poly-L-lysine to induce interferon was determined in Rhesus or cynomolgous monkeys. Four young adult monkeys were injected iv with the different compounds at 3 mg/kg. Blood samples were taken 8 and 24 hr after the first injection. The sera of these samples were titered for interferon. Of the two poly(ICL) compounds, the one with the higher concentration of poly-L-lysine (FR-3) induced significantly higher levels ($P < 0.01$) of interferon than did FR-1 (Table I). On the other hand, poly(ICLC), which contained the CMC molecule, induced significantly higher levels ($P < 0.05$) of serum interferon than did the complexes without CMC (Table I).

In individual monkeys at the 3 mg/kg dose level, titers as high as 5000 units/ml serum were seen with poly(ICLC). With FR-3, serum interferon levels did not exceed 3200 units/ml. The FR-3 compound, on an average, induced about five times as much interferon as did the FR-1 preparation at this dose of drug. These differences are significantly different statistically.

It should also be noted that when a second dose of drug was given to monkeys 24 hr after the first, peak serum interferon levels were higher after the second dose than after the first. This phenomenon has been observed in several present experiments and has been reported before with poly(ICLC) (17). It may represent priming.

**Discussion.** Preparations of poly(I)·poly(C) stabilized with two different amounts of poly-L-lysine without carboxymethylcellulose have been described. The poly(ICL) complex with a ratio for $PO_4$:$NH_2$ of 1:0.77 is the more resistant to hydrolysis by RNase-A and is an effective inducer of interferon in monkeys.

With the two complexes examined, as the amount of poly-L-lysine increased, the resistance to hydrolysis by RNase increased and primate interferon-inducing capacity increased. Surprisingly, however, the $T_m$ decreased as the amount of poly-L-lysine increased, suggesting that resistance to hydrolysis is a more important predictor of the ability to induce interferon in primates than is thermodynamic stability.

In clinical trials in humans, the primary toxicities of poly(ICLC) have been fever (100% of patients), lymphopenia (20% of patients), and hypotension (5% of patients). These toxicities qualitatively resemble those seen in patients treated with exogenous interferon. Poly(ICL) is less toxic in mice than is poly(ICLC), as determined by $LD_{50}$ values. Current studies are comparing side effects of poly(ICL) with those of poly(ICLC) in monkeys.

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