# The Effect of Altering the Size of Poly C on the Toxicity and Antigenicity of Poly I:C (36934)

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Previous studies from these laboratories (1, 2) recorded that the interferon and resistance-inducing capacities of poly I:C (rIn:rCn) were markedly affected by the size of poly I:C and of its component homopolymers. The present report is an extension of these studies and shows that acute toxicity for mice and antigenicity for rabbits of poly I:C can be markedly reduced by altering the size of the poly C component of the complex without affecting induced antiviral activity. These findings are of importance in developing an optimally acceptable poly I:C for clinical studies in man.

*Materials and Methods. Sources of reagents.* Polyriboinosinic acid (poly I, rIn) and polyribocytidylic acid (poly C, rCn) were prepared by Drs. E. E. Harris and C. H. Hoffman of the Merck Developmental Research Group. Ribonuclease A (chromatographically purified grade) was obtained from Mann Research Laboratories, New York, NY. Macaloid (purified grade of Hectorite) was obtained from Beroid Division of National Lead Co., Houston, TX.

*Preparation of poly I:C containing enzymatically degraded poly C.* One liter of a 1 mg/ml solution of poly C ($S_{20,w} = 6.0$) in phosphate buffered saline solution (PBS) (0.006 *M* sodium phosphate, 0.15 *M* sodium chloride, pH 7.0) was incubated with 0.001 μg/ml of pancreatic ribonuclease A at 25°. Aliquots (100 ml) were removed at various time periods between 1 and 4 hr incubation. Enzymatic action in the 100 ml aliquots was stopped by addition of 0.033% final concentration of sodium dodecyl sulfate (3) and 2 mg of Macaloid (4). Separation of poly C was carried out by shaking vigorously for 5 min with an equal volume of 88% phenol that had been equilibrated with PBS. Phenol extraction was repeated after adding a further 2 mg of Macaloid. Two volumes of cold absolute ethanol were then added and precipitation of the poly C was allowed to proceed overnight at −20°. The precipitate, after washing with 95% ethanol and absolute ethanol, was dried in a vacuum desiccator over potassium hydroxide pellets. Each enzymatically degraded poly C preparation was reconstituted at a concentration of 4 mg/ml in PBS containing 0.01 *M* magnesium chloride. The poly C solutions were added to equal volumes of high molecular weight poly I ($S_{20,w} = 14.5$) at 4.3 mg/ml in PBS and complexing to form poly I:C was allowed to proceed at room temperature.

*Physical and biochemical determinations.* The degraded poly C samples were chromatographed on Sepharose 4B (Pharmacia, Uppsala, Sweden). Poly C solution (0.5 ml) containing 9 OD units (268 nm) was applied to a 1.5 × 36 cm column and eluted with PBS. The void volume of the column was determined using high molecular weight poly I:C ($7.8 \times 10^6$ daltons). The procedures for determining ribonuclease sensitivity, relative viscosity, hypochromicity, hyperchromicity, and $T_m$ of poly I:C complexes were described earlier (1).

*Biological determinations.* Acute toxicity of the poly I:C samples was measured by intraperitoneal injection into 20–25 mice, each weighing 14–16 g; deaths were recorded for a 7 day period. Methods for assaying for induction of interferon and resistance to viral infection were described previously (1). To measure antigenicity, groups of ten 4–5 lb New Zealand white rabbits were injected intravenously at weekly intervals for 5 wk with

TABLE I. Physical and Biological Properties of Poly I:C Prepared with Enzymatically Degraded Poly C.

| Poly C | | Poly I:C complex[a] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Physical assay | | | | Biological assay in mice | | | |
| | | | | | | Acute toxicity (% deaths at mg/kg dose) | | | |
| Treatment with RNase time (min) | $V_e/V_0$ ratio (chromatography on Sepharose 4B) | $E_{1\%}^{248nm}$ | % Hypochromicity on mixing | Relative viscosity | RNase sensitivity (increase in OD/min at 248 nm × $10^3$) | 270 | 135 | 68 | 34 | Minimum protective dose, PVM in mice[b] ($\mu$g/mouse) |
| 0 | Polydisperse, 1.0 | 132 | 37.4 | 3.30 | 1.0 | — | 90 | 55 | 15 | 1 |
| 60 | 2.47 | 137 | 35.3 | 2.79 | 3.7 | — | 14 | 4 | 0 | 1 |
| 80 | 2.55 | 136 | 35.8 | 2.75 | 6.5 | 15 | 0 | 0 | 0 | 1 |
| 90 | 2.57 | 136 | 35.8 | 2.58 | 7.4 | 0 | 5 | 0 | 0 | 1 |
| 105 | 2.59 | 138 | 35.0 | 2.52 | 8.5 | 15 | 5 | 0 | 0 | 1 |
| 120 | 2.61 | 141 | 33.2 | 2.10 | 9.2 | 15 | 0 | 0 | 0 | 1 |
| 140 | 2.64 | 146 | 31.1 | 2.20 | 9.3 | 0 | 0 | 0 | 0 | 1 |
| 160 | 2.68 | 149 | 29.5 | 1.90 | 12.3 | 0 | 0 | 0 | 0 | >4 |
| 240 | — | 163 | 23.0 | 1.60 | 13.7 | ND[c] | ND | ND | ND | >4 |

[a] The Poly I used had an $S$ value of 14.5.

[b] Minimum intranasal dose which protected at least 50% of the treated mice against fatal infection with 30 $LD_{50}$ of PVM (pneumoniae virus of mice).

[c] ND = not done.

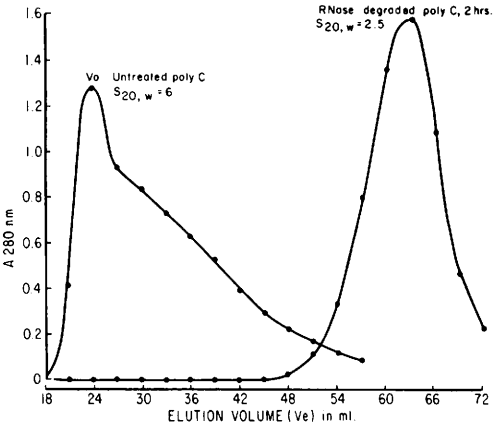


FIG. 1. Chromatography on Sepharose 4B of poly C and RNase degraded poly C.

1 ml of a 1 mg/ml solution of untreated poly I:C or with poly I:C prepared with poly C that had been treated with ribonuclease for 2 hr. A final injection was made 2 wk after the fifth dose and blood samples were taken prior to the first injection and 1 wk after the last. The serum samples were adsorbed at 4° with sheep erythrocytes, inactivated at 56° for 30 min, and the titer of complement-fixing (CF) antibody against poly I:C was determined according to Osler, Strauss and Mayer (5).

*Results. Effect on the physical properties of poly I:C complex of degrading the poly C component.* Table I shows that the poly C was progressively reduced in molecular size by incubating with ribonuclease (RNase) as evidenced by increase in the ratio of the elution volume ($V_e$) to the void volume ($V_0$) on Sepharose chromatography. Further, Fig. 1 shows that the product of RNase degradation was eluted from Sepharose in a sharper, less heterodispersed peak than the undergraded material. The properties of the poly I:C complex were markedly affected by degrading the poly C component. Progressive shortening of the poly C chain (Table I) was accompanied by increase in extinction coefficient, reduction in relative viscosity and hypochromicity on mixing, and an increase in sensitivity to destruction by RNase. Figure 2 shows that there was less hypochromicity on complexing of poly I:C prepared from degraded compared with untreated poly C. Additionally, Fig. 2 shows poly I:C prepared with degraded poly C dissociated over a broader temperature range and displayed a lower $T_m$ (58°) compared with complex made with untreated poly C ($T_m$ 62°).

*Effect on toxicity and protective efficacy in mice of poly I:C complex containing enzymatically degraded poly C component.* Table I shows that there was significant reduction in acute toxicity in mice by degrading the poly C component with RNase. At the 135 mg/kg body weight dosage, *e.g.*, treatment with RNase for 80 min or longer reduced the death rates from 90 to 0%. Such effect was shown for the other dose levels as well. High viscosity precluded testing the untreated and RNase-treated poly C in poly I:C at higher

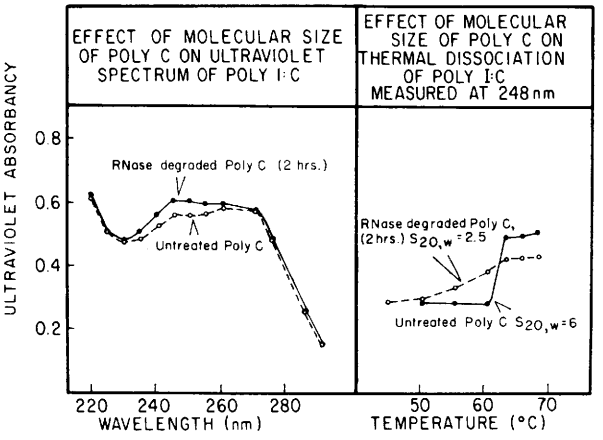


FIG. 2. Effect of reduction in molecular size of Poly C on uv absorption spectrum and thermal dissociation curve on poly I:C complex.

TABLE II. Induction of Interferon and Host Resistance by Poly I:C Prepared with Untreated Compared with RNase Degraded Poly C.

| Interferon inducer | Interferon induction in rabbits: Dose (μg/rabbit) | Interferon induction in rabbits: Serum titer | Interference in PRK cells, minimum effective dose (μg/ml) |
|---|---|---|---|
| Poly I:C | 2.5 | 320, 640 | 0.003 |
| | 0.6 | 40, 5 | |
| | 0 | <5, <5 | |
| Poly I:C-RNase[a] | 2.5 | 10, 20 | 0.003 |
| | 0.6 | <5, <5 | |
| | 0 | <5, <5 | |

[a] Poly C was degraded 120 min with $10^{-9}$ g/ml ribonuclease A and complexed with poly I.

than 270 mg/kg.

Even though the size of the poly C was greatly reduced after RNase treatment ($S_{20,w}$ reduced from 6.0 to 2.5 in 120 min), there was full retention of protective efficacy of complex prepared from poly C treated as long as 140 min. This drastic reduction in toxicity for mice with no apparent loss in protective effect produced a markedly improved toxicity:activity ratio. Further, as is shown in Table II, there was no detectable increase in the dose requirement of poly I:C to induce resistance in primary rabbit kidney cell culture against vesicular stomatitis virus using poly C degraded for 120 min with RNase compared with untreated homopolymer. Such treatment for a 120 min period did, however (Table II), cause apparent reduction in the ability of poly I:C to stimulate induction of interferon in rabbits.

*Effect on antigenicity in rabbits of poly I:C complex prepared with enzymatically degraded poly C.* Table III shows that poly C degraded for 120 min with RNase yielded poly I:C complex that was markedly less antigenic for rabbits than was the complex prepared using untreated homopolymer. This was evidenced by reduction in antibody response rate (100 vs 40%) and geometric mean antibody titer (100 vs 3.2).

*Discussion.* Controlled reduction in molecular size of poly C by ribonuclease treatment effected, within defined limits, favorable properties of the complex formed, on addition to poly I. Thus, treatment with RNase of poly C for periods up to 140 min caused a marked decrease in toxicity for mice and antigenicity for rabbits. There was no detectable decrease in protection against PVM in mice, or induction of resistance to VSV in cell culture. There was some reduction in titer of interferon induced in rabbits. Essentially, poly C could be reduced in molecular size to a sedimentation coefficient of 2.5*S* ($1.6 \times 10^4$ daltons) without reduction in *in vivo* and *in vitro* protection. Continued degradation of the poly C beyond 140 min reduced the biological activity, probably be-

TABLE III. Antigenicity for Rabbits of Poly I:C Prepared with Untreated Compared with RNase Degraded Poly C.

| Rabbit nos. | Poly I:C prepared using: | Poly I:C antibody titer: Individual titers | Poly I:C antibody titer: Geometric mean |
|---|---|---|---|
| 1–9 | Untreated poly C | 80, 320, 80, 160, 10, 320, 40, 320, 80 | 100 |
| 10–19 | RNase degraded[a] poly C | 20, 20, 20, 10, <10, <10, <10, <10, <10, <10 | 3.2 |

[a] Poly C was degraded 120 min with RNase.

cause of reduction in size to the point that a stable helical structure with poly I did not result.

Studies of the effect of reducing the size of poly I on the toxic and protective efficacies of poly I:C complex were not carried out because reduction in the average sedimentation coefficient of poly I below 9*S* resulted in a sharp reduction in *in vitro* activity and in loss in protective efficacy in mice (2). Additionally, there was no significant alteration in mouse toxicity of poly I:C when the sedimentation coefficient of the poly I was reduced from 14.5 to 9.2.

The requirement for a large poly I and the possibility of reducing the size of poly C without reducing antiviral activity was reported earlier by us (2) and confirmed by Pitha *et al.* (6). At variance with these reports were the findings of Niblack and McCreary (7) that reduction of the molecular size of the poly C below 100,000 greatly reduced the antiviral activity of poly I:C. This discrepancy may be explained by the latter workers' use of thermally degraded poly C which results in a broad polydispersity of molecular size in contrast of RNase degraded homopolymer which is more homogeneous in size distribution.

The toxicity of poly I:C for experimental animals, especially the dog, is well documented (8, 9). Such manifestations, other than fever, have not been observed in trials in man, even with large dosage given daily (12) Parenteral administration of poly I:C may induce antibody in rabbits and certain strains of mice (10, 11) but no such induction of antibodies by poly I:C has been found in man (12). The lack of any toxic effect for man has prompted continued optimism concerning its eventual use in human medicine. The further reduction in toxicity and antigenicity of poly I:C through enzymatic degradation of poly C adds another parameter for increased safety of poly I:C should this prove necessary.

*Summary.* Appropriate reduction in size of poly C by degradation with ribonuclease prior to complexing with poly I effected marked reduction in toxicity for mice and antigenicity for rabbits of poly I:C without detectable reduction in *in vivo* or *in vitro* protection against viral infection. There was slight reduction in interferon-inducing capacity for rabbits. Such procedure adds another possible means for increasing the safety of poly I:C in eventual clinical application.

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