Interferon Production Linked to Toxicity of Polyriboinosinic Acid-Polyribocytidylic Acid

ERIK DE CLERCQ,¹ WILLIAM E. STEWART II, AND PIERRE DE SOMER

Rega Institute for Medical Research, University of Leuven, 3000 Leuven, Belgium

Received for publication 21 April 1972

Different procedures have been used in attempts to increase the production of interferon by polyriboinosinic acid-polyribocytidylic acid (poly (rI)·poly (rC)) in mice: simultaneous injection of lead acetate, cycloheximide, or actinomycin D and prior injection of Freund's adjuvant, chlorite-oxidized oxyamylose (COAM), endotoxin, or *Brucella abortus*. In the experimental conditions tested, lead acetate, cycloheximide, Freund's adjuvant, and COAM brought about a parallel increase in interferon production and toxicity (lethality) of poly (rI)·poly (rC); actinomycin D, endotoxin, and *B. abortus* increased the lethality of poly (rI)·poly (rC) without a concomitant raise of its interferon-inducing capacity. Our results indicate that no significant increase in interferon production (or antiviral activity, as far as the antiviral activity is accounted for by interferon production) without an accompanying increase in toxicity can be achieved with poly (rI)·poly (rC) and that it might be impossible to increase its therapeutic ratio (ratio of maximum tolerated dose to minimum effective dose).

In view of the potential importance of polyriboinosinic acid-polyribocytidylic acid (poly (rI)·poly (rC)) as an interferon inducer and antiviral agent in clinical medicine, numerous attempts have been undertaken to increase its antiviral activity or decrease its toxicity, or both.

Several procedures are known to increase the in vivo interferon-inducing capacity of poly (rI)·poly (rC): (i) complex formation of the polynucleotide with high-molecular-weight polycations such as diethylaminoethyl (DEAE)-dextran (9, 10) or poly-D-lysine (18, 19); (ii) simultaneous injection of lead acetate (21); (iii) simultaneous injection of cycloheximide (6, 26); and (iv) prior injection of Freund's adjuvant (7) or polyacetal carboxylates such as chlorite-oxidized oxyamylose (COAM) (H. B. Levy, Int. Colloq. on Interferon and Interferon Inducers, Leuven, Belgium, 1971). Some of these procedures have also been shown to enhance the toxicity of poly (rI)·poly (rC) in vivo: simultaneous injection of lead acetate (1); other conditions known to produce enhancement of toxicity are simultaneous injection of actinomycin D (17) and adrenalectomy (27).

We have now directly compared the effects of several of these and other factors (lead acetate, cycloheximide, actinomycin D, Freund's ad-

juvant, COAM, endotoxin and *Brucella abortus*) on both toxicity and interferon production by poly (rI)·poly (rC) in mice and found that, with all systems tested, no increase in interferon production could be achieved without a concomitant rise in toxicity. Our results strongly suggest that the in vivo interferon-inducing and toxic effects of poly (rI)·poly (rC) are linked phenomena and that it might be impossible to improve the therapeutic ratio (ratio of maximum tolerated dose to minimum effective dose) of poly (rI)·poly (rC).

MATERIALS AND METHODS

Animals. Female NMRI mice, weighing 10 to 12 g, were used throughout our experiments. They were housed in an air-conditioned room at 25 C and given food and water ad libitum.

Compounds. Poly (rI)·poly (rC) was prepared by mixing the individual homopolymers (both purchased from P-L Biochemicals, Inc., Milwaukee, Wis.) in equal amounts (2.5 mg/ml) in buffered saline (0.15 M NaCl) and allowing them to react for 1 hr at 45 C. Samples of the stock solutions were stored at -20 C. Before use a sample was thawed and diluted in phosphate-buffered saline to the appropriate concentration. Poly (rI)·poly (rC) was injected intravenously.

Lead acetate was dissolved in distilled water at 5 mg/ml; cycloheximide (purchased from Sigma Chemical Co., St. Louis, Mo.) was also dissolved in distilled water at 2.5 mg/ml. COAM was synthesized by P. Claes of our Institute (5), dissolved in saline at 20

¹ "Aangesteld Navorsers" of the Belgian National Fonds voor Wetenschappelijk Onderzoek.

mg/ml and stored at 4 C. Freund's adjuvant, a suspension of killed and dried *Mycobacterium butyricum* in a mixture of 8.5 ml of paraffin oil (Bayol F) and 1.5 ml of mannide monooleate (Arlacel), was purchased from Difco Laboratories, Detroit, Mich. *Brucella abortus* (attenuated strain 19, used for vaccination of cattle) was obtained as a lyophilized preparation from R.I.T., Genval, Belgium. Immediately before use the bacteria were suspended in saline at 2.5×10^{10} bacteria per ml. *Salmonella enteritidis* endotoxin was obtained from Difco. It was stored at 0.25 mg/ml in saline at 4 C. Actinomycin D was purchased from Schwarz-Mann, Orangeburg, N.Y., dissolved in distilled water, and stored at 50 μ g/ml at 4 C.

Interferon assay. Blood samples were obtained at 2 or 8 hr after administration of poly (rI)·poly (rC); sera were assayed for interferon by a plaque-reduction technique in L 929 cells using bovine vesicular stomatitis virus (Indiana strain) as the challenge virus (8).

Toxicity. Mortality was scored daily for 10 days. Deaths due to poly (rI)·poly (rC) were all recorded within 48 hr after injection of the polynucleotide.

RESULTS

Lethality and interferon production were determined with different doses of poly (rI)·poly (rC) in normal mice and mice treated with lead acetate or Freund's adjuvant (Fig. 1). To assess the effects of lead acetate and Freund's adjuvant on interferon production by poly (rI)·poly (rC), blood samples were taken at 2 hr after injection of the polynucleotide, as serum interferon levels reach their peak value at this time, both in control mice and mice treated with lead acetate or Freund's adjuvant. Lead acetate injected intravenously at 1 mg per mouse im-

FIG. 1. Effect of lead acetate and Freund's adjuvant on toxicity and interferon production by poly (rI)·poly (rC). Lead acetate was injected intravenously at 1 mg per 0.2 ml per mouse immediately before injection of poly (rI)·poly (rC). Freund's adjuvant was injected intraperitoneally at 0.2 ml per mouse 2 days before injection of poly (rI)·poly (rC). Poly (rI)·poly (rC) was injected intravenously at the indicated doses per 0.2 ml per mouse. Circulating interferon titers were measured 2 hr after injection of poly (rI)·poly (rC). Each group had 12 mice.

mediately before poly (rI)·poly (rC) decreased the minimum effective dose of poly (rI)·poly (rC) (the dose stimulating the production of 100 units of interferon per 4 ml of serum) from 0.1 to 0.005 μ g per mouse and decreased the 50% lethality dose (LD₅₀) from 200 to 5 μ g per mouse. Thus, the activity-to-toxicity ratio of poly (rI)·poly (rC) did not change more than twofold following lead acetate treatment. Similarly, this ratio remained essentially unaltered by previous treatment with Freund's adjuvant; 0.2 ml of Freund's adjuvant injected intraperitoneally 2 days before poly (rI)·poly (rC) decreased the minimum effective dose of poly (rI)·poly (rC) from 0.1 to 0.02 μ g per mouse and the LD₅₀ dose from 200 to 50 μ g per mouse. Neither lead acetate alone nor Freund's adjuvant alone caused mortality or stimulated interferon production at the doses used.

Lethality and interferon production by poly (rI)·poly (rC) were also determined in mice treated with cycloheximide or COAM (Fig. 2). These mice were tested for circulating interferon 8 hr after injection of poly (rI)·poly (rC). Blood samples were taken at 8 hr (instead of 2 hr) for two reasons: (i) in mice treated with cycloheximide, serum interferon levels reach their peak value at 8 hr after injection of the polynucleotide and, (ii) in mice treated with COAM, residual interferon production, due to COAM itself, which is injected 24 hr before poly (rI)·poly (rC), is minimal at 8 hr after injection of the polynucleotide. Both COAM injected intra-

FIG. 2. Effect of COAM and cycloheximide on toxicity and interferon production by poly (rI)·poly (rC). COAM was injected intraperitoneally at 5 mg per 0.25 ml per mouse 24 hr before injection of poly (rI)·poly (rC). Cycloheximide was injected intraperitoneally at 0.5 mg per 0.2 ml per mouse simultaneously with the injection of poly (rI)·poly (rC). Poly (rI)·poly (rC) was injected intravenously at the indicated doses per 0.2 ml per mouse. Circulating interferon titers were measured 8 hr after injection of poly (rI)·poly (rC). Each group had 12 mice.

peritoneally at 5 mg/mouse 24 hr before poly (rI)·poly (rC) and cycloheximide injected intraperitoneally at 0.5 mg/mouse simultaneously with poly (rI)·poly (rC) increased interferon production and lethality of the polynucleotide to approximately the same extent: the minimum effective dose of poly (rI)·poly (rC) was reduced from 5 μ g to 0.2 μ g per mouse by cycloheximide and to 0.1 μ g per mouse by COAM; the LD₅₀ dose was reduced from 200 μ g to 20 μ g per mouse by cycloheximide and to 10 μ g per mouse by COAM. Neither COAM alone nor cycloheximide alone caused mortality. Serum interferon titers obtained with cycloheximide and COAM amounted to less than 30 U/4 ml (8 hr after intraperitoneal injection of cycloheximide) and to 60 to 80 U/4 ml (32 hr after intraperitoneal injection of COAM).

In addition to the aforementioned procedures, other systems were tried to increase the biological activities of poly (rI)·poly (rC): *B. abortus* injected intravenously at 5×10^9 living bacteria per mouse, 24 hr before poly (rI)·poly (rC); *Salmonella enteritidis* endotoxin injected intravenously at 50 μ g per mouse 6 hr before poly (rI)·poly (rC); actinomycin D injected intraperitoneally at 10 μ g per mouse simultaneously with poly (rI)·poly (rC). *B. abortus*, endotoxin, and actinomycin D increased the lethality of poly (rI)·poly (rC) without a concomitant raise of its interferon-inducing capacity. *B. abortus* increased the lethality of a dose of 100 μ g of poly (rI)·poly (rC) per mouse from 10 to 80%, as did endotoxin, whereas actinomycin D brought about the same shift in lethality as observed with cycloheximide (see Fig. 2), but neither *B. abortus* nor endotoxin nor actinomycin D brought about a significant increase in the interferon response to poly (rI)·poly (rC), whether interferon production was measured 2 hr (with *B. abortus* and endotoxin) or 8 hr (with actinomycin D) after injection of the polynucleotide.

DISCUSSION

There is ample evidence that poly (rI)·poly (rC) is a toxic substance sharing many of the toxic properties of bacterial endotoxins. Poly (rI)·poly (rC) is lethal for mice at relatively low dosages (Fig. 1 and 2), provokes a local Shwartzman phenomenon (1), is highly pyrogenic (13) and embryotoxic (2) in rabbits, exerts a toxic effect on the hemopoietic stem cells in mice (11), induces allogeneic (runt-like) disease in mice and rats (thymic atrophy, spleen hypoplasia, lymphopenia) (12), and accelerates the onset of autoimmune disease in New Zealand mice (20). Ocular toxicity (lens opacification, iris hy-

peremia, aqueous flare) has been reported with poly (rI)·poly (rC) in rabbits (16) and cerebellar toxicity (edema, hemorrhage, ataxia, due to destruction of the endothelial cells of the small blood vessels) has been reported in chickens (25).

Attempts have been made to increase the activity-to-toxicity ratio of poly (rI)·poly (rC) by decreasing its molecular weight, but the antiviral activity appeared to decline with decreases in homopolymer molecular weight at approximately the same rate as the acute toxicity (15). The data presented here clearly indicate that with poly (rI)·poly (rC) no significant separation of toxicity from interferon production (or antiviral activity, since interferon production accounts for the whole antiviral activity of poly [rI]·poly [rC] in certain well defined experimental conditions [8]) can be achieved.

Four different procedures enhanced the interferon-inducing capacity of poly (rI)·poly (rC): prior injection of Freund's adjuvant or COAM and simultaneous injection of lead acetate and cycloheximide. Although these procedures may alter a comparable basic parameter in interferon-producing cells, they are thought to increase interferon production by different mechanisms: Freund's adjuvant, by a stimulatory effect on the phagocytic activity of the lymphoreticular system (7); cycloheximide, by inhibition of the synthesis of a regulatory protein (repressor) which prevents interferon production (24), or, by stimulation of the release of previously cleared interferon (4). The mechanism by which lead acetate and COAM increase the interferon response to poly (rI)·poly (rC) is not clear. The enhancing effect of lead acetate on the toxicity of endotoxin has been ascribed to an inactivation of the sulfhydryl groups of enzymes involved in the detoxification of endotoxin (3). The priming effect of COAM on the activity of poly (rI)·poly (rC) might be related to a stimulatory effect on some functional activities of the lymphoreticular system; other polycarboxylates such as polymethacrylic acid (E. De Clercq and P. De Somer, unpublished data) and pyran copolymer (14) have also been shown to sensitize animals for the interferon-inducing and lethal effects of endotoxin. All four systems used (Freund's adjuvant, lead acetate, cycloheximide, and COAM) increased interferon production and toxicity of poly (rI)·poly (rC) in parallel. From these results it seems likely that the antiviral activity of poly (rI)·poly (rC) cannot be increased without a concomitant increase in toxicity.

Three other procedures (actinomycin D, endotoxin, and *B. abortus*) increased the lethality of poly (rI)·poly (rC) without increasing the

interferon response to the polynucleotide. Actinomycin D was injected simultaneously with poly (rI)·poly (rC), whereas endotoxin and *B. abortus* were injected 6 and 24 hr, respectively, before the polynucleotide. It is possible, however, that some modifications in these time intervals might have caused an increase in interferon production proportional to the increased toxicity.

It has been proposed (22) that, since interferon-treated cells are able to produce interferon in response to levels of poly (rI)·poly (rC) too low to induce interferon in normal cells, interferon treatment might improve the therapeutic ratio of this inducer. However, our recent findings that interferon-treated cells are more susceptible to the toxicity of poly (rI)·poly (rC) (23) appears again to reinforce the present conclusion that the antiviral activity of poly (rI)·poly (rC) cannot be increased without a parallel increase in its toxicity.

ACKNOWLEDGMENTS

This investigation was supported by a grant from the Belgian F.G.W.O. (Fonds voor Geneeskundig Wetenschappelijk Onderzoek). The excellent technical assistance of A. Van Lierde is acknowledged.

LITERATURE CITED

- Absher, M., and W. R. Stinebring. 1969. Endotoxin-like properties of poly I·poly C, an interferon stimulator. *Nature (London)* **223**:715-717.
- Adamson, R. H., and S. Fabro. 1969. Embryotoxic effect of poly I·poly C. *Nature (London)* **223**:718.
- Bertok, L. 1968. Effect of sulfhydryl compound on the lead acetate-induced endotoxin hypersensitivity of rats. *J. Bacteriol.* **95**:1974-1975.
- Chester, T. J., E. De Clercq, M. R. Nuwer, and T. C. Merigan. 1972. In vivo release of previously cleared interferon by cycloheximide. *Infect. Immunity* **5**:383-388.
- Claes, P., A. Billiau, E. De Clercq, J. Desmyter, E. Schonne, H. Vanderhaeghe, and P. De Somer. 1970. Polyacetal carboxylic acids: a new group of antiviral polyanions. *J. Virol.* **5**:313-320.
- De Clercq, E., and T. C. Merigan. 1970. Stimulation or inhibition of interferon production depending on time of cycloheximide administration. *Virology* **42**:799-802.
- De Clercq, E., M. R. Nuwer, and T. C. Merigan. 1970. Potentiating effect of Freund's adjuvant on interferon production by endotoxin or poly rI·poly rC. *Infect. Immunity* **2**:69-76.
- De Clercq, E., M. R. Nuwer, and T. C. Merigan. 1970. The role of interferon in the protective effect of a synthetic double-stranded polyribonucleotide against intranasal vesicular stomatitis virus challenge in mice. *J. Clin. Invest.* **49**:1565-1577.
- Dianzani, F., G. Rita, P. Cantagalli, and S. Gagnoni. 1969. Effect of DEAE-dextran on interferon production and protective effect in mice treated with the double-stranded polynucleotide complex polyinosinic-polycytidylic acid. *J. Immunol.* **102**:24-27.
- Finter, N. B. 1970. Studies with an interferon inducer (polyinosinic-polycytidylic acid) in mice, p. 325-333. In "L'Interféron." Ministère de la Santé Publique et de la Sécurité Sociale, (ed.), Colloques de l'Institut National de la Santé et de la Recherche Médicale, n° 6. (Paris).
- Jullien, P., and J. De Maeyer-Guignard. 1971. Toxic effect of polyriboinosinic-polyribocytidylic acid in mouse hemopoietic stem cells. *Int. J. Cancer* **7**:468-475.
- Leonard, B. J., E. Eccleston, and D. Jones. 1969. Toxicity of interferon inducers of the double stranded RNA type. *Nature (London)* **224**:1023-1024.
- Lindsay, H. L., P. W. Trown, J. Brandt, and M. Forbes. 1969. Pyrogenicity of poly I·poly C in rabbits. *Nature (London)* **223**:717-718.
- Munson, A. E., and W. Regelson. 1971. Sensitization to endotoxin by pyran copolymer. *Proc. Soc. Exp. Biol. Med.* **137**:553-557.
- Niblack, J. F., and M. B. McCreary. 1971. Relationship of biological activities of poly I·poly C to homopolymer molecular weights. *Nature N. Biol. (London)* **233**:52-53.
- Ostler, H. B., J. O. Oh, C. R. Dawson, and W. L. Burt. 1970. Toxicity of poly I·poly C for rabbit eyes. *Nature (London)* **228**:362-364.
- Pieroni, R. E., A. E. Bundeally, and L. Levine. 1971. The effect of actinomycin D on the lethality of poly I:C. *J. Immunol.* **106**:1128-1129.
- Rice, J. M., W. Turner, M. A. Chirigos, and N. R. Rice. 1970. Enhancement by poly-d-lysine of poly I:C-induced interferon production in mice. *Appl. Microbiol.* **19**:867-869.
- Rice, J. M., W. Turner, M. A. Chirigos, and G. Spahn. 1971. Dose responsiveness and variation among inbred strain of mice in production of interferon after treatment with poly (I)·poly (C) [poly-d-lysine] complexes. *Appl. Microbiol.* **22**:380-386.
- Steinberg, A. D., S. Baron, and N. Talal. 1969. The pathogenesis of autoimmunity in New Zealand mice. I. Induction of antinuclear acid antibodies by polyinosinic-polycytidylic acid. *Proc. Nat. Acad. Sci. U.S.A.* **63**:1102-1107.
- Stinebring, W. R., and M. Absher. 1970. Studies on the pool of interferon available for release by non-viral agents, p. 63-69. In "L'Interféron." Ministère de la Santé Publique et de la Sécurité Sociale, (ed.), Colloques de l'Institut National de la Santé et de la Recherche Médicale, n° 6. (Paris).
- Stewart, W. E. II, L. B. Gosser, and R. Z. Lockart, Jr. 1971. Priming: a nonantiviral function of interferon. *J. Virol.* **7**:792-801.
- Stewart, W. E. II, E. De Clercq, A. Billiau, J. Desmyter, and P. De Somer. 1972. Increased susceptibility of cells treated with interferon to the toxicity of polyriboinosinic-polyribocytidylic acid. *Proc. Nat. Acad. Sci. U.S.A. In press.*
- Vilcek, J., and M. H. Ng. 1971. Post-transcriptional control of interferon synthesis. *J. Virol.* **7**:588-594.
- Young, P. A., J. J. Taylor, M. C. Yu, and E. Eyerlyman. 1970. Morphological changes in chick cerebellum induced by poly I·poly C. *Nature (London)* **228**:1191-1192.
- Youngner, J. S., and J. V. Hallum. 1968. Interferon production in mice by double-stranded synthetic polynucleotides: induction or release? *Virology* **35**:177-179.
- Zedeck, M. S., H. Marquardt, S. S. Sternberg, M. Fleisher, and L. D. Hamilton. 1970. Role of adrenal cortical function in toxicity of polyriboinosinic-polyribocytidylic acid and its component homopolymers. *Proc. Nat. Acad. Sci. U.S.A.* **67**:180-184.