Induction of Interferon in Human Subjects by Poly I:C (35454)

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The induction of interferon and of resistance to viral infections by complexed (double-stranded) polyribonucleotides, including poly I:C (rI_n:rC_n), was first reported from these laboratories in 1967 (1–4) and has since been confirmed by many workers (5–8). The further objective of our group has centered on the application of interferon inducers such as poly I:C to human and animal medicine. A previous report by our group (9) recorded the induction of circulating interferon in a small number of patients with far-advanced cancer given poly I:C by the intravenous route. The present report describes the detailed laboratory findings in studies of 20 human subjects given the polynucleotide. The findings in tests for induction of antibody against polynucleotides are also included. The detailed clinical results will be recorded elsewhere.[1]

Materials and Methods. Poly I:C. The individual polynucleotides used in the studies were prepared by Drs. E. E. Harris and C. H. Hoffman, Merck Developmental Research. Four preparations of poly I:C were employed. The individual lots of polynucleotides which were used were confined to sharply defined physical and biological characteristics so that the results obtained in continuing studies would be consistent and reproducible. The sedimentation coefficients (in phosphate buffered saline) of the polyriboinosinic (rI_n) and polyribocytidylic acids (rC_n) were 9.2 to 14.5 and 6.1 to 9.2, respectively. Sedimentation coefficients reported in prior publications (9, 10) were performed in cacodylate buffer and hence the values reported here are slightly different from those given earlier. The T_m range of the complexed polynucleotides (rI_n:rC_n) was 64–65°. The relative viscosity ranged from 3.1 to 3.9. The four preparations of complex polynucleotides had sedimentation coefficients of 16.2 to 18.0 (in phosphate buffered saline). The poly I:C lots were prepared aseptically in pyrogen-free phosphate buffered saline solution. The hypochromicity after complexing ranged from 37.1 to 39.0%. The hyperchromicity on thermal dissociation ranged from 72.0 to 73.5%. All preparations were active in submicrogram amounts in inducing host resistance to viral infections *in vitro* and in microgram amounts *in vivo*.

Assay for interferon activity. Samples of human serum were assayed for interferon content on monolayers of primary human amnion cells by the plaque reduction method using vesicular stomatitis virus (VSV) challenge. One unit of interferon was the reciprocal of the highest initial dilution of serum which, in 1-ml amount, effected at least a 50% reduction in VSV plaques compared with untreated controls.

Characterization of human interferon. Trypsin sensitivity. Serum diluted 1:4 using serum-free Eagle's minimal essential medium (Grand Island Biological Co.) was incubated for 18 hours at 35° with crystalline trypsin (Sigma Chemical Co.) solution at 50 μg/ml and pH 7.0–7.2. The trypsin activity was neutralized by adding an equivalent amount of soybean trypsin inhibitor which was devoid of antiviral activity. Serum samples without added trypsin or inhibitor were tested in a similar manner for control purpose. The final samples were assayed for interferon activity after adjustment to equivalent serum concentrations.

pH stability and dialyzability. Serum sam-

[1] Young, C. W. *et al.*, in preparation.

TABLE I. First Interferon Response Among 20 Cancer Patients Given Poly I:C Intravenously.

Patient			Poly I:C dose	Interferon titer of serum (units) at hr:					
No.	Age	Histopathologic diagnosis	(μg/kg)	0	2	12	24	48	72
614230	61	Reticulum cell sarcoma	2	0	8	0	0	—[a]	—
254292	28	Hodgkin's disease	25	0	—	—	32	—	—
275260	31	Nasopharyngeal carcinoma	50	0	0	8	8	0	0
253197	56	Carcinoma of breast	50	0	—	4	8	8	0
272979	57	Mesothelioma	100	0	8	16	8	8	8
270269	23	Acute monoblastic leukemia	500	0	—	—	8	—	—
271745	30	Hodgkin's disease	1000	0	0	—	8	—	—
265468	56	Carcinoma of cervix	2000	0	0	0–4	8	4	0
616649	66	Carcinoma of prostate	4000	0	0	4	8	—	0
614634	58	Carcinoma of bladder	25	4	—	8	16	—	—
613294	18	Embryonal carcinoma	100	4	—	—	16	4	8
613148	25	Carcinoma of testis	500	4	—	—	16	—	—
116592	50	Leukosarcoma	500	4	4	8	8	4	8
672043	24	Leukosarcoma	1000	4	4	8	16	—	—
273384	45	Malignant melanoma	10	4	4	0	0	—	—
613039	52	Laryngeal carcinoma	25	0	0	0	0	0	0
273050	22	Hodgkin's disease	25	0	0	—	0	—	—
273282	40	Cervical carcinoma	50	0	0	0	0	0	0
212266	25	Hodgkin's disease	50	0	—	—	0	—	—
274987	39	Malignant melanoma	2000	0	—	0	0	0	—

[a] Not done.

ples were dialyzed at 4° for 24 hr against 100 vol of buffer at the pH levels indicated in Table IV, followed by similar dialysis against buffer at pH 7.0. The final samples were assayed for interferon activity.

Host species specificity. Interferon titers of human sera were determined in plaque reduction assays employing VSV in primary human amnion, RK_{13} (stable rabbit kidney cell line) and primary mouse embryo cell cultures.

Viral species specificity. Interferon titers of human sera were determined in plaque reduction assays in primary human amnion cell cultures employing vesicular stomatitis, Semliki Forest, and Sindbis viruses.

Sedimentability. Human serum samples were centrifuged at 35,000 rpm (approx 82,000g) in a Spinco 40 rotor for 1 hr at 4°. The supernates and the untreated serum samples were assayed for interferon activity.

Antibody against polynucleotides. Tests for presence of complement-fixing (CF) antibody against poly I:C and heat-denatured deoxyribonucleic acid (DNA) were carried out by the complement-fixation procedure of Osler *et al.* (11). The positive antisera used in the tests for control purpose were prepared in rabbits employing procedures described by Nahon *et al.* (12) and Plescia *et al.* (13). Calf thymus DNA was denatured by heating at 1 mg/ml concentration in phosphate buffered saline solution (0.15 *M* NaCl, 0.006 *M* sodium phosphate, pH 7.0) at 120–125° for 15 min followed by rapid chilling in an ethanol–dry ice bath. The denatured DNA was mixed with an equal volume of methylated bovine serum albumin (1 mg/ml in phosphate buffered saline solution) and 1-ml volumes were given weekly for 5 successive weeks by the intravenous route to 4- to 5-lb New Zealand white rabbits. One additional injection was given 2 weeks later. The rabbits were bled prior to injection (control serum) and 1 week following the last DNA-albumin injection. Antisera against poly I:C were prepared by the same method as against DNA. In the tests for nucleic acid, 4 units of positive antiserum and 5 units of complement were employed.

TABLE II. Repeated Interferon Induction Following Intravenous Injection of Poly I:C in 3 Patients.

Patient no.	Poly I:C dose (μg/kg)	Successful induction	Time interval since last induction (days)	Interferon titer of serum (units) at hr: 0	2	12	24	48	72
272979	100	First	—	0	8	16	8	8	8
	150	Second	7	0	4	8	8	4	0
	200	Third	7	4	4	8	16	4	16
	250	Fourth	7	0	4	8	8	8	4
	300	Fifth	7	4	—[a]	—	16	8	4
	350	Sixth	7	4	4	—	8	8	4
613294	100	First	—	4	—	—	16	4	8
	200	Second	6	4	0	—	16	4	8
	400	Third	7	0	4	—	8	4	—
253197	50	First	—	0	—	4	8	8	—
	50	Second	3	0	—	—	16	—	—

[a] Not done.

The assay was highly sensitive and capable of detecting approximately 0.025 to 0.05 μg/ml of either denatured DNA or poly I:C. In the tests for antibody in patient's serum, 8 units of denatured DNA (0.2 μg), or 4 units of poly I:C (0.1 μg) were used with 5 units of complement. All sera were inactivated at 56° for 30 min and were adsorbed with washed sheep erythrocytes prior to test. Appropriate positive and negative rabbit sera were included in all the tests for control purpose. All CF tests were read for 50% hemolysis end point.

Clinical trials. Several studies have shown that poly I:C may exhibit therapeutic and prophylactic activities against virus-induced and transplant tumors in animal species (14–17). Such activity may be due to induction of interferon, to stimulation of ordinary immune mechanisms, or to other undefined activities of the poly I:C. The trials in man were designed to test for activity against cancer in clinical regimens which were appropriate to that purpose. Samples of blood were taken prior to, during, and at various intervals following poly I:C treatment for purpose of assay for interferon. Such retrospective surveillance permitted definition of certain of the characteristics of interferon induction, refractoriness, and duration of interferon response following primary and repeat injection of poly I:C in human subjects. All patients had far advanced cancer.

Results. Interferon induction in man. Twenty subjects with advanced cancer of various histopathologic type were given 2 to 4000 μg/kg of poly I:C by the intravenous route. Serum samples for interferon assay were taken prior to injection and at various time periods up to 72 hr thereafter. Table I shows that 14 of the patients responded with production of antiviral substance in the serum which was identified as interferon in selected serum samples (see below). Nine of the patients responded from a zero initial titer and 5 from a preexisting titer of 4. Six failed to respond. Whether there was induction, and magnitude and duration of response did not appear to be strongly dose-related within the dose range of drug tested. Interferon appeared as early as 2 hr after poly I:C injection and persisted in some patients for at least 72 hr. Peak production of interferon was usually at 12 to 48 hr after drug administration.

Reinduction. Persons given poly I:C in repeated injections were capable of reinduction after an adequate time period. Unfortunately, the data were insufficient to fix precisely the number of injections and amount of poly I:C required to bring about hyporesponsiveness and to define the duration of the refractory period. Table II shows, however, that successful reinductions were obtained

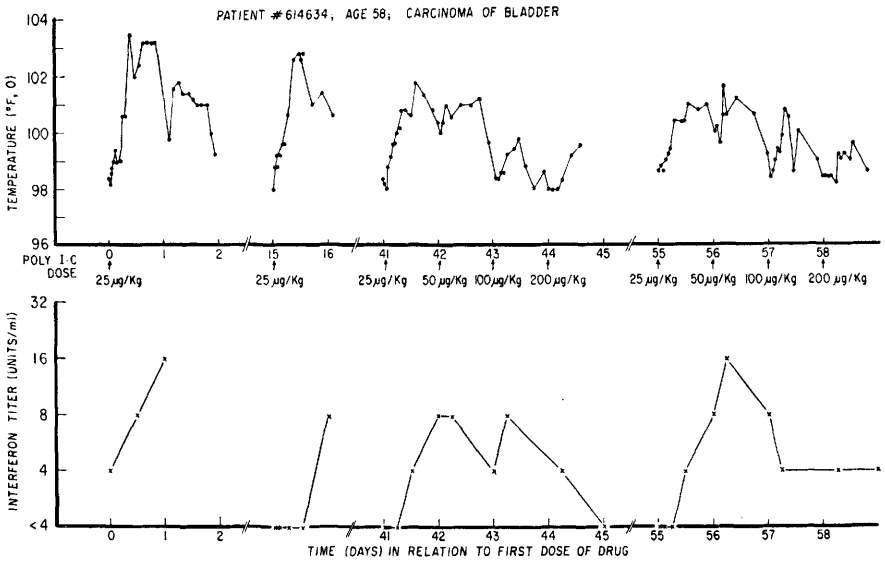

FIG. 1. Febrile and interferon responses in a human subject to repeat intravenous administration of poly I:C.

upon repeated injection at 6- to 7-day intervals in two patients (272979, 613294) and within 3 days in one patient (253197). Patient 614634 (Fig. 1) showed hyporesponsiveness to interferon induction after the fourth dose (200 µg/kg) of poly I:C in a series of increasing daily doses (days 41–45). The patient became fully responsive again after a 10-day period, after which the same pattern of induction and subsequent refractoriness was repeated (days 55–58). It appeared that although refractoriness was established in man, the duration was relatively short.

Clinical response. The most consistently repeatable response to intravenous injection of poly I:C in man was a febrile reaction (see example in Fig. 1). The temperature elevation in the patients ranged from 0 to 7°F, reaching a peak 6–15 hr after injection. The febrile response generally occurred together with interferon induction, though the responses occurred independently in some patients. There was no correlation with dosage. Extensive laboratory observations revealed no disturbance of liver, kidney, or bone marrow functions. No effects on clotting factors were noted and no limiting toxic effect was noted in clinical symptomatology. The detailed findings will be reported elsewhere.[1]

Lack of antipolynucleotide antibody response. Antibody response to poly I:C or to DNA in the human subjects was considered possible. Serum samples available from 19 patients were tested for CF antibody against poly I:C and heat-denatured DNA according to the procedure outlined in the section on Materials and Methods. Serum samples were available from 7 patients who had received multiple doses of poly I:C and who were bled during a sufficient time period to reasonably permit an antibody response. The time periods for poly I:C injection and for serum sampling are given in Table III. Six of the 7 patients showed an interferon response. None of these 7 patients, nor any of the remaining 12 patients, showed antibody against poly I:C or to heat-denatured DNA when tested at serum dilutions of 1:5 and greater.

Identification of interferon in human sera. The antiviral activity in selected serum samples of patients who had received poly I:C was identified as interferon based on the usual five criteria, *viz.*, narrow host species specificity, broad spectrum antiviral activity, destruction by trypsin, stability over a broad range of pH, and a macromolecule of relatively small size as indicated by lack of sedimentation at 82,000*g* for 1 hr and nondialyzability. Representative findings are shown in

TABLE III. Time Periods of Poly I:C Injection and Blood Tests for Antibody Against Poly I:C and DNA in Human Subjects.[a]

Patient no.	Doses of poly I:C		Time periods (days) for serum sampling after initial poly I:C dose
	Amounts (μg/kg)	Time period (days)	
613294	100, 200, 400	6	0, 3, 6, 7, 9, 13, 14, 15
271745	500, 1000, 1000	7	0, 3, 7, 8
272979	50, 100, 150, 200	41	0, 1/4, 1, 41
116592	500, 100 T.D.	17	0, 1, 2, 3, 17, 18
616649	4000, 6750	2	0, 1, 2, 3, 5, 6, 7
614634	25, 25, 25, 50, 100	55	0, 16, 17, 41, 43, 59
273282	50, 100, 25	3	0, 1, 3, 13

[a] None of the subjects exhibited antibodies to poly I:C or denatured DNA when tested by complement fixation at a serum dilution of 1:5.

Table IV.

Discussion. The intravenous injection of poly I:C in man results in the appearance of an antiviral substance in the serum. This substance has been identified as interferon based on nondialyzability, pH stability, sensitivity to trypsin, host species specificity, broad antiviral spectrum and nonsedimentability at 82,000*g*. The response in man to poly I:C with the production of circulating interferon resembles that of the rabbit, mouse, and rat and is unlike the monkey, dog, and guinea pig which fail to develop detectable circulating interferon following intravenous injection of poly I:C.

TABLE IV. Characterization of Interferon in Sera from Human Subjects Given Poly I:C Intravenously.

Assay and treatment	Interferon titer (units)
Host species specificity	
Primary human amnion	32, 16
Rabbit kidney line RK_{13}	<4, <4
Primary mouse embryo	<4, <4
Antiviral spectrum	
Vesicular stomatitis virus (VSV)	16
Semliki forest virus	32
Sindbis virus	16
Trypsin sensitivity (50 μg/ml)	
Treated	<4
Untreated control	16
pH stability range (including dialysis)	
pH 2.0	8
pH 10.0	8
pH 7.0 (control)	8
Sedimentation	
82,000*g*, 1 hr	16, 8
Untreated control	16, 8

The peak of interferon production occurred at 12-48 hr after intravenous injection of poly I:C. Circulating interferon was detectable as early as 2 hr and as late as 72 hr after a single injection of poly I:C. In this small group of patients, there was no correlation of interferon titer with age or with dosage. The peak titers of interferon achieved in this study were not as high as those reported by Hill *et al.* (18) but this may reflect assay differences.

Repeated successful induction of interferon was demonstrated in certain patients when the polynucleotide was given at 6–7-day intervals and in one patient at 3-day intervals. Repeated daily injections did lead to a condition of hyporesponsiveness (refractory period) which had disappeared after a 10-day rest period. It was not possible to define the refractory period more precisely under the conditions of these regimens. The mouse (our own unpublished data) given a high dosage of poly I:C (200 μg/mouse) showed refractoriness to interferon induction for a period of 4 days. DuBuy *et al.* (19) have demonstrated that the onset of the hyporeactive state and its duration is dose-dependent in mice. Smaller doses (25 μg/mouse) caused refractory periods of only 12–18 hr.

The paucity of clinical reactions in man,

other than fever, following intravenous administration of poly I:C in doses as high as 4 mg/kg is surprising in view of the toxic manifestations observed in dogs, rabbits, mice, and rats (10, 20–22). The increases in serum SGOT and alkaline phosphatase which were seen in dogs and rabbits did not occur in the human subjects. The leukopenia which was noted in dogs and rabbits was not seen in man. Though poly I:C may be regarded as relatively nontoxic for man, cognizance must be taken that the patients in the present study did have far advanced cancer and this might have obscured minor toxic effects which might be observed in healthy human subjects.

Poly I:C has been found to be a potent antigen in NZB, NZW, and B/W strains of New Zealand mice (23, 24). It induces the formation of antibodies to poly I:C in NZB and NZW mice but not to native or denatured DNA. These mice are uniquely susceptible to the formation of antinucleic acid antibodies, especially the B/W female strain which forms such antibodies spontaneously. NZB and B/W mice also develop a spontaneous autoimmune disorder which closely resembles human systemic lupus erythematosus. The development of autoimmune disease (glomerulonephritis) was accelerated in B/W female mice by multiple injections of poly I:C. Poly I:C may act as an immunologic adjuvant in these mice since polynucleotides have been shown to have such activity by Braun and Nakano (25). It is noteworthy in this context that sera from certain patients with systemic lupus erythematosus have been reported to contain antibodies that react with double-stranded polynucleotides (23, 26). We have shown in our laboratory (unpublished) that poly I:C, given parenterally without carrier protein or adjuvant, is antigenic in New Zealand white rabbits, inducing the formation of antibodies to poly I:C, poly I and to other double-stranded ribonucleic acids but not to denatured DNA. On the other hand we have been unable to elicit antibody formation in ICR mice to either double-stranded RNA or denatured DNA by parenteral injections of poly I:C (without carrier protein or adjuvant). Talal and Steinberg (24) have reported similar experiences with C_3H/He, $C_{57}B1/6$ and BALB/c mice. The human subjects in our clinical trial did not demonstrate antibodies to poly I:C or denatured DNA initially and did not develop such antibodies after treatment with poly I:C. Within the limits of the trial, human subjects, therefore, behaved like ICR, C_3H/He, $C_{57}B1/6$, and BALB/c mice and not like NZ mice or rabbits. Caution must be used in interpreting these results because of possible immunologic deficiencies in patients with cancer.

Summary. Poly I:C (rI_n:rC_n), a synthetic double-stranded polynucleotide previously demonstrated to be a potent inducer of interferon and host resistance to viral infection in cell culture and in animals, has been successfully used to induce interferon in human beings. Fourteen of 20 patients with advanced cancer developed interferon after a single intravenous administration of poly I:C. The interferon was identified by the usual criteria of pH stability, host species specificity, broad antiviral spectrum, inactivation by trypsin, and nonsedimentability under defined conditions. Several of the patients were capable of repeated induction of interferon by poly I:C at intervals of 3 to 7 days. Evidence of refractoriness to induction occurred only after repeated daily injections of poly I:C. The only consistent clinical manifestation of poly I:C administration was a febrile response. None of the patients tested developed demonstrable CF antibodies against either poly I:C or denatured DNA during the course of treatment.

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