INDUCERS OF INTERFERON AND HOST RESISTANCE, II. MULTISTRANDED SYNTHETIC POLYNUCLEOTIDE COMPLEXES

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Communicated by Max Tishler, June 5, 1967

Studies in our laboratories during the past several years have been directed toward finding a highly active interferon inducer which would be worthy of clinical evaluation. It was found in these studies that capacity for inducing interferon by ribonucleic acids depends on (*a*) multistrandedness of RNA and (*b*) in certain instances, freedom from inhibitory proteins.

The present paper reports the discovery that certain multistranded polynucleotide complexes are highly active in microgram amounts in inducing interferon and host resistance *in vivo* and *in vitro*. Other reports in the present series describe the isolation and demonstration of similar activity of double-stranded RNA from *Penicillium funiculosum* (HeI-RNA)[1] and from reovirus type 3.[2]

Materials and Methods.—(1) Polynucleotides were purchased from Mann Research Laboratories, New York, New York; Miles Laboratories, Elkhart, Indiana; and Sigma Chemical Co., St. Louis, Missouri. The designated polymers were mixed in equimolar concentration in phosphate-buffered saline, pH 7.0 (0.006 *M* sodium phosphate, 0.15 *M* NaCl), and complex formation, as determined by hypochromic effect, developed within the first minutes after mixing.[3, 4] Homopolymer solutions were stored at −20°C. In this paper the nomenclature of Inman and Baldwin will be followed.[5] Thus, I, C, A, U, X, G, and DHU will signify homopolymers of hypoxanthine (for which the ribonucleoside is inosine), cytosine, adenine, uracil, xanthine, guanine, and dihydrouracil. CU, IU, AC, GU, and AG will signify copolymers of C and U, I and U, A and C, G and U, and A and G. Octanucleotides of A and U are signified by 8A and 8U. A dephosphorylated trinucleotide of I is indicated by 3I. CpA, CpG, GpU, and CpC signify dephosphorylated dinucleotides of C and A, C and G, G and U, and C and C. *A colon between polynucleotides signifies that complex formation has occurred, as shown by hypochromic effect.* A mixed solution of polymers showing no hypochromic effect is indicated by (+) between polynucleotides. (2) *Assays* for interferon induction in rabbits, for interferon characterization, viz., species-specificity, trypsin sensitivity, molecular weight, and isoelectric point, and for induction of host resistance in mice were described previously.[1] Other pertinent methods are presented in the text.

Results.—(1) *Induction of interferon in rabbits by complexed polynucleotides:* (a) *Induction:* Table 1 shows that I:C induced interferon in rabbits when as little as a 0.5-μg dose was given intravenously per rabbit. A:U and I+CpC were less active and induced interferon less consistently in repeat tests. The following were found to be inactive at doses between 50 and 200 μg per rabbit: G, X, I, C, A, U, DHU, CU, IU, AC, GU, AG, 8A, 8U, 3I, CpC, CpA, CpG, GpU, cytosine, cytidine, cytidylic acid, hypoxanthine, inosine, inosinic acid, guanine, A+C, C+U, I+U, X+C, A:I, G+C, A+DHU, A+8U, U+8A, 8A+8U, C+3I, I+CpA, I+GpU, C+GpU, I+CpG, C+CpG, I+cytidylic acid, C+inosinic acid,

TABLE 1

INDUCTION OF INTERFERON IN RABBITS BY INTRAVENOUS INJECTION OF COMPLEXED POLYNUCLEOTIDES

Polynucleotide	Dose* per rabbit (μg)	Interferon titers of individual rabbits
I:C	2.0	>640, >640
I:C	0.5	20, 40
I:C	0.25	<5, <5
I only	25	<5, <5
C only	20	<5, <5
A:U	200	10, 20
A:U	100	10, 20
A:U	25	20, 40
A:U	6.25	5, 10
A only	200	<5, <5
U only	200	5, 5
I+CpC	100	>640, >640
I+CpC	10	<5, <5
CpC only	50	<5, <5
Untreated controls	0	<5, <5

* Total weight of substance given in single 0.5-ml dose intravenously.

I+cytidine, C+inosine, I+cytosine, C+guanine, C+hypoxanthine, AC+IU, AG+CU, and GU+AC.

(b) *Kinetics of induction in rabbits:* The kinetics for interferon induction by I:C are shown in Figure 1. A significant level of interferon appeared by one hour, reached a peak shortly thereafter, and declined significantly by four to six hours.

(2) *Characterization of interferon induced in rabbits by complexed polynucleotides:* The viral inhibitory substances in the sera of rabbits injected with the active complexed polynucleotides were identified as interferon based on their biological and biochemical properties. (a) *Host species-specificity:* The rabbit serum interferon noted in Table 2 was prepared as previously described.[1] Mouse interferon was prepared from groups of 25 mice each, 16–18 gm in weight, injected intravenously with 10.5 μg of I:C in 0.2-ml doses. The mice were bled in two hours, the sera pooled, sterilized by ultraviolet irradiation, and titrated by the plaque reduction technique against VSV challenge. Where the presence of residual inducer in serum samples might interfere with the species-specificity determination, the interferon was purified by chromatography on CM-Sephadex.[6] It is seen in Table 2 that resistance was evoked in the homologous cell systems by the corresponding interferons but not in heterologous cell culture systems. (b) *Trypsin sensitivity:* Table 3 shows that the activity of the rabbit serum interferon was destroyed by trypsin. (c) *Molecular weight:* The molecular weight of rabbit serum interferon induced by I:C was determined by gel filtration through Sephadex G-200 and was found to range from 49,000 to 52,000.[1] (d) *Isoelectric point:* The isoelectric point of rabbit serum interferon induced by I:C was measured by chromatography on CM-Sephadex and was found to be pH 6.8–6.9.[1]

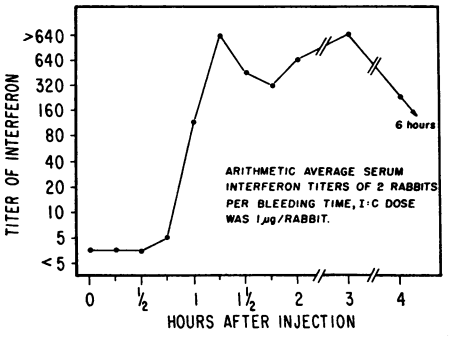

FIG. 1.—Kinetics of serum interferon induction after single I:C intravenous injection.

TABLE 2

SPECIES-SPECIFICITY OF INTERFERON INDUCED IN ANIMALS BY INTRAVENOUS INJECTION OF COMPLEXED POLYNUCLEOTIDES

Inducer	Anima	Interferon Titer Based on Plaque Assay in Cell Cultures: Rabbit kidney	Mouse embryo	Chick embryo
I:C	Rabbit	>2048	<16	ND
I+CpC	"	>2048	<8	ND
A:U	"	512	<8	<32
I:C	Mouse	<16	32	ND
I:C	"	ND	128	<8

TABLE 3

TRYPSIN SENSITIVITY OF INTERFERON* IN SERUM OF RABBITS INDUCED BY INTRAVENOUS INJECTION OF COMPLEXED POLYNUCLEOTIDES

Interferon inducer	Treatment	Interferon titer
I:C	None (control)	256
	Trypsin† (50 μg/ml)	<16
I+CpC	None (control)	1024
	Trypsin (50 μg/ml)	8

* Partially purified by CM-Sephadex chromatography.
† Crystalline, 4 hr at 35°C.

(3) *Induction of interferon in vitro by I:C:* Trypsinized spleen cells from six-week-old rabbits were suspended in Eagle's spinner medium (Grand Island Biologicals, Grand Island, New York) containing 10 per cent agamma calf serum. Incubation was in 25-ml Bellco spinner flasks at 35°C at a concentration of about 10^7 cells per ml. Sampling for interferon production was made at several time intervals after addition of the polynucleotides. The samples were centrifuged at 2000 rpm for ten minutes to remove cells and titrated for reduction of VSV plaque formation on monolayers of RK-13 cells (stable line rabbit kidney cells). RK-13 cells were used because they were not protected against VSV plaque formation by the concentrations of I:C employed, but were protected by rabbit interferon. Table 4 shows that 10.5 μg I:C/ml induced production of interferon by 7.5 hours.

TABLE 4

KINETICS FOR INTERFERON INDUCTION BY I:C IN RABBIT SPLEEN CELL SUSPENSION *in vitro*

Expt. no.	Polynucleotide (conc./ml)	Time of sampling (hr)	Interferon titer
1	I:C (10.5 μg)	0	<2
		2.0	32
		7.5	128
		19.0	128
	Untreated control	7.5	8*
2	I:C (10.5 μg)	7.5	>512
	I (11 μg)	7.5	32*
	C (10 μg)	7.5	16*
	Untreated control	7.5	16*

* Nonspecific inhibitory substances which appear upon incubation.

I and C polymers alone were inactive. Titers of inhibitor of 1:8 to 1:32 were occasionally found in this culture system without treatment. The identity of the interferon was established by tests for species-specificity, trypsin-sensitivity, isoelectric point, and molecular weight. These results will be presented in more detail elsewhere.[7]

TABLE 5

THRESHOLD AMOUNT OF COMPLEXED POLYNUCLEOTIDES REQUIRED TO INDUCE RESISTANCE TO VSV IN PRIMARY RABBIT KIDNEY MONOLAYERS *in vitro*

Polynucleotide	Threshold amount (μg/ml)
I:C	<0.00125
I	>11
C	>10
A:U	0.0015
A	>11
U	>10

(4) *Induction of interference in monolayers of primary rabbit kidney cell culture:* Cell monolayers were incubated for 18 hours with medium containing the polynucleotide under test. After removal of this medium, interference with VSV replication was measured by the plaque-reduction assay.[1] Table 5 shows that extremely small amounts of I:C and A:U were required to effect 50 per cent plaque reduction, whereas individual homopolymers had no effect.

(5) *Induction of host resistance in mice to viral infection by I:C:* The complex induced resistance to both pneumonia virus of mice (PVM) and Columbia SK viruses in mice.[8] Table 6 shows that per cent survival of the mice was greatly enhanced by I:C.

TABLE 6

INDUCTION OF RESISTANCE IN MICE TO VIRAL INFECTION BY I:C

Challange virus	Total dose per mouse (μg)	No. survived/ no. infected	Survival (%)
Columbia SK*	525	12/14	86
	263	14/15	93
	131	12/15	80
	Buffer (control)	2/45	4
PVM†	31.5	18/21	86
	15.8	14/16	88
	7.9	12/20	60
	Buffer (control)	0/17	0

* I and C, each alone, at 550 μg and 500 μg/dose, respectively, were inactive.
† I and C, each alone, at 16.5 μg and 15.0 μg/dose, respectively, were inactive. PVM, pneumonia virus of mice.

(6) *Physical and chemical properties of I:C inducer:* (a) *Formation of I:C and prevention of complexing:* I and C polymers complexed readily as shown by the marked hypochromic effect noted in Figure 2 and elsewhere.[4] The thermal transition mid-point for I:C, as measured in a Beckman DB-G spectrophotometer equipped with a *Tm* analyzer and recorder, was 60.5°C in phosphate-buffered saline, ionic strength 0.1. This is in excellent agreement with the value obtained by Chamberlin and Patterson[9] for I:C. However, when the amino groups of the C were bound by prior treatment with 0.4 per cent formaldehyde (C-HCHO), the mixed polynucleotides were no longer capable of hydrogen bonding and gave an ultraviolet absorption spectrum which was equal to the summation of the individual absorptions of the two polynucleotides (Fig. 2). Whereas 26 μg of I:C readily induced interferon in rabbits, 26 μg of I+C-HCHO solution did not induce interferon. Deamination of C was carried out with 1.0 *M* sodium nitrite at pH 4.0 for one hour at 0°C. Excess nitrous acid was quenched by addition of urea, and low-molecular-weight products were removed by dialysis. The I+deaminated C mix-

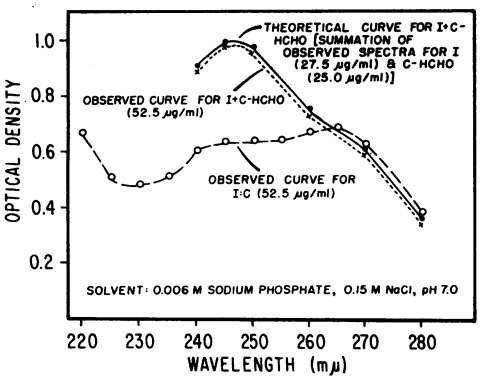

FIG. 2.—I:C formation and its prevention by prior treatment of C with 0.4% formaldehyde.

ture was inactive at 26 μg in interferon induction. Prior treatment of the C (50 μg/ml) with bovine pancreatic RNase (1 μg/ml) for four hours at 25°C effected a degradation of the C to shorter chain lengths as evidenced by increase in optical density during incubation. The RNase-degraded C did not interact with I as evidenced by lack of hypochromicity, and the solution (26 μg) was inactive as an interferon inducer.

(b) *Degradation by RNase:* I:C at 40 μg/ml was incubated in a quartz cuvette at 25°C with bovine pancreatic RNase (0.4 μg/ml) at pH 7.0 and the resulting degradation of the complex was followed by measuring the increase in optical density with a recording spectrophotometer equipped with a controlled heater compartment. Figure 3 shows that I:C was degraded by RNase. Under the same conditions, double-stranded RNA from type 3 reovirus virions was not appreciably affected. The RNase-degraded I:C had reduced capacity to induce interferon in rabbits.

(c) *pH dissociation of I:C:* I (27.5 μg/ml), C (25.0 μg/ml), and I:C (52.5 μg/ml) in phosphate buffered saline, pH 7.0, were titrated with increments of alkali. As shown in Figure 4, an abrupt hyperchromic effect at pH 9.5–10.0 occurred for I:C, but not for I and C alone. Unlike the absorption spectrum at neutral pH, the absorption spectrum of I+C solution at pH 10.8 was identical to that calculated from the uncomplexed I and C at pH 10.8. Thus, at pH 9.5–10.0 the I:C underwent dissociation to form a solution of uncomplexed I and C. Mixtures of I and C at the same concentrations but at pH 7.5 and 10.8 were titrated for interferon induction in rabbits. The material at pH 7.5 gave interferon induction at 0.33 μg while that at 10.8 gave induction in the rabbit at 1.31 μg. This was inter-

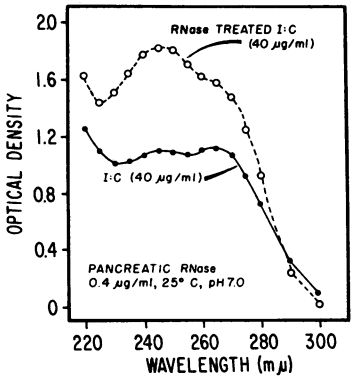

FIG. 3.—Degradation of I:C by RNase.

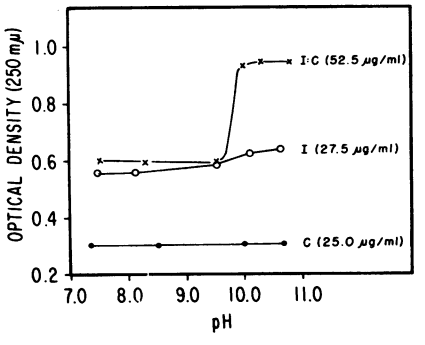

FIG. 4.—Alkaline titration of polynucleotides.

preted to indicate that complexing of I and C did take place in the environment of the rabbit bloodstream but to a lesser degree than when the two substances were premixed at optimal pH. This interpretation was supported by the demonstration that I and C did not detectably form a complex in distilled water. Yet 26 μg of the mixed polynucleotides in distilled water induced interferon in rabbits, suggesting that complexing did take place in the rabbit blood.

Discussion.—The present report shows that microgram amounts of I:C, A:U, and I+CpC induced interferon in rabbits; the I:C being the most active complex found to date. A wide variety of other individual or mixed homopolymers, copolymers, oligonucleotides, nucleotides, nucleosides, and free bases were inactive. The interferon induced in rabbits by I:C exhibited characteristics usually ascribed to interferon, including species-specificity, trypsin sensitivity, isoelectric point near neutral, and relatively low molecular weight (49,000–52,000). I:C also induced interferon and resistance to virus in cell cultures *in vitro* and resistance against Columbia SK and PVM infections in mice. A:U also induced resistance in cell culture. Complexing as well as polymer length was important to biological activity. The I:C complex was rapidly formed at room temperature in salt solution but was not formed in distilled water. The thermal transition mid-point for I:C was 60.5°C, indicating a helical structure of I:C as described by Chamberlin and Patterson.[9] Effective complexing was prevented by binding of amino groups of C by formaldehyde or removal by deamination. Biological activity was destroyed and complex formation prevented by shortening the chain length of C by treatment with RNase prior to mixing. I:C was dissociated at high pH (9.5 or >), reducing biological activity. I:C was vulnerable to degradation by RNase at 25°C with reduction of biological activity, in contrast to double-stranded RNA from type 3 reovirus virions.

An essential element for biological activity for I:C, for A:U, and possibly also for I+CpC was complex formation. The chemical constitution of the homopolymer was important since other mixtures of homopolymers failed to show biological activity, even though hypochromicity was exhibited (viz., A:I). The requirement of double-strandedness for biological activity was previously shown for the RNA (HeI-RNA) isolated from extracts of *P. funiculosum*[1] and for the uniquely double-stranded RNA from type 3 reovirus virions, presented in another publication by us.[2]

Summary.—Complexes of polyinosinic acid and polycytidylic acid (I:C), polyadenylic acid and polyuridylic acid (A:U), and a mixture of polyinosinic acid and cytidylyl-cytidine (I+CpC) were active in microgram amounts in inducing interferon in rabbits. A variety of homopolymers and copolymers tested singly and in combination were inactive, as were a large number of oligonucleotides, dinucleotides, nucleotides, nucleosides, and free bases. I:C also induced resistance to viral infection in mice and evoked interferon and resistance to viruses in cell culture. Details relating to the conditions for complexing, the nature of the complexes, and the requirements for biological activity are presented. This demonstration of the biological activity of complexed homopolynucleotides is new and perhaps provides a new basis for understanding induction of interferon and resistance to viral infection.

Valuable technical assistance was given by J. Liedtke, K. Young, and J. Armstrong.

[1] Lampson, G. P., A. A. Tytell, A. K. Field, M. M. Nemes, and M. R. Hilleman, these PROCEEDINGS, **58**, 782 (1967).

[2] Tytell, A. A., G. P. Lampson, A. K. Field, and M. R. Hilleman, these PROCEEDINGS, in press.

[3] Felsenfeld, G., and A. Rich, *Biochim. Biophys. Acta*, **26**, 457 (1957).

[4] Davies, D. R., and A. Rich, *J. Am. Chem. Soc.*, **80**, 1003 (1958).

[5] Inman, R. B., and R. L. Baldwin, *J. Mol. Biol.*, **5**, 172 (1962).

[6] Lampson, G. P., A. A. Tytell, M. M. Nemes, and M. R. Hilleman, *Proc. Soc. Exptl. Biol. Med.*, **121**, 377 (1966).

[7] Field, A. K., A. A. Tytell, G. P. Lampson, and M. R. Hilleman, in preparation.

[8] These tests were carried out by Dr. M. Nemes.

[9] Chamberlin, M. J., and D. L. Patterson, *J. Mol. Biol.*, **12**, 410 (1965).