Induction of the Interferon Mechanism by Single-Stranded RNA: Potentiation by Polybasic Substances (34310)

A. BILLIAU,[1] C. E. BUCKLER, F. DIANZANI,[2] C. UHLENDORF, AND S. BARON

U. S. Department of Health, Education and Welfare, Public Health Service, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20014

The single-stranded synthetic RNAs, polycytidylic (poly-rC) and polyinosinic (poly-rI) acids, can induce interferon-like resistance in various cell species (1). However, individual preparations varied widely in their activity. Possible explanations for this variation include differences in absorption by cells and destruction by cellular ribonuclease (1).

In order to test this hypothesis we investigated the possibility that substances which are known to improve cellular uptake of RNA, or to inhibit RNase activity, would enhance the resistance-inducing effect of single-stranded poly-rI, poly-rC, and poly-rI:rC. Specifically we tested methylated albumin, protamine sulfate, and DEAE-dextran which were reported to both enhance uptake (2–7) and to inhibit degradation by ribonuclease (3, 7). Neomycin and streptomycin which inhibit ribonuclease activity (8) were also tested.

Materials and Methods. Compounds. Single-stranded polyinosinic acid (poly-rI) and polycytidylic acid (poly-rC) were purchased from three different manufacturers: (i) P-L Biochemicals, Inc., Milwaukee, Wisconsin, (ii) Miles Chemical Co., Elkhart, Indiana, and (iii) Biopolymers, Chagrin Falls, Ohio. The compounds were dissolved in phosphate buffered saline containing 100 units/ml of penicillin and 100 μg/ml of streptomycin. Stock solutions containing 1000 μg/ml were kept frozen at —20°. Double-stranded poly-rI:rC was prepared by mixing equal volumes of stock solutions of poly-rI and poly-rC and adding $MgCl_2$ up to a final concentration of 5×10^{-3} *M* Neomycin sulfate (Mycifradin®) was purchased from Upjohn Co., Kalamazoo, Michigan and streptomycin from E. R. Squibb & Sons, New York, New York. Methylated albumin was prepared according to Mandell and Hershey (9). Protamine sulfate was obtained from Nutritional Biochemicals Co., Cleveland, Ohio. DEAE-dextran of molecular weight 2×10^6 was produced by the Pharmacia Co.

Assay of cellular resistance to viral infection. In order to test induction of cellular resistance by synthetic RNAs, sets of 2 tissue culture tubes were incubated with medium containing the appropriate concentration of the compounds. Unless indicated otherwise, the enhancers were added to the tissue cultures in twice the final concentration desired (0.5 ml/tube). The inducers (poly-rI, poly-rC, or poly-rI:rC) were added in an equal volume, also in twice the final concentration. Appropriate control tubes were included, i.e., tubes without inducers or enhancers, and tubes with only the enhancers. After 24 hr incubation at 37°, the cultures were decanted, washed once or twice with Earle's balanced salt solution and challenged with vesicular stomatitis virus (VSV) or Semliki Forest virus (SFV) at an approximate multiplicity of infection of 20 PFU/cell. After an adsorption period of 1 hr, the excess virus was removed by washing the cultures three times with 2 ml of Earle's balanced salt solution. The cultures were refed with maintenance medium and further incubated for another 16–24 hr. Each set of cultures was then harvested. VSV yields were determined by plaque assay on mouse CCL-1 cells and SFV yields were determined by plaque assay on

[1] Aspirant of the Belgian NFWO. Present address Rega Instituut, University Leuven, Belgium.

[2] University of Siena, Siena, Italy.

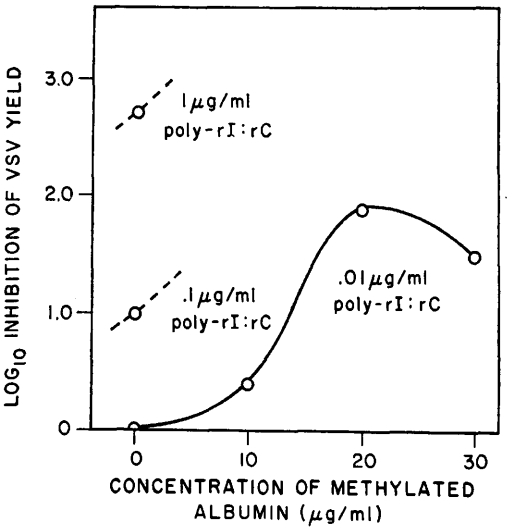

FIG. 1. Influence of methylated albumin on the induction of viral resistance by poly-rI:rC in rabbit kidney cells.

chick embryo cells. Resistance to virus infection was measured as decrease of virus yield on tubes treated with the compounds as compared to those not treated. Reduction in virus yield of a 0.5 $\log_{10}$ is significant to the $p<0.05$ level. For the study of DEAE-dextran the procedure was modified to an 8 hr preincubation period with DEAE-dextran before application of the RNA inducers. In all cases the polybasic substances alone had no effect on virus replication.

Results. Influence of methylated albumin, protamine and DEAE-dextran on the induction of cellular resistance of VSV by synthetic RNAs. In a first experiment, three sets of rabbit kidney cultures were fed with medium containing respectively 0.01, 0.1, and 1 μg/ml of poly-rI:rC. Three other sets were treated with 0.01 μg/ml of poly-rI:rC plus 10, 20, or 30 μg/ml of methylated albumin. Development of cellular resistance was assayed as indicated in "Materials and Methods." Methylated albumin by itself did not noticeably affect the virus yield. As shown in Fig. 1 methylated albumin increased the biological activity of poly-rI:rC by 20 times.

In a similar way we tested whether marginally active preparations of single-stranded poly-rC or poly-rI would become active in the presence of methylated albumin. It was found that 100 μg/ml of poly-rC alone (P-L Biochemicals, Inc.) was needed to induce measurable resistance (0.8 $\log_{10}$ reduction of VSV yield). When tested in the presence of 20 μg/ml of methylated albumin, 10 μg/ml of poly-rC could induce measurable resistance (0.8 $\log_{10}$), while 30 or 100 μg/ml induced very strong resistance (2.3 and 2.6 $\log_{10}$, respectively). Poly-rC purchased from Miles Chemical Co. or from Biopolymers were completely inactive when tested at the 100 μg/ml level. In the presence of methylated albumin, Miles' poly-rC developed only suggestive activity (0.4 $\log_{10}$) while the Biopolymers material induced measurable resistance (0.8 $\log_{10}$).

In the case of poly-rI 20 μg/ml of methylated albumin failed to convert inactive batches of 100 μg/ml of poly-rI (P-L Biochemicals, Miles and Biopolymers) into active inducers; 30 μg/ml of methylated albumin potentiated 100 μg/ml of poly-rI only from P-L Biochemicals. However at 50 or 100 μg/ml, methylated albumin formed heavy precipitates with poly-rI and exerted a cytopathic effect on the cultures.

The influence of protamine sulfate was tested in different cell types; African green monkey kidney (AGMK) cells, chick-, mouse-, and rat embryo cells. Duplicate cultures were incubated with either poly-rI:rC at final concentrations of 0.01, 0.1, or 1 μg/ml or poly-rC at final concentrations of 10 or 100 μg/ml, either with or without protamine (200 μg/ml) included in the medium. Table I shows that AGMK-, chick embryo-, and mouse embryo cells could not be induced by 1 μg/ml of poly-rI:rC. However when protamine was included, strong resistance was observed, even in the cultures which only received 0.1 μg/ml of poly-rI:rC. Rat embryo cells were readily induced by all concentrations of poly-rI:rC tested. However, at 0.1 μg/ml, poly-rI:rC only induced a small amount of resistance while in the presence of protamine a strong antiviral state developed. Protamine alone had no effect.

Table I also shows that there was no antiviral effect of a preparation of poly-rC (P-L Biochemicals) at 100 μg/ml and actually marginal enhancement of virus replica-

TABLE I. Influence of Protamine on the Development of Resistance in Different Cell Cultures Treated with Poly-rC or Poly-rI:rC.

Inducer	Conc (μg/ml)	Resistance (log_{10} inhibition of VSV yield)									
		Rabbit kidney		Chick embryo		Mouse embryo		Rat embryo[a]		African green	
		Control	Protamine	Control	Protamine	Control	Protamine	Control	Protamine	Control	Protamine
Poly-rI:rC	1	>2.2	>2.2	0	2.2	0.3	2.6	>2.2	>2.3	0.3	1.8
	0.1	0.9	>2.2	−0.3	0.7	0	1.1	>2.2	2.3	0	1.2
	0.01	0.1	1.9	−0.1	−0.1	0	0.3	0.6	1.7	0	0.5
Poly-rC (P-L Biochemicals)	100	−0.6	2.2	0.2	0.3	0.4	2.4	0.4	2.0	0.2	0
	10	−0.3	0.1	—	—	−0.1	0.1	−0.2	0	—	—

[a] Secondary rat embryo cultures.

tion in rabbit kidney cells. When protamine was included in the medium, mouse embryo-, rabbit kidney-, and rat embryo cells developed moderately high resistance.

Table II summarizes experiments performed with DEAE-dextran as the enhancing agent. As shown, this polybasic substance also enhanced the antiviral effect of poly-rC in a manner similar to its previously reported enhancement of the antiviral effect of poly-rI:rC. Toxicity of DEAE-dextran for other cell cultures precluded experiments with other cell types.

Influence of the basic antibiotics, neomycin and streptomycin, on the induction of cellular resistance to VSV by synthetic RNAs. Single-stranded poly-rI and poly-rC from three different manufacturers were examined for their resistance inducing effect in rabbit kidney cultures, either in the presence or absence of neomycin (Table III). At a concentration of 100 μg/ml none of the RNAs induced measurable resistance to VSV infection. When neomycin (300 μg/ml) was added all the compounds, except Biopolymers' poly-rI became highly active. Figure 2 shows that the optimal enhancing concentration for neomycin was 200–300 μg/ml. Figure 2 shows the results of two experiments with overlapping points.

Double-stranded poly-rI:rC was also en-

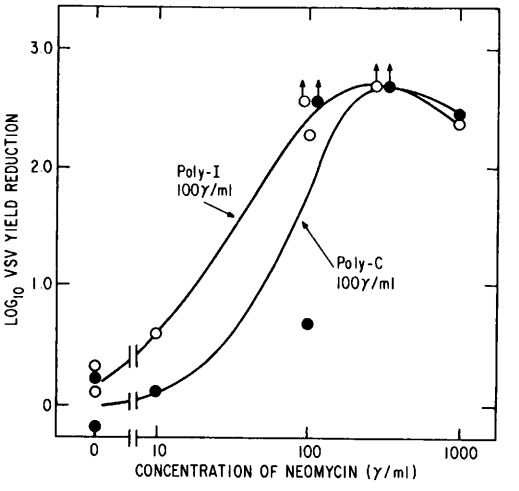

FIG. 2. Influence of neomycin on the development of resistance in rabbit kidney cells treated with single-stranded polynucleotides.

TABLE II. DEAE-dx Enhancement of Resistance Induced by Poly-rI:rC or by Poly-rC.

		Log_{10} inhibition of virus yield			
		SFV in CCL-1 mouse L cells		VSV in mouse embryo cells	
Inducer	Conc (μg/ml)	Control	DEAE-dx[a]	Control	DEAE-dx[a]
Poly-rI:rC	10.0	0	2.1	0.2	2.2
	1.0	0	1.5	0.1	1.9
	0.1	ND	ND	0.1	1.1
Poly-rC	100.0	0.1	1.4	0.1	2.2
	50.0	0	0.9	0.3	0.7
	10.0	0	0	0.3	0.3

[a] DEAE-dx was kept in contact with the cells (400 μg/ml) for 8 hr at room temperature. Then the cultures were washed and treated with poly-rI:rC or poly-rC overnight.

hanced by neomycin. In the presence of neomycin, only 0.00012 μg/ml of poly-rI:rC were needed to induce resistance in rabbit kidney cells, while in the absence of the antibiotic the smallest active dose was 0.01 μg/ml.

In the next experiment the possibility was tested that cell species which are relatively insensitive to the resistance-inducing effect of poly-rI:rC and almost completely refractory to single-stranded RNAs, would become sensitive when the inducers were applied in the presence of neomycin. Table IV shows that both in chick- and mouse embryo cells, neomycin enhanced poly-rI:rC. It is also clear that poly-rC (P-L Biochemicals) which, in the absence of neomycin, was inactive at a concentration of 100 μg/ml, became an active inducer in the presence of neomycin. Only 10–30 μg/ml of poly-rC was needed to induce significant virus resistance in insensitive cells in the presence of basic substances.

Like neomycin, steptomycin is an aminosugar and it also inhibits RNase activity (8) although to a lesser degree than does neomycin. Therefore it seemed not unlikely that it might also enhance the resistance-inducing capacity of nucleic acids. All tissue culture media used in the reported experiments contained streptomycin in a concentration of 100 μg/ml. The single-stranded RNA preparations used in this experiment induced significant antiviral activity under these standard condtions. Streptomycin (100 μg) did

TABLE III. Influence of Neomycin (300 μg/ml) on the Development of Resistance in Rabbit Kidney Cultures Treated with Single-Stranded Polynucleotides.

			Log_{10} inhibition of VSV yield	
Inducer	Manufacturer	Conc (μg/ml)	Control	Neomycin
Poly-rC	P-L Biochemicals	300	1.3	>2.7
		100	0.2	>2.7
		30	—0.1	1.7
		10	0	0.2
	Miles Chemical Co.	100	—0.1	1.6
	Biopolymers	100	—0.1	0.8
Poly-rI	P-L Biochemicals	100	0.3	>2.7
		10	0	0.6
	Miles Chemical Co.	100	—0.2	2.7
	Biopolymers	100	—0.1	0.1

TABLE IV. Effect of Neomycin (Neo) on the Induction of Resistance to VSV in Chick- and in Mouse Embryo Cells by Poly-rC and Poly-rI:rC.

			Resistance (log_{10} inhibition of VSV yield)		
				Neo (μg/ml)	
Embryo cell system	Inducer	Conc (μg/ml)	Control	300	1000
Mouse	Poly-rC	300	0.1	3.4	—
	(P-L)	100	0.1	3.1	—
		10	0	0.6	—
	Poly-rI:rC	10	2.0	3.1	—
	(P-L)	1	0.2	0.8	—
Chick	Poly-rC	300	0	—	2.5
		100	—0.1	—	1.9
		30	—0.2	—	0.5
		10	—0.3	—	0
	Poly-rI:rC	100	2.9	—	3.1
		10	1.1	—	2.8
		1	0	—	2.7
		0.1	—0.1	—	1.0
		0.01	0	—	0

not enhance the antiviral activity of the single-stranded RNAs. When this concentration was increased to 200 or 1000 μg/ml, both single- and double-stranded synthetic RNAs became markedly more active (Table V).

Influence of enhancers on the production of interferon. In order to help support the assumption that resistance observed in the foregoing experiments was interferon-like, an experiment was designed to measure interferon induction by poly-rI:rC, either in the absence or presence of enhancers. Specifically, sets of rabbit kidney cultures were incubated with poly-rI:rC at a concentration of either 1 or 10 μg/ml. One group of cultures contained no enhancers; a second one contained 200 μg/ml of neomycin and a third one contained 200 μg/ml of protamine. After 3 hr of incubation at 37°, the cultures were washed three times with Earle's balanced salt solution and refed with maintenance medium. The supernatant was harvested for interferon determination after 24 hr more of incubation. Table VI shows that cultures incubated with poly-rI:rC and protamine produced 10 times more interferon than those receiving poly-rI:rC only. Neomycin had a similar, though less pronounced enhancing effect. It is unlikely that sufficient poly-rI:rC remained after washing to confer an interferon-like effect in the harvested fluids. The interferon produced was distinguishable from any possible residual poly-rI:rC left in the cultures by the fact that its antiviral activity

TABLE V. Effect of Streptomycin on the Induction of Resistance to VSV in Rabbit Kidney Cells by Poly-rC, Poly-rI, and Poly-rI:rC.

		Resistance (log_{10} inhibition of VSV yield); streptomycin (μg/ml)		
Inducer	Conc (μg/ml)	100	200	1000
Poly-rI (Miles)	100	0.6	0.6	1.4
Poly-rC (P-L)	100	0.6	1.4	>2.3
Poly-rI:rC	0.1	>2.8	2.3	2.8
	0.01	0.6	0.8	1.5

TABLE VI. Enhancement of Interferon Production by Neomycin and Protamine Sulfate in Rabbit Kidney Cultures Stimulated with Synthetic Double-Stranded RNA (Poly-rI:rC).[a]

Inducer	Conc (μg/ml)	Enhancer		
		None	Neomycin 200 μg/ml	Protamine 200 μg/ml
Poly-rI:rC	10	16[b]	32	160
	1	<5	18	80

[a] Rabbit cultures were incubated for 3 hr with poly-rI:rC with, or without, the enhancers. After three washes the cultures were refed, respectively, with medium containing the enhancers or with control medium. Interferon was harvested after 24 hr more of incubation.

[b] Interferon (units/ml).

was not increased by either neomycin or protamine. Moreover the inhibitor was species specific in that it did not induce resistance in rat cells. Under the same conditions 100 μg/ml of poly-rI or poly-rC induced less than 10 units/ml of interferon. This is consistent with the lower induction of antiviral activity by the single-stranded homopolymers as compared with the double-stranded poly-rI:rC.

Discussion. The present findings demonstrate that the polybasic substances methylated albumin, neomycin, streptomycin, protamine sulfate and DEAE-dextran markedly enhance the induction of the interferon mechanism by single-stranded and by double-stranded synthetic RNAs. These polybasic substances are known to combine with RNA, to protect RNA against ribonuclease (2, 4, 7, 8, 10), and to increase cellular uptake of RNA (2–7). The present information does not allow a choice among these possible explanations for the enhancing effect of the polybasic substances.

The demonstration that all the synthetic single-stranded preparations of poly-rI and poly-rC induced the interferon mechanism in the presence of the polybasic substances is strong evidence against the hypothesis that only double-stranded RNA is capable of inducing the interferon mechanism (11). Further support for the interferon inducing ability of single-stranded RNA comes from our preliminary observation that yeast RNA (Sigma) can induce the interferon mechanism in the presence of polybasic enhancers. Taken together these findings are in agreement with the reports by Isaacs and his coworkers (12–15) and by others (16, 17) that preparations of naturally occuring single-stranded RNA induce interferon and interferon-like resistance. The implication is that single-stranded parental RNA from virus is the stimulus for induction of interferon in infected cells. The difficulties with weak induction and irreproducibility in the previous experimental systems may now be overcome with the use of the polybasic enhancers. It will be of great interest to reexamine Isaacs' proposal that foreign nucleic acids are the essential stimuli for induction of the interferon system. In addition to these theoretical considerations the present findings point to the possibility that the polybasic enhancers may find practical application as adjuncts to RNA for the control of viral infections of animals and man.

Summary. A study was undertaken to determine whether the interferon-stimulating capacity of single-stranded RNA could be enhanced by substances which are known to combine with RNA, to enhance uptake of RNA into cells and to inhibit degradation of RNA by ribonuclease. The results demonstrated that the polybasic substances diethylaminoethyl-dextran (DEAE-dextran), methylated albumin, neomycin, streptomycin, and protamine sulfate markedly enhanced the induction of the interferon mechanism by single-stranded and by double-stranded synthetic RNAs. The demonstration that all the synthetic single-stranded preparations of polyinosinic acid and polycytidylic acid induced the interferon mechanism in the presence of the polybasic substances is strong evidence against the hypothesis that only double-

stranded RNA is capable of inducing the interferon mechanism. In addition to the theoretical implications the present findings point to the possibility that the polybasic substances may be useful to enhance the antiviral action of RNAs for the control of viral infections.

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