SEPHAROSE-BOUND POLY(I).POLY(C) : INTERACTION WITH CELLS AND INTERFERON PRODUCTION

Lucien Bachner**, Erik De Clercq* and Minh Nguy Thang**

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

and

** Institut de Biologie Physico-Chimique, Paris, France.

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Summary : Poly(I).poly(C) covalently coupled to a matrix by one point fixation through its 3' terminal stimulated both antiviral activity and interferon production in primary rabbit kidney (PRK) cells. This effect could not be accounted for by free polynucleotide released from the matrix into the medium. Penetration of the polynucleotide into the cells does not appear to be necessary for interferon production. A limited amount of matrix-bound poly(I).poly(C) was associated with the cells. Since it was sensitive to extraneous ribonuclease treatment, this poly(I).poly(C) was believed to be localized at the cell surface. Preliminary findings suggest that the binding of the polynucleotide to the cell is not directly proportional to the amount of interferon induced.

INTRODUCTION

Poly(I).poly(C) covalently attached to solid carriers, either through the 5' phosphate moiety (1) or by multiple fixation points (2,3), has been reported to stimulate the production of interferon.

However, during the incubation period, various degrees of leakage of the covalently bound complex from the matrix were observed. Thus, it was not ruled out that some soluble material released, and not the bound poly(I).poly(C) itself, was responsible for the induction of interferon. In this communication we present data obtained with another type of matrix bound poly(I).poly(C), namely poly(I) covalently coupled through the oxidized 3' terminal to agarose beads, then hybridized with $[^{14}C]$poly(C), under conditions of minimum release of bound polynucleotides. Comparative results with free and bound poly(I).poly(C) as regards interferon production, antiviral activity and association of the inducer with the cells are presented.

MATERIALS AND METHODS

Synthesis of poly(I).poly(C) and $[^{14}C]$poly(C)

All polynucleotides were synthesized with E.coli pure polynucleotide

phosphorylase covalently attached to agarose beads (Thang, Platel & Bachner, unpublished). The enzyme was purified as previously described (4), except the 55° treatment step. Separate samples of matrix-bound enzymes were used for each type of polynucleotide made and the enzyme could be recycled for repeated preparations. The polymerization was followed by phosphate release and stopped when it attained plateau. The enzyme was separated from the reaction mixture by centrifugation. The polynucleotide was recovered by precipitation with ethanol. Contaminants of nucleoside diphosphate were eliminated by filtration of the polymer solutions through a sephadex G-50 column.

Covalent coupling of polynucleotide to sepharose

Poly(I) was covalently bound through the 3' terminal to the "blocked hydrazide resin" according to Robberson and Davidson (5). An excess of periodate-oxidized poly(I) (57 A_{260} nm units) was added to hydrazide agarose in 0.1M sodium acetate buffer. A rotator allowed gently stirring. The uptake of poly(I) was followed by spectrophotometric measurements of the supernatant solution. After 150 min., the resin was saturated with both covalently coupled poly(I) and some non specific bound polymer. The non-covalently bound poly(I) was washed out with 0.1M bicarbonate pH 9. The resin was finally rinsed with 6 mM phosphate buffer (pH 7) containing 150 mM NaCl. Under these conditions, 14 A_{260} nm units remained attached to the resin as quantified by alkaline hydrolysis of fixed polymer and by subtraction of uncoupled polymer found in the total washes.

Hybridization of poly(C) to sepharose bound poly(I)

Poly(I) and poly(C) or [^{14}C]poly(C) used in this work were first verified to be able to form in solution a 1:1 complex by mixing curves experiments. To a given amount of poly(I) coupled to sepharose, a double amount of [^{14}C]poly(C) was added. The mixture was left at 4° overnight under continuous slow stirring. Then, the free poly(C) was separated by centrifugation and the poly(C) adsorbed to the resin surface but not hybridized to poly(I) was washed out by 0.1M bicarbonate buffer pH 9. The amount of poly(C) remained associated to sepharose-coupled poly(I) was quantified by subtraction of poly(C) found in the total washes estimated by absorbance at 268 nm or by controlled RNase degradations. The bound poly(C) was found to be equivalent to bound poly(I).

No release of the poly(I).poly(C) complex from the resin was detected after standing 24 hr in phosphate-NaCl buffer.

The origin of the products was as follows : IDP and CDP from Yamasa Shoyu, Japan; [^{14}C] CDP from Amersham, Great Britain; actinomycin D and pancreatic ribonuclease (bovine pancreatic ribonuclease A, 5 x crystallized) from Sigma

TABLE I

ANTIVIRAL ACTIVITY AND INTERFERON INDUCTION BY SEPHAROSE-BOUND POLY(I).POLY(C*) IN PRK CELLS

I. Inhibition of vesicular stomatitis virus-induced cytopathogenicity in PRK cells+

	Minimal inhibitory concentration (μg/ml)
Sepharose-bound poly(I).poly(C*)	0.03
Uncoupled poly(I).poly(C)	0.003

II. Interferon production in PRK cells "superinduced" with cycloheximide and actinomycin D‡

	Dose (μg/ml)	Interferon titer (U/ml) Exp. 1	Exp. 2
Sepharose-bound poly(I).poly(C*)	1	300	720
Uncoupled poly(I).poly(C)	1	1000	1000
	10	6000	10000

+ PRK cells were exposed to serial concentrations of the polynucleotides [dissolved in MEM (Eagle's minimal essential medium) for 24 hr at 37°], at which time the cells were challenged with vesicular stomatitis virus (6). Virus-induced cytopathogenicity was recorded 1 day later. The minimal inhibitory concentration corresponds to the concentration required to inhibit virus-induced cytopathogenicity by 50 %.

‡ PRK cells were exposed to the polynucleotide dissolved at the indicated concentration in MEM for 1 hr at 37°, washed (3 x with MEM), treated with cycloheximide (2 μg/ml) for 3 hr, washed again (3 x with MEM), treated with actinomycin D (3 μg/ml) for 30 min., washed again (3 x with MEM) and further incubated with MEM + 3 % calf serum for 20 hr, at which time the supernatant fluid was withdrawn for interferon titration.

Chemical Co., St. Louis, USA; Sephadex G-50 and sepharose 4B from Pharmacia, Sweden.

All tests were performed in primary rabbit kidney (PRK) cell cultures grown to confluency in 60 mm Falcon plastic petri dishes. Antiviral activity was measured in PRK cells challenged with vesicular stomatitis virus, and interferon production was determined in PRK cells "superinduced" with cyclo-

heximide and actinomycin D, as described previously (6). The methodology for the preparation of cell homogenates and determination of acid-insoluble radioactivity has also been described (7).

RESULTS

As shown in Table 1, the duplex formed between [^{14}C]poly(C) and the poly(I) covalently coupled through the 3' terminal to blocked hydrazide sepharose, was quite effective in stimulating antiviral activity as well as interferon production. In this experiment, that corresponded to Exp. 1 in Table II, there was only 1 % of radioactivity material released into the incubation medium during the induction period. This amount of free polynucleotide (circa 0.015 µg/ml), assumed that it was still in duplex form, was largely insufficient to account for the titers of interferon obtained. Thus, the production of interferon observed with sepharose-bound poly(I).poly(C*) (Table I) can be considered as a direct effect of the bound poly(I).poly(C*).

In the next experiments, the site of action of the inducer was examined. Therefore, the association of the matrix-bound poly(I).poly(C*) with the cells was studied. The amount of acid-insoluble radioactivity associated with the cells after 1 hr incubation with sepharose-bound poly(I).poly(C*) corresponded to approximately 0.8 % (Exp. 1), 1.6 % (Exp. 2) and 1.3 % (Exp. 3) of the total amount of acid-insoluble material exposed to the cells (Table II). These amounts of cell-associated radioactivity did not markedly differ from those observed with cells which had been exposed to free poly(I).poly(C*) : approximately 1.7 % (Exp. 2) and 2.1 % (Exp. 3) of the total input material became associated with cells after their contact with free poly(I).poly(C*) (TableII).

That the radioactive material associated with the cells after 1 hr incubation with either free of sepharose-coupled poly(I).poly(C*) did not reflect degraded polymer re-utilized into host cell RNA synthesis was insured by studying the polymer-cell interaction with PRK cells which had been treated with actinomycin D (1 µg/ml) for 1 hr before their exposure to poly(I).poly(C*). No differences in cell-associated radioactivity were found between cells which had been treated with actinomycin D and cells which had not been treated (Table III). At the dosage used, actinomycin D suppressed host cell RNA synthesis (as measured by ^{3}H-uridine incorporation) by approximately 97 %.

To determine whether the radioactive material taken up by the cells after 1 hr incubation with free or sepharose-bound poly(I).poly(C*) was localized at

* Poly(I).poly(C*) refers to poly(I) complexed with [^{14}C]-labelled poly(C).

TABLE II

ACID-INSOLUBLE RADIOACTIVITY ASSOCIATED WITH PRK CELLS AFTER INCUBATION WITH SEPHAROSE-BOUND OR FREE POLY(I).POLY(C*)

		Radioactivity (cpm)				
		Sepharose-bound poly(I).poly(C*)			Free poly(I).poly(C*)	
		Exp. 1	Exp. 2	Exp. 3	Exp. 2	Exp. 3
(1)	Total amount of acid-insoluble radioactivity exposed to the cells	15,129	25,137	28,375	32,594	23,302
	Amount of free poly(I).poly(C*) in (1)	64	272	1,189	31,593	22,452
(2)	Total amount of acid-insoluble radioactivity recovered in supernatant fluid after 1 hr incubation of the cells with poly(I).poly(C*)	15,762	19,737	20,520	23,490	18,509
	Amount of free poly(I).poly(C*) in (2)	221	1,819	1,454	24,075	18,524
(3)	Acid-insoluble radioactivity associated with the cells after 1 hr contact with poly(I).poly(C*)	125	417	370	571	485

PRK cells were exposed to the polynucleotide at 1 µg/ml MEM/petri dish for 1 hr at 37°. All counts are expressed per petri dish. The amounts of free poly(I).poly(C*) in (1) and (2) were determined in the supernatants of samples which had been centrifuged for 15 min. at 2000 rpm. Cell-associated radioactivity was measured after extensive washing of the cells (8 x with MEM). The consecutive washings may have removed some poly(I).poly(C*) material that was too loosely bound to the cells. This would explain the differences in cpm between (1) and (2).

the cell surface or in the interior of the cell, PRK cells were submitted to an additional incubation with pancreatic ribonuclease A after their exposure to poly(I).poly(C*) (Table IV). Cells which had been exposed to sepharose-bound poly(I).poly(C*) lost approximately 80 % of cell-associated radioactivity upon an additional treatment with 100 µg/ml of pancreatic ribonuclease. A similar sensitivity of cell-bound poly(I).poly(C) to extraneous ribonuclease treatment has previously been noted with cells exposed to rabbit red blood cell-bound poly(I).poly(C) (8). However, cells which were exposed to free poly(I).poly(C*)

TABLE III

TOTAL VERSUS ACID-INSOLUBLE RADIOACTIVITY ASSOCIATED WITH PRK CELLS AFTER EXPOSURE TO SEPHAROSE-BOUND POLY(I).POLY(C*)

	Total radioactivity (cpm)	Acid-insoluble radioactivity (cpm)
Total amount of polymer exposed to the cells	8553	8682
Radioactivity associated with normal cells after 1 hr contact with poly(I).poly(C*)	167	153
Radioactivity associated with actinomcyin D-treated cells after 1 hr contact with poly(I).poly(C*)	297	190

PRK cells were pretreated with actinomycin D (1 µg/ml MEM/petri dish) or control medium for 1 hr at 37°, and immediately thereafter exposed to the polynucleotide at 1 µg/ml MEM/petri dish for 1 hr at 37°. All counts are expressed per petri dish. Cells were extensively washed (5 x with MEM) after their incubation with poly(I).poly(C*).

lost very little of their acid-insoluble radioactivity after an additional incubation period with or without pancreatic ribonuclease (Table IV).

DISCUSSION

Poly(I).poly(C) covalently coupled to agarose beads retained a high capacity of inducing interferon production in PRK cells under conditions where only 1 % of the bound polymer was released into the medium. This leakage corresponded to too low a concentration of free poly(I).poly(C) in the medium to account for the interferon induction effect. In chick embryo cells, Taylor-Papadimitriou and Kallos observed even a 20-fold higher interferon production with poly(I).poly(C) bound to sepharose through multiple points attachment, than with free poly(I).poly(C), under conditions where there was 3 % leakage of the bound polymer. The surprising fact reported in their study (3) was the "stability" of poly(I).poly(C) in 0.3M KOH at 37° for 16 hr. This would imply that each nucleotide was covalently bound to the matrix or that the alkaline hydrolysate

TABLE IV

RIBONUCLEASE SENSITIVITY OF ACID-INSOLUBLE RADIOACTIVITY ASSOCIATED WITH PRK CELLS AFTER INCUBATION WITH SEPHAROSE-BOUND OR FREE POLY(I).POLY(C*)

	Radioactivity (cpm)		
	Sepharose-bound poly(I).poly(C*)		Free poly(I).poly(C*)
	Exp. 1	Exp. 2	Exp. 2
Total amount of acid-insoluble radioactivity exposed to the cells	8,120	28,044	23,734
Acid-insoluble radioactivity associated with the cells after 1 hr contact with poly(I).poly(C*)	406	184	273
Acid-insoluble radioactivity associated with the cells after 1 hr contact with poly(I).poly(C*) followed by an additional incubation for 30 min. with :			
MEM	99	168	282
MEM + pancreatic ribonuclease A (100 µg/ml)	80	38	276
MEM + 10^{-3} EDTA	120	134	261
MEM + 10^{-3} EDTA + pancreatic ribonuclease A (10 µg/ml)	83	85	255

PRK cells were exposed to the polynucleotide at 1 µg/ml MEM/petri dish for 1 hr at 37°. All counts are expressed per petri dish. Cells were extensively washed (8 x with MEM) after their incubation with poly(I).poly(C*).

was readsorbed to the agarose. At any event, we conclude that in PRK cells sepharose-bound poly(I).poly(C) as such is able to induce interferon. The superficial localization of the polynucleotide associated with the cells, as indicated by RNase degradation assay, suggests that penetration of the polynucleotide into the cell is not necessary. Hence, the signal of induction is probably received somewhere at the cell surface.

This raises the question as to the nature of such receptor site. Association of a limited amount of polynucleotide with the cell surface might either suggest

that a stable inducer-receptor complex is formed, or simply be interpreted as irrelevant to the interferon induction process. One should keep in mind that non-specific interactions between the cell surface components and polyanions take place easily. The fact that the amounts of polynucleotide, associated with the cells, do not strictly conform to the input concentrations, does not seem to plead in favor of the existence of a stable inducer-receptor complex. The association of the polynucleotide with the receptor site may be weak. More detailed kinetic studies on both polymer-cell interaction and subsequent interferon production will be needed to localize such site(s).

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