A CONTINUOUS HIGH MOLECULAR WEIGHT BASE-PAIRED STRUCTURE IS NOT AN ABSOLUTE REQUIREMENT FOR A POTENTIAL POLYNUCLEOTIDE INDUCER OF INTERFERON

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Received 21 January 1977

1. Introduction

It is generally agreed that one of the most stringent requirements for the interferon inducing capacity of polynucleotide inducers is double-strandedness [1]. This definition is frequently accompanied by other operational criteria, namely high molecular weight, high T_m (thermal stability), resistance to nuclease degradation and presence of 2′-hydroxyl groups. Considering these criteria, one would expect many double-stranded RNA complexes to be equally efficient polynucleotide inducers.

Indeed, complementary polynucleotides duplexes such as poly(A) · poly(U), poly(I) · poly(C) have quite similar helical parameters, the same sugar puckering [2], a sufficient high T_m and they are known to have a high degree of resistance to nucleolytic degradation. Yet, only poly(I) · poly(C) behaves invariably as a potent inducer while poly(A) · poly(U) is definitely less effective. Poly(C) · poly(G) has been found effective by some but not by others. Strandwise rearrangement could be proposed to explain the low activity of poly(A) · poly(U). However, strandwise rearrangement is highly improbable under the ionic conditions and temperature used for interferon induction. Phase diagram of the poly(A) · poly(U) [3] and poly(I) · poly(C) [4] complexes clearly show that, in the domain considered, only double-stranded complexes occur, provided one starts with an equimolar mixture of complementary polynucleotides.

In recent studies [5,6] the double-stranded complexes poly(c^7A) · poly(U), poly(c^7A) · poly(rT), poly(c^7A) · poly(br^5U), poly(L) · poly(C) and poly(L) · poly(br^5C) were found to be inactive in interferon induction, although these complexes partly or completely fulfilled the criteria mentioned above. A very recent investigation [7] of the conformation of polynucleotides and polynucleotide analogs concluded that interferon induction is dependent on the

Abbreviations: poly(C^7A) – poly(7-deazaadenylic acid), poly(L) – polylaurusin, poly(br5U) – poly(5-bromouridylic acid), poly(br5C) – poly(5-bromo-cytidylic acid)

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recognition of a particular spatial and steric organization of a double-stranded RNA. On the other hand, it was reported that 'single-stranded' polynucleotides, in particular poly(I), could, in some conditions, elicit an interferon response [8,9]. Other reports (see Discussion) have emphasized the importance of the poly(I) strand in the poly(I) · poly(C) complex. What structural feature the interferon inducing ability of polynucleotide precisely depends on, has not been established.

In this paper, we describe the properties of two poly(I) preparations which demonstrated an unusually high interferon inducing activity both in vitro (cell cultures) and in vivo (animals).

Methods

2.1. *Polynucleotide synthesis*

Polynucleotides poly(I), poly(C), poly(A), poly(U) and poly(^{14}C) were synthesized by agarose-bound purified *Escherichia coli* polynucleotide phosphorylase (agarose–PNPase) as described previously [10]. The enzyme was prepared by a modified procedure described previously [11].

All batches of poly(I) synthetized in the laboratory were pepared with the same batch of IDP. Poly(I) 09, 10 and 11 were the products of the first, second and third cycle of polymerization catalyzed by the same agarose–PNPase. Poly(I) CV1 and CV2 were the products of the first and second cycle of polymerization catalyzed by another batch of agarose–PNPase. Poly(I) 315, 332 and 338, synthesized by the same methods, were provided by Choay Laboratories, Paris, France. Other polynucleotides were purchased from various commercial sources: Miles Laboratories Elkhart, Indiana, P-L Biochemicals Milwaukee, Wisconsin, Choay Laboratories Paris, France, Mycophage dsRNA (*Penicillium* BRL-5907) was obtained from D. N. Planterose, Beecham Research Laboratories Betchworth, Surrey, England. Bacteriophage f2 dsRNA was supplied by Dr L. Borecky, Institute of Virology, Slovak Academy of Sciences, Bratislava, Czechoslovakia.

2.2. *Serological analyses*

Rabbit antibodies specific for double-stranded RNA were induced by poly(A) · poly(U)-methylated BSA complexes [12] and immuno-specifically purified from precipitates made with poly(I) · poly(C) [13]. Anti-poly(I) antiserum was obtained by similar immunization of rabbits with commercial poly(I) (P-L Biochemicals). Quantitative micro-complement fixation assays were performed as described elsewhere [14].

2.3. *Interferon induction*

Interferon induction in PRK (primary rabbit kidney cells) was measured as described previously [5].

2.4. *Nucleoside diphosphates*

Nucleoside diphosphates IDP, CDP, ADP, UDP, GDP were purchased from Yamasa, Shoyu, Japan. The purety of IDP was controlled in the laboratory by paper chromatography and PEI-cellulose thin-layer chromatography [15]. No contaminant nucleoside diphosphates were detected.

3. Results

3.1. *Interferon induction in PRK cells*

In several experiments carried out in PRK cells over a more than 2-year period, poly(I) 09 and CV2 reproducibly exhibited about one-third of the interferon inducing capacity of poly(I) · poly(C) (table 1).

Table 1
Comparative interferon inducing activity of different polymers in PRK cells superinduced with cycloheximide and actinomycin D

Polymers[a]	Interferon titer[b] (U/ml)
Poly(I) 09	2100 (1000– 6000)
Poly(I) 10	⩽ 10
Poly(I) 11	⩽ 10
Poly(I) CV1	⩽ 10
Poly(I) CV2	2600 (2000– 6000)
Poly(I) 315	⩽ 10
Poly(I) 332	⩽ 10
Poly(I) 338	⩽ 10
Poly(I) P-L	⩽ 10
Poly(A) · poly(U)	1000 (300– 3000)
Poly(I) · poly(C)	7100 (3000–20 000)
Mycophage (Penicillium) dsRNA	110 (60– 200)
Bacteriophage (f2) dsRNA	120 (30– 200)

[a] All polymers were assayed at 10 μg/ml
[b] Mean values (in parentheses, range of individual results) for at least 12 observations (only 3 for mycophage dsRNA and bacteriophage dsRNA)

Table 2
Effect of different treatments on interferon inducing activity of poly(I) 09 in PRK cells superinduced with cycloheximide and actinomycin D

Treatment[a]	Interferon titer (U/ml)
Untreated	3000 (1000–6000)
Dialyzed	3000
Heated at 60°C for 5 min, then cooled	3000
Filtered through millipore (0.025 μM)	3000
Treated with RNAase T_1	< 10
Poly(I) · poly(C) P-L	10 000
Poly(I) · poly(C) P-L, treated with RNAase T_1	6000

[a] Poly(I) 09 was first diluted to 10 μg/ml in Eagle's minimal essential medium (MEM), treated as indicated and then tested for interferon induction. When treated with RNAase T_1, the polymers were first diluted to 10 μg/ml in MEM + 1 mM EDTA containing 0.1 μg/ml RNAase T_1, incubated for 1 h at 37°C and then applied onto the cells.

Both preparations were slightly but consistently more active than poly(A) · poly(U). They were significantly more effective than bacteriophage and mycophage dsRNA. Other poly(I) preparations (10, 11, CV1, 315, 332, 338 and P-L) were devoid of any interferon inducing activity (table 1). However, all poly(I) samples gained full interferon inducing activity when poly(C) was added to form poly(I) · poly(C).

Neither dialysis nor heating followed by rapid cooling affected the interferon inducing capacity of poly(I) (table 2). No loss of activity was noted upon filtration of poly(I) through millipore (pore size 25 nm), suggesting that an aggregated form of poly(I) such as that described by Maurizot and Boubault [17] was not responsible for the activity. Treatment with RNAase T_1 abolished the interferon inducing activity of poly(I).

The two poly(I) preparations which were active in PRK cells have also been shown to induce interferon in various other assay systems: e.g., mouse L cells (in vitro) and rabbits (in vivo) (De Clercq et al., manuscript in preparation).

3.2. *Chemical purity of poly(I)*

The fact that nearly homogenous *E. coli* PNPase, covalently bound to solid support, was used for the synthesis of poly(I) excluded virtually any protein contaminants originating from the enzyme preparation. The enzyme/nucleotide ratio (w/w) was approximately $1/10^4$ and there was no significant loss of enzymatic activity after several cycles of polymerization.

The final product poly(I) did not contain any nucleotide other than IMP, as assessed by the following criteria. Paper chromatography or PEI-cellulose thin-layer chromatography of the alkaline hydrolyzate of poly(I) showed the presence of IMP only, even when 12 A_{260} units of hydrolysis was applied. When the product of complete phosphorolysis of poly(I) catalyzed by polynucleotide phosphorylase in the presence of highly radioactive [^{32}P]orthophosphate was analysed by the same methods, one found only ^{32}[P]IDP. The sensitivity of this technique permitted to ascertain that the purity of poly(I) was greater than 99.5%.

No detectable divalent ions such as Mg^{2+}, Mn^{2+} or Ca^{2+} were found in poly(I) samples by atomic absorption spectroscopy.

3.3. *Physical properties*

All batches poly(I), inducers or not, had the known ultraviolet spectra and CD-spectra. Even when the CD-spectra were recorded by raising rapidly the ionic strength to detect the cryoform of poly(I) [18], using two couples of inducer–non-inducer, namely poly(I) CV2 and 338 and poly(I) 09 and 10, no significant difference could be observed between an interferon inducer poly(I) and a non-inducer poly(I) (fig.1a,b;

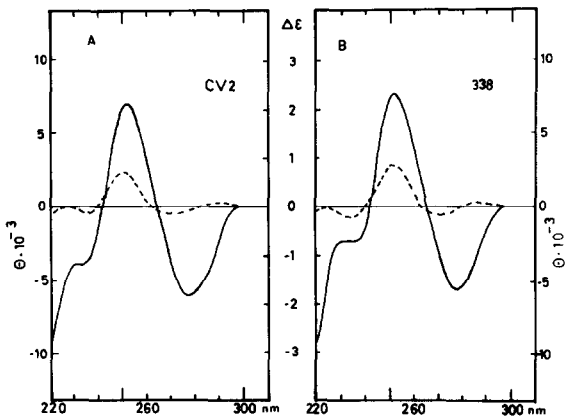

Fig.1. CD-Spectra of poly(I) as a function of ionic strength: (A) poly(I) CV2, (B) poly(I) 338 (——) in water, (- - - -) recorded 1 h after addition of NaCl to 1.0 M.

spectra on poly(I) 09 and 10 are not shown). This indicated that the poly(I) samples examined could be considered as identical as far as their overall structure is concerned. However, conformational heterogeneity has been observed among many poly(I) samples (see below). Further evidence for a structural similarity was provided by studies on complex formation between poly(C) and poly(I), poly(A) and poly(I), and poly(A) · poly(U) and poly(I). The mixing curves obtained comparatively for poly(I) 09 and poly(I) 10 (figs.2a,b,c) clearly pointed to a similarity of their structural features.

The T_m, measured in 1 M NaCl [18], of most poly(I) samples including the interferon inducer poly(I) CV2 and the non-inducer poly(I) CV1, was

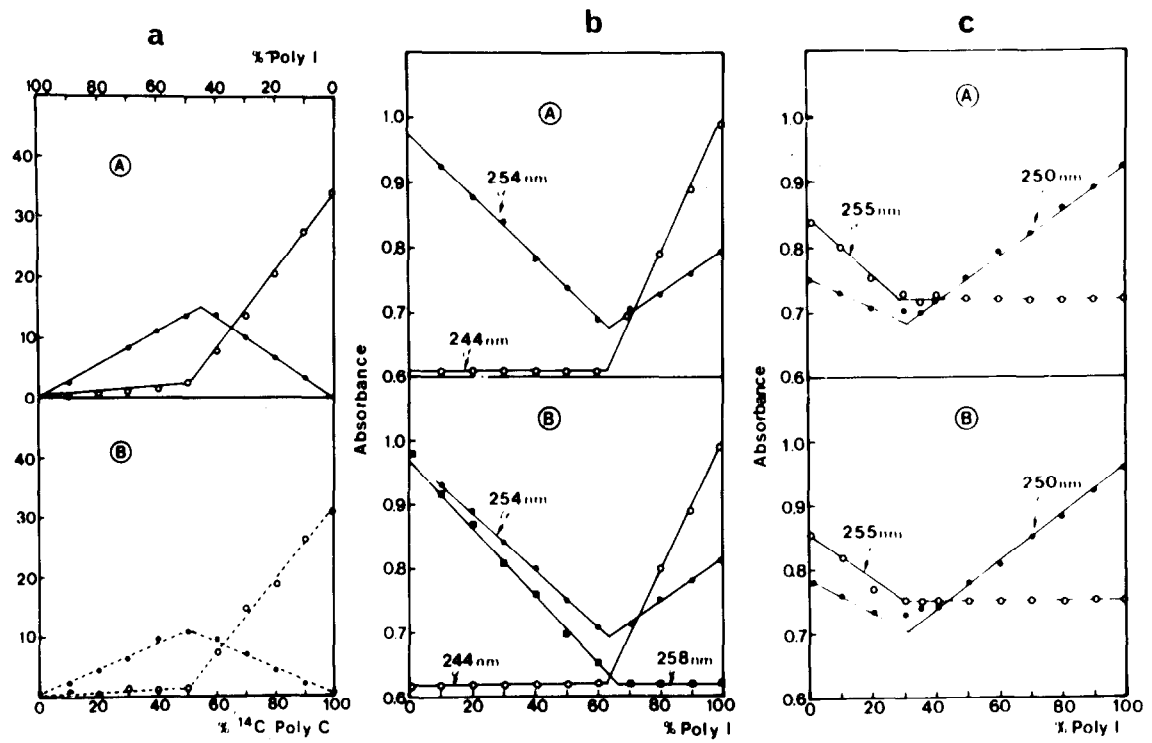

Fig.2. Complex formation between poly(I) and complementary polynucleotide: (A) inducer poly(I) 09, (B) non-inducer poly(I) 10. (a) Poly(I) and poly(C). Poly(I) was mixed with poly(^{14}C) in 6 mM phosphate buffer pH 7, containing 1.5 mM NaCl and equilibrated at 4°C for 20 h. Pancreatic RNAase (from Worthington) was added at a concentration of 1 μg/ml and the mixture was incubated at 20°C for 90 s. The polynucleotides were then precipitated with $HClO_4$ 5% (v/v). The radioactivity in the supernatant corresponded to unpaired poly(^{14}C) degraded by RNAase (o——o) and the cpm in the washed precipitate to that of the poly(I) · poly(^{14}C) complex. (b) Poly(I) and poly(A). Poly(I) was mixed with poly(A) in 10 mM sodium cacodylate pH 7 containing 0.09 M NaCl and equilibrated at 4°C for 20 h. Complex formation was measured at 244 nm, 254 nm and 258 nm. (c) Poly(I) and poly(A) · poly(U). Poly(A) · poly(U) was previously formed under standard conditions. Poly(A) · poly(U) was then mixed with poly(I) as in (b). Triplex formation was monitored at 250 nm and 255 nm.

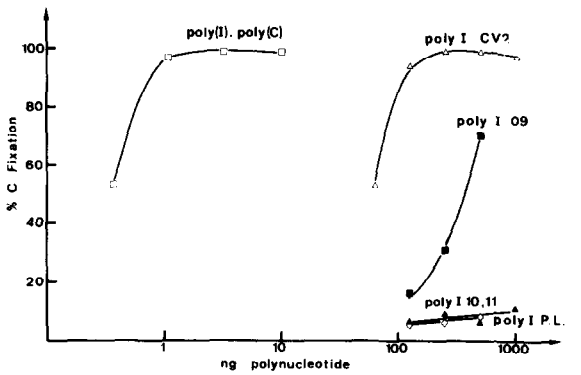

Fig.3. Complement fixation of varying quantities of inducer and non-inducer poly(I) with immunospecifically purified antibodies to double-stranded RNA (final dilution 1/300).

around 42°C although one non-inducer [poly(I) 338] had a T_m = 45°C.

3.4. *Reactions with antibodies*

Significant differences were observed in the recognition of our poly(I) samples by antibodies to either double-stranded RNA or to poly(I). The inducer poly(I) CV2 and 09 did react with antibodies to double-stranded RNA, while non-inducer samples did not (fig.3). Much more poly(I) CV2 than the standard poly(I) · poly(C) was required for a given level of complement fixation. Even higher concentrations of poly(I) 09 than that of poly(I) CV2 was needed for a significant complement fixation. The meaning of these differences will be discussed in a next communication (B. D. Stollar et al., manuscript in preparation). From a comparison of these amounts, it appeared that about 1.5% of the poly(I) CV2 reacted as if it were double-stranded. In view of the high interferon inducing activity of this material, this result raised the question of whether only 1.5% of the molecules of the poly(I) are particularly efficient in induction while most of the structure is identical with non-inducer poly(I), or whether the whole poly(I) CV2 population has a different structure, with some local regions of helical configuration appropriate for these antibodies.

Experiments with anti-poly(I) antibodies indicated that, in fact, a large part of the poly(I) CV2 population differed from non-inducer samples. In complement fixation assays, non-inducer poly(I) preparations gave 50–90% complement fixation with a 1/3000 final serum dilution, while poly(I) CV2 required four-times as much serum to give even 30–50% complement fixation. For further exploration of this question, the IgG fraction of anti-poly(I) antiserum was prepared and covalently linked to Sepharose 4B to provide an affinity column. The column was specific for poly(I) and did not bind any of poly(A) · poly(U), poly(I) · poly(C), poly(A), yeast RNA or native or denatured DNA. A column that was able to bind most of a sample of non-inducer poly(I) bound only 20% of a similar sample of poly(I) CV2. Even the bound portion in the latter case was weakly retained, since half of it could be washed off with PBS; only a much smaller fraction of the bound non-inducer poly(I) was washed off with PBS. These results eliminate the possibility that the activity of poly(I) CV2 was due to a 1.5% content of double-stranded molecules in a population otherwise identical with the non-inducers. Details of these experiments will be published elsewhere.

4. Discussion

The structure of poly(I) remains badly understood. It is assumed [18] that this polynucleotide is a single-stranded, rather poorly stacked helix at low ionic strength. At higher salt concentrations (> 0.1 M NaCl), its structure depends on the ionic strength, the divalent ions, the temperature and the time factor. In PBS buffer and MEM medium, at least part of the poly(I) should be multi-stranded.

To the best of our knowledge with regard to the mode of action of *E. coli* polynucleotide phosphorylase, it is not conceivable that poly(I) samples, prepared under identical conditions with an unique batch of IDP could have an overall structure different from one preparation to another. However, we did find conformational heterogeneity among poly(I) preparations, as defined by their interaction with anti-dsRNA antibodies (Stollar et al., in preparation).

It may be assumed therefore that the particular structural element in inducer poly(I) which is recognized by anti-dsRNA antibodies and by the hypothetical interferon receptor site, is introduced accidentally in poly(I) after its synthesis. A helical region formed by complementary base-pairing is very

unlikely since biochemical analysis showed more than 99.5% purity in IMP moieties. Also, X-ray diffraction studies (S. Arnott) of the inducer poly(I) 09 did not reveal a pattern different from the usual poly(I) pattern.

In the absence of a precise physico-chemical basis for the particularly high interferon inducing activity of poly(I) 09 and CV2, we tentatively suggest that a portion of the poly(I) could be self-structured in a highly stable conformation. Such conformation may be introduced by local nucleation or covalent linkage between two bases. The bridging agent could be an unknown chemical agent or a photo-chemical product. Thus, a single-stranded poly(I) could be activated* and become an inducer for interferon production according to the following scheme:

(A)

Several reports point to a differential importance of poly(I) and poly(C) in the process of interferon induction by poly(I) · poly(C) [19–24]. When poly(I) and poly(C) are administered separately to the cells, they reunite at the cell-surface [23]. A significantly greater interferon response has been obtained in cell-cultures exposed to poly(I) followed by poly(C) than in cell-cultures exposed to poly(C) followed by poly(I), and this increased interferon response appeared to be related to a firmer binding of poly(I) poly(C) to the cell [24]. These findings suggest that in the induction of interferon by poly(I) · poly(C), poly(I) would play a central role. In fact, poly(C) may be replaced by a distantly related analog (poly(1-vinylcytosine)) without siginificant loss of (in vitro) activity [25].

Accordingly, one could extend scheme (A) to poly(I) · poly(C):

$$\text{poly(I)} \cdot \text{poly(C)} \xrightarrow{x} \text{poly(*I)} \cdot \text{poly(C)} \quad \text{(B)}$$

and postulate that an rI sequence stabilized in a suitable conformation by a factor x fits in the receptor site of the cell membrane and initiates the process of interferon production. The remaining part of the polynucleotide would serve for attachment. Factor x may be a cellular component in the case of interferon induction by poly(I) · poly(C), or, in the case of poly(I) it may be DEAE–dextran, protamine or neomycin [8–9] or nucleation or the hypothetic bridging agent. This hypothesis is now under investigation using model polynucleotides.

Acknowledgements

We thank S. Arnott for the X-ray examination of poly(I) 09, D. Thiele for the atomic absorption spectrometer measurements, and P. F. Torrence and W. Guschlbauer for stimulating discussions and Janine Putzeys for editorial help. This investigation was supported by grants from the French DGRST, Comité Génie Biologique et Médical (Convention No. 75-7-0894) and CNRS, Groupe de Recherche No. 18, the Belgian FGWO (krediet No. 30048.75) and Fonds Derde Cyclus (Katholieke Universiteit Leuven, project no. OT/I/50), and grant No. PCM 76-11496 from the US National Science Foundation.

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