Antiviral activity of polynucleotides: copolymers of inosinic acid and N2-dimethylguanylic or 2-methylthioinosinic acid

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ABSTRACT

Complexes of poly(C) with copolymers of inosinic acid containing various amounts of mismatched bases (m_2^2G or ms^2I) have been examined for direct resistance to virus infection, interferon induction and toxicity in two different cell cultures (primary rabbit kidney cells and mouse L-929 cells). Complexes in which 20 % of the hypoxanthine bases were replaced by m_2^2G or ms^2I were partially active whereas complexes in which 40 % or more of the hypoxanthine bases were replaced by the odd bases were entirely inactive. The decrease in biological activity observed upon intrusion of m_2^2G or ms^2I in the poly(I) strand of poly(I).poly(C) closely paralleled the amount of odd bases introduced irrespective of the system employed to assess the biological activity (resistance to virus infection, interferon induction or toxicity).

INTRODUCTION

The antiviral activity and interferon inducing capacity of polynucleotides depends on a variety of structural features some of which have been fairly well characterized : (1°) a sufficiently high molecular size, (2°) a highly ordered secondary structure, (3°) double-strandedness; (4°) adequate resistance to degradation by nucleases, (5°) free 2'-OH groups, (6°) an intact purine ring (at least for some complexes), and (7°) a C-N glycoside bond (1 and references cited therein, 2-8).

The necessity for a highly ordered secondary structure was originally deduced from the T_m dependence of the antiviral activity of double-stranded RNA complexes (9). Both the concept of a highly ordered secondary structure and of T_m dependence have occasionally been challenged (10,11). Yet, the

Abbreviations used : m_2^2G , N2-dimethylguanylic acid; ms^2I , 2-methylthioinosinic acid; T_m , thermal stability (melting temperature); CPE, cytopathogenic effect; PRK, primary rabbit kidney; MEM, (Eagle's) minimal essential medium; PBS, (Dulbecco's) phosphate buffered saline; CCID₅₀, cell culture infecting dose 50.

necessity for a fully base-paired, hence stable secondary structure can hardly be doubted in view of the remarkable decrease in antiviral activity observed with poly(I).poly(C) upon strand interruption or introduction of odd bases (12). With poly(I).poly(C) the requirements of strand continuity and base-pairing appear to be more stringent for the poly(I) than for the poly(C) strand, since decrease of the size of poly(I) or introduction of odd bases (A, U, G) into the poly(I) strand led to a significantly greater loss of antiviral activity than similar modifications of the poly(C) strand (12-15).

These studies have now been extended to copolynucleotides of inosinic acid (I) containing various ratios of N²-dimethylguanylic acid (m_2^2G) or 2-methylthioinosinic acid (ms^2I). The I, m_2^2G and I, ms^2I copolymers and their mixtures with poly(C) were examined for direct inhibition of viral CPE (cytopathogenicity), induction of interferon and cytotoxicity in either PRK- (primary rabbit kidney) cell cultures or mouse L-929 cell cultures. The interferon induction system (16) was also employed to study the interactions of (1°) poly(I, m_2^2G) and poly(I, ms^2I) with poly(A).poly(U) and of (2°) poly(I) with mixtures of poly(I, m_2^2G) + poly(C) and of poly(I, ms^2I) + poly(C). N²-Dimethylguanine and 2-methylthiohypoxanthine are odd or mismatched bases as compared to hypoxanthine, since they cannot form a hydrogen-bound complex with cytosine (17,18).

MATERIALS AND METHODS

The synthesis and physicochemical characteristics of poly($I_{84}, m_2^2G_{16}$), poly($I_{60}, m_2^2G_{40}$), and poly($I_{31}, m_2^2G_{69}$) have been described previously (17). The synthesis and properties of poly(ms^2I) have also been documented (18). For the synthesis of poly(I_4, ms^2I_1) and poly(I_3, ms^2I_2), 40 μ moles (or 30 μ moles, respectively) IDP and 10 μ moles (or 20 μ moles, respectively) ms^2IDP were incubated together with 6.25 μ moles $MnCl_2$, 62.5 μ moles Tris-HCl (pH 8.5) and 4.8 units of *E. coli* polynucleotide phosphorylase (total volume 12.5 ml) at 60° for 10 h. The mixture was extracted with isoamyl alcohol-chloroform (1:1) and applied to a Sephadex G-25 column. The polynucleotide fraction appearing first was collected and dialysed successively against 0.25M NaCl-0.01M Tris-HCl (pH 7.5), 0.02M EDTA-0.01M Tris-HCl (pH 7.0) and distilled water.

All I, m_2^2G and I, ms^2I copolymers were characterized as having chain lengths of more than 100 nucleotide units: (1°) gel filtration through Sephadex G-25 or G-50 showed the appropriate elution profile and (2°)

alkaline digestion of the product yielded nucleoside/nucleotide at a ratio smaller than 1:100.

Poly(I) and poly(C) were purchased from P-L Biochemicals, Milwaukee, Wisconsin, and poly(A) and poly(U) were purchased from Miles Laboratories, Elkhart, Indiana. Sedimentation values ($S_{20,w}$) : poly(I), 9.0; poly(C), 10.0; poly(A), 8.0; poly(U), 3.5-7.4.

The $I_{m_2}G$ and $I_{ms^2}I$ copolymers were dissolved in 0.1M Tris-HCl, 0.2M NaCl (pH 7.0) at 1 mg/ml and stored at 4°. Poly(I), poly(C), poly(A) and poly(U) were dissolved in Dulbecco's phosphate buffered saline (PBS) at 1 mg/ml. Poly(A).poly(U) was prepared by mixing equal volumes of the poly(A) and poly(U) solution, both at 1 mg/ml, for 1 h at 37°. The stock solutions of poly(I), poly(C) and poly(A).poly(U) were stored at -20°.

Mixtures of poly(C) with poly($I_{84,m_2}G_{16}$), poly($I_{60,m_2}G_{40}$), poly($I_{31,m_2}G_{69}$), poly($I_{4,ms^2}I_1$), poly($I_{3,ms^2}I_2$), poly(ms^2I) or poly(I) were prepared as follows : stock polymer solutions were first diluted to 100 µg/ml in PBS; equal volumes of these solutions were then mixed, incubated for 1 h at 37° and then stored at 4°. All experiments were performed with mixtures which had been kept at 4° for at least 4 days. Hypochromicity (read at 245 nm) of the mixtures poly(C) + poly($I_{84,m_2}G_{16}$), poly(C) + poly($I_{60,m_2}G_{40}$), poly(C) + poly($I_{31,m_2}G_{69}$), poly(C) + poly($I_{4,ms^2}I_1$), poly(C) + poly($I_{3,ms^2}I_2$), poly(C) + poly(ms^2I) and poly(C) + poly(I) was 19.7, 6.1, 0, 15.2, 11.8, 0 and 14.2 %, respectively.

The whole methodology for measuring direct resistance to virus infection and interferon production in PRK cells superinduced with metabolic inhibitors (cycloheximide, actinomycin D) has been described previously (3). The procedure for comparing interferon induction to toxicity in interferon-treated L-929 cell cultures has also been described (2,19,20). Further details are referred to in the footnotes to the Tables.

RESULTS AND DISCUSSION

PRK cells are among the most sensitive cells to assess the antiviral activity and interferon inducing capacity of double-stranded RNAs and their responsiveness is even enhanced if they are treated (superinduced) with cycloheximide and actinomycin D some time after their exposure to the double-stranded RNA (21,22).

In accord with previous results (3), poly(I).poly(C) inhibited vesicular stomatitis virus-induced CPE in PRK cells at a concentration as low as 0.003

TABLE 1

INDUCTION OF RESISTANCE TO VESICULAR STOMATITISVIRUS INFECTION IN PRK CELLS

Polynucleotide	Minimum inhibitory concentration*	
	as such	mixed with poly(C)
Poly(I ₈₄ ,m ₂ G ₁₆)	> 10	0.03
Poly(I ₆₀ ,m ₂ G ₄₀)	> 10	> 10
Poly(I ₃₁ ,m ₂ G ₆₉)	> 10	> 10
Poly(I ₄ ,ms ² I ₁)	> 10	0.3
Poly(I ₃ ,ms ² I ₂)	> 10	> 10
Poly(ms ² I)	> 10	> 10
Poly(I)	> 10	0.003

* Required to inhibit viral CPE by 50 %. The polynucleotides (prepared in MEM at 10, 1, 0.1 ... µg/ml) were incubated on the cells (in tubes at 37°) for 20 h at which time the supernatant fluids were removed and the cells were challenged with vesicular stomatitis virus at 500 CCID₅₀ per tube.

TABLE 2

INDUCTION OF INTERFERON IN PRK CELLS SUPERINDUCED WITHCYCLOHEXIMIDE AND ACTINOMYCIN D*

Polynucleotide	Interferon titer (units/ml)	
	as such	mixed with poly(C)
Poly(I ₈₄ ,m ₂ G ₁₆)	≤ 10	3000
Poly(I ₆₀ ,m ₂ G ₄₀)	≤ 10	≤ 10
Poly(I ₃₁ ,m ₂ G ₆₉)	≤ 10	≤ 10
Poly(I ₄ ,ms ² I ₁)	≤ 10	300
Poly(I ₃ ,ms ² I ₂)	≤ 10	≤ 10
Poly(ms ² I)	≤ 10	≤ 10
Poly(I)	≤ 10	10000

* Cells (in petri dishes) were exposed to 10 µg/ml of the polynucleotides (in MEM) at 37° for 1 h, successively treated with cycloheximide (2 µg/ml) for 3 h and actinomycin D (3 µg/ml) for 30 min, and then allowed to release their interferon into the supernatant fluid.

µg/ml (Table 1) and, at 10 µg/ml, poly(I).poly(C) stimulated the production of 10,000 units/ml of interferon in PRK cells superinduced with cycloheximide and actinomycin D (Table 2). None of the single homo- or copolynucleotides was active in inhibiting viral CPE or inducing interferon at the highest concentration tested (10 µg/ml) (Tables 1 & 2). When mixed with poly(C), only poly(I₈₄,m₂²G₁₆) and poly(I₄,ms²I₁) exhibited an appreciable activity (Tables 1 & 2). Mixtures of poly(C) with either poly(I₆₀,m₂²G₄₀), poly(I₃₁,m₂²G₆₉), poly(I₃,ms²I₂) or poly(ms²I) were definitely inactive.

The extent of the antiviral activity of the poly(I,m₂²G) + poly(C) and poly(I,ms²I) + poly(C) mixtures was the same, whether their antiviral activity was measured by interferon induction (Table 2) or direct inhibition of viral CPE (Table 1). These data add strength to the concept that the antiviral activity of synthetic polynucleotides, at least if they are exposed to the cells ~ 20 h before virus challenge, is fully accounted for by interferon induction (23-25). If these polynucleotides are able to induce resistance to virus infection at concentrations at which they fail to induce demonstrable interferon in the culture fluids, intracellular interferon formation should be considered as the most plausible explanation for their antiviral effects (24,25).

Similar results to those in PRK cells were obtained in L-929 cells primed with homologous interferon (Table 3). L-cells do not respond to the interferon inducing activity of double-stranded RNAs unless they have been pre-treated (primed) with interferon (19 and references cited therein). In these cells mixtures of poly(C) with poly(I₈₄,m₂²G₁₆) and poly(I₄,ms²I₁) were about three to eight times less active in inducing interferon than poly(I).poly(C). All other mixtures were completely inactive (Table 3). Interferon-treated L-929 cells are known to be highly susceptible to the cytotoxic effects of poly(I).poly(C) (2,19,20). As shown in Table 3, mixtures of poly(C) with copolymers of I and m₂²G or ms²I were less toxic for interferon-primed L-929 cells than poly(I).poly(C), and the degree of toxicity they elicited closely paralleled their interferon inducing capacity. Thus, introduction of mismatched bases in poly(I) did not significantly alter the interferon induction/toxicity ratio of poly(I).poly(C) in interferon-treated L-cells. It has also been impossible to uncouple the toxic and interferon inducing properties of poly(I).poly(C) in interferon-treated L-cells by reduction of the molecular size of either poly(I) or poly(C), or both (2).

The interferon inducing activity of poly(A).poly(U) in PRK cells is markedly depressed if poly(I) is added to the cells 1 h before or after, or

TABLE 3
INDUCTION OF INTERFERON AND TOXICITY IN L-929 CELLS
PRIMED WITH INTERFERON*

Mixture of poly(C) with ...	Interferon titer (units/ml)	Toxicity
Poly(I ₈₄ ,m ₂ G ₁₆)	76	++
Poly(I ₆₀ ,m ₂ G ₄₀)	< 3	-
Poly(I ₃₁ ,m ₂ G ₆₉)	< 3	-
Poly(I ₄ ,ms ² I ₁)	35	++
Poly(I ₃ ,ms ² I ₂)	< 3	-
Poly(ms ² I)	< 3	-
Poly(I)	260	++++

* Cells (in petri dishes) were first treated with mouse interferon (100 units/ml) for 16 h at 37°, then exposed to 10 µg/ml of the polynucleotides (in MEM), for 1 h at 37° and further processed for interferon production and toxicity as described previously (19).

TABLE 4
INTERACTIONS AMONG POLYNUCLEOTIDES AS MONITORED BY INTERFERON
INDUCTION IN PRK CELLS SUPERINDUCED WITH CYCLOHEXIMIDE
AND ACTINOMYCIN D

Polynucleotide	Interferon titer (units/ml)	
	mixed with poly(A).poly(U)*	first mixed with poly(C), then poly(I) added
Poly(I ₈₄ ,m ₂ G ₁₆)	200	15000
Poly(I ₆₀ ,m ₂ G ₄₀)	250	7500
Poly(I ₃₁ ,m ₂ G ₆₉)	200	3000
Poly(I ₄ ,ms ² I ₁)	250	3000
Poly(I ₃ ,ms ² I ₂)	450	6000
Poly(ms ² I)	1000	4500
Poly(I)	60	7500
Control	2000	7500

* Poly(A).poly(U) was mixed with either poly(I₈₄,m₂G₁₆), poly(I₆₀,m₂G₄₀), at a final concentration of 10 and 5 µg/ml (in MEM), respectively, incubated for 1 h at 37° and then applied to the cells.

+ Poly(I) was mixed with mixtures of either poly(I₈₄,m₂G₁₆) + poly(C), poly(I₆₀,m₂G₄₀) + poly(C), ... at a final concentration of 5 and 10 µg/ml (in MEM) respectively, incubated for 1 h at 37° and then applied to the cells.

together with the duplex (16). This decrease in activity is due to the formation of the triple-stranded complex poly(I).poly(A).poly(U) as evidenced by ultraviolet mixing curves, thermal melting profiles, sucrose gradient ultracentrifugation and ribonuclease sensitivity experiments (26). Accordingly, poly(I) brought about a significant (30-fold) reduction of the interferon inducing capacity of poly(A).poly(U) when mixed with the duplex (Table 4). Poly(I, m_2^2 G) caused a eight- to ten-fold reduction, irrespective of the I to m_2^2 G ratio of the preparation; poly(I₄, ms^2 I₁) caused also a eight-fold reduction, but poly(I₃, ms^2 I₂) was less effective and poly(ms^2 I) did not cause a significant reduction of the activity of poly(A).poly(U) (Table 4). In as far as interferon induction data can be employed to evaluate interactions among polynucleotides (16), the data presented in Table 4 suggest that all I, m_2^2 G and I, ms^2 I copolymers interact with poly(A).poly(U) but less well than poly(I). The interaction between poly(I) and poly(A).poly(U) has been visualized as the formation of the triplex poly(I).poly(A).poly(U) (23). It should now be verified whether poly(I, m_2^2 G) and poly(I, ms^2 I) are also able to form such triplexes.

Not only triple-strand formations but also displacement reactions can be monitored by interferon induction studies (16) and a typical example of the latter is : poly(A).2 poly(I) + 2 poly(C) $\longrightarrow$ poly(A) + 2 poly(I).poly(C). This displacement reaction and many others have been suggested by interferon induction data (27) and confirmed by either sucrose gradient sedimentation, thermal stability profiles or ribonuclease sensitivity experiments (27-29). The equilibrium of these displacement reactions appears to be shifted towards the formation of the complex with the higher T_m. According to this rule, one may expect that the mixtures of poly(C) with poly(I, m_2^2 G) or poly(I, ms^2 I) which are all less effective than poly(I).poly(C) in stimulating interferon production (Table 2) would attain an activity comparable to that of poly(I).poly(C) upon addition of poly(I). As shown in Table 4, the interferon inducing activity of poly(C) + [poly(I, m_2^2 G) or poly(I, ms^2 I)] + poly(I) mixtures fell within the range of activity of poly(I).poly(C) itself. Thus, all poly(I, m_2^2 G).poly(C) and poly(I, ms^2 I).poly(C) complexes existing at 37°, exchanged their "imperfect" I-strand for intact poly(I), hereby forming the poly(I).poly(C) duplex.

CONCLUSION

Complexes of poly(C) with copolymers of I and m_2^2 G or ms^2 I were all less effective than poly(I).poly(C) in inducing interferon, resistance to virus

infection and toxicity. Only those copolymers in which no more than 20 % of the I-residues were replaced by m_2^2G or ms^2I residues retained a significant part of their activity, when complexed with poly(C). Copolymers in which 40 % or more of the I-residues were substituted by m_2^2G or ms^2I were completely inactive, when mixed with poly(C).

These data emphasize the necessity for a stable secondary, fully base-paired double-stranded structure for the induction of interferon by synthetic polynucleotides. Poly($I_{84}, m_2^2G_{16}$) is the sole I, m_2^2G copolymer which can form a double-stranded complex with poly(C) (17), and, quite in accord with its activity (Tables 1-3), the T_m of poly($I_{84}, m_2^2G_{16}$).poly(C) is only slightly inferior to that of poly(I).poly(C) (in 0.1M Na^+ , 39.5° and 43°, respectively) (17). That poly($I_{84}, m_2^2G_{16}$) is able to form a relatively stable complex with poly(C), that acts as an effective interferon inducer despite the intrusion of mismatched bases, suggests that the m_2^2G residues are retained in the double helical array without forming loops, when they are present in small amounts (ratio $m_2^2G/I \leq 16:84$). Meanwhile, they would stabilize the double helical structure by vertical stacking forces (17). The same interpretation may, of course, apply to poly(I_4, ms^2I_1).

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