ANTI-COMPLEMENT ACTIVITY OF POLYNUCLEOTIDES

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Summary

A biologic assay system, based on complement (C') inhibition, is described to unravel structural differences among polynucleotides. The C' system appears particularly suitable to distinguish (1) homo- from copoly-ribonucleotides, (2) deoxyribo- from 2'-OH and other 2'-modified polynucleotides, and (3) single homopolynucleotides from double- or triple-stranded complexes.

From these studies a number of polynucleotides emerged with potent anti-C' activities, worthy of further investigation. The most active polymers were $(G)_n$ (polyguanylic acid), $(dCcl)_n$ [poly(2'-chloro-2'deoxycytidylic acid)] and $(dUz)_n$ [poly(2'-azido-2'-deoxyuridylic acid)].

INTRODUCTION

Yachnin (1) first noted that $(I)_n$* exhibited strong anticomplementary (anti-C') activity. Other homopolyribonucleotides [$(A)_n$, $(C)_n$, $(U)_n$] were devoid of such activity and $(I)_n$ lost its anti-C' activity when annealed to either $(C)_n$ or $(A)_n$ (1). $(I)_n$ was also found to be an effective in vivo

*Abbreviations: C', complement; $(A)_n$, polyadenylic acid; $(U)_n$, polyuridylic acid; $(C)_n$, polycytidylic acid; $(I)_n$, polyinosinic acid; $(G)_n$, polyguanylic acid; $(X)_n$, polyxanthylic acid; $(rT)_n$, polythymidylic acid; $(br^5C)_n$, poly(5-bromocytidylic acid); $(m^3U)_n$, poly(3-methyluridylic acid); $(dA)_n$, poly(2'-deoxyadenylic acid); $(dT)_n$, poly(2'-deoxythymidylic acid); $(dC)_n$, poly(2'-deoxycytidylic acid); $(dI)_n$, poly(2'-deoxyinosinic acid); $(dG)_n$, poly(2'-deoxyguanylic acid); $(dUf)_n$, poly(2'-fluoro-2'-deoxyuridylic acid); $(dUz)_n$, poly(2'-azido-2'-deoxyuridylic acid); $(dCcl)_n$, poly(2'-chloro-2'-deoxycytidylic acid); $(dCz)_n$, poly(2'-azido-2'-deoxycytidylic acid); $(A,G)_n$, $(U,G)_n$, $(U,I)_n$, $(I,G)_n$, random copolymers of adenylic and guanylic acid, of uridylic and guanylic acid, of uridylic and inosinic acid, and of inosinic and guanylic acid, respectively.

inhibitor of C' activity when administered to rats (2) and it was further ascertained that $(I)_n$ owed its anti-C' activity to a (reversible) inactivation of C'1 and/or an irreversible inactivation of C'4 (2-4).

Yachnin found $(G_{85},U_{15})_n$ five times more potent in inhibiting C' activity than an I,U copolymer of similar base composition (1) and hypothesized from these data that $(G)_n$ may be more potent than $(I)_n$ in anti-C' activity. Yet, $(G)_n$ proved only 1/7 as potent as $(I)_n$ (5), a paradoxical behavior which was ascribed to the multi-stranded structure of $(G)_n$ (5). The possibility that the relative activities of $(I)_n$ and $(G)_n$ may have been influenced by differences in molecular size [$s_{20,w}$ values of the $(I)_n$ and $(G)_n$ preparations employed : 5.6S and 3.2S, respectively] was not considered.

We have now compared the anti-C' properties of a large variety of polynucleotides, including homo(ribo- or deoxyribo-)polynucleotides, copolynucleotides, double- and triple-stranded homopolyribonucleotide complexes, homopolyribonucleotides of varying molecular size and 2'-OH modified homopolynucleotides. These studies are interesting for two reasons. First, they may reveal the existence of new polynucleotides with potent anti-C' activity, which, coupled to other biologic properties of these polynucleotides, might possibly be exploited in certain biomedical situations. Second, the complement system may emerge as a useful biologic probe for identifying structural and conformational differences among polynucleotides.

MATERIALS AND METHODS

The following homo- and copolynucleotides were obtained form Miles Laboratories (Elkhart, Indiana) : $(A)_n$ ($s_{20,w}$ = 7.2S); $(U)_n$ ($s_{20,w}$ 7.4S; $(C)_n$ ($s_{20,w}$ = 5.7S); $(I)_n$ ($s_{20,w}$ = 4.8S); $(G)_n$ ($s_{20,w}$ = 13.3S); $(X)_n$ ($s_{20,w}$ = 4.7S); $(dA)_n$ ($s_{20,w}$ = 3.0S); $(dT)_n$ ($s_{20,w}$ = 5.3S); $(dC)_n$ ($s_{20,w}$ = 8.8S); $(dI)_n$ ($s_{20,w}$ = 3.5S); $(dG)_n$ ($s_{20,w}$ = 5.4S); $(A,G)_n$ (A:G ratio 1.2/1; MW > 100,000); $(U,G)_n$ (U:G ratio 1.2/1; $s_{20,w}$ = 4.1S); $(U,I)_n$ (U:I ratio 1/1; MW > 100,000) and $(I,G)_n$ (I:G ratio 1/1; $s_{20,w}$ = 3.6S). $(I)_n$ preparations of different molecular size ($s_{20,w}$ = 2.5, 4.39, 6.13, 7.94, 10.6 and 12.5S, respectively) were purchased from P-L Biochemical (Milwaukee, Wisconsin). The following polynucleotides were obtained according to previously described methodology : $(br^5C)_n$ ($s_{20,w}$ = 11.5S) (6,7); $(m^3U)_n$ ($s_{20,w}$ =

>6S) (8); $(rT)_n$ ($s_{20,w}$ = 6.9S) (9); $(dUf)_n$ ($s_{20,w}$ = 12.3S) (10); $(dUz)_n$ ($s_{20,w}$ = 12.6S) (11); $(dCcl)_n$ ($s_{20,w}$ = 10.5S) (12) and $(dCz)_n$ ($s_{20,w}$ = 8.6S) (13).

All polynucleotides were dissolved at either 4 or 1 mg/ml in PBS (phosphate buffered saline : 0.14M NaCl, 0.01M Na_2HPO_4, 2mM KH_2PO_4, 2.5mM KCl, 1mM $CaCl_2$, 0.5mM $MgCl_2.6\ H_2O$; pH 7.0) except (br^5C), $(m^3U)_n$, $(rT)_n$, $(dUf)_n$ and $(dUz)_n$, which were dissolved at 1 mg/ml in Tris buffer (0.1M Tris-HCl, 0.2M NaCl; pH 7.0). The resulting solutions were stored at -20° and 4°, respectively. The homopolyribonucleotide complexes were prepared by mixing appropriate stoichiometric quantities of the homopolymers in PBS. These complexes were stored at 4°.

Sheep erythrocytes, rabbit antibody to sheep erythrocytes and guinea pig complement were purchased from B-D Merieux (Marcy-L'Etoile, France), Institut Pasteur (Paris, France) and Difco Laboratories (Detroit, Michigan), respectively.

Bacto-complement (C') was rehydrated by adding the specified amount of Bacto complement reconstituting fluid. A 1:10 dilution of this complement (in PBS) (ca. 160-200 $C'H_{50}$ /ml) was incubated for 1 h at 37° in the presence of varying polynucleotide concentrations as indicated in Table 1. The remaining C' activity was determined by a simple hemolytic assay technique, following the principles of Kabat and Mayer (14). The initial C' sample (1/10 dilution) was further diluted (1/2, 1/4,...), and to 0.25 ml of these dilutions, an equal volume of a 0.5% suspension of sensitised erythrocytes was added, and hemolysis was scored after 30 min incubation at 37°. The hemolytic C' titer was defined as the highest dilution of sample that caused more than 90% lysis of the sensitized red blood cells.

RESULTS

All results are presented in Table 1. In keeping with Yachnin's data (1-5), we found that $(I)_n$ markedly inhibited hemolytic C' activity, whereas $(A)_n$, $(U)_n$ and $(C)_n$ failed to do so, even at concentrations up to 400 µg/ml. Unlike Yachnin's $(G)_n$ ($s_{20,w}$ = 3.2S) (5), the $(G)_n$ preparation used herein ($s_{20,w}$ = 13.3S) proved clearly more potent than $(I)_n$ in blocking C' activity. $(X)_n$ also exhibited anti-C' activity, albeit to a lesser extent than $(I)_n$.

That the molecular size may play a critical role in the anti-C' activity of homopolymers such as $(I)_n$ and $(G)_n$ was ascertained by a comparative investigation of the anti-C' potency of $(I)_n$ preparations of different molecular size. The anti-C' activity slightly decreased when the molecular size decreased from 12.5 to 4.39S but abruptly fell when the molecular size further diminished to 2.5S.

TABLE I. Anti-C' Potency of Polynucleotides

	Hemolytic C' titer			
	C' (diluted 1/10 in PBS) incubated for 1 h at 37° in the presence of varying polynucleotide concentrations* (μg/ml)			
	10	40	100	400
Homopolyribonucleotides				
$(A)_n$	-	-	320	320
$(U)_n$	-	-	320	320
$(C)_n$	-	-	320	320
$(I)_n$	160-320	40	20	<10
$(G)_n$	160	<10	<10	-
$(X)_n$	320	160-320	40-80	<10
$(I)_n$ preparations of different molecular size				
$s_{20,w}$ = 2.5	-	-	160-320	-
4.39	-	-	80	-
6.13	-	-	40-80	-
7.94	-	-	40-80	-
10.6	-	-	40	-
12.5	-	-	20-40	-
Copolyribonucleotides				
$(A,G)_n$	-	40	10	-
$(U,G)_n$	-	-	320	-
$(U,I)_n$	-	-	160-320	-
$(I,G)_n$	-	-	160	-
Homopolyribonucleotide duplexes				
$(A)_n \cdot (U)_n$	-	-	320	-
$(I)_n \cdot (C)_n$	-	-	320	-
$(I)_n \cdot (br^5C)_n$	-	-	320	-
$(A)_n \cdot (X)_n$	-	-	160-320	-
$(U)_n \cdot (X)_n$	-	-	320	-
$(I)_n \cdot (X)_n$	-	-	80-160	-

Table I (continued)

Homopolyribonucleotide triplexes				
$(A)_n \cdot 2\ (U)_n$	-	-	320	-
$(A)_n \cdot 2\ (I)_n$	-	-	160	-
$(A)_n \cdot (U)_n \cdot (I)_n$	-	-	80	-
$(A)_n \cdot (U)_n \cdot (X)_n$	-	-	320	-
Homopolydeoxyribonucleotides				
$(dA)_n$	-	-	320	-
$(dT)_n$	-	-	320	-
$(dC)_n$	-	-	320	-
$(dI)_n$	-	-	320	-
$(dG)_n$	-	-	320	-
Pyrimidine-modified polynucleotides				
$(br^5C)_n$	-	-	320	-
$(m^3U)_n$	-	-	320	-
$(rT)_n$	-	-	320	-
2'-OH modified polynucleotides				
$(dUf)_n$	-	-	160	80
$(dUz)_n$	160	<10	<10	-
$(dCcl)_n$	40	<10	<10	-
$(dCz)_n$	80	40	-	-
$(A)_n \cdot (dUz)_n$	-	320	-	-
Control PBS	320	320	320	320

* For complexes composed of two or three homopolynucleotides the polynucleotide concentration refers to each homopolynucleotide separately.

As shown before (1), U,G and U,I copolymers (with a U/G or U/I base ratio = ∿1) were devoid of anti-C' activity. Though very active in their homopolymer form, inosinic acid and guanylic acid were almost inactive when present in copolymer form. Surprisingly, $(G)_n$ retained a significant part of its activity when adenylic acid was inserted in the G strand.

When complexed to either $(C)_n$ or $(br^5C)_n$ (16), $(I)_n$ completely lost its

anti-C' activity. The complement system would also seem appropriate to monitor $(X)_n$-derived complexes (15) as evidence by the decline in anti-C' potency of $(X)_n$ when mixed with either $(A)_n$, $(U)_n$ or $(I)_n$. In spite of its low thermal stability [40° in 0.15M Na^+ (15)], $(I)_n \cdot (X)_n$ exhibited a reduced anti-C' activity as compared to either $(I)_n$ or $(X)_n$, suggesting that, in our assay conditions, the complex did not dissociate.

As would be anticipated if $(I)_n$ and $(X)_n$ added to $(A)_n \cdot (U)_n$ to form the triplexes $(A)_n \cdot (U)_n \cdot (I)_n$ (16) and $(A)_n \cdot (U)_n \cdot (X)_n$ (17), both $(I)_n$ and $(X)_n$ lost a significant part of their anti-C' properties when mixed with $(A)_n \cdot (U)_n$.

Unlike $(I)_n$ and $(G)_n$, $(dI)_n$ and $(dG)_n$ proved ineffective in inhibiting C' activity, pointing to the importance of the 2'-hydroxyl group in the anti-C' activity of $(I)_n$ and $(G)_n$. It should be recognized, however, that the $(dI)_n$ preparation employed had a rather low sedimentation value (3.5S).

Whereas modifications of the pyrimidine base did not endow $(C)_n$ or $(U)_n$ with anti-C' properties, introduction of an azido or halogeno (chloro or fluoro) substituent into the C-2' position of the ribose moiety of either $(C)_n$ or $(U)_n$ rendered these polymers remarkably effective in inhibiting C' activity. It was further ascertained that the anti-C' activity of $(dUz)_n$ resided in its homopolymer structure, since (i0 $(dUz)_n$ lost its anti-C' activity when annealed to $(A)_n$, and ii) 2'-azido-2'-deoxyuridine (dUz) its 3'-acetyl derivative and 5'-monophosphate were totally devoid of anti-C' activity, even at concentrations up to 1 mg/ml (data not shown).

DISCUSSION

Both interferon induction and reactivity with specific antibody had proven to be useful probes for detecting conformational and structural differences among polynucleotide complexes which are rather difficult to resolve by standard physicochemical means. Both probes readily recognize modifications at the furanose C-2' position of either of the two strands of polynucleotide duplexes, such as $(A)_n \cdot (U)_n$ and $(I)_n \cdot (C)_n$ (18 and references cited therein). In addition, with the aid of interferon induction and/or immunoreactivity one can (i)

distinguish double-stranded RNA and RNA-DNA hybrids (19), (ii) demonstrate double-stranded RNA *in situ* in virus-infected cells (20), (iii) detect odd bases in double-helical RNA (21), (iv) differentiate triple-stranded structures built on $(A)_n$ from others built on $(dA)_n$ (22) and (v) monitor polynucleotide displacement reactions as well as transitions from double-stranded to triple-stranded RNA complexes (8,16,23,24).

As described in the present report the complement system represents a third useful biologic probe to identify specific structural determinants in polynucleotides:

1. Since $(I)_n$, $(X)_n$, $(dUz)_n$ lose their anti-C' activity when complexed tc a complementary homopolymer [*viz.* poly(A)], it appears as though measurement of C' activity may be exploited as a rapid screen for complex formation between complementary homopolymers, provided one of the homopolymers possesses anti-C' activity. As exemplified for the systems $(I)_n + (A)_n \cdot (U)_n$ and $(X)_n + (A)_n \cdot (U)_n$, the C' assay may evaluate whether a given homopolymer is capable of adding to a double-stranded complex to form the triple-stranded derivative. In this sense, the C' assay would seem as useful as interferon induction (23) to detect transition of double- to triple-stranded forms.
2. Other structural determinants that are picked up by the C' assay are molecular size and base heterogeneity. To be fully effective in inhibiting C' activity the polynucleotide should exceed a threshold molecular size of approximately 4-5S. A similar dependence on molecular weight has previously been established for other biologic properties of polynucleotides such as interferon induction (27). As noted before (1), the anti-C' activity of $(I)_n$ and $(G)_n$ is disturbed if odd bases (e.g. uridylic acid) are inserted in the nucleotide strand. Hence, determination of anti-C' activity may provide rapid information regarding the molecular size and homogeneity of newly prepared polynucleotides.

Even if the unjustified assumption is made that the C' inhibiting polynucleotides described herein function at the same site(s) as $(I)_n$, there presently emerges no unifying rationale to explain the ability of a given nucleic acid to

inactivate C'. Thus although $(I)_n$ and $(G)_n$ most likely possess a multistranded (four-stranded) (25) secondary structure under the assay conditions, other inhibitors, such as $(dUz)_n$, do not (11,26). If it were not for the observed C' inhibitory action of $(dCcl)_n$ and $(dCz)_n$, it might be hypothesized that the -NH-C=O functionality as found in the purine ring of $(I)_n$ and the pyrimidine ring of $(dUz)_n$ is of importance. Still an additional complicating factor is the dependence on the substituent at the 2' position; 2'-H substituted polymers are inactive whereas other 2' substituents (N_3Cl) are active as C' inhibitors.

Apart from the fundamental aspects related to the structural parameters that govern the anti-C' activity of polynucleotides, the novel anti-C' compounds reported herein may prove useful in situations where C', in association with other factors, leads to tissue injury (e.g. allograft rejection, inflammatory reactions, autoimmune diseases,etc.). An additional advantage of the 2'-azido and 2'-halogeno polynucleotides is that they inhibit the DNA polymerase activity and other functions of oncornaviruses (29,30). This is in contrast with the commonly used immunosuppressive agents which are known to increase susceptibility to virus infection, and, in some cases, even activate tumor virus expression (31).

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