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An Integrated and Comparative Study of the Antiviral Effects and Other Biological Properties of the Polyinosinic Acid-Polycytidylic Acid and Its Mismatched Analogues

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SUMMARY

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Extensive investigations were made of the biological properties of the polynucleotide duplex, $rI_n \cdot rC_n$, along with two modified duplexes having mismatched base pairs, $rI_n \cdot r(C_{12},U)_n$ [as well as $rI_n \cdot r(C_{13},U)_n$] and $rI_n \cdot r(C_{29},G)_n$ [as well as $rI_n \cdot r(C_{20},G)_n$]. These duplexes were studied using a variety of biological systems to determine their efficacy in producing antiviral activity (e.g., interferon) relative to their accompanying secondary biological properties (e.g., acute toxicity, pyrogenicity, and mitogenic activity). These duplexes were studied on a comparative basis in the mouse for their ability to elicit protection against a lethal viral challenge, their acute toxicity, and their relative mitogenic effects on splenic cells. In the intact rabbit, circulating interferon and pyrogenicity were studied, and complementary studies were done with rabbit kidney cells in culture. In human neonatal fibroblasts in culture, these duplexes were studied for their antiviral protection and relative levels of interferon production. Our results indicate that the mismatched polynucleotide duplexes are comparable in their antiviral properties to $rI_n \cdot rC_n$ but possess much less pronounced secondary effects than the $rI_n \cdot rC_n$ molecule. The utility of these duplexes in elucidating mechanisms of biological responses to

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double-stranded RNA is discussed along with the presentation of a theoretical framework for design and development of polynucleotide duplexes having a therapeutic efficacy greater than $rI_n \cdot rC_n$.

INTRODUCTION

The effectiveness of the double-stranded synthetic polynucleotide duplex, $rI_n \cdot rC_n$,[2] as an antiviral substance, specifically as an interferon inducer, has been recognized for about 8 years (1–5). However, the potential use of $rI_n \cdot rC_n$ as a chemotherapeutic agent against viral disease has been constrained by the other biological effects, many of which can be considered "toxic" (4, 6).

Briefly, the other biological effects include pyrogenicity in the rabbit (7), diminution of hematopoietic stem cells in mice and rats (7), and stimulation of autoimmune disease (8). In dogs, toxicity includes reduced hematopoiesis and necrosis of various specialized cells (7, 9). In man, the most consistent toxic response is fever (10, 11). Recently we have also noted coagulation defects after intravenous administration of $rI_n \cdot rC_n$.[3] Much effort has been made to improve the efficacy of $rI_n \cdot rC_n$, particularly by increasing the antiviral activity of these compounds (5, 12). In general, these attempts have not resulted in a significant dissociation of the antiviral from the specific secondary effects being measured; that is, an increase in antiviral protection usually results in proportional increments in the other biological properties (5).

In our preceding publication (13) we constructed two mismatched polynucleotide duplexes, $rI_n \cdot r(C_{13},U)_n$ and $rI_n \cdot r(C_{20},G)_n$. In these two duplexes the complex was interrupted by the unpaired bases (uracil or guanine) located in the rC_n strand. These two mismatched duplexes were found to be comparable in antiviral activity to the perfectly matched $rI_n \cdot rC_n$ duplex when tested in human neonatal fibroblasts in culture. These two mismatched duplexes, however, were hydrolyzed 5–8 times faster by nucleases. We proposed at that time that these two mismatched duplexes might have better therapeutic efficacy than $rI_n \cdot rC_n$.

In the present investigation we have tested the antiviral properties and several secondary effects of these two types of mismatched duplexes [$rI_n \cdot r(C_{12}, U)_n / rI_n \cdot r(C_{13}, U)_n$ and $rI_n \cdot r(C_{20},G)_n / rI_n \cdot r(C_{29},G)_n$] both in human neonatal fibroblasts and in two intact animal systems; at each point comparisons have been made with the perfectly matched $rI_n \cdot rC_n$ molecule. In the mouse, relative protection by these inducers against a lethal viral challenge (Semliki Forest virus), their acute toxicity (resulting in mortality), and their relative mitogenic effects on splenic cells have been investigated. In addition, in the intact rabbit, circulating interferon and pyrogenicity have been carefully studied, and complementary studies have been done with rabbit kidney cells in culture. Our data suggest that these mismatched polynucleotide duplexes are comparable in their antiviral properties to $rI_n \cdot rC_n$ but possess much less pronounced secondary effects than the $rI_n \cdot rC_n$ molecule (4). The results not only warrant further study in appropriate animal models, but also suggest that early clinical investigations might be worthwhile.

A theoretical framework for the design and development of polynucleotide duplexes having a therapeutic efficacy greater than $rI_n \cdot rC_n$ is also discussed.

MATERIALS AND METHODS

Solutions. Buffer A was used for both physical and biological studies so that the results could be compared directly. Eagle's minimal essential medium (14) was prepared to contain fetal calf serum as specified, and glutamine (2 mM), penicillin G (200 units/ml), and streptomycin (200 μg/ml) were added immediately before use.

[2] The abbreviations used are: $rI_n \cdot rC_n$, polyinosinic acid·polycytidylic acid; $rI_n \cdot r(C_{13},U)_n$, polyinosinic aicd·copolymer of cytidylic and uridylic acid with a base ratio of 13:1, respectively, and so on; buffer A, 0.15 M NaCl, 0.01 M sodium phosphate (pH 7.2), and 0.001 M $MgCl_2$.

[3] A. Freeman, J. O'Malley, and W. A. Carter, unpublished observations.

Polynucleotide duplexes. The nucleoside diphosphates, enzymes, and polynucleotides were: CDP, Schwarz BioResearch and P-L Biochemicals; UDP, Miles Laboratories; GDP, Schwarz BioResearch; and *Micrococcus luteus* polynucleotide phosphorylase (EC 2.7.7.8), P-L Biochemicals. The polyinosinic acid and polycytidylic acid used were obtained from P-L Biochemicals.

The copolymer $r(C_{12},U)_n$ was synthesized using the following mixture with an incubation period of 4.5 hr at 37°: CDP, 36 mM; UDP, 7 mM; Tris-Cl, 0.15 M, pH 8.2; $MgCl_2$, 0.01 M; EDTA, 0.4 mM; and polynucleotide phosphorylase, 2 mg/ml. The copolymer $rI_n \cdot r(C_{29},G)_n$ was synthesized using the following mixture with an incubation period of 5 hr at 37°: CDP, 38 mM; GDP, 2 mM; Tris-Cl, 0.15 M, pH 8.2; $MgCl_2$, 0.01 M; EDTA, 0.4 mM; and polynucleotide phosphorylase, 2 mg/ml. The purification procedure was the same for both polymer preparations. The incubation mixture was deproteinized three times with phenol, as previously described (13). The resulting aqueous phase was then dialyzed extensively against NaCl (50 mM)–EDTA (5 mM), NaCl (5 mM)–EDTA (0.5 mM), and finally distilled H_2O. This procedure yielded polymers (38% yield with respect to starting substrates) which contained no nucleoside diphosphates, as judged by paper chromatography, or detectable amounts of protein, by the Lowry assay (15).

The base ratios of both copolymers were determined by exhaustive hydrolysis to nucleoside monophosphates, followed by treatment with bacterial alkaline phosphatase. The resulting nucleosidic solution was analyzed by paper chromatography to determine the base ratio. $r(C_{12},U)_n$ (17.4 A_{265} units, at a concentration of 43 units/ml) was hydrolyzed with 0.3 N KOH at 37° for 23 hr. The pH of the solution was then lowered to 8.5 by bubbling CO_2 through the solution. Alkaline phosphatase (1 mg/ml) was added to the solution and incubated at 37° for 6 hr. The entire mixture was applied to Whatman No. 1 paper and developed with 1-butanol–formic acid–H_2O (77:10:13). The ultraviolet-absorbing material was eluted from the paper, and the base ratio was determined to be 12 C:1 U. $r(C_{29},G)_n$ (20 A_{265} units, at 67 units/ml) was hydrolyzed in a mixture containing Tris-Cl (0.1 M, pH 8.2), EDTA (1 mM), RNase A (1 μg/ml), RNase T_1 (0.013 mg/ml), and alkaline phosphatase (0.27 mg/ml). This mixture was incubated at 37° for 28 hr, then applied to paper and developed in 2-propanol–ammonia–water (7:1:2). The ultraviolet-absorbing materials eluted from the chromatogram indicated a base ratio of 29 C:1 G.

The sedimentation coefficients of $r(C_{12},U)_n$ and $r(C_{29},G)_n$ in buffer A were 7.2 S and 7.6 S, respectively; the corresponding values of the commercial preparations of rI_n and rC_n in buffer A were 6.3 S and 6.7 S, respectively.

The polynucleotide duplexes $rI_n \cdot rC_n$, $rI_n \cdot r(C_{12},U)_n$, and $rI_n \cdot r(C_{29},G)_n$ were prepared by the following procedure. Each single-stranded component of a complex was prepared from a concentrated stock to yield a solution of 1 mM base residue in buffer A. The extinction coefficients of $r(C_{12},U)_n$ and $r(C_{29},G)_n$ were assumed to be identical with that of rC_n. These 1 mM component solutions were sterilized by passage through a Millipore filter (0.22 μm). The solutions were handled under sterile conditions thereafter. The polynucleotide duplex was formed by mixing 1:1 stoichiometric amounts of the complementary strands, to yield a solution of duplex with a concentration of 1 mM total base. Annealing of the complementary strands was facilitated by chilling the solution at 4° for 90 min. The formation of the duplex was verified by the ultraviolet spectrum of the solution. The solution complexes were then stored at −70°.

The duplexes of $rI_n \cdot r(C_{13},U)_n$ and $rI_n \cdot r(C_{20},G)_n$ were the preparations described and characterized in our preceding publication (13).

Viruses. Bovine vesicular stomatitis virus, New Jersey serotype, was harvested from infected mouse L-cells to yield a titer of $1–10 \times 10^8$ plaque-forming units/ml. The virus stock, usually diluted 100-fold, was stored at −70°. Semliki Forest virus obtained from the Center for Disease Con-

trol, Atlanta (R1-1 MP 11 PR 3564-73), was diluted 1000 fold, and 0.01 ml was injected intracerebrally into suckling ICR Swiss mice. Virus in brain tissue was harvested after 5 days and diluted 10-fold in minimal essential medium with 20% fetal calf serum, and the LD_{50} was determined by titration in adult ICR Swiss mice. The virus stock was diluted 100-fold in minimal essential medium with 20% fetal calf serum and stored in aliquots at −70°.

Antiviral activities in human neonatal fibroblasts. Human neonatal fibroblasts were grown as monolayers in 75-cm^2 plastic flasks with minimal essential medium containing 10% fetal calf serum. Cells were exposed to polynucleotide duplexes in buffer A for 1 hr and then washed three times before reincubation in fresh medium at 37°. Intracellular protection was determined colorimetrically (16, 17). Generally a multiplicity of infection of approximately 0.15 plaque-forming unit/cell was used. Percentage of protection was defined as the ratio of dye uptake by viable cells in the infected viral culture to dye uptake by viable cells in a mock-infected culture. The colorimetric titration (in duplicate or triplicate) was performed 28 hr after infection. For measurement of virus yield reduction, 18 hr after exposure to polynucleotide duplexes, cells were challenged with vesicular stomatitis virus (multiplicity of infection, 0.15) and incubated for 28 hr. Virus titer was then determined (in duplicate) in mouse cells by plaque titration.

Antiviral activities in rabbit kidney cells. Primary rabbit kidney cell cultures were grown as monolayers in minimal essential medium containing 10% fetal calf serum, and these cultures were treated with polynucleotides for 1 hr. The antiviral activities were measured by intracellular protection against vesicular stomatitis virus challenge, as described in the section above on human cells (16, 17), and by extracellular interferon titers. Interferon was harvested from the extracellular fluids 18 hr after the exposure to the polynucleotide duplex and measured colorimetrically, using vesicular stomatitis virus as the challenge virus. Assays were carried out in duplicate or triplicate.

Mitogenic activities measured in murine splenic cells. Splenic cells from a single 12-week-old female CD-1 mouse were suspended in McCoy's modified medium 5A containing *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer (Flow Labs, Rockville, Md.) with and without human serum (5%) at a concentration of 4 × 10^6 cells/ml. To aliquots (0.5 ml) of the cell suspension (in sextuplicate), polynucleotide duplexes (0.2 ml of a 1 mM solution in buffer A) were added. The controls contained cells with aliquots (0.2 ml) of either buffer A or phytohemagglutinin (Wellcome Research Laboratories) (a 5 μg/ml solution prepared in buffer A). The mixtures were incubated for 30 min at 37°, after which 0.3 ml of McCoy's medium 5A with human serum was added. The final concentration of human serum was 5%. The cultures were incubated at 37° in a humidified 7.5% CO_2 atmosphere. After 45 hr, 1 μCi of [^{3}H]thymidine (specific activity, 6.7 Ci/mmole; Schwarz/Mann) in 0.5 ml of medium was added to half the cultures. Following further incubation at 37°, triplicate cultures were harvested 6 hr later and incorporation of label was determined by liquid scintillation counting according to Bach *et al.* (18). The other half of the cultures were incubated for a total of 141 hr before introduction of [^{3}H]thymidine for an incorporation period of 6 hr.

Semliki Forest virus infection of mice. Semliki Forest virus solutions of the LD_{75} dose (0.2 ml of a 1 × 10^{-5} dilution) were injected intraperitoneally into groups of eight mice. The polynucleotide duplexes (0.5 ml) were also injected intraperitoneally 1 hr prior to virus infection and daily for 3 days after the infection. Control animals, i.e., without virus infection but with polynucleotide injection, and animals with virus infection but without polynucleotide injection, were also included in these experiments. Animal deaths were recorded daily, and mortality rates were calculated after a 7-day period.

Interferon induction in rabbits in vivo. Male albino rabbits (2–3 kg) were injected intravenously with 1 ml of polynucleotide duplexes dissolved in buffer A. Serum was collected 4, 6, and 8 hr after injection, di-

luted in minimal essential medium containing 2% fetal calf serum, and assayed for interferon.

Mouse mortality caused by polynucleotide duplexes. Polynucleotide duplexes in 0.3 ml of buffer A were injected intravenously into groups of five mice (each weighed about 15 g), and the mortality was determined after 24 hr. Buffer A was injected into control animals.

Pyrogenicity in rabbits caused by polynucleotide duplexes. A single dose of polynucleotide in 0.3 ml of buffer A was injected intravenously into male albino rabbits (aproximately 3 kg; B. and H. Rabbitry, Rockville, Md.) after the animals had first been observed to have a stable temperature for at least 1 hr. The temperature of individual rabbits after injection was recorded every 12 min up to 24 hr by a Yellow Springs Telethermometer and recorder. The mean values of temperature deviation from at least four animals were plotted. On the average, one out of eight animals developed erratic temperatures unrelated to the injection (which might have been due to handling), and records from these animals were not used. Buffer A was injected into the control animals.

RESULTS

Following our earlier work (13) on the mismatched polynucleotide duplexes $rI_n \cdot r(C_{13},U)_n$ and $rI_n \cdot r(C_{20},G)_n$, we prepared two new duplexes, $rI_n \cdot r(C_{12},U)_n$ and $rI_n \cdot r(C_{29},G)_n$, in gram quantities sufficient for testing their antiviral properties and ability to evoke measurable changes in other physiological events. Some of these events could manifest themselves as overtly "toxic" reactions; however, the term toxicity is obviously difficult to define, both conceptually and experimentally. In this study we simply assumed that interferon induction was the event to be preserved, and asked whether the additional biological reactions (operationally defined as a measure of "toxicity") must necessarily covary. That is, could antiviral activity be uncoupled from the multiplicity of other reactions? The data are presented in the following three sections.

Confirmation of antiviral properties of $rI_n \cdot r(C_{12}, U)_n$ and $rI_n \cdot r(C_{29}, G)_n$ in the system of human neonatal fibroblasts. In Table 1 the antiviral effects of the newly prepared, mismatched $rI_n \cdot r(C_{12},U)_n$ and $rI_n \cdot r(C_{29},G)_n$ duplexes are compared with those of the perfectly matched $rI_n \cdot rC_n$ in human neonatal fibroblasts. The results of three separate experiments (A, B, and C), measuring intracellular protection, virus yield, and interferon release, show that the antiviral effects of these two newly prepared, imperfectly matched duplexes are comparable to those of the perfectly matched $rI_n \cdot rC_n$. These results (Table 1) thus fully confirm our previous observation (13) and provide the foundation for further testing of the biological properties of $rI_n \cdot r(C_{12},U)_n$ and $rI_n \cdot r(C_{29},G)_n$.

The mouse model. The antiviral effects and the toxicity of $rI_n \cdot r(C_{12},U)_n$ [as well as $rI_n \cdot r(C_{13},U)_n$, prepared previously] and $rI_n \cdot r(C_{29},G)_n$ [as well as $rI_n \cdot r(C_{20},G)_n$, prepared previously] were compared with those of $rI_n \cdot rC_n$ at both the cellular and the whole animal level.

Table 2 describes the antiviral effect of $rI_n \cdot rC_n$ and the four mismatched analogues as measured by protection of mice against Semliki Forest virus. The results show that the antiviral effects of $rI_n \cdot r(C_{12}, U)_n$ and $rI_n \cdot r(C_{13},U)_n$ were identical with that of $rI_n \cdot rC_n$ at the three doses studied (100, 25, and 10 μg/injection). The antiviral effects of $rI_n \cdot r(C_{20},G)_n$ and $rI_n \cdot r(C_{29}, G)_n$ were also the same at doses of 100 and 25 μg, but appeared to be slightly lower than those of $rI_n \cdot rC_n$ and of $rI_n \cdot r(C_{12},U)_n$/ $rI_n \cdot r(C_{13},U)_n$ at the 10-μg dose level.

Table 3 describes the acute toxicity of $rI_n \cdot rC_n$ and its two mismatched analogues as measured by the mortality of mice after intravenous injection of a relatively large dosage of the polynucleotide duplex. At the 400 μg/animal dose, in two separate experiments (A and B), 80% of the animals died when injected with $rI_n \cdot rC_n$, only 40% died when injected with $rI_n \cdot r(C_{13},U)_n$, and none died when injected with $rI_n \cdot r(C_{20},G)_n$. At the level of 350 μg/animal only 20% of the mice died when injected with $rI_n \cdot rC_n$, and at this dosage none died with an $rI_n \cdot r(C_{13}, U)_n$ injection. The data show that $rI_n \cdot r(C_{20}, G)_n$ is definitely less acutely toxic than

TABLE 1

Antiviral effects of $rI_n \cdot rC_n$ and its mismatched analogues as measured with human neonatal fibroblasts

Results are from three separate experiments, A, B, and C.

Polynucleotide duplex	Concentration			Intracellular protection			Virus yield			Reduction in virus yield			Interferon titer
	A	B	C	A	B	C	A	B	C	A	B	C	
	μM	*μM*	*μM*	%	%	%	*PFU*	*PFU*	*PFU*	*×10²*	*×10²*	*×10²*	
None	0	0	0	0	0		8.0×10^8	6.7×10^{13}	3.0×10^8	0	0	0	
$rI_n \cdot rC_n$			0.1			33			2.0×10^8			0.015	<1
	1		1	89		100	1.5×10^6		1.2×10^5	5.3		25	4
	10		10	98		100	1.4×10^5		1.2×10^4	57		250	6
	100	100	100	100		100	3.0×10^4	2.5×10^8	1.2×10^4	270	2,700	300	26
		500			74			5.0×10^6			130,000		
$rI_n \cdot r(C_{12},U)_n$			0.1			87			3.0×10^7			0.1	<1
	1		1	52		100	6.4×10^7		1.4×10^5	0.13		21	7
	10		10	83		100	2.2×10^7		3.2×10^5	0.36		9.4	<1
	100	100	100	100	22	100	7.5×10^5	1.0×10^{11}	8.0×10^4	11	6.7	38	10
		500			50			1.3×10^7			50,000		
$rI_n \cdot r(C_{29},G)_n$			0.1			49			5.2×10^7			0.058	<1
	1		1	47		100	5.9×10^7		3.2×10^5	0.14		9.40	5
	10		10	86		100	1.1×10^6		2.0×10^4	7.3		150	7
	100		100	100		100	5.0×10^4		1.2×10^4	160		250	15

TABLE 2

Antiviral effects of $rI_n \cdot rC_n$ and its mismatched analogues as measured by protection of mice against Semliki Forest virus (SFV)

The polynucleotide duplex was administered in 0.5 ml by intraperitoneal injection 1 hr prior to and daily for 3 days after virus infection with approximately an LD_{75} dose of virus. In experiments with virus infection, each group consisted of eight mice; in experiments without virus infection, each group consisted of five mice. No mortality was observed in experiments with injection of the polynucleotides without virus infection.

Virus	Polynucleotide duplex	Mortality			
		100 μg	25 μg	10 μg	
				Expt. A	Expt. B
		%	%	%	%
SFV	None	75	75	87.5	75.0
None	$rI_n \cdot rC_n$	0			
SFV	$rI_n \cdot rC_n$	0	0	12.5	12.5
None	$rI_n \cdot r(C_{12},U)_n$	0			
SFV	$rI_n \cdot r(C_{12},U)_n$		0	12.5	12.5
None	$rI_n \cdot r(C_{13},U)_n$	0			
SFV	$rI_n \cdot r(C_{13},U)_n$	0	0	12.5	12.5
None	$rI_n \cdot r(C_{20},G)_n$	0		62.5	12.5
SFV	$rI_n \cdot r(C_{20},G)_n$	0	0	50.0	12.5
None	$rI_n \cdot r(C_{29},G)_n$	0			
SFV	$rI_n \cdot r(C_{29},G)_n$		0	12.5	25.0

$rI_n \cdot rC_n$ and possibly less toxic than $rI_n \cdot r(C_{13},U)_n$. Also, the $rI_n \cdot r(C_{13},U)_n$ duplex appears to be less acutely toxic than $rI_n \cdot rC_n$. It should be noted that this test for acute toxicity, as measured by mortality, is really an "all-or-none" phenomenon which can be effectively studied only within a relatively narrow concentration range. Therefore we developed additional test systems for chronic toxicity to monitor the possible undesirable effects of $rI_n \cdot rC_n$ and the mismatched analogues at low dosages and over a much wider range of polymer concentration; these studies will be the subject of a separate paper.

As an additional test of potentially significant biological events elicited by a single dosage of RNA duplexes, we also measured the mitogenic effects on murine splenic cells, which would signify stimulation of the immune system. Table 4 describes one of several typical experiments in which measurements were made at two time points (45 and 141 hr) in both the presence and absence of added serum in the culture medium during initial exposure to the different compounds. In these experiments addition of buffer A served as a negative control in providing a background value of [^{3}H]thymidine incorporation, and treatment with phytohemagglutinin served as a positive control in demonstrating the viability and responsiveness of these cells in culture (19–21). The early mitogenic effect of $rI_n \cdot rC_n$ at 290 μM concentration was clearly demonstrated in this experiment by measuring the [^{3}H]thymidine incorporation after 45 hr of incubation. The results also showed that

TABLE 3

Acute toxicity of $rI_n \cdot rC_n$ and its mismatched analogues as measured by mortality of mice

The polynucleotide duplex was administered by intravenous injection in 0.3 ml to groups of five mice. Mortality was determined after 24 hr.

Polynucleotide duplex	Mortality			
	400 μg		350 μg	300 μg
	Expt. A	Expt. B		
	%	%	%	%
$rI_n \cdot rC_n$	80	80	20	20
$rI_n \cdot r(C_{13},U)_n$	40	40	0	
$rI_n \cdot r(C_{20},G)_n$	0	0		
Buffer A	0	0		

TABLE 4

Mitogenic effects of $rI_n \cdot rC_n$ and its mismatched analogues on murine splenic cells as measured by [^{3}H]thymidine incorporation

Splenic cells (4×10^6 cells/ml) from a 12-week-old female CD-1 mouse were suspended in medium, with and without human serum (5%). After addition of compounds to the cultures, cells were incubated for 45 hr before addition of [^{3}H]thymidine to half the cultures. After 6 hr of incubation with [^{3}H]thymidine, triplicate cultures were harvested and incorporation was determined. The remaining cultures were incubated continuously for a total of 141 hr before addition of [^{3}H]thymidine for a 6-hr period of labeling. For other details, see MATERIALS AND METHODS.

Compound	After 45 hr of incubation				After 141 hr of incubation			
	Without serum		With serum		Without serum		With serum	
	Average radioactivity incorporated	Stimulation index	Average radioactivity incorporated	Stimulation index	Average radioactivity incorporated	Stimulation index	Average radioactivity incorporated	Stimulation index
	cpm		*cpm*		*cpm*		*cpm*	
Buffer A	411	1.0	1,000	1.0	270	1.0	307	1.0
PHA[a]	40,540	98.6	39,860	39.8	2,100	7.8	1,370	4.5
$rI_n \cdot rC_n$[b]	7,670	18.7	6,035	6.0	1,110	4.1	423	1.4
$rI_n \cdot r(C_{12},U)_n$[b]	1,975	4.8	2,750	2.8	1,148	4.3	773	2.5
$rI_n \cdot r(C_{29},G)_n$[b]	1,620	3.9	2,790	2.8	1,690	6.3	340	1.1

[a] PHA(phytohemagglutinin) was dissolved in buffer A at a concentration of 5 μg/ml.

[b] Polynucleotide duplexes were added to a final concentration of 290 μM.

the mismatched complexes $rI_n \cdot r(C_{12},U)_n$ and $rI_n \cdot r(C_{29},G)_n$ were only one-third to one-half as mitogenic by comparison. In other words, the mismatched complexes caused much less arousal of the immune system as judged by their direct specific mitogenic effects. Presumably this differential effect might also be seen in intact animals with circulating RNA duplexes gaining access to splenocytes within the reticuloendothelial network (see DISCUSSION).

The rabbit model. The antiviral effects of $rI_n \cdot rC_n$ and the two mismatched analogues, $rI_n \cdot r(C_{12},U)_n$ and $rI_n \cdot r(C_{29},G)_n$, were first measured with rabbit kidney cells in culture. As shown in Table 5, the data on intracellular protection and on extracellular interferon indicate that these two mismatched analogues are as effective as $rI_n \cdot rC_n$.

The antiviral effects of $rI_n \cdot rC_n$ were then compared with those of the two mismatched analogues for relative ability to induce circulating interferon in the intact animal. In Table 6 the time course of serum interferon levels after an intravenous injection of $rI_n \cdot rC_n$ at 2 μg/kg is shown. While there was some variation among the six animals, the results generally indicated a relatively high level at 4 and 6 hr, followed by a decline at 8 hr. These data are also pertinent to subsequent pyrogen-

TABLE 5

Antiviral effects of $rI_n \cdot rC_n$ and its mismatched analogues as measured in rabbit kidney cells

The cells were treated with polynucleotide duplexes for 1 hr.

Polynucleotide duplex	Concentration	Intracellular protection	Interferon titer
	μM	%	
Buffer A		0	<1
$rI_n \cdot rC_n$	0.1	42	<216
	1	100	<216
	10	100	800
	100	100	1300
$rI_n \cdot r(C_{12},U)_n$	0.1	24	<216
	1	90	216
	10	94	1300
	100	99	3500
$rI_n \cdot r(C_{29},G)_n$	0.1	74	<216
	1	98	440
	10	98	680
	100	100	1700

icity studies, discussed below. Table 7 describes typical results in a series of experiments in which the induction of circulating interferon by $rI_n \cdot rC_n$ (at a 1 μg/kg dose) was compared with that of the two mismatched analogues, $rI_n \cdot r(C_{12},U)_n$ and $rI_n \cdot r(C_{29},G)_n$. Again, against a background of some variation from animal to animal, one can note that both the mismatched analogues induced measurable interferon titers (compare Tables 6 and 7). The $rI_n \cdot r(C_{29},G)_n$ molecule appeared to be a very effective inducer, indeed as good as $rI_n \cdot rC_n$, if not better, while $rI_n \cdot r(C_{12},U)_n$ appeared somewhat less active. Thus we note a slight difference in antiviral activity between the two mismatched duplexes when they are carefully compared in the mouse and rabbit.

As an additional test of another significant biological response, we measured pyrogenicity after intravenous injection of the polynucleotide duplexes (7). This test system is remarkably sensitive to the presence of double-stranded RNA (4, 7), and therefore we could establish correlations, if any, between the levels of circulating interferon and the intensity of the fever response. In addition, the pyrogenicity measurement appeared particularly pertinent to problems which might be encountered with the clinical evaluation of these specific polynucleotide duplexes.

Figure 1A describes the rise in body temperature after intravenous injection of four dosages of $rI_n \cdot rC_n$ in rabbits. Very little effect was observed at doses of 0.001 and 0.01 μg/kg of $rI_n \cdot rC_n$; however, a significant rise was observed at doses of 0.1 and 1 μg/kg. This increase in temperature reached a maximum at about 3–4 hr and

TABLE 6

Induction of circulating interferon in rabbits by $rI_n \cdot rC_n$

Six male albino rabbits (2–3 kg) were injected intravenously with 2 μg/kg of $rI_n \cdot rC_n$ or buffer A and were bled 4, 6, and 8 hr after injection.

Rabbit	Treatment	Interferon titers after injection		
		4 hr	6 hr	8 hr
1	Buffer A	<10		<10
2	$rI_n \cdot rC_n$	1200	2900	220
3	$rI_n \cdot rC_n$	1800	2700	1800
4	$rI_n \cdot rC_n$	1800	2800	1500
5	$rI_n \cdot rC_n$	1100	4400	620
6	$rI_n \cdot rC_n$	2200	2900	900

TABLE 7

Induction of circulating interferon in rabbits by $rI_n \cdot rC_n$, $rI_n \cdot r(C_{12},U)_n$, and $rI_n \cdot r(C_{29},G)_n$

Male albino rabbits weighing approximately 2 kg each were injected intravenously with 1 μg/kg of $rI_n \cdot rC_n$ or analogues. The animals were bled 4, 6, and 8 hr after injection, and serum interferon titers were determined.

Inducer[a]	Rabbit	Interferon titers after injection			
		Expt. A			Expt. B (4 hr)
		4 hr	6 hr	8 hr	
$rI_n \cdot rC_n$	1	950	650	290	230
	2	2000	620	210	<100
	3				160
	4				300
$rI_n \cdot r(C_{12},U)_n$	1	105	160	<100	<100
	2	200	115	140	<100
	3	130	500	120	<100
	4	350	170	<100	<100
$rI_n \cdot r(C_{29},G)_n$	1	290	260	130	220
	2	2700	1200	260	200
	3	950	1900	110	260
	4	1000	430	500	210

[a] The interferon titers of rabbits injected with buffer A were less than 10 (see Table 6).

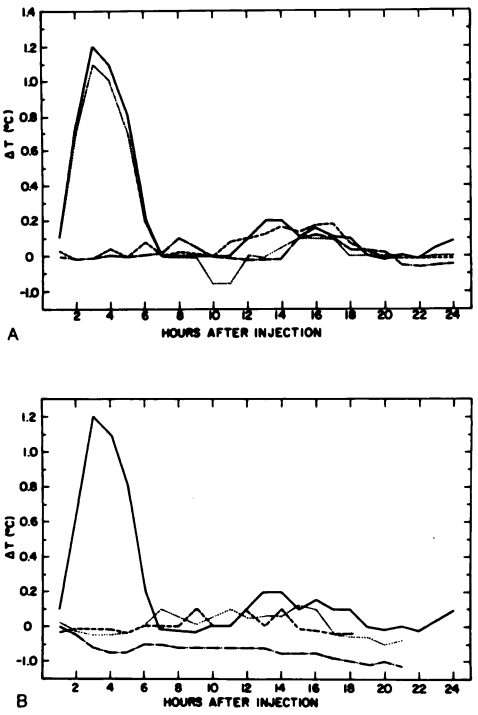

FIG. 1. *Pyrogenicity of $rI_n \cdot rC_n$ in rabbits*

A. Temperature changes in rabbits after different intravenous doses of $rI_n \cdot rC_n$. Each curve is the average of four animals receiving 0.001 (-·-·), 0.01 (- - -), 0.1 (····), or 1.0 μg/kg (——) of the polynucleotide duplex.

B. Temperature changes in rabbits after 1 μg/kg intravenous doses of $rI_n \cdot rC_n$ (——), rI_n(····), rC_n (- - -), or buffer A (-·-·). Each curve is the average of four animals, except for the buffer A control curve (two animals) (see MATERIALS AND METHODS).

declined to normal levels at about 7–8 hr. Between the 13th and 17th hours a second wave of temperature increase was noted, a phenomenon more noticeable (see below) at higher dosages. The extent of increase in body temperature was similar at the 0.1 and 1 μg/kg doses. Figure 1B shows that, at the 1 μg/kg dose, only the $rI_n \cdot rC_n$ duplex could consistently induce fever; the single-stranded rI_n and rC_n molecules were inert.

Figures 2 and 3 describe the comparative pyrogenic effects of $rI_n \cdot rC_n$ and the two mismatched analogues, $rI_n \cdot r(C_{13},U)_n$ and $rI_n \cdot r(C_{20},G)_n$ (Figs. 2A and 3A), as well as $rI_n \cdot r(C_{12},U)_n$ and $rI_n \cdot r(C_{29},G)_n$ (Figs. 2B and 3B). The data clearly indicate that at doses of both 1 and 0.1 μg/kg these two types of mismatched duplexes have little, if any, ability to induce fever as compared to the $rI_n \cdot rC_n$ molecule. Indeed, only at a dose level of 5 μg/kg (Fig. 4) could a small inductive effect ($\Delta T \cong 0.3$–$0.5°$) of $rI_n \cdot r(C_{13},U)_n$ and $rI_n \cdot r(C_{20},G)_n$ be consistently observed. At this dosage (5 μg/kg) the extent of temperature rise ($\Delta T \cong 1.5$–$1.6°$) induced by $rI_n \cdot rC_n$ was much higher and a second wave of fever was clearly evident (Fig. 4). Since the pyrogenicity caused by these two mismatched duplexes at the 5 μg/kg level (Fig. 4) appeared to be considerably lower than the pyrogenicity induced by $rI_n \cdot rC_n$ at a 0.1 μg/kg dose (Fig. 1), it could be estimated that the pyrogenic properties of the $rI_n \cdot r(C_{13},U)_n$ and $rI_n \cdot r(C_{20},G)_n$ duplexes were probably 50–100-fold lower than that of $rI_n \cdot rC_n$.

It should be particularly noted that at doses of 1 and 2 μg/kg the time curve of circulating interferon titers in these ani-

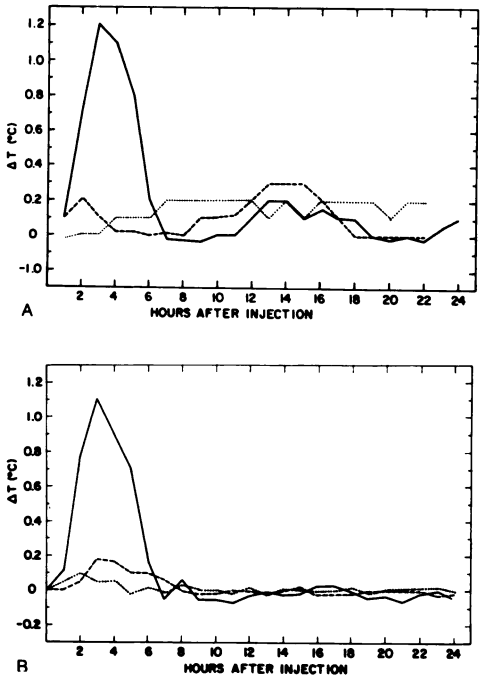

FIG. 2. *Temperature changes in rabbits after 1.0 μg/kg intravenous doses of polynucleotide duplexes*

Each curve is the average of four animals. A. ——, $rI_n \cdot rC_n$; - - -, $rI_n \cdot r(C_{13},U)_n$; ····, $rI_n \cdot r(C_{20},G)_n$. B. ——, $rI_n \cdot rC_n$; - - -, $rI_n \cdot r(C_{12},U)_n$; · · · ·, $rI_n \cdot r(C_{29},G)_n$.

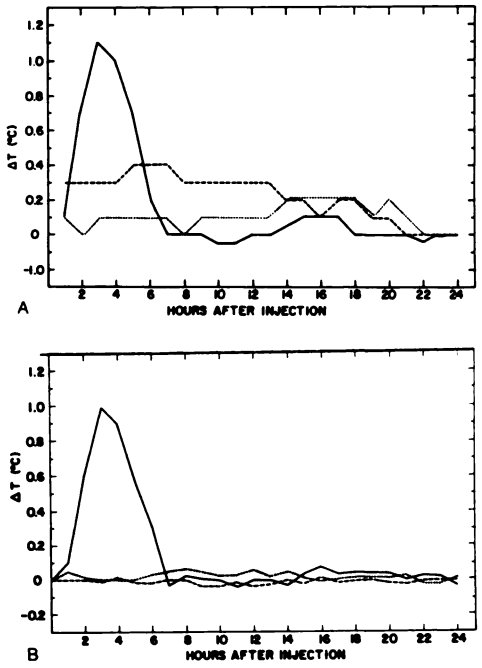

FIG. 3. *Temperature changes in rabbits after 0.1 μg/kg intravenous doses of polynucleotide duplexes.*
Each curve is the average of four animals. A. ——, $rI_n \cdot rC_n$; - - -, $rI_n \cdot r(C_{13},U)_n$; ····, $rI_n \cdot r(C_{20},G)_n$. B. ——, $rI_n \cdot rC_n$; - - -, $rI_n \cdot r(C_{12},U)_n$; · · · ·, $rI_n \cdot r(C_{29},G)_n$.

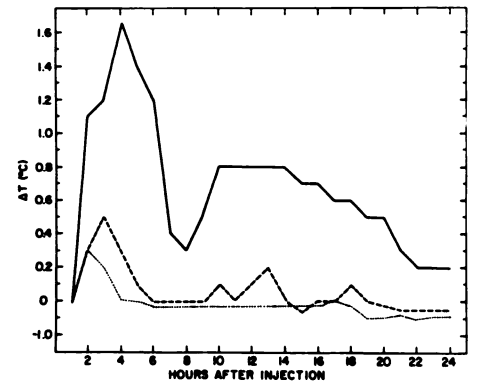

FIG. 4. *Temperature changes in rabbits after 5 μg/kg intravenous doses of $rI_n \cdot rC_n$ (——), $rI_n r(C_{13}, U)_n$ (- - -), and $rI_n \cdot r(C_{20},G)_n$ (····)*
Each curve is the average of four animals.

mals had already been described (Tables 6 and 7). Thus both the intensity of fever and the circulating interferon level induced by these polynucleotide duplexes could be evaluated under identical conditions.

DISCUSSION

Under RESULTS we compared the antiviral properties of $rI_n \cdot rC_n$ and its mismatched analogues, $rI_n \cdot r(C_{12},U)_n / rI_n \cdot r(C_{13},U)_n$, and $rI_n \cdot r(C_{20},G)_n / rI_n \cdot r(C_{29},G)_n$, in two cellular systems (human neonatal fibroblasts and rabbit kidney cells) and in two animal systems (protection of mice and rabbits). The data clearly support and extend our previous conclusion (13) that these two types of mismatched analogues are as effective, or very nearly so, as the perfectly matched $rI_n \cdot rC_n$ in their antiviral properties. We have also compared the "toxicity" of $rI_n \cdot rC_n$ and these two mismatched analogues in one cellular system (mitogenic effect on murine splenic cells) and two animal systems (acute toxicity/mortality in mice and pyrogenicity in rabbits). The results substantiate our previous suggestion (13) that these mismatched duplexes are in fact significantly less able to trigger additional biological responses than $rI_n \cdot rC_n$. Our suggestion originated from the experimental observation that these mismatched duplexes, $rI_n \cdot r(C_{13},U)_n$ and $rI_n \cdot r(C_{20},G)_n$, are much more sensitive to degradation by nucleases (13) and therefore might be removed much more readily than $rI_n \cdot rC_n$.

In experiments in which the polynucleotide duplexes were introduced to the animal at a very low dose (1–0.1 μg/kg), as in the pyrogenicity tests with rabbits, the difference in "toxicity" between the $rI_n \cdot rC_n$ and its mismatched duplexes was striking, i.e., about 50–100-fold less for the mismatched duplexes. In this situation we reason that the injected polynucleotide duplexes are probably degraded and removed rapidly by the animal. In agreement with this proposition, in experiments in which splenic cells were exposed to a fairly high concentration of polynucleotide duplexes, the difference in "toxicity" was only 3–4-fold in favor of the mismatched duplexes. Finally, in the acute toxicity experiments, in which a relatively massive amount of polynucleotide duplex (400 μg) was injected intravenously into the mouse, the differences between $rI_n \cdot rC_n$ and the two mismatched duplexes became smaller and

distinctions were more difficult to evaluate.

There are at least two intrinsic problems in providing a quantitative interpretation of data derived from the acute toxicity/mortality experiments. (a) When an overwhelming amount of any potentially toxic substance is introduced into the organism, the system of natural resistance (such as the disposal and repair processes) tends to break down and become inoperative. Therefore this type of experiment does not reflect the tolerance of the organism's protective mechanism with its natural disposal and repair processes functioning under more normal conditions. (b) Since a large amount of polynucleotide duplexes is needed in the tests, and the response (death) is all-or-none in nature, only a very narrow range of concentrations can be effectively tested. In view of these serious intrinsic problems, we have doubts about the usefulness of the data from acute toxicity/mortality measurements, particularly as they relate to the possible chemotherapeutic application of these polynucleotide duplexes. Since the advantage of the mismatched duplexes is based on proper functioning of the disposal process in the animal, the data for acute toxicity/mortality at very high dosages may not really evaluate properly the therapeutic efficacy of mismatched polynucleotide duplexes.

In the mouse model the protection of the animal against a lethal viral challenge and the test for mitogenic effects of the splenic cells are, in contrast, more clearly relevant to the chemotherapeutic application of these polynucleotide duplexes. First, we have confirmed the report of Dean *et al.* (22) that $rI_n \cdot rC_n$ is a potent mitogen for lymphocytes and spleen cells in the mouse. Collaro *et al.* (23) reported that intraperitoneal injection of $rI_n \cdot rC_n$ into the mouse can cause an immediate and transient cytopathic action on the reticuloendothelial system, enlargement of the spleen, and a selective stimulating action on antibody-producing cells. Recently the effects of $rI_n \cdot rC_n$ and interferon preparations on the immune system of the host have received much attention (for discussions, see refs. 1-5, 24). Our preliminary results on human splenic cells mimic those reported here for the murine system and will be described elsewhere.

In the rabbit we have confirmed and extended the study of Philips *et al.* (7) on the pyrogenicity of $rI_n \cdot rC_n$. Certainly this test is exquisitely sensitive (for $rI_n \cdot rC_n$ at the dose of 0.1 μg/kg), although the biological origin of the fever is not entirely clear (4). A particular merit of the rabbit model is that pyrogenicity and the induction of circulating interferon can be measured concurrently at the same dose level of polynucleotide duplex in the same animal.

It should be emphasized that the four test systems established in these two animal models may be useful in signaling cellular targets and therapeutic potential in man. Thus the protection against viral disease, the responsiveness of lymphoid tissue, the induction of circulating interferon, and the level of febrile responsiveness are measurements directly pertinent to the establishment of therapeutic efficacy of these polynucleotide duplexes. The data from the two animal models warrant an early clinical investigation of these mismatched duplexes in comparison with $rI_n \cdot rC_n$.

The current study also helps to establish a theoretical framework for designing a polynucleotide duplex as an interferon inducer having greater therapeutic efficacy than $rI_n \cdot rC_n$. In general, we can classify the biological responses to polynucleotide duplexes into two categories, i.e., the desirable effects (e.g., antiviral activities) and the undesirable effects of "toxicity." There exists a variety of clearly undesirable effects, such as inhibition of the synthesis of protein (25), DNA (26), and RNA (27) (for a review, see ref. 4). The strategy in designing a polynucleotide duplex of greater efficacy rests on the basic premise that either the *structural requirements* or the *temporal requirements* of the desirable and the undesirable responses are not identical. Substantial efforts have been made to modify the bases (28, 29), backbone (30, 31), and sizes (32) (for a comprehensive review, see ref. 5). The limited

success of these approaches may stem partly from the relative unavailability of pertinent systems for testing a spectrum of physiological responses to a specific RNA duplex, and partly from the relative absence of theoretical guidance for an experimental approach in significantly modifying the therapeutic ratio.

Emphasis has been placed by our laboratories on the possible differences in temporal requirements of the antiviral vs. the other biological responses. This approach is based on the observation (33) that the time requirement for triggering the antiviral and interferon response by $rI_n \cdot rC_n$, at least for cells in culture, is very short indeed (within a few minutes or less at 37°). Therefore there is a strong possibility that the "time constants" in triggering the desirable response and in the induction of the other physiological responses may be sufficiently different to permit the design and development of an interferon inducer of superior efficacy to $rI_n \cdot rC_n$ (4). Our preceding paper (13) was predicated on this premise, and our present results confirm the apparent advantages of this approach.

Several additional comments are in order. Obviously this specific strategy will be successful for the development of a superior interferon inducer only when the "time constant" or the temporal requirement of the antiviral response is shorter than that of the other responses. Moreover, this approach may have general applicability to the study of basic mechanisms concerning the biological effects of double-stranded RNAs. For example, it is unlikely that all the biological responses to double-stranded RNA will have the same "time constants." Therefore, at least in theory, a series of double-stranded RNAs with varying degrees of relative sensitivity to enzymatic degradation can be constructed to examine a number of biological responses. The variations in temporal requirements may actually reflect relative differences in location, as well as in structure, of "receptor" sites which promote the other biological responses. For instance, it seems reasonable to presume that the brief time for triggering the antiviral and interferon response is related to "receptor" sites, perhaps on the outer face of the plasma membrane. On the other hand, many receptor sites for other biological responses may be located inside the cell and, indeed, within the nucleus.

The approach developed in the current study, based on differences in temporal requirements, may therefore yield valuable insights about both the structure and location of "receptors" which induce a spectrum of biological responses on exposure to polynucleotide duplexes. A recent hypothesis (4) suggests that aspects of morbidity normally encountered in viral infection may in fact occur through the molecular mediation of double-stranded RNA. Our current studies provide an experimental approach to this type of question and, at the same time, a possible opportunity to evolve a chemotherapeutic compound of interest.

Previous efforts have been made to prepare polynucleotide duplexes with much more resistance to enzymatic hydrolysis; thus far such complexes have not been found to have a therapeutic efficacy greater than $rI_n \cdot rC_n$ (5). According to our own theoretical analysis, these resistant complexes should not, a priori, possess any advantage over $rI_n \cdot rC_n$. In fact, from considerations of the temporal requirements, these duplexes are likely to be less selective since they persist longer in biological fluids than $rI_n \cdot rC_n$, thus having a greater probability of inducing additional physiological responses. Such an approach may only be successful in the event that the chemical or structural modification that confers resistance of the duplex to enzymatic degradation also happens to provide a structure more specific for interferon induction and, similarly, is noninductive of the other biological responses. Such an event seems rather unlikely by chance alone, particularly in view of the large spectrum of specific physiological responses to double-stranded RNA molecules. Thus we feel that the success of an approach based on specific structural requirements (as opposed to temporal re-

quirements) may be accomplished only by rigorous theoretical guidance with systematic procedures; such an approach is currently being followed in our laboratories (34).

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