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

III. Chronic Effects and Immunological Features

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SUMMARY

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Extensive investigations were made of the chronic biological properties of the polyinosinic·polycytidylic acid ($rI_n \cdot rC_n$) duplex, along with two duplexes having mismatched base pairs, $rI_n \cdot r(C_{12},U)_n$ and $rI_n \cdot r(C_{29},G)_n$, and a double-stranded RNA (BRL 5907) obtained from virus-like particles in *Penicillium* cultures. Earlier we had shown that mismatched polynucleotide duplexes are comparable in their antiviral properties to $rI_n \cdot rC_n$ but possess much less pronounced immediate secondary effects than the $rI_n \cdot rC_n$ molecule. In this work the relative antiviral activities against encephalomyocarditis virus infection in mice of these four duplexes were compared and $rI_n \cdot r(C_{29},G)_n$ was found to be somewhat less active than the other double-stranded RNAs. In experiments on mice with a 7-day repeat dose, $rI_n \cdot rC_n$ and BRL 5907 caused a spectrum of toxic effects: a substantial loss of body weight, acute lymphopenia, and thrombocytopenia; on day 8, neutrophil leukocytosis, anemia, splenomegaly, and thymic atrophy were also noted. In contrast, $rI_n \cdot r(C_{29},G)_n$ was less toxic and did not promote thymic atrophy; $rI_n \cdot r(C_{12},U)_n$ effected even fewer secondary biological events since it did not promote anemia or a change in body weight, modify the neutrophil count, or induce the thymic atrophy,

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effects characteristic of $rI_n \cdot rC_n$ and BRL 5907. All inducers, including the mismatched duplexes, induced some degree of thrombocytopenia, acute lymphopenia, and splenomegaly by day 8. Splenomegaly may be due to the mitogenic potential of double-stranded RNA on splenocytes, a reaction known not to be completely abrogated by purposeful mismatching of bases in RNA. Thus these studies established an order of capacity for triggering specific secondary toxic responses: BRL 5907 $\cong$ $rI_n \cdot rC_n$ > $rI_n \cdot r(C_{29},G)_n \gg rI_n \cdot r(C_{12},U)_n$. Using radioactive $rI_n \cdot rC_n$ as a probe, serum antibody was assayed in both mice and rabbits following repeated administration of the three synthetic double-stranded RNAs: $rI_n \cdot rC_n$ appeared the most immunogenic of the three. The order of immunogenic potential for mismatched duplexes, tested only by cross-reaction, was BRL 5907 > $rI_n \cdot rC_n$ > $rI_n \cdot r(C_{29},G)_n \gg rI_n \cdot r(C_{12},U)_n$. Interestingly, antibody raised against the naturally occurring RNA could interact effectively with antibody raised specifically against $rI_n \cdot rC_n$, thereby supporting the notion that extensive cross-reactivity is shared by antibodies raised against the double-stranded RNAs with the various sequences. Companion studies *in vitro*, which evaluated the relative rates of polymer degradation with pancreatic RNase, confirmed that mismatching of the base pairs in the duplex RNA, as exemplified by $rI_n \cdot r(C_{12},U)_n$ and $rI_n \cdot r(C_{29},G)_n$, would enhance the rate of polymer degradation. The natural double-stranded RNA (BRL 5907) was degraded much more slowly than the others. Thus this mechanism appears to be one of the factors in the differential effects observed *in vivo*. Together with our earlier work, the current results more firmly establish the importance of the "kinetic term" in modifying the therapeutic ratios of double-stranded RNAs in intact biological systems and illustrate how the spectrum of biological responses to double-stranded RNAs can be modulated by this strategy.

INTRODUCTION

The effectiveness of double-stranded polynucleotide duplexes as antiviral reagents is generally well known: both the synthetic duplex polyinosinic acid-polycytidylic acid and the naturally occurring dsRNA[1] from culture filtrates of *Penicillium chrysogenum*, termed BRL 5907, have as their major mode of antiviral action the induction of interferon (1–6). However, the potential use of these molecules as chemotherapeutic agents against viral disease has been constrained by other biological effects, many of which are often considered "toxic" (4, 7).

These other biological effects include pyrogenicity in rabbits (8), diminution of hematopoietic stem cells in mice and rats (8), and stimulation of autoimmune disease (9). In dogs, toxicity includes reduced hematopoiesis and necrosis of various specialized cells (8, 10). In humans, the most consistent toxic response is fever (11, 12). Recently we also noted coagulation defects after intravenous administration of $rI_n \cdot rC_n$.[2] Much effort has been made to improve the efficacy of these duplexes, particularly of $rI_n \cdot rC_n$, by increasing its antiviral activity, for example (5, 13). In general, these attempts have not resulted in a significant dissociation of the antiviral and specific secondary effects being measured; that is, an increase in antiviral protection usually resulted in a proportional increment in the other biological properties (5).

In our first publication on this subject (14) 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 mis-

[1] The abbreviations used are: dsRNA, double-stranded RNA; $rI_n \cdot rC_n$, polyinosinic acid·polycytidylic acid duplex; $rI_n \cdot r(C_{12},U)_n$, mismatched polynucleotide duplex containing unpaired uracil bases located in the polycytidylic acid strand; EMC virus, encephalomyocarditis virus.

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

matched duplexes might have better therapeutic efficacy than $rI_n \cdot rC_n$.

In our second communication (15) we tested the antiviral properties and several secondary effects, relatively short-term, of these two types of mismatched duplexes [$rI_n \cdot r(C_{12},U)_n$ and $rI_n \cdot r(C_{13},U)_n$], as well as $rI_n \cdot r(C_{20},G)_n$ and $rI_n \cdot r(C_{29},G)_n$, both in human neonatal fibroblasts and in two intact animal systems; at each point comparisons were made with the perfectly matched $rI_n \cdot rC_n$ molecule. In the mouse we showed that the mismatched inducers were similar in relative protection against a lethal viral challenge (Semliki Forest virus) although their acute toxicity (resulting in mortality within 24 hr) and their relative mitogenic effects on splenic cells were significantly less. In addition, in the intact rabbit, we carefully studied circulating interferon and pyrogenicity and conducted complementary studies with rabbit kidney cells in culture. These results also indicated that the synthetic mismatched polynucleotide duplexes were comparable in their antiviral properties to $rI_n \cdot rC_n$ but possessed much less pronounced secondary effects than the $rI_n \cdot rC_n$ molecule (4). These results seemed to warrant further study, particularly of a type involving more chronic toxicity evaluations with repeated administration of dsRNAs; such studies might also logically embrace some of the immune responses characteristically seen with dsRNA.

We now report studies designed to further our understanding of the possible impact of mismatching bases in dsRNA on the spectrum of biological events encountered; additionally, we have included a naturally occurring dsRNA for direct comparison. For these studies, the synthetic dsRNAs were coded.

Finally, the theoretical framework for the design and development of polynucleotide duplexes with greater therapeutic efficacy than $rI_n \cdot rC_n$, developed in our preceding text (15), is discussed in light of the current results.

MATERIALS AND METHODS

Solutions

Buffer A consisted of 0.15 M NaCl, 0.01 M sodium phosphate (pH 7.2), and 0.001 M $MgCl_2$; SSC solution contained 0.015 M NaCl and 0.0015 M trisodium citrate, pH 7.0.

Polynucleotide Duplexes

Polynucleotides were those described in our previous report (15).

Tritium-labeled $rI_n \cdot rC_n$ was purchased from Miles Laboratories (code 57.326, lot 2) and had a specific radioactivity of 19.3 μCi/μmole. It was used at a concentration of approximately 2 μg/ml in borate-buffered NaCl, pH 8.3. Tritiated BRL 5907 was isolated from a fermentation after addition of [^{3}H]adenosine to the culture medium and had a specific activity of 132 μCi/μmole.

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 $r(C_{29},G)_n$ 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 (14). 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 phosphates, as judged by paper chromatography, or detectable amounts of protein, by the Lowry assay (16).

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 solution was then lowered to pH 8.5 by bubbling CO_2 through

it 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 s values of $r(C_{12},U)_n$ and $r(C_{29},G)_n$ in buffer A were 7.2 and 7.6 S, respectively; those of the commercial preparations of rI_n and rC_n in buffer A were 6.3 and 6.7 S, respectively. All values were obtained by analytical ultracentrifugation.

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 1 mM in 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) and 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 placing 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°.

Melting characteristics and ribonuclease degradation of $rI_n \cdot rC_n$ and the two mismatched analogues. The melting characteristics of $rI_n \cdot rC_n$ and the two mismatched analogues were measured simultaneously in 1-cm cells in a Pye-Unicam SP1800 spectrophotometer equipped with an SP877 electrically heated cell holder and an SP876 Accuran temperature program controller. The solution temperature was elevated by 0.5°/min, and the absorbance was recorded every 2 min. Hyperchromicity was measured between 30° and 77° at 250 nm. In SSC (pH 7.0) $rI_n \cdot r(C_{12},U)_n$ exhibited a T_m value (temperature at which 50% of the total hyperchromicity is achieved) of 60° and 71.5% hyperchromicity; $rI_n \cdot r(C_{29},G)_n$, a T_m of 61° and 73% hyperchromicity; and $rI_n \cdot rC_n$, a T_m of 63.5° and 77% hyperchromicity. BRL 5907 displayed a T_m of 94° and 40% hyperchromicity measured at 260 nm between 30° and 100°.

For studying RNase degradation, each of the dsRNAs (approximately 85 μg) was dissolved in 0.15 M NaCl (2.5 ml) and its spectrum was recorded in a 1-cm cell in a Pye-Unicam SP1800 spectrophotometer. The time taken for half the final hyperchromicity to be developed ($T_{1/2\ Hc}$) gives a measure of the rate of degradation (Table 1A). In a second experiment, the nucleic acid to enzyme ratio was increased to 110:10 (w/w) to give slightly longer $T_{1/2\ Hc}$ values (see Table 1B). The results of Table 1 confirm an earlier finding (14) with the two original preparations of mismatched bases: their propensity for accelerated degradation by pancreatic RNase. The degradation of the natural material, BRL 5907, was markedly slower than that of the other duplexes.

The instrument was then set to record the absorbance of the four samples at 250 nm at 1-min intervals, and the tempera-

TABLE 1

Degradation of $rI_n \cdot rC_n$, $rI_n \cdot r(C_{12},U)_n$, $rI_n \cdot r(C_{29},G)_n$, and BRL 5907 by pancreatic RNase

Sample	Hyperchromicity	$T_{\frac{1}{2}Hc}$
	%	*min*
A. Measured at 17 hr		
$rI_n \cdot r(C_{12},U)_n$	74	2
$rI_n \cdot r(C_{29},G)_n$	80	2
$rI_n \cdot rC_n$	77	3.5
BRL 5907	42	50
B. Measured at 23 hr		
$rI_n \cdot r(C_{12},U)_n$	75	5
$rI_n \cdot r(C_{29},G)_n$	82	8.5
$rI_n \cdot rC_n$	86	11
BRL 5907	53	230

ture was left at 25°. Bovine pancreatic ribonuclease (Miles Laboratories) (10 μg in 10 μl of 0.01 M sodium acetate, pH 6.0) was added to each reference and sample cell. The recording was started as soon as possible, and after the first 10 min the absorbance was measured at 10-min intervals for up to 90 min. Then a final measurement was made after 17 hr. To ensure that the nucleic acids were fully degraded after RNase treatment, 10 N NaOH (approximately 10 μl) was added to each cell; no further degradation was noted.

Viruses

A lethal strain of encephalomyocarditis virus was purchased from Searle, and male CD_1 mice of the Charles River strain were used. These mice weighed approximately 20 g each at the start of the experiments and had been accommodated in the animal house at Brockham Park for at least 1 week before use.

The dsRNAs were stored in buffer A at concentrations of 1.0, 0.1, 0.01, and 0.001 mg/ml. The solutions were stored at 4° between experiments, and the experiments were conducted in double-blind fashion.

The drugs were administered by intraperitoneal injection in a volume of 0.1 ml/mouse in buffer A. Groups of 10 mice were used, and 1 day after drug administration the mice were infected by intraperitoneal injection of 0.1 ml of a virus suspension (day 0). The infectivity of EMC virus was titrated at the same time in groups of 10 mice, using five or six 10-fold dilutions of virus.

To assess results, animals were inspected at a regular time each day and deaths were recorded over a period of 13 days. In each group the reciprocals of the day of death for each mouse were summed and averaged, and the reciprocal of this was used as the mean survival time for the group. The total group deaths in the virus titrations were plotted ($\log_{10}$ dilution on probit x linear graphs). From this, we calculated the LD_{50} of the virus, from which, in turn, the challenge levels were calculated.

Comparative 7-Day Intraperitoneal Repeat Dose Toxicity Study in Mice

Animals. Male HaM/ICR strain mice (Charles River, U. K.) were randomly allocated to five groups of six animals.

Design and dosage. BRL 5907 was used as a solution of 22–24 mg/ml in NaCl, and $rI_n \cdot rC_n$ and its mismatched analogues, as solutions of 1.7 mg/ml in buffer A. Each stock solution was diluted with 0.9% NaCl so that a constant volume of 0.1 ml/10 g could be administered to each group intraperitoneally. The four compounds were each tested at a dose level of 4 mg/kg/day for 7 consecutive days, and a control group was treated with 0.9% NaCl solution at 0.1 ml/10 g. The animals were bled from the retro-orbital sinus on days 2 and 8 (24 hr after the previous dose) for measurement of total white blood cells and differential, microhematocrit, and platelet count. After bleeding on day 8, the animals were killed and the thymus, spleen, and adrenal glands were removed and weighed.

Immunogenicity and Antigenicity of $rI_n \cdot rC_n$ and Its Mismatched Analogues

$rI_n \cdot rC_n$ and its two mismatched analogues were placed in solution at 1.7 mg/ml (buffer A) and were diluted in sterile 0.9% NaCl to a final concentration of 1 mg/ml. This solution was used for injection throughout the experiments; BRL 5907 was also used at a concentration of 1 mg/ml in sterile NaCl.

Female Balb/C mice (Southern Biologicals, Ltd.) were 8–10 weeks of age and weighed 19–23 g; New Zealand white female rabbits (Ranch Rabbits, Ltd.) weighed 2.4–3.0 kg at the start of the experiment. Guinea pigs were Hartley strain animals of either sex, bred in the animal breeding unit of Beecham Laboratories.

Immunization. Mice received repeated weekly intraperitoneal injections of 0.1-ml volumes (100 μg) of the test solutions and one intravenous injection of 0.2 ml via the lateral tail vein. Blood samples (approximately 0.4 ml) were obtained from the retro-orbital sinus under ether anesthesia. Rabbits were injected intravenously weekly with 0.5 ml (500 μg) into the mar-

ginal ear vein, and bleedings, of approximately 5 ml of blood, were made also from this vein, without anesthesia.

Antibody assay. Sera were tested for ability to bind [^{3}H]$rI_n \cdot rC_n$. Briefly, 100 μl of serum (or serum diluted in borate-buffered NaCl, pH 8.3) were incubated with approximately 200 ng of $rI_n \cdot rC_n$ contained in 100 μl. The bound antigen was then precipitated with 40% saturated ammonium sulfate. After washing in 40% saturated ammonium sulfate, the precipitate was solubilized and then diluted in 12 ml of scintillation fluid, and activity was measured in a Packard scintillation counter. From this activity the percentage of added antigen bound was calculated. For further quantitation of antibody, the serum was titrated using a suitable range of dilutions. The 33% binding point was determined graphically and used to calculate antigen-binding capacity (ABC_{33}) per milliliter of serum. Cross-reactivity was also tested using the ability of the different antigens to induce a passive cutaneous anaphylaxis reaction in guinea pigs. Briefly, 0.1-ml volumes of serum, diluted in NaCl, were injected intradermally into the shaved flanks of two animals. Between 18 and 20 hr later the reaction was revealed by intravenous injection of 250 μg of antigen together with 0.3 ml of 5% pontamine sky blue dye. After a further 14–20 min the sites were examined for positive reactions, and the diameters of the sites were measured using a Bencard skin reaction gauge.

RESULTS

Following the lead of our earlier work (14) on the mismatched polynucleotide duplexes $rI_n \cdot r(C_{13},U)_n$ and $rI_n \cdot r(C_{20},G)_n$, we made two new preparations of $rI_n \cdot r(C_{12},U)_n$ and $rI_n \cdot r(C_{29},G)_n$ duplexes in gram quantities sufficient for testing their antiviral properties and ability to evoke measurable changes in other physiological events. These preparations were used in our recent (15) and this current study. As we have noted earlier, other physiological events induced by polynucleotide duplexes could manifest themselves as overtly "toxic" reactions, but we have commented that the term "toxicity" is obviously difficult to define, both conceptually and experimentally. In this study, as in the immediately preceding one (15), 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.

Extension of Antiviral Properties of $rI_n \cdot r(C_{12},U)_n$ and $rI_n \cdot r(C_{29},G)_n$ in EMC Virus-Infected Mice

Earlier we had shown that the newly prepared mismatched complexes $rI_n \cdot r(C_{12},U)_n$ and $rI_n \cdot r(C_{29},G)_n$ are active as measured by the protection of mice against Semliki Forest virus (15). $rI_n \cdot r(C_{29},G)_n$, however, appeared to be slightly less active than $rI_n \cdot rC_n$ and $rI_n \cdot r(C_{12},U)_n$ at the 10-μg dose level. These results are supported and extended by the current ones.

In the first experiment (Table 2) the LD_{50} of EMC virus titrated at $10^{-6.2}$. Challenge was made at 10^{-5} dilution, thus equivalent to $LD_{50} \approx 16$. The second experiment was again performed at a relatively low viral dosage. The LD_{50} of EMC virus was $10^{-6.3}$; thus a 10^{-5} dilution provided $LD_{50} \approx 20$. The third experiment was carried out at an LD_{50} of 200 (10^{-4} dilution of virus stock), or relatively high viral dosage.

It is immediately apparent that each dsRNA tested did afford protection against this lethal viral challenge, and, as expected, the protective effects became more apparent at lower viral dosage (16–20 LD_{50}) and were also dependent on drug concentration.

While there are many ways in which comparisons can be made in studies of this sort, we used several methods, including an estimate of the protective dose for 50% of the animals and a calculation of the total number of survivors under each experimental condition. Using these methods, it was possible to show that $rI_n \cdot r(C_{29},G)_n$, while possessing distinct an-

TABLE 2

Effects of dsRNAs on EMC virus infection

Each group consisted of 10 animals unless otherwise stated.

Polynucleotide duplex	Dose	16 × LD_{50}			20 × LD_{50}			200 × LD_{50}		
		Deaths	ST[a]	ST ratio[b]	Deaths	ST	ST ratio	Deaths	ST	ST ratio
	mg/kg		*days*			*days*			*days*	
$rI_n \cdot r(C_{12},U)_n$	5.0	1	70	12.3	1/9	83	18.3	4	13	3.5
	0.5	3	21	3.7	3	23	5.1	1/8	32	8.6
	0.05	3	14	2.5	2	16	3.6	8	5.8	1.6
	0.005	6	11	2.0	8	6.5	1.4	10	5.2	1.4
$rI_n \cdot rC_n$	5.0	1	70	12.3	2	33	7.3	2	52	14
	0.5	2	32	5.6	1	60	13.2	6	8.5	2.3
	0.05	5	12	2.0	1	80	17.6	9	5.0	1.4
	0.005	7	5.8	1.0	5	8.9	2.0	10	4.5	1.2
$rI_n \cdot r(C_{29},G)_n$	5.0	3	15	2.7	0	>130	>29	4/9	13	3.5
	0.5	5	13	2.2	3	18	3.9	5/9	9.1	2.5
	0.05	7	7.1	1.3	4/7	7.8	1.7	6/7	5.1	1.4
	0.005	8	6.0	1.1	9	4.5	1.0	10	4.7	1.3
BRL 5907	5.0	1	100	17.5	2/9	24	5.3	3	16	4.4
	0.5	4	15	2.5	3	19	4.1	4	14	3.7
	0.05	4	11	1.8	2	28	6.2	7	6.8	1.8
	0.005	8	5 5.6	1.0	4	14	3.1	9	5.0	1.4
Infected controls		8	5.7	1.0	8	4.5	1.0	10	3.7	1.0

[a] ST survival time.

[b] ST ratio = survival time of the test group divided by survival time of the infected control group.

tiviral properties, probably did not display the same level of specific activity as $rI_n \cdot rC_n$, $rI_n \cdot r(C_{12},U)_n$, and BRL 5907. In these current tests the latter three double-helical RNAs behaved quite similarly, so that further distinctions were unwarranted.

Comparative 7-Day Intraperitoneal Repeat Dose Toxicity Study in Mice

In our preceding publication, we confirmed previous work (17) that a single dosage of RNA duplexes in the mouse was sufficient to elicit a potent mitogenic response in murine splenic cells in addition to the antiviral effects. However, the early mitogenic response (at 45 hr) normally elicited by $rI_n \cdot rC_n$ (0.3 mM) was reduced by one-half to two-thirds by $rI_n \cdot r(C_{12},U)_n$ and $rI_n \cdot r(C_{29},G)_n$. In other words, the mismatched complexes caused much less arousal of the immune system as judged by their direct mitogenic effects. The current study was carried out to determine whether the relative diminution of this specific response induced by the mismatched complexes would extend to other responses of the intact mouse to the presence of dsRNAs in various available molecular conformations. Although our earlier data suggested that the mismatched analogues were apparently less acutely toxic (as judged by mortality), the "all-or-none" feature of this phenomenon clearly did not permit quantitative distinctions. We also reasoned that the acute toxicity-mortality experiments (15) had a second intrinsic problem: whenever an overwhelming amount of any potentially toxic substance is introduced into an organism, the system of natural resistance (such as the disposal and repair processes) tends to break down and become inoperative. Therefore, since the previous acute mor-

tality experiments might not necessarily have reflected the tolerance of the organism's protective mechanism with its natural disposal and repair processes functioning under normal conditions, current experiments were designed to approach this important objective more closely.

In the 7-day repeat dosage study, BRL 5907 and $rI_n \cdot rC_n$ caused a spectrum of toxic effects previously seen with BRL 5907, and considered to be characteristic of dsRNA in this test. This comprised a marked but transient loss in body weight, acute lymphopenia and thrombocytopenia, and, on day 8, neutrophil leukocytosis, anemia, splenomegaly, and thymic atrophy (Fig. 1 and Table 3). This was the first such occasion for direct comparison of the response to administration of a naturally occurring and a synthetically derived dsRNA; BRL 5907 and $rI_n \cdot rC_n$ appeared to be equally inductive of cell damage. The acute lymphopenia and thymic atrophy, seen in their most pronounced forms with animals receiving either the naturally occurring or the synthetic dsRNA (perfect duplexes), are reminiscent of the cytopathic effect of dsRNA on lymphocytes frequently seen *in vitro* (see ref. 4 for review). These animals showed the typical symptomatology associated with their recorded loss in body weight, namely, hunched posture and general inactivity. In additional analyses (not shown) at day 8, adrenal weight in the BRL 5907 group was significantly increased ($p < 0.05$).

$rI_n \cdot r(C_{29},G)_n$ was of intermediate toxicity, as it did not appear to cause thymic atrophy (Table 3) or loss of weight. $rI_n \cdot r(C_{12},U)_n$ was substantially less toxic, since it also had a modest effect on body weight (Fig. 1) and at day 8 was found to have caused neither anemia, nor changes in neutrophil count or thymic weight (Table 3). In other respects the mismatched duplexes were qualitatively and quantitatively similar to BRL 5907 and $rI_n \cdot rC_n$. Specifically, the effects on relative spleen weight (Table 3) noted with both mismatched analogues were reminiscent of our earlier study (15), in which these same complexes, while reducing the level of mitogenic response of murine splenocytes (*in vitro*) relative to $rI_n \cdot rC_n$, did not abolish it altogether. These results further suggest that enlargement of this organ might be moderated in part by the direct mitogenic stimulus measurable *in vitro*. Lymphocytopenia, also seen on day 2 with each of the molecular preparations, could be related to a similar biochemical event, possibly associated with the redistribution of these cells into the splenic compartment. By day 8, all groups receiving dsRNAs showed some evidence of red cell regeneration, including evidence of polychromasia and anisocytosis. Similarly, thrombocytopenia, customarily seen within 48 hr (also in humans[2]), occurred in all groups.

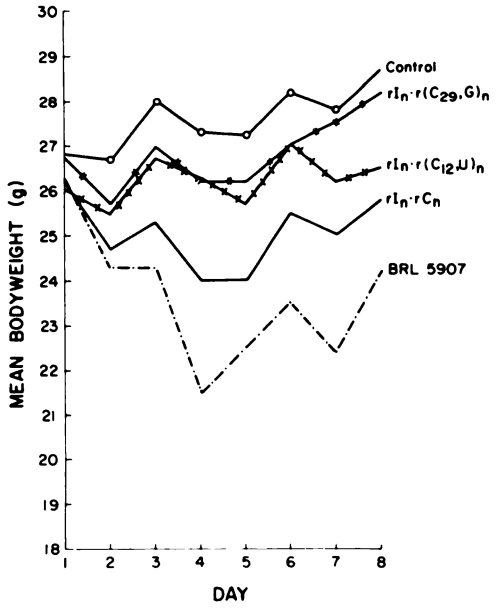

FIG. 1. *Mean body weight of mice receiving daily injections of matched and mismatched polynucleotide duplexes*

The experimental procedure is described under MATERIALS AND METHODS.

Immunogenicity and Antigenicity of $rI_n \cdot rC_n$ and Its Mismatched Analogues

The immunological reactivity of dsRNAs was noted earlier by others (18, 19), including the triggering of antibody formation against dsRNA, the adjuvant effect of double-helical RNA on a number of immune responses (for recent summary, see ref. 20), and the possible role of these

TABLE 3

Spectrum of toxicity seen in mice receiving repeated administration of a naturally occurring RNA, $rI_n \cdot rC_n$, and its two mismatched analogues

Each polynucleotide duplex and the naturally occurring BRL 5907 were administered at a dose of 4 mg/kg/day. Values are percentage change compared with the control group; numbers in parentheses indicate the ranked order of toxicity of each compound for a particular parameter, where 1 = least effect and 4 = greatest effect. *p* values were determined by Student's *t*-test (modified for groups with unequal variance, where appropriate).

Parameter	BRL 5907	$rI_n \cdot r(C_{12}, U)_n$	$rI_n \cdot rC_n$	$rI_n \cdot r(C_{29}, G)_n$
	%	%	%	%
Body weight gain, day 4	−20 (4)	−0.5 (1)	−10 (3)	−3.5 (2)
Body weight gain, day 8	−15 (4)	−5 (2½)	−4 (2½)	−1 (1)
Lymphocytes, day 2	−76[a] (4)	−41 (1)	−49[b] (2)	−63[b] (3)
Thrombocytes, day 2	−18 (1½)	−18 (1½)	−32[a] (3½)	−30[c] (3½)
Neutrophils, day 8	+45 (3)	−3 (1)	+68 (4)	+32 (2)
Packed cell volume, day 8	−19 (4)	+1 (1)	−9[a] (3)	−6 (2)
Relative spleen weight, day 8	+41[b] (2½)	+36[a] (1)	+56[c] (4)	+40[b] (2½)
Relative thymus weight, day 8	−10 (3½)	−3 (1½)	−8 (3½)	+6 (1½)
Total rank score	26½	10½	25½	17½
Over-all rank order	=3	1	=3	2

[a] $p < 0.01$.
[b] $p < 0.05$.
[c] $p < 0.001$.

molecular events in host recovery from viral infection (4). Little is known of the temporal requirements, i.e., how long the double helix of RNA must be preserved, for triggering antibody formation against this specific conformation of RNA. Our studies were thus directed in part toward determining whether the kinetic term, which we have shown to be important in the differential production of other various aspects of the physiological response to dsRNA, might also be significant in eliciting an antibody response in mice and rabbits. The time course of serum binding activity for $[^3H]rI_n \cdot rC_n$ (groups of 12 mice) is shown in Table 4 for each of the interferon inducers, $rI_n \cdot rC_n$, $rI_n \cdot r(C_{12},U)_n$, $rI_n \cdot r(C_{29},G)_n$, and BRL 5907. Production of antibody against the tritiated probe was most rapid initially with $rI_n \cdot rC_n$, although by the end of the dosage period (8 weeks) antibody induced by $rI_n \cdot r(C_{29},G)_n$ was quite similar. Antibody induction by $rI_n \cdot r(C_{12},U)_n$ remained modest to very low. For comparative purposes, we also determined the time course of serum binding activity for $[^3H]rI_n \cdot rC_n$ in Balb/C mice immunized with BRL 5907 (Table 4). Although this latter binding clearly represented only cross-reactive antibody for the polynucleotide duplex, significantly it was evidently at a very high level (binding up to 60% of the tritiated $rI_n \cdot rC_n$ probe by 8 weeks) throughout the dosage period. While these antibody levels clearly did not provide an absolute comparison, since sera were assayed only against $[^3H]rI_n \cdot rC_n$, we can deduce that there was substantial cross-reactivity. This notion was supported by the cross-reactivity of BRL 5907 antibody against $rI_n \cdot rC_n$ (*vis-à-vis* the subtle differences in conformations of the two mismatched complexes when compared with $rI_n \cdot rC_n$) and the results of a passive cutaneous anaphylaxis study, which are noted below. In addition, results similar to those in Balb/C mice were obtained with outbred groups of mice (CFLP). Additional comparisons of BRL 4907 and $rI_n \cdot rC_n$ have recently been reported by Cunnington and Naysmith (21).

Because the amounts of antibody seen in the mouse were relatively low, we undertook a more detailed study in the rabbit, in which quantitation and specificity studies of the dsRNA antibody would be more feasible.

The time course of antibody reactive

TABLE 4

Binding activity for [^{3}H]$rI_n \cdot rC_n$ in sera of Balb/C mice given weekly injections of $rI_n \cdot rC_n$, its mismatched analogues, or BRL 5907

For details, see MATERIALS AND METHODS. In the experiments with $rI_n \cdot rC_n$ and its mismatched analogues, 18,868 dpm (212 ng/test) of antigen were added. In the BRL 5907 experiment, 18,524 dpm (208 ng/test) of antigen were added.

Time	Added antigen bound by 33 μl of serum			
	$rI_n \cdot r(C_{12}, U)_n$	$rI_n \cdot r(C_{29}, G_n)$	$rI_n \cdot rC_n$	BRL 5907
wk	*% ± SE*	*% ± SE*	*% ± SE*	*%*
0[a]	3.8 ± 0.5	3.7 ± 0.8	4.6 ± 0.4	2.2
1	7.5 ± 0.7	7.7 ± 1.8	17.1 ± 2.6[b]	29.5
2	7.5 ± 1.0	9.6 ± 1.5	15.1 ± 2.9[c]	44.2
6	13.9 ± 3.5	18.2 ± 6.3	4.7 ± 0.5	48.5
8	6.1 ± 0.5	14.0 ± 6.2	12.6 ± 3.3	63.4

[a] Before inoculation.

[b] $rI_n \cdot rC_n > rI_n \cdot r(C_{12},U)_n$ and $rI_n \cdot r(C_{29},G)_n$; $p < 0.01$.

[c] $rI_n \cdot rC_n > rI_n \cdot r(C_{12},U)_n$; $p < 0.05$.

with [^{3}H]$rI_n \cdot rC_n$ in groups of four or five rabbits is given in Fig. 2. It is clear that substantial amounts of antibody were induced by both $rI_n \cdot rC_n$ and $rI_n \cdot r(C_{29},G)_n$, but that $rI_n \cdot rC_n$ was markedly superior as an immunogen. In strong contrast, $rI_n \cdot r(C_{12},U)_n$ gave rise to little or no antibody formation during the entire 9-week dosage period.

Quantitation of antibody levels in individual rabbits at 7 weeks was also carried out, and the results (Table 5) supported the evidence of the time course study concerning the relative levels of responses to each compound (Fig. 2). In addition, we noted that the amount of anti-[^{3}H]$rI_n \cdot rC_n$ antibody observed (up to an ABC_{33} of 50 μg/ml) was quite analogous to those expected for BRL 5907 in this species. Specificity studies (data not shown) were also performed with six of these sera by means of passive cutaneous anaphylaxis in guinea pigs (details are presented under MATERIALS AND METHODS). The results of these passive cutaneous anaphylaxis reactivity tests indicated that antibody raised against $rI_n \cdot rC_n$ recognized the mismatched duplexes to a similar degree within limits of the test.

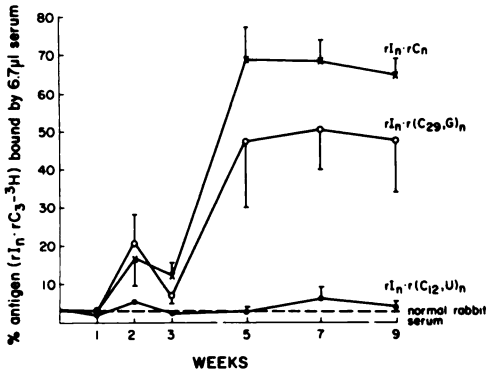

FIG. 2. *Differential production of antibody against dsRNA by repeated administration of $rI_n \cdot rC_n$ and its mismatched analogues in rabbits*

Each point represents the mean of four or five sera ± standard error (vertical bars). No significant statistical differences were seen until 3 weeks [$rI_n \cdot rC_n$, $rI_n \cdot r(C_{12},U)_n$, ($p < 0.01$)]; at 5 weeks, $rI_n \cdot rC_n$, $rI_n \cdot r(C_{29},G)_n$, $rI_n \cdot r(C_{12},U)_n$ ($p < 0.05$); at 7 weeks, $rI_n \cdot rC_n$, $rI_n \cdot r(C_{29},G)_n$, $rI_n \cdot r(C_{12},U)_n$, ($p < 0.01$); at 9 weeks, $rI_n \cdot rC_n$, $rI_n \cdot r(C_{29},G)_n$, $rI_n \cdot r(C_{12},U)_n$, ($p < 0.01$).

TABLE 5

Antigen-binding capacity (ABC_{33}) of rabbit sera obtained 7 weeks after commencement of administration of $rI_n \cdot rC_n$ and its mismatched analogues

Immunogen	Rabbit	ABC_{33}	Mean ABC
		μg/ml	*μg/ml*
$rI_n \cdot r(C_{12},U)_n$	86	4.2	
	95	1.4	2.62
	96	<1	
	105	4.3	
$rI_n \cdot r(C_{29},G)_n$	88	30.4	
	101	10.8	
	102	17.2	17.00
	107	21.0	
	108	5.6	
$rI_n \cdot rC_n$	97	41.3	
	98	29.1	42.47
	99	58.2	
	100	41.3	

DISCUSSION

In this study we have extended the antiviral properties of $rI_n \cdot rC_n$ and its mismatched analogues $rI_n \cdot r(C_{12},U)_n$ and $rI_n \cdot r(C_{29},G)_n$ to include EMC virus infection of mice at relatively high and low viral challenge dosages (Table 2). The data lend support our previous conclusion (14,

15) that these two types of mismatched analogues are active and similar to perfectly matched $rI_n \cdot rC_n$ in their antiviral properties. As noted before, $rI_n \cdot r(C_{29},G)_n$ may be slightly less active in the mouse. The "toxicity" of $rI_n \cdot rC_n$ was compared with that of these two mismatched analogues as well as the naturally occurring dsRNA BRL 5907 in the mouse following repeated administration of dsRNA for 7 days (Fig. 1 and Table 3). These results extend our previous observations in the mouse model (15), by demonstrating that features such as gross change in body weight and sustained changes in hematological indices need not accompany repeated administration of dsRNAs, specifically, those with subtle structural alterations. These results reinforce the suggestion from our previous paper (15) that these mismatched duplexes are in fact significantly less able than $rI_n \cdot rC_n$ to trigger additional biological responses. Our initial suggestion originated in the experimental observation that the 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 (14) and therefore might be removed much more readily than $rI_n \cdot rC_n$; the biochemical basis for this sensitivity was again demonstrated in the current paper (see MATERIALS AND METHODS), using two lots of newly prepared mismatched duplexes.

The studies on the toxicity and RNase degradation of $rI_n \cdot rC_n$ and BRL 5907, compared with those of the mismatched duplexes of $rI_n \cdot rC_n$, lend some support to the scientific basis of our earlier prediction on the relative toxicities of these duplexes. In the earlier experiments, in which the polynucleotide duplexes were introduced into the animal at a very low dose (1–0.1 μg/kg), as in the pyrogenicity tests with rabbits, the difference in "toxicity" between $rI_n \cdot rC_n$ and its mismatched duplexes was very striking, i.e., about 40–100 times less for the mismatched duplexes. In this situation, we reasoned that the injected polynucleotide duplexes were probably degraded and removed rapidly by the animal. As expected from this conclusion, 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 which preceded this current report, in which a relatively massive amount of polynucleotide duplex (400 μg) was injected intravenously on one occasion into the mouse, the differences between $rI_n \cdot rC_n$ and the two mismatched duplexes became much less and distinctions were more difficult to evaluate. For instance, at a 400-μg dose, 80% of the animals died upon receiving $rI_n \cdot rC_n$, while 40% died upon receiving $rI_n \cdot r(C_{13},U)_n$ and none died upon receiving $rI_n \cdot r(C_{20},G)_n$ (15). At a 350-μg dose, 20% died upon receiving $rI_n \cdot rC_n$, while none died after receiving $rI_n \cdot r(C_{13},U)_n$. A careful evaluation of the difference between the "toxicity" of $rI_n \cdot rC_n$ and the mismatched analogues could not be made because of the relatively narrow range of concentration used and the apparent all-or-none response. Thus, in view of the intrinsic problems of acute toxicity-mortality data (15), the current experiments (Fig. 1 and Table 3) seem potentially more useful as they might relate to the possible chemotherapeutic application of these polynucleotide duplexes. The possible advantage of the mismatched duplexes is based on proper functioning of the disposal process in the animal; the current data on chronic toxicity at lower dosages may more closely mimic the degree of other biological responses which would occur with mismatched nucleic acid duplexes used in a chemotherapeutic situation.

Finally, we turned in the current report to immunological studies designed to determine whether the kinetic term (which we have shown to be important in other aspects of the differential physiological response to dsRNA) might also be significant in eliciting antibody responses to double-helical RNA in mice and rabbits. Earlier we had confirmed the report of Dean *et al.* (17) that $rI_n \cdot rC_n$ is a potent mitogen for lymphocytes and spleen cells in the mouse. Collaro *et al.* (18) had reported that intraperitoneal injection of $rI_n \cdot rC_n$ into the mouse caused an immediate, transient cytopathic action on the reticuloendothelial system, enlargement of the spleen, and

selective stimulation of antibody-producing cells. Recently the effect of $rI_n \cdot rC_n$ on the immune system of the host has received much attention (see ref. 4). We found that the mismatched complexes were only one-third to one-half as mitogenic as $rI_n \cdot rC_n$ (15). Very recent results[2] on human splenic cells mimic those reported in the murine system (15).

We now have shown that the time course of the development of serum binding activity for $[^3H]rI_n \cdot rC_n$ is rapid in mice (Table 4) and rabbits (Fig. 2) receiving weekly injections of aqueous $rI_n \cdot rC_n$ or the naturally occurring dsRNA, BRL 5907. [Interestingly, antibody raised against BRL 5907 in the mouse reacts with $[^3H]rI_n \cdot rC_n$ (Table 4), supporting the notion that antibody against dsRNA recognizes primarily over-all helical structure rather than base sequence or subtle conformational features as exemplified by the mismatched complexes.] By contrast, the rate of antibody formation in the mouse against the mismatched complexes was much slower, although by the end of the dosage period (8 weeks) cross-reactive antibody induced by $rI_n \cdot r(C_{29},G)_n$ was similar to that induced by $rI_n \cdot rC_n$. The levels of cross-reactive antibody induced by $rI_n \cdot r(C_{12},U)_n$ remained low throughout, perhaps because of the low immunogenic potential of short stretches of normal helical structure.

In the rabbit, we were able to do more quantitative studies, some providing insight into the specificity of the antibodies raised. The time courses of antibody formation against the various dsRNAs, monitored by the $[^3H]rI_n \cdot rC_n$ probe, were similar in the case of the mismatched bases (Fig. 2), but quantitative studies of antibody levels (Table 5) confirmed the lower immunogenic potential of the mismatched duplexes. Perhaps most impressively, $rI_n \cdot r(C_{12},U)_n$ gave rise to little or no apparent antibody formation during the entire 9-week period. $rI_n \cdot rC_n$ was clearly a more potent immunogen than its mismatched analogues, and $rI_n \cdot r(C_{29},G)_n$ was of intermediate potency. Passive cutaneous anaphylaxis tests in the guinea pig, employed as an index of antigenic relatedness of the three synthetic interferon inducers, showed a strong similarity, if not identity, in their behavior. A particular merit of experiments in rabbits is that, by comparing the current results with those previously published (15), one can establish a table of minimum polynucleotide concentrations necessary to achieve a set of biological responses, e.g., circulating interferon, a pyrogenic response, and antibody formation. Presumably these could also be measured concurrently at the same dose level of polynucleotide duplex in the same animal and thereby integrate each of the results within a specific host.

It should be emphasized that the six test systems established in the two species, mouse and rabbit, may be useful in signaling cellular targets and therapeutic potential in man. Thus the protection against viral disease, the immediate responsiveness of lymphoid cells (mitogenic response), the induction of circulating interferon, the level of febrile responsiveness, and, as reported now, the cumulative responses of hematological and lymphoid populations (including antibody formation against dsRNA) to repeated polymer administration are measurements directly pertinent to the establishment of therapeutic efficacy of these polynucleotide duplexes. Collectively, the data from the two species indicate that these mismatched duplexes, $rI_n \cdot r(C_{12},U)_n$ and $rI_n \cdot r(C_{29},G)_n$, are comparable in their antiviral properties to $rI_n \cdot rC_n$ but have fewer secondary effects. The rather flat dose-response curve in the EMC virus test (Table 2) may have contributed to the similarity in antiviral behavior; however, the over-all conclusion is strengthened by our earlier data with Semliki Forest virus infection in the same species (15). The current study further helps to establish a theoretical framework for designing a polynucleotide duplex as an interferon inducer having greater therapeutic efficacy than $rI_n \cdot rC_n$. However, for species with greatly differing serum RNase activities (e.g., mice and humans), the optimum sensitivity of the duplex to RNase should be expected to vary. In general, we have classified the biological responses to polynucleotide duplexes into

two categories: desirable effects (e.g., antiviral activities) and undesirable effects, or "toxicity." There exists a variety of clearly undesirable effects, such as inhibition of the synthesis of protein (22), DNA (23), and RNA (24) (for a review, see ref. 4). Particularly interesting, and perhaps at the molecular basis of many of cellular responses noted in the current paper, is the catalytic ability of properly and extensively matched (more than about 50 base pairs) dsRNA to inhibit the initiation of protein synthesis (25).

The strategy in designing a polynucleotide duplex of greater efficacy rests on the basic premise that either the structural or the temporal requirements of the desirable and the undesirable responses are not identical. Substantial efforts have been made in modification of the bases, backbone, and sizes of dsRNA (see ref. 5 for review). The rather limited yield of these approaches may stem, in part, from the previous relative unavailability of pertinent systems for testing a spectrum of physiological responses to a specific RNA duplex and, in part, from the relative absence of theoretical guidance for an experimental approach in significantly modifying the therapeutic ratio.

Emphasis has been placed in our current program on the possible differences in temporal requirements between the antiviral and the other biological responses. This approach is based on the observation (26) 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 at 37°). Therefore there is a strong possibility that the "time constant" in triggering the desirable response and the "time constant" in induction of the other physiological responses might be sufficiently different to permit the design and development of an interferon inducer of efficacy superior to $rI_n \cdot rC_n$ (4). Our preceding papers were predicated on this premise, and the present results seem to confirm the apparent advantages of this approach.

Several additional comments are in order. Obviously, as we have previously noted, this specific strategy will be successful for the development of a superior interferon inducer only when the "time constant," or 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 dsRNAs. For example, it is unlikely that all biological responses to dsRNA will have the same "time constants." Therefore, at least in theory, a series of dsRNAs with varying degrees of relative sensitivity to enzymatic degradation can be constructed to examine a number of biological responses. 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, e.g., the molecular target for dsRNA which results in catalytic inhibition of protein synthesis.

The approaches developed in the current paper, based on differences in temporal requirements, may therefore ultimately yield some insights into both the structure and location of "receptors" which induce the spectrum of biological responses on exposure to polynucleotide duplexes. A recently proposed hypothesis (4) suggests that many aspects of host morbidity normally encountered in local or systemic viral infection may in fact occur through the molecular mediation of dsRNA. Such recent experiments as those indicating the very high specific activity of dsRNA as an inhibitor of protein synthesis (25) and those ascribing the cell-killing potential of vesicular stomatitis virus specifically to its content of dsRNA (27) support this idea. Our current studies provide additional experimental approaches to this type of major question in viral pathogenesis and, at the same time, an interesting opportunity to evolve a chemotherapeutic compound of interest.

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