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Antiviral Activity of Poly(7-deazainosinic acid)-Derived Complexes *in Vitro* and *in Vivo*

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SUMMARY

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The antiviral activities and/or interferon-inducing abilities of poly(I)·poly(C), poly(I)·poly(br^{5}C), poly(c^{7}I)·poly(C), and poly(c^{7}I)·poly(br^{5}C) have been assessed in a variety of cell cultures (human, rabbit, and mouse) and animals (rabbits, mice). In cultured cells the compounds were also examined for inhibition of host cell macromolecule synthesis. Lethal effects were looked for in mice sensitized with lead acetate. Although the extent of antiviral activity of the polymers varied from one assay system to another, the following order of (decreasing) activity appeared applicable to all systems: poly(I)·poly(br^{5}C) $\geqq$ poly(I)·poly(C) $>$ poly(c^{7}I)·poly(br^{5}C) $\geqslant$ poly(c^{7}I)·poly(C). Poly(I)·poly(C) and poly(I)·poly(br^{5}C) did not differ markedly in antiviral activity, interferon-inducing ability, or toxicity. However, in human skin fibroblasts, poly(I)·poly(br^{5}C) was distinctly superior as an interferon inducer, especially at lower doses. Poly(c^{7}I)·poly(C) was the least active of the four polymers and was also the most potent inhibitor of host cell macromolecule synthesis. Poly(c^{7}I)·poly(br^{5}C) equaled or even surpassed poly(I)·poly(C) in interferon-inducing activity in human skin fibroblasts; in animals, however, it proved definitely less active. In determining the antiviral behavior of the complexes studied, two effector mechanisms should be taken into account: interferon induction and inhibition of macromolecule biosynthesis. Which of these parameters will predominate would ultimately depend on the fate of the double-stranded complex after it has reacted with the cell.

INTRODUCTION

Poly(c^{7}I)[1] is a poly(I) analogue, whose synthesis and physical characteristics have recently been described by Torrence *et al.* (1) and Ikehara *et al.* (2). Poly(c^{7}I) forms 1:1 stoichiometric complexes with both poly(C) and poly(br^{5}C), one of which [poly(c^{7}I)·poly(br^{5}C)] proved to be at least as effective as poly(I)·poly(C) in inducing

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[1] The abbreviations used are: poly(c^{7}I), poly(7-deazainosinic acid); poly(br^{5}C), poly(5-bromocytidylic acid); poly(I), polyinosinic acid; poly(C), polycytidylic acid. Poly(I)·poly(C) represents the 1:1 complex formed from equimolar quantities of poly(I) and poly(C). Similar notation is used to indicate the other polynucleotide duplexes. Additional abbreviations are: VSV, vesicular stomatitis virus; HSF, human skin fibroblasts.

interferon and direct resistance to virus infection in primary rabbit kidney cell cultures (1). The interferon-inducing activity of poly(c^7I)·poly(C), poly(c^7I)·poly(br^5C), and their parent compounds, poly(I)·poly(C) and poly(I)·poly(br^5C), has now been explored in a number of assay systems, both *in vitro* (human and mouse cell cultures) and *in vivo* (mice, rabbits). Activity to toxicity ratios were assessed in lead acetate-treated mice. To account for their different behavior as antiviral agents, poly(c^7I)·poly(C) and its congeners were also examined for inhibition of macromolecule (RNA, DNA, protein) synthesis in cultured cells.

MATERIALS AND METHODS

Preparation and characterization of polynucleotides. These procedures have been described (1). s_{20} values were as follows: poly(c^7I), 4.8 S; poly(br^5C), 10.3 S; poly(I), 9.4 S; poly(C), 10.0 S. The complexes were prepared by mixing appropriate quantities of the homopolymers in distilled water. After lyophilization, the resulting material was dissolved in 0.1 M Tris-HCl–0.2 M NaCl (pH 7.0) at a concentration of 1 mg of polymer per milliliter (ml) and stored at 4°. Before testing, the polymers were further diluted to the appropriate concentrations either in Eagle's minimal essential medium, for the cell culture experiments, or in Dulbecco's phosphate-buffered NaCl, for the animal experiments.

Interferon production in human skin fibroblast cell cultures. A "superinduction" technique similar to that previously described for primary rabbit kidney cells (1) was employed to measure interferon induction in NS and FS-4 cells, two human diploid cell lines, the latter of which was kindly provided by Dr. J. Vilcek. The human diploid cells were exposed to 10, 1, or 0.1 μg/ml of the polymers for 1 hr, and to cycloheximide (10 μg/ml) for 6 hr. Actinomycin D (1 μg/ml) was added 4 hr after the onset of this treatment. At 6 hr the metabolic inhibitors were removed and the cells were washed and replenished with production medium (Eagle's minimal essential medium plus 2% calf serum; 2 ml/Petri dish). Approximately 20 hr after the cells had been incubated with production medium, the culture fluids were harvested and titrated for interferon as described previously (3, 4).

Interferon production in rabbits. Rabbits weighing approximately 1 kg were injected intravenously with 10 or 1 μg of the polymers (in 1 ml of phosphate-buffered NaCl). Blood samples were taken 1, 2, 4, 7, 12, and 24 hr thereafter, and the serum was assayed for interferon by reduction of vesicular stomatitis virus cytopathogenicity in PRK cell cultures (3).

Interferon production in interferon-primed mouse L-929 cell cultures. Confluent L-929 cell cultures in 60-mm plastic petri dishes were exposed to mouse interferon (100 units/ml) in minimal essential medium plus 3% calf serum (2 ml/Petri dish) for 16 hr, washed three times with minimal essential medium and then incubated with various concentrations (0.016, 0.08, 0.4, 2, and 10 μg/ml) of the polymers (in minimal essential medium, 1 ml/Petri dish) for 1 hr at 37°. The cells were washed again and further incubated with minimal essential medium plus 3% calf serum (4 ml/Petri dish) for 20 hr. At this time the supernatant fluids were withdrawn for interferon titration. Interferon titers were measured by a plaque reduction technique in L-929 cells with VSV as challenge virus, 50% virus plaque reduction corresponding to 1 unit of interferon.

Interferon production and toxicity in mice. Randomly bred female NMRI mice weighing 9–11 g were injected intravenously with various doses of the polymers, ranging from 0.002 to 100 μg (in 0.2 ml of phosphate-buffered NaCl) per mouse (10 mice per group for toxicity studies, four mice per group for interferon production studies). Lead acetate [$Pb(OOC-CH_3)_2$] (1 mg in 0.2 ml of distilled water) was injected intravenously immediately before the administration of the polymers. For measuring interferon production, blood samples were taken 3 hr after injection of the polymers and the serum interferon titers were determined by VSV plaque reduction in L-929 cells.

Induction of resistance to virus infection in cell cultures. PRK, HSF (either NS or

FS-4), HeLa, L-929, RK13, and VERO cell cultures (in tubes) were exposed to various concentrations of the polymers (10, 1, or 0.1 μg/ml in minimal essential medium plus 3% calf serum; 1 ml/tube) for 24 hr at 37°. The supernatant fluids were then removed and the cells were challenged with VSV (100 $CCID_{50}$/tube). Virus-induced cytopathogenicity was recorded as soon as it reached 100% in the control cell cultures.

Effect on RNA synthesis in cell cultures. Confluent PRK, HeLa, and VERO cell cultures (in plastic Petri dishes) were exposed to 10 μg/ml of the polymers (in minimal essential medium plus 3% calf serum; 2 ml/Petri dish) for 24 hr at 37°, washed three times with minimal essential medium, and then incubated for 30 min at 37° with [5-³H]uridine (1 μCi/ml of minimal essential medium; 1 ml/Petri dish). Acid-insoluble radioactivity was determined as described previously (5).

RESULTS

Depending on the assay system used to explore the interferon-inducing properties of the polymers, the following order of decreasing activity was noted. (a) Poly(I)·poly(br⁵C) > poly(c⁷I)·poly(br⁵C) ≅ poly(I)·poly(C) > poly(c⁷I)·poly(C) in human diploid cells, superinduced with cycloheximide and actinomycin D (Fig. 1). (b) Poly(I)·poly(br⁵C) ≅ poly(I)·poly(C) > poly(c⁷I)·poly(br⁵C) > poly(c⁷I)·poly(C) in rabbits (Fig. 2). The same order of activity was obtained whether the rabbits were injected with 1 μg or 10 μg of polymer. (c) Poly(I)·poly(C) > poly(c⁷I)·poly(C) > poly(I)·poly(br⁵C) > poly(c⁷I)·poly(br⁵C) in interferon-primed L-929 cells, when exposed to relatively high doses (2–10 μg/ml) of the polymers (Fig. 3). (d) Poly(I)·poly(C) > poly(I)·poly(br⁵C) > poly(c⁷I)·poly(br⁵C) > poly(c⁷I)·poly(C) in mice sensitized with lead acetate (Fig. 4A). A similar order of activity was witnessed in mice not treated with lead acetate (data not shown).

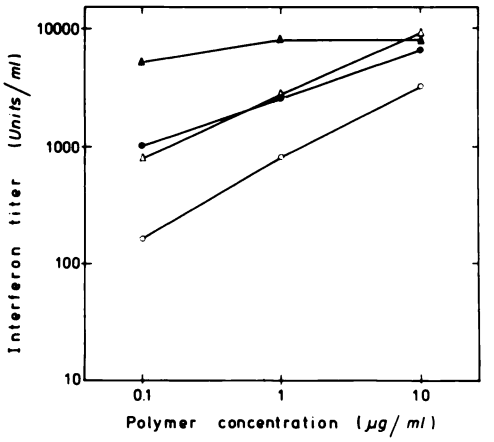

FIG. 1. *Interferon induction in HSF cell cultures superinduced with cycloheximide and actinomycin D*

▲, poly(I)·poly(br⁵C); △, poly(I)·poly(C); ●, poly(c⁷I)·poly(br⁵C); ○, poly(c⁷I)·poly(C). The results shown refer to those obtained in NS cells. Similar results were obtained in FS-4 cells.

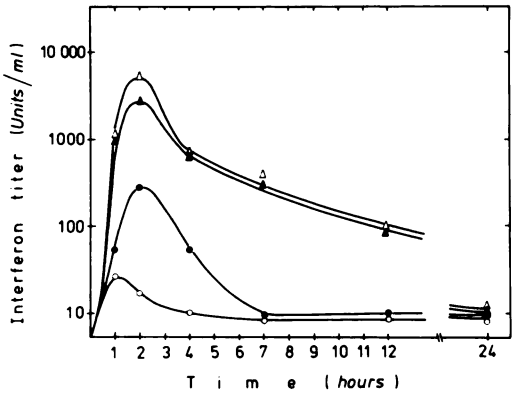

FIG. 2. *Interferon induction in rabbits*

▲, poly(I)·poly(br⁵C); △, poly(I)·poly(C); ●, poly(c⁷I)·poly(br⁵C); ○, poly(c⁷I)·poly(C). The polymers were injected at 1 μg/rabbit.

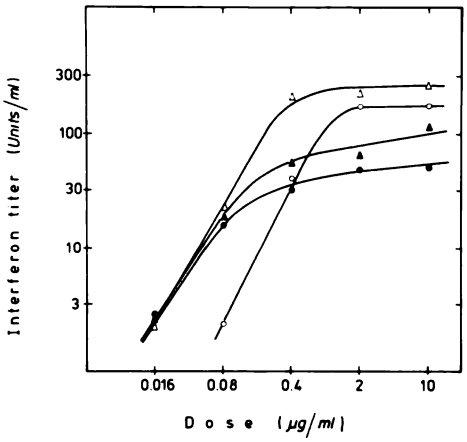

FIG. 3. *Interferon induction in interferon-primed L-929 cell cultures*

▲, poly(I)·poly(br⁵C); △, poly(I)·poly(C); ●, poly(c⁷I)·poly(br⁵C); ○, poly(c⁷I)·poly(C).

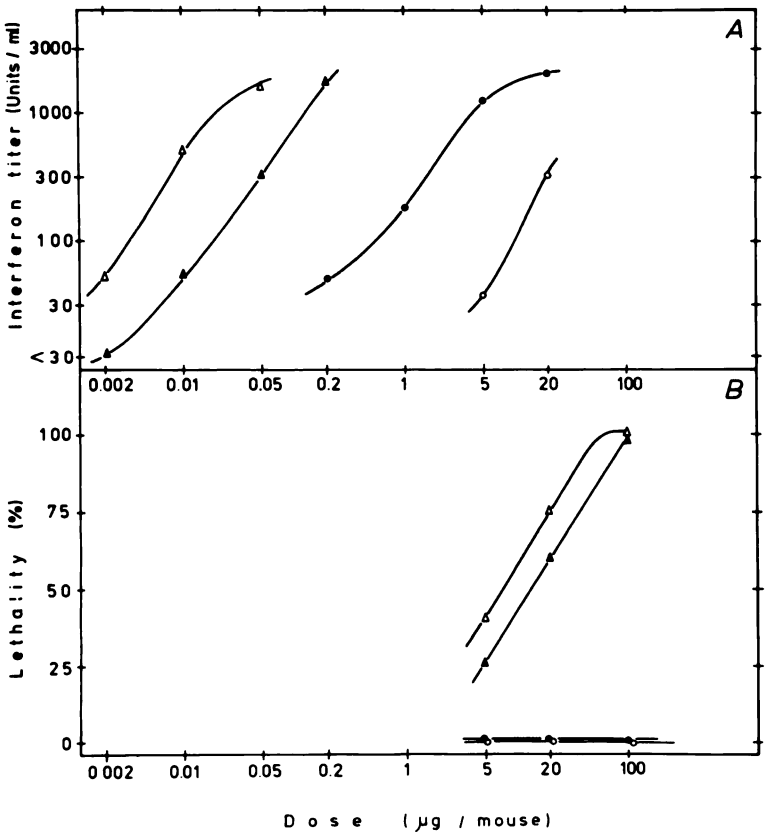

FIG. 4. *Interferon induction (A) and lethality (B) in lead acetate-treated mice*
▲, poly(I)·poly(br^{5}C); △, poly(I)·poly(C); ●, poly(c^{7}I)·poly(br^{5}C); ○, poly(c^{7}I)·poly(C).

In lead acetate-treated mice the order of toxicity appeared to reflect the order of activity (Fig. 4B). However, with poly(c^{7}I)·poly(br^{5}C) and poly(c^{7}I)·poly(C), toxicity could not be properly assessed, since both polymers failed to cause any lethal effect at the highest dose tested (100 µg/mouse).

In HSF cells (Fig. 1), poly(I)·poly(br^{5}C), poly(c^{7}I)·poly(br^{5}C), and poly(I)·poly(C) did not differ markedly in interferon-inducing activity when tested at 10 µg/ml. As the concentration of polymer was decreased, differences in activity became more pronounced, and at 0.1 µg/ml poly(I)·poly(br^{5}C) proved clearly more effective than poly(I)·poly(C) and poly(c^{7}I)·poly(br^{5}C).

Poly(I)·poly(C) renders the cell resistant to virus infection, even at polymer concentrations which do not lead to the release of detectable amounts of interferon in the cell culture medium. It has been postulated that the resistance induced by such low poly(I)·poly(C) concentrations is mediated by endogenous interferon production (6–10). The minimal concentration of poly(I)·poly(C) required to induce cellular resistance to virus infection varies considerably from one cell type to another (Table 1). In accord with previous findings (11), the cell cultures studies could be classified as follows [in order of decreasing sensitivity to the antiviral activity of poly(I)·poly(C)]: PRK > HSF > L-929 ≅ RK13 > HeLa > VERO. The minimal inhibitory concentrations recorded for poly(I)·poly(br^{5}C) corresponded well to those recorded for poly(I)·poly(C) (Table 1). Poly(c^{7}I)·poly(br^{5}C) and poly(c^{7}I)·poly(C), however, behaved quite differently from poly(I)·poly(br^{5}C) and poly-

(I)·poly(C). In PRK cells, poly(c^{7}I)·poly(C) was significantly less effective than, but poly(c^{7}I)·poly(br^{5}C) was equally effective as, poly(I)·poly(br^{5}C) and poly(I)·poly(C). In HSF (whether NS or FS-4), both poly(c^{7}I)·poly(br^{5}C) and poly(c^{7}I)·poly(C) were less effective than poly(I)·poly(br^{5}C) and poly(I)·poly(C). In HeLa cells, however, the poly(c^{7}I)-derived complexes displayed a greater antiviral effect than their unsubstituted counterparts. In VERO cells, none of the compounds showed appreciable activity.

How may the differences in the antiviral behavior of poly(c^{7}I)·poly(br^{5}C) and poly(c^{7}I)·poly(C), as compared to poly(I)·poly(br^{5}C) and poly(I)·poly(C), be accounted for? In view of the recently described antimetabolic properties of poly(c^{7}I) (5), it became mandatory to examine the effects of poly(c^{7}I)·poly(br^{5}C) and its congeners on host cell DNA, RNA, and protein synthesis. The effect on RNA synthesis is presented in Table 2. In PRK cells, only poly(c^{7}I)·poly(C) was found to inhibit RNA synthesis; in HeLa cells both poly(c^{7}I)·poly(br^{5}C) and poly(c^{7}I)·poly(C) were found inhibitory; and, in VERO cells, none of the compounds impaired RNA synthesis. Similar data (not shown) were obtained when DNA synthesis (monitored by [*methyl*-^{3}H]thymidine incorporation) and protein synthesis (monitored by L-[4-^{3}H]leucine incorporation) were measured.

DISCUSSION

The interferon-inducing activities of poly(c^{7}I)·poly(C), poly(c^{7}I)·poly(br^{5}C), and their unmodified parent compounds, poly(I)·poly(C) and poly(I)·poly(br^{5}C), have been explored in several assay systems: primary rabbit kidney cells, human diploid fibroblasts, mouse L-929 cells, rabbits, and mice. Although differences were observed in the relative order of activity of the compounds, depending on the assay system used, it appeared that in most, if not all, systems poly(c^{7}I)·poly(C) was the least effective of the four compounds tested, that poly(I)·poly(C) and poly(I)·poly(br^{5}C) were usually similar in activity, and that poly(c^{7}I)·poly(br^{5}C) showed

TABLE 1

Induction of resistance to virus infection in various cell cultures

Compound	Minimal inhibitory concentration[a]						
	HSF (NS)	HSF (FS-4)	HeLa	L-929	PRK	RK13	VERO
				μg/ml			
Poly(I)·poly(br^{5}C)	0.01	0.001	10	1	0.001	3	>10
Poly(I)·poly(C)	0.01	0.001	10	0.3	0.001	0.3	>10
Poly(c^{7}I)·poly(br^{5}C)	0.3	0.03	1	10	0.001	≧10	>10
Poly(c^{7}I)·poly(C)	0.6	0.06	1	>10	0.1	≧10	>10

[a] Required to reduce virus-induced cytopathogenicity by 50%. Cells were challenged with VSV (100 $CCID_{50}$/tube) after they had been exposed to the compounds for 24 hr. Data represent average values for at least three experiments.

TABLE 2

Effect on RNA synthesis in various cell cultures

Compound	Dose	[5-^{3}H]Uridine incorporated into RNA[a]					
		HeLa		PRK		VERO	
	μg/ml	*cpm/Petri dish*	*% control*	*cpm/Petri dish*	*% control*	*cpm/Petri dish*	*% control*
Poly(I)·poly(br^{5}C)	10	33,157 ± 975	115.0	10,263 ± 684	117.1	39,936 ± 4	109.6
Poly(I)·poly(C)	10	26,834 ± 1,579	93.1	8,496 ± 84	97.0	36,176 ± 1,876	99.3
Poly(c^{7}I)·poly(br^{5}C)	10	20,700 ± 1,417	71.8	8,356 ± 453	95.4	38,260 ± 207	105.0
Poly(c^{7}I)·poly(C)	10	18,475 ± 1,190	64.1	4,964 ± 146	56.6	38,549 ± 1,735	105.8
Control		28,831 ± 691	100	8,763 ± 229	100	36,429 ± 665	100

[a] Measured after the cells had been exposed to the compounds for 24 hr. Data represent average values ± standard deviations for four to six observations.

variable behavior. The interferon-inducing activity of poly(c^{7}I)·poly(br^{5}C) equaled or even surpassed that of poly(I)·poly(C) in PRK (1) and HSF cells (Fig. 1). Yet poly(c^{7}I)·poly(br^{5}C) was less effective than poly(I)·poly(C) in all other assay systems (rabbits, mice, and mouse L-929 cells).

What factors are responsible for the differences in activity among the poly(I)·poly(C) analogues and for the fluctuations in the order of activity from one assay system to another? The differences in activity among poly(I)·poly(C), poly(I)·poly(br^{5}C), poly(c^{7}I)·poly(C), and poly(c^{7}I)·poly(br^{5}C) cannot be attributed to differences in the molecular weight of the constituent homopolynucleotides, since all polymers closely approached or surpassed the threshold molecular size (4–5 S) required for maximal interferon production (12): poly(I), 9.4 S; poly(C), 10.0 S; poly(c^{7}I), 4.8 S; poly(br^{5}C), 10.3 S.

The relative inactivity of poly(c^{7}I)·poly(C) as an interferon inducer would seem related to its low thermal stability [T_m = 49°, as compared to 67°, 86°, and 89° for poly(I)·poly(C), poly(c^{7}I)·poly(br^{5}C), and poly(I)·poly(br^{5}C), respectively (1)]. This low T_m may also explain why poly(c^{7}I)·poly(C) inhibits DNA, RNA, and protein synthesis in PRK cells at a concentration (10 μg/ml) at which poly(c^{7}I)·poly(br^{5}C) fails to do so. The antimetabolic activity of poly(c^{7}I)·poly(C) is apparently due to the poly(c^{7}I) component of the complex. Poly(c^{7}I), as well as its monomer, c^{7}I (7-deazainosine), has previously been shown to inhibit DNA and RNA synthesis in a number of virus-cell systems (5). Furthermore, poly(c^{7}I) has been shown to abolish the interferon-inducing capacity of poly(I)·poly(C) in human diploid fibroblasts.[2] Poly(c^{7}I)·poly(br^{5}C), which is significantly more stable than poly(c^{7}I)·poly(C) (1), would dissociate less readily into its components, and hence exert a less pronounced antimetabolic effect, than poly(c^{7}I)·poly(C).

The antiviral behavior of the poly(c^{7}I)-derived complexes appears to be governed by two effector mechanisms: interferon induction and antimetabolic activity. Depending on the polymer-cell system, one or the other effector mechanism will be favored. For poly(c^{7}I)·poly(C), the antimetabolic activity would generally predominate. For poly(c^{7}I)·poly(br^{5}C), the antimetabolic activity may supersede the interferon-inducing capacity in some assay systems but not in others.

Whether the poly(c^{7}I) duplexes act as interferon inducers or antimetabolites may be determined by (a) the nuclease activity of the cell in question (degrading the polymers to c^{7}IMP) and (b) the ability of that particular cell to convert c^{7}IMP to c^{7}AMP (tubercidin monophosphate). Evidence has been accumulated that suggests that the antimetabolic activity of c^{7}I depends on its conversion to c^{7}A (tubercidin) nucleotides (13).

By virtue of the antimetabolic properties of their poly(c^{7}I) component, poly(c^{7}I)·poly(C) and poly(c^{7}I)·poly(br^{5}C) may exhibit some antiviral activity in cells which are refractory to the interferon-inducing activity of double-stranded RNAs. Hence poly(c^{7}I)·poly(C) and poly(c^{7}I)·poly(br^{5}C) exerted an inhibitory effect on VSV multiplication in HeLa cells (Table 1), and this inhibitory effect was paralleled by a suppressive effect on host cell RNA synthesis (Table 2). That poly(c^{7}I)·poly(C) and poly(c^{7}I)·poly(br^{5}C) did not display antiviral activity in VERO cells (Table 1) is no surprise. VERO cells are known to be refractory to interferon induction. They also proved inert to the suppressive effects of poly(c^{7}I)·poly(C) and poly(c^{7}I)·poly(br^{5}C) on RNA synthesis (Table 2).

PRK cell cultures, especially when "superinduced" with metabolic inhibitors, are extremely sensitive to the interferon-inducing capacity of homopolyribonucleotide duplexes (3). As evidenced for poly(c^{7}I)·poly(br^{5}C), caution should be exercised in extrapolating data on the interferon-inducing capacity of polynucleotides from one assay system to another. The activity of polynucleotides in cultured cells does not necessarily reflect their activity in the whole animal. In rabbits, poly(c^{7}I)·poly(br^{5}C) was definitely less effective

[2] B. F. Torrence and J. Vilcek, unpublished observations.

than poly(I)·poly(C) (Fig. 2), although it surpassed the activity of poly(I)·poly(C) in PRK cell cultures (1).

Does substitution of CH for N-7 in the hypoxanthine ring or substitution of bromine at C-5 of cytosine result in any uncoupling of the toxic and interferon-inducing properties of poly(I)·poly(C)? To approach this problem, activity to toxicity ratios were determined in lead acetate-treated mice. The activity to toxicity ratios of poly(I)·poly(C) and poly(I)·poly(br^{5}C) did not differ markedly. The activity to toxicity ratios of poly(c^{7}I)·poly(br^{5}C) and poly(c^{7}I)·poly(C) could not be properly estimated, because of lack of toxicity of poly(c^{7}I)·poly(br^{5}C) and poly(c^{7}I)·poly(C) at the highest dosage tested. Whether the toxicity *in vivo* of poly(I)·poly(C) and other double-stranded RNAs can be dissociated from their interferon-inducing activity is still a debated question. Hence Niblack and McCreary (14), De Clercq *et al.* (15), and Black *et al.* (16) did not find significant separation of toxicity from activity with a series of poly(I)·poly(C) preparations of different molecular weight or with different procedures intended to increase interferon production. However, Lampson *et al.* (17) found a decrease in toxicity of poly(I)·poly(C), without a concomitant decrease in antiviral activity, by reducing the molecular size of poly(C), and Ts'o *et al.* (18) reported dissociation of antiviral activity from toxicity with poly(I)·poly(C) analogues having mismatched base pairs [e.g., poly(I)·poly(C_{13},U) and poly(I)·poly(C_{20},G)].

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