Preferential Inhibition by Homopolyribonucleotides of the Methylation of Ribosomal Ribonucleic Acid and Disruption of the Production of Ribosomes in a Rat Tumor

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SUMMARY

The literature indicates that some mechanism other than the interferon or host-mediated immune enhancement might also be responsible for an antitumor effect of polyinosinate-polycytidylicate [poly(I)-poly(C)]. We have examined the effect of this drug on the synthesis of ribosomes and other macromolecules in a rat tumor, the Novikoff ascites hepatoma. The nucleolus was one of the primary targets affected by the administration of poly(I)-poly(C) in vivo. A progressive decline of the activity of nucleolar ribosomal RNA methylases began within 2 hr, followed by a decline of the nucleolar RNA content. The activity of nucleolar RNA polymerase was inhibited only at later time intervals. Labeling of tumor macromolecules in vivo revealed that the methylation of ribosomal RNA and the production of ribosomes, particularly the small subunits, were immediately and progressively affected, followed by inhibition of the synthesis of DNA, RNA, and protein at later times. In addition, poly(I)-poly(C) also induced disaggregation of polyribosomes and restricted the movements of nuclear RNA to cytoplasm and of cytoplasmic protein to nucleus. These in vivo effects of poly(I)-poly(C) on tumor cells were observed neither on the host livers nor on livers of normal rats.

Studies on isolated nucleoli showed that the in vitro addition of polyinosinate and several other compounds actively inhibited tumor ribosomal RNA methylases but were devoid of inhibitory effect against liver ribosomal RNA methylases; these results augment other studies in the literature in suggesting a selective effect of the polyinosinate moiety on tumor cells.

We conclude from this study that initial impairment of the methylation of ribosomal precursor RNA, following exposure of tumor cells to poly(I)-poly(C), is responsible for the destruction of ribosomes, preferentially the small subunits, during the maturation processes. Failure to provide new ribosomes thus triggers the events limiting the growth of tumor cells.

INTRODUCTION

Poly(I)-poly(C) has been shown to block the induction of tumor by various agents (16, 18, 19), and to inhibit the growth of experimental tumors (1, 4, 11, 20–22). Furthermore, it has been reported that poly(I)-poly(C) can also inhibit the replication of normal cells characterized by rapid growth such as embryonic tissues (1), regenerating liver (15), or hormone-induced proliferation of salivary epithelium (37). Attempts to elucidate the mechanism of antitumor effect by this compound are complicated by the wide variety of biological effects exerted by this compound in animals. Some of these effects, notably the induction of interferon (12, 17), the activation of macrophages (2), and the enhancement of immune response (10), have been considered to play an important role in the destruction of tumor cells in vivo. However, the correlation between the induction of interferon and the antitumor effect of interferon inducers has not been consistent (4, 9, 35, 43). The demonstration that poly(I)-poly(C) was active against certain cells in culture (29) and against solid tumors (20, 21), and was as effective against tumors of immunosuppressed hosts (4, 11), indicated that some mechanism other than host-mediated immune enhancement is responsible for the antitumor effect of poly(I)-poly(C). Thus, the antitumor effect of poly(I)-poly(C) cannot be satisfactorily attributed solely to the interferon-induced or host-mediated mechanisms. It is our intention to explore still unknown mechanisms of the antitumor effect mediated by poly(I)-poly(C).

This report provides evidence that the nucleolus of rat Novikoff ascites hepatoma was one of the primary targets affected by the administration of poly(I)-poly(C) in vivo. The inhibition of the methylases was the earliest detectable event, followed by inhibition of the synthesis of DNA, RNA, and protein at later time intervals. It appears that initial impairment of the methylation of ribosomal precursor RNA, following exposure of tumor cells to poly(I)-poly(C), is responsible for the destruction of ribosomes, preferentially the small subunits at early time intervals, during maturation processes. Failure to provide new ribosomes thus triggers the events limiting the growth of tumor cells. Evidence is also provided to indicate that rRNA methylases are probably regulated differently during normal and malignant growth. The sensitivity of tumor enzymes and insensitivity of liver enzymes toward several inhibitory compounds suggests that the use of rRNA
methylase inhibitors as antitumor agents may provide favorable differential effects on tumor cells.

MATERIALS AND METHODS

Materials. [3H]S-Ado-Met (4.5 Ci/mmmole) and [methyl-3H]-L-methionine (5 Ci/mmmole) were obtained from Amer sham/Searle Corp., Arlington Heights, Ill. NaH232PO4 (0.5 Ci/mmmole) was obtained from New England Nuclear, Boston, Mass. [5-3H]CTP (12.6 Ci/mmole) and [5-3H]-orotic acid (5 Ci/mmole) were obtained from Schwarz/Man n, Orangeburg, N. Y. Poly(1)-poly(C), sodium salt (SSv, > 12), and poly(I) and polyguanylate (both M.W. > 100,000) were obtained from P-L Biochemicals, Inc., Milwaukee, Wis. Novikoff ascites hepatoma cells were grown in 150- to 175-g Sprague-Dawley rats. The experi ments were conducted on the 4th or 5th day after transplantation of the tumor cells, when about 4 to 6 ml of packed cells per rat were obtained.

Preparation of Nucleoli. Nucleoli from Novikoff ascites hepatoma were prepared as described previously (24, 25). Nucleoli from host or normal rat liver were prepared by a similar procedure. Rats were sacrificed by decapitation, and livers were perfused in situ with cold 0.25 m sucrose, 5 mM MgCl2, and 0.01 M Tris acetate (pH 7). The operations thereafter were conducted in ice-bath temperature. The livers were pressed through a stainless steel Harvard tissue press (Harvard Apparatus Co., Dover, Mass.) to remove connective tissues. The minced livers were homogenized with 3 volumes (assuming 1 g tissue to be 1 ml) of 0.01 M Tris acetate (pH 7), 10 mM MgCl2, and 0.2 mM CaCl2. The homogenate was filtered through 8 layers of cheesecloth and pressed through a chilled French pressure cell (American Instrument Co., Silver Spring, Md.) at 4000 to 6000 psi. The pressed homogenate was made 0.25 m sucrose and was centrifuged at 900 x g for 10 min. The sediment was suspended by homogenization with a glass-Teflon Potter-Elvehjem homogenizer in 4 volumes (with respect to starting tissue volume) of 2.1 m sucrose, 5 mM MgCl2, and 0.01 m Tris acetate (pH 7), and was centrifuged at 88,700 x g for 15 min to sediment nucleoli.

In most cases, very clean nucleolar preparations from tumor cells could be obtained easily. The preparations were free of contamination by nuclei or large subcellular fragments, except that slight contamination by amorphous and fibrous particles was occasionally observed. The nucleolar preparations from liver were also free of contamination by nuclei or large subcellular fragments, although the contamination by fibrous materials was more noticeable than in the preparations from tumor cells. The RNA/DNA ratios of the tumor nucleolar preparations were 1.46 ± 0.05, whereas those of the whole livers were 2.8 to 3.3.

Analyses of the Effect of Poly(I)-Poly(C) on the Synthesis of Macromolecules in Tumor Cells and Host Liver. Rats bearing Novikoff ascites hepatoma were given single i.p. injections of poly(I)-poly(C) (5 to 6 mg/kg) dissolved in 0.9% NaCl solution. After various periods of exposure, 50 μCi of [32P]phosphate or 0.3 mCi of [methyl-3H]methionine, together with 1 μmole of adenosine and guanosine, were injected per rat, again i.p. The rats were sacrificed 3 hr later, and tumor cells and livers were removed for the analyses of incorporation of radioactive precursors into macromolecules.

Tumor cells were washed 3 times with 0.14 m NaCl and 0.01 m Tris-chloride (pH 7.8) by low-speed centrifugation (300 x g, 5 min). Fractionation of tumor cells into nuclear and cytoplasmic fractions was accomplished by homogenization of tumor cells in 6 volumes of 0.01 m Tris-chloride (pH 7.8)-0.01 m NaCl-1.5 mM MgCl2 containing 0.1% Triton X-100 in a Potter-Elvehjem homogenizer with a loose-fitting Teflon pestle until most of the cells were broken. Nuclei were sedimented at 600 x g for 5 min and resuspended in one-half the amount of 0.01 m Tris-chloride (pH 7.8)-0.01 m NaCl-1.5 mM MgCl2 used. One-tenth volume of 3.3% deoxycholate-6.7% Triton X-100 was added to the nuclear suspension, stirred by Vortex mixer, and centrifuged at 600 x g for 5 min to yield pure nuclei. The nuclei were free of cytoplasmic tags and contamination by whole cells. The RNA/DNA ratios of these nuclear preparations were 0.38 to 0.55, whereas those of the tumor cells were 2.9 to 3.3. The combined supernatant (S20) was further centrifuged at 20,000 x g for 15 min to yield the supernatant postmitochondrial extract (S20).

For the fractionation of liver, the rats were sacrificed by decapitation, and the excised livers were rinsed with isotonic sucrose solution. The livers were pressed through a Harvard tissue press, then were homogenized in 5 volumes of 0.25 m sucrose, 5 mM MgCl2, and 0.01 m Tris acetate (pH 7), and were centrifuged at 600 x g for 10 min to separate cytoplasm from nuclei. Nuclei were further purified by suspension of the 600 x g sediment in 5 volumes (with respect to starting tissue volume) of 2.2 m sucrose, 5 mM MgCl2, and 0.01 m Tris acetate (pH 7) and were centrifuged at 88,700 x g for 15 min. The RNA/DNA ratios of the purified liver nuclear preparations were 0.2 to 0.24, whereas those of whole livers were 2.8 to 3.3.

For the determination of DNA synthesis through incorporation of 32P into DNA, an aliquot of purified nuclear preparations was precipitated and washed with 0.4 N PCA. The precipitate was digested with 0.3 M KOH at 37° for 18 hr. The alkaline solution was acidified to 0.4 N PCA. The precipitate containing DNA and protein was washed once with cold 0.4 N PCA. The DNA was hydrolyzed in 0.4 N PCA at 100° for 15 min. The hot PCA-soluble supernatant was analyzed for DNA content and 32P.

For the determination of RNA synthesis through incorporation of 32P into RNA, an aliquot of tumor cells or liver homogenate was deproteinized with phenol, according to procedures described previously (25). The ethanol-precipitated RNA was washed with cold 0.4 N PCA and then hydrolyzed in 0.3 N KOH at 37° for 18 hr. The alkaline hydrolysate was acidified to 0.4 N PCA. The PCA-soluble supernatant was taken for the determination of RNA content and 32P.

For the determination of RNA methylation through incorporation of methyl-3H into RNA, an aliquot of tumor cells, subcellular fractions, or liver homogenate was deproteinized with phenol as mentioned above. The RNA was incubated in 0.5 m Tris-chloride (pH 8.8) at 37° for 1 hr,
precipitated, and washed in 0.4 N PCA, and then was hydrolyzed in 0.3 N KOH at 37° for 18 hr. The alkaline hydrolysate was acidified to 0.4 N PCA. The PCA-soluble supernatant was taken for the determination of RNA content and ³H.

For the determination of protein synthesis through incorporation of [methyl-³H]methionine into protein, an aliquot of tumor cells or liver homogenate was precipitated with 0.4 N PCA and extracted with 0.4 N PCA at 100° for 10 min. An aliquot of NaOH-soluble protein was precipitated with 5% TCA and collected on a Millipore filter for the determination of ³H; another aliquot was taken for the determination of protein according to the method of Lowry et al. (27).

Since the amount of ascites fluid and tumor cells and probably the amount of labeled precursor taken up varies from rat to rat, it is difficult to compare precisely the relative activities of the labeled macromolecules. Wherever possible, data were referred to an amount of cells containing 1 mg of DNA. In order to further normalize the data, the cold 0.4 N PCA-soluble radioactivity (cpm/mg of DNA) of the washed tumor cells or washed liver was measured in every case, and the activities of macromolecular synthesis (cpm incorporated per mg of RNA, DNA, or protein) were then adjusted proportionally to represent the same amount of acid-soluble radioactivity as the control. Thus, the effect of poly(I)-poly(C) on the uptake of precursor or the pool size of acid-soluble intermediates has been cancelled out in the data reported.

For the study of polysomal profiles, 3 ml of S₂₀ fraction were layered onto 24 ml of 15 to 30% (w/w) sucrose gradient containing 0.02 M Tris-chloride (pH 7.8), 2 mM MgCl₂, and 0.05 M KCl on top of a 2-ml cushion of 50% sucrose, and the gradient was centrifuged at 15,000 rpm for 16 hr in an SW 25.1 rotor. The gradient was then fractionated into 1-ml fractions by an automatic fractionator (Instrumentation Specialties Co., Lincoln, Neb.). The ribonucleaseprotein was filtered onto Millipore filters (0.45 µm), washed with cold 5 mM MgCl₂, and dried for the measurement of radioactivity (34).

For the study of RNA profiles, 3 ml of S₂₀ fraction were made 0.3% with respect to Sarcosyl NL 97 (K & K Laboratories, Inc., Plainview, N. Y.) and were deproteinized by shaking with an equal volume of phenol at room temperature as described (25). The RNA was layered onto 28 ml of 10 to 40% (w/w) sucrose gradient containing 0.01 M NaCl, 1 mM EDTA, and 0.01 M sodium acetate (pH 5.1) and was centrifuged at 22,500 rpm for 16 hr in an SW 25.1 rotor. The gradient was then fractionated into 1-ml fractions. The RNA was precipitated with 5% TCA, filtered onto Millipore filters (0.45 µm), washed with 5% TCA, and dried for the measurement of radioactivity.

Analysis of Methylated Nucleotides. The analysis of base-methylated mononucleotides of tRNA liberated by alkaline hydrolysis was carried out as previously described (24).

Assay of RNA, DNA, Protein, and Radioactivity. RNA was determined by the orcinol reaction (14), DNA was determined by Burton’s diphenylamine reaction (8), and protein by the procedure of Lowry et al. (27). The determination of radioactivity on Millipore filters or in aqueous solution was as previously described (26).

Assay of Nucleolar RNA Polymerase, rRNA Methylases, and Cell-soluble tRNA Methylases. To study the effect of poly(I)-poly(C) on the nucleolar enzymes involved in the synthesis of ribosomes and cell-soluble tRNA methylases, rats bearing Novikoff ascites hepatoma were given 5 to 6 mg poly(I)-poly(C) per kg i.p. Poly(I)-poly(C) was dissolved (1 mg/ml) in 0.9% NaCl solution by incubation at 37° for 1 hr, with occasional vigorous shaking. Nucleoli and high-speed cellular extract (S₂₀₆) from control rats, as well as from poly(I)-poly(C)-treated rats, were prepared as described previously (24, 26). Isolated nucleoli were incubated in the complete RNA-synthesizing medium (24), either with [³H]CTP for the assay of RNA polymerase or with [³H]S-Ado-Met for the assay of rRNA methylase activities. [³H]S-Ado-Met, stored in dilute acid solution to prevent decomposition, was neutralized with NH₄OH before use. The assay of tRNA methylases in S₂₀₆ was conducted as described previously (26).

RESULTS

The Nucleolus as the Primary Target following the Administration of Poly(I)-Poly(C) in vivo. It has been reported by Margolis and Levy (29) that exposure of cultured cells to poly(I)-poly(C) under certain conditions led to the inhibition of the synthesis of ribosomal precursor RNA. The nucleolus of Novikoff ascites hepatoma is apparently also one of the primary targets affected by the administration of poly(I)-poly(C) into the rat in vivo. The effect of poly(I)-poly(C) on the tumor nucleolus was studied by exposing Novikoff ascites hepatoma to poly(I)-poly(C) in vivo, followed by the isolation of nucleoli and assay in vitro of the activity of enzymes involved in the synthesis of ribosomes. The results summarized in Table 1 indicate that the rRNA methylases showed a definite decline in activity within 2 hr and progressively declined in activity as exposure time increased. The RNA content of isolated nucleoli diminished within 4 hr and also progressively decreased in amount. In contrast, the activity of RNA polymerase was altered relatively little for the 1st 4 hr of exposure to poly(I)-poly(C). Although a considerable decrease of RNA polymerase activity was observed between 4 and 8 hr and thereafter, the degree of inhibition was never as great as that of rRNA methylases. The same dosage of poly(I)-poly(C) given to a normal rat did not cause an appreciable change of the activity of liver nucleolar enzymes.

The tumor nucleolar RNA formed in the presence of poly(I)-poly(C) showed various degrees of degradation on sucrose gradients. An example is shown in Chart 1. Upon incubation of isolated nucleoli in vitro, both nonlabeled and labeled RNA from in vivo poly(I)-poly(C)-treated tumor nucleoli underwent extensive degradation, indicating that RNA formed under the influence of poly(I)-poly(C) was more unstable than control RNA. The preferential inhibi-
Antitumor Effects of Poly(I). Poly(C)

Table 1

The effect of poly(I).poly(C) in vivo on the nucleolar RNA content, on activities of nucleolar enzymes involved in the synthesis of rRNA, and on activities of cell-soluble tRNA methylases

Rats (150 to 160 g/rat) bearing Novikoff ascites hepatoma on the 4th day (8 and 16 hr of exposure) or 5th day (2 and 4 hr of exposure) were given i.p. dose of poly(I).poly(C) (5 to 6 mg/kg, dissolved in 0.9% NaCl solution) and were killed at the end of the indicated time periods. Poly(I).poly(C) was also given i.p. to a normal rat (liver, 16 hr) at the same dosage. Nucleoli and ultracentrifugal cell extract (S_{20w}) were prepared from control tumors cells and liver, and from poly(I).poly(C)-treated tumor cells and liver. Control livers were from normal rats. Nucleolar RNA content: Aliquots of nucleoli were incubated in the complete RNA-synthesizing medium (25) either with 0.2 mM [^3H]CTP or 2.2 μM [^3H]S-Ado-Met for the assay of RNA polymerase or rRNA methylase activities, respectively. The incubation was conducted at 30° for 15 min. The nucleoli were precipitated with cold 0.4 M PCA, neutralized, extracted with 2 M NaCl at 100°, and the nucleic acids were chilled and reprecipitated with cold 0.4 M PCA. RNA was hydrolyzed in 0.3 N KOH at 37° for 18 hr, and DNA was precipitated by acidifying to 0.4 N PCA for the separate determination of RNA, DNA, and radioactivity. The RNA polymerase activity of control tumor nucleoli was in the range of 11.3 ± 1.8 nmoles of [^3H]CMP incorporated into RNA per mg DNA, and the rRNA methylase activity was in the range of 0.27 ± 0.006 n mole of methyl[^3H] incorporated into RNA per mg DNA. The corresponding values for liver control nucleoli were 2.26 ± 0.045 and 0.043 ± 0.005 nmoles, respectively.

Cell-soluble tRNA methylase activity: The assay of tRNA methylases was conducted as described previously (26). The standard assay mixture in a volume of 0.25 ml contained 0.05 M Tris-chloride (pH 7.8), 0.04 M NH_4F, 1 mM dithiothreitol, 1 mM ATP, 50 μg E. coli tRNA (sodium salt), 0.9 μM [^3H]S-Ado-Met, and an amount of S_{20w} containing 85 to 100 μg of protein. The tRNA methylase activity of tumor control was in the range of 39 to 45 pmoles of methyl[^3H] incorporated into tRNA per mg protein. The data are the average of 2 to 3 determinations, where expressed as mean ± S.E., and represent a single determination elsewhere.

<table>
<thead>
<tr>
<th>Time of exposure to poly(I)-poly(C)</th>
<th>Nucleolar RNA content</th>
<th>Nucleolar enzyme activity</th>
<th>Cell-soluble tRNA methylase activity % of control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RNA/DNA</td>
<td>% of control</td>
<td>RNA polymerase (% of control)</td>
</tr>
<tr>
<td>Tumor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 hr, control</td>
<td>1.46 ± 0.05^a</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>2 hr</td>
<td>1.38 ± 0.07</td>
<td>94</td>
<td>96 ± 3.5</td>
</tr>
<tr>
<td>4 hr</td>
<td>1.24 ± 0.08</td>
<td>85</td>
<td>93 ± 3.8</td>
</tr>
<tr>
<td>8 hr</td>
<td>1.09 ± 0.03</td>
<td>75</td>
<td>59 ± 1.5</td>
</tr>
<tr>
<td>16 hr</td>
<td>0.60 ± 0.05</td>
<td>41</td>
<td>30 ± 0.2</td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 hr, control</td>
<td>0.65 ± 0.05</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>16 hr</td>
<td>0.68</td>
<td>104</td>
<td>104</td>
</tr>
</tbody>
</table>

^a Mean ± S.E.

We have previously shown that poly(I) was capable of inhibiting both nucleolar rRNA methylases and tRNA methylases in vitro (24). In contrast to the progressive decline of the activity of nucleolar rRNA methylases, the activity of cell-soluble tRNA methylases, when assayed on 100 mCi of[^3H]methionine for 3 hr. Nucleoli were prepared and divided into 2 equal parts. One part was suspended in 0.5 ml of 0.05 M Tris (pH 7.8), 0.25 M sucrose, 5 mM MgCl_2, 0.04 M NH_4F, and 0.9 mg of bentonite per ml, and incubated at 30° for 15 min. RNA was prepared from the control nucleoli (a, nonincubated; b, incubated) and from nucleoli from the poly(I)-poly(C)-treated rat (c, non-incubated; d, incubated) by phenol deproteinization (25) and resolved in 10 to 40% sucrose gradients containing 0.01 M Tris (pH 7.4), 0.1 M LiCl, 0.01 M EDTA, and 0.2% Sarcosyl NL 97 by centrifugation at 45,000 rpm for 4 hr in an SW 50 rotor.

Chart 1. The effect of poly(I)-poly(C) on the stability of nucleolar RNA. One rat bearing Novikoff ascites hepatoma was given i.p. dose of poly(I)-poly(C) (5 mg/kg, dissolved in 0.9% NaCl solution) for 16 hr. The poly(I)-poly(C)-treated rat and control rat were then labeled with 0.3 mCi of[^3H]methionine for 3 hr. Nucleoli were prepared and divided into 2 equal parts. One part was suspended in 0.5 ml of 0.05 M Tris (pH 7.8), 0.25 M sucrose, 5 mM MgCl_2, 0.04 M NH_4F, and 0.9 mg of bentonite per ml, and incubated at 30° for 15 min. RNA was prepared from the control nucleoli (a, nonincubated; b, incubated) and from nucleoli from the poly(I)-poly(C)-treated rat (c, non-incubated; d, incubated) by phenol deproteinization (25) and resolved in 10 to 40% sucrose gradients containing 0.01 M Tris (pH 7.4), 0.1 M LiCl, 0.01 M EDTA, and 0.2% Sarcosyl NL 97 by centrifugation at 45,000 rpm for 4 hr in an SW 50 rotor.
heterologous Escherichia coli tRNA, was found to be diminished only slightly at the early time point (2 hr). When exposure time was extended to 8 hr or longer, the specific activity of tRNA methylases was slightly enhanced. The same results were also obtained when an assay of tRNA methylases was conducted in the presence of a stimulatory concentration of NH₄Cl (0.2 M). An analogous observation has been reported that the methylation of rRNA and tRNA was non-coordinately inhibited when cells were infected with foot-and-mouth disease virus (3, 40).

The Effect of Poly(I)-Poly(C) on the Synthesis of Macromolecules in Tumor Cells and Host Liver. There was a technical difficulty in using rat-carried tumor cells for the measurement of the rate of synthesis of macromolecules. The synthetic rate was affected by the age and concentration of the tumor cells and the physiological condition of the rat, such as the amount of ascites hemorrhage. In general, younger tumor cells, more concentrated tumor cells, and less hemorrhagic conditions tended to permit a higher rate for the synthesis of macromolecules. To minimize non-uniformity, rats carrying approximately the same amount of ascites fluid were selected among many transplanted rats for the experiments, and labeling with radioactive precursors was conducted on the 5th day after transplantation. Data were further adjusted to the control level of acid-soluble radioactive intermediates. The possible differences in the uptake of radioactive precursors and their conversion to acid-soluble radioactive intermediates were thereby greatly reduced. There was, however, an apparently significant (although difficult to quantitate) increase of the acid-soluble radioactivity (both 32P and [methyl-3H]methionine derivatives) stimulated by poly(I)-poly(C). The increase was 20 to 25% for the 2-hr exposure and 6 to 18% for the 8 to 16-hr exposure.

As shown in Table 2, the 3-hr incorporation of [32P]phosphate into DNA in tumor cells was initially stimulated, and then was inhibited after more than 8 hr of exposure to poly(I)-poly(C). The stimulation of DNA synthesis is in accord with the observation of Brown and Coffey (6) that poly(I) was capable of stimulating DNA synthesis in isolated nuclei and soluble chromatins. The incorporation of phosphate into RNA and of methionine into protein was immediately and gradually inhibited to almost the same extent. The effect of poly(I)-poly(C) on the methylation of RNA was similar to that on RNA synthesis; however, the inhibition of RNA methylation was greater than that of RNA synthesis.

In contrast to the inhibitory effects of poly(I)-poly(C) on macromolecular synthesis in tumor cells, Table 2 shows that the synthesis of macromolecules in host liver in vivo was not inhibited. In a separate experiment, when poly(I)-poly(C) was given i.p. to a normal rat, followed 16 hr later by labeling of RNA with 0.2 mCi of [3H]orotic acid for 3 hr, there was only 11% inhibition of liver RNA synthesis in the treated rat. These results, considered together with the differential effect of poly(I)-poly(C) on the activities of tumor and liver nucleolar enzymes (Table 1), suggest that the effect of poly(I)-poly(C) is relatively specific for these tumor cells in vivo. In this in vivo system, it was not possible to make direct comparisons of effects on liver and ascites tumor, because the same accessibility of the drug to the liver is not assured. However, it has previously been documented in other systems that poly(I)-poly(C) exerts a greater effect on tumor cells and rapidly proliferating cells than on normal resting cells (1, 15, 18, 20), and in a later section we present results of experimentations in vitro in which these physiological factors were avoided. Evidently, the relatively specific antitumor effect of poly(I)-poly(C) could not be attributed to selective uptake of this compound into the tumor (20).

The inhibition of protein synthesis by poly(I)-poly(C) correlates with the effect of poly(I)-poly(C) on the polysomal profiles, as shown in Chart 2A. In these experiments, Novikoff ascites cells were vigorously homogenized in order to obtain clean nuclei. As a result, polysomes were not well preserved. Nevertheless, the amount of polysomes as measured by UV absorption was noticeably diminished, and the incorporation of 32P into the polysomal fraction was markedly decreased in response to the treatment of the rat with poly(I)-poly(C). The effect was more prominent in those cells exposed to poly(I)-poly(C) for longer periods of time.

Table 2
The effect of poly(I)-poly(C) on the synthesis of macromolecules in tumor cells and host liver

<table>
<thead>
<tr>
<th>Time of exposure to poly(I)-poly(C) before labeling (hr)</th>
<th>DNA synthesis (32P)</th>
<th>RNA synthesis (32P)</th>
<th>RNA methylation (methyl-3H)</th>
<th>Protein synthesis (methyl-3H)</th>
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<tbody>
<tr>
<td>Control</td>
<td>Tumor 13,350</td>
<td>Liver 590</td>
<td>Tumor 45,000</td>
<td>Liver 12,000</td>
</tr>
<tr>
<td>2*</td>
<td>121 ± 5.8*</td>
<td>103 ± 8.4</td>
<td>93 ± 4.3</td>
<td>118 ± 5.9</td>
</tr>
<tr>
<td>8*</td>
<td>33 ± 2.4</td>
<td>110 ± 8.9</td>
<td>45 ± 3.3</td>
<td>97 ± 6.8</td>
</tr>
<tr>
<td>16*</td>
<td>22 ± 2.2</td>
<td>96 ± 4.5</td>
<td>32 ± 2.0</td>
<td>108 ± 4.1</td>
</tr>
</tbody>
</table>

* The data are expressed as % of control activity.

** Mean ± S.E.
to disaggregate polysomes, the appearance of radioactive ribonucleoprotein in the 45 S peak of the cytoplasm, where RNA's prepared from this S9 fraction also revealed considerably different profiles among control and poly(I)-poly(C)-treated specimens, as shown in Chart 2B. The labeling of rRNA was more greatly inhibited relative to the labeling of 4 to 6 S RNA, and the labeling of 18 S rRNA was more greatly inhibited relative to the labeling of 28 S rRNA. In addition, the radioactive peak sedimenting between 4 S and 18 S characteristic of cytoplasmic mRNA was diminished in RNA extracted from cells exposed to poly(I)-poly(C) for 8 hr or longer. It appears that poly(I)-poly(C) has preferential inhibitory effect toward the production of ribosomes, and more selectively on the production of small subunits. Labeling of RNA with [methyl-3H]methionine produced a similar trend (Table 3), namely, that a diminished formation of methylated cytoplasmic rRNA is observed after 2 hr of treatment by poly(I)-poly(C). Selective inhibition of the methylation of small subunits was apparent only during early intervals (2 to 1 hr) of exposure.

The Effect of Poly(I)·Poly(C) on the Intracellular Movement of Macromolecules in Tumor Cells. Poly(I)-poly(C) caused not only the inhibition of macromolecular synthesis in tumor cells but also aberrations in movement of macromolecules from the site of synthesis to their destination. As shown in Tables 3 and 4, the relative amount of radioactive RNA in the cytoplasm and the relative amount of radioactive protein in the nucleus progressively decreased in response to increasing exposure time.

The Effect of Poly(I)·Poly(C) Administered in Vivo on the Base Methylation of tRNA. One of the remarkable features of poly(I) as tRNA methylase inhibitor is its selective inhibition of adenine methylase when directly included in the in vitro enzyme assay (24). The analysis of tRNA methylated with [methyl-3H]methionine in vivo (Table 5) indicates that poly(I)-poly(C) caused a small but noticeable preferential inhibition of the methylation of tRNA adenine during early time intervals of exposure to poly(I)-poly(C), although this inhibition of the methylation of tRNA adenine was no longer detectable when cells were exposed to poly(I)-poly(C) for 8 hr or longer. An assay of cell-soluble tRNA methylases, prepared from tumor cells treated in vivo with poly(I)-poly(C), on heterologous E. coli tRNA (Table 1) also revealed similar results. Preferential inhibition of the adenine methylase was detectable only in enzyme extracts prepared from tumor cells treated for a short time (2 hr), but not for a longer time (10 hr).

Different Responses of Tumor and Liver Nucleolar rRNA Methytransferases toward Compounds Affecting Their Activities. The results shown in Tables 1 and 2 indicate that poly(I)-poly(C) in vivo exerted its inhibitory effect selectively on tumor cells. We regard this selective effect to be a very important feature of this type of antitumor agent. The problem is to determine what causes this kind of selective action. Since tumor rRNA methylases are apparently one of the primary targets affected by the administration of poly(I)-poly(C) in vivo, we therefore carried out a study to determine whether there was any difference in the behavior of enzymes in nucleoli from tumor or liver. The results
**The effect of poly(I)-poly(C) on the methylation of RNA in tumor cells**

Rats bearing Novikoff ascites hepatoma were given poly(I)-poly(C) and were subsequently labeled with [methyl-3H]methionine, as described in Tables 1 and 2. Tumor cells were fractionated into nuclear and cytoplasmic fractions as described in “Materials and Methods.” RNA’s were purified by deproteinization with phenol and precipitation with ethanol; tRNA was separated from rRNA, in the case of cytoplasmic RNA preparation, by precipitation with ethanol; rRNA was recovered from 2 M NaCl supernatant by ethanol precipitation and was further incubated in 0.5 M Tris-chloride (pH 8.8) at 37° for 1 hr. The determination of specific activity of methylated RNA and the correction based on the same amount of acid-soluble radioactivity were as described in Table 2. The data represent the average of 2 or 3 separate determinations expressed as mean ± SE.

<table>
<thead>
<tr>
<th>Time of exposure to poly(I)·poly(C) before labeling (hr)</th>
<th>Total RNA methylation as % of control activity</th>
<th>Methylated cytoplasmic RNA as % of total methylated RNA</th>
<th>Methylated cytoplasmic tRNA % of control activity</th>
<th>Methylated cytoplasmic tRNA activity as % of total methylated cytoplasmic RNA</th>
<th>Methylated cytoplasmic rRNA activity as % of control activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>58 ± 1.8</td>
<td>100%</td>
<td>47 ± 3.4</td>
<td>100%</td>
</tr>
<tr>
<td>2</td>
<td>86 ± 1.7</td>
<td>53 ± 2.5</td>
<td>78 ± 4.8</td>
<td>52 ± 2.7</td>
<td>0.98 ± 0.01</td>
</tr>
<tr>
<td>4</td>
<td>45 ± 1.9</td>
<td>50 ± 0.9</td>
<td>44 ± 3.0</td>
<td>54 ± 1.6</td>
<td>0.73 ± 0.08</td>
</tr>
<tr>
<td>8</td>
<td>37 ± 2.1</td>
<td>42 ± 2.2</td>
<td>37 ± 1.3</td>
<td>63 ± 1.6</td>
<td>0.89 ± 0.05</td>
</tr>
</tbody>
</table>

(a) 3,900 cpm/mg RNA.
(b) 11,000 cpm/mg RNA.
(c) 1,520 cpm/mg RNA.

**Table 4**

The Effect of poly(I)-poly(C) on the transport of macromolecules in tumor cells

The experiments were the same as described in Table 2. However, the tumor cells were fractionated into cytoplasmic and nuclear fractions, as described in “Materials and Methods.” The assay of [methyl-3H]methionine-labeled protein among cytoplasmic and nuclear fractions was as described in “Materials and Methods.”

<table>
<thead>
<tr>
<th>Time of exposure to poly(I)-poly(C) before labeling (hr)</th>
<th>Cytoplasmic [% of total [32P]RNA labeled protein as % of total [32P]RNA]</th>
<th>[methyl-3H]methionine-labeled protein as % of total [3H]protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>0, control</td>
<td>54</td>
<td>73</td>
</tr>
<tr>
<td>2</td>
<td>51</td>
<td>77</td>
</tr>
<tr>
<td>4</td>
<td>43</td>
<td>80</td>
</tr>
<tr>
<td>8</td>
<td>37</td>
<td>83</td>
</tr>
</tbody>
</table>

(Table 6) show that many compounds that are inhibitory against tumor nucleolar RNA methylases were without inhibitory effects against liver nucleolar RNA methylases. On the contrary, there were various degrees of stimulation, possibly resulting from inhibition of RNase activity. The sensitivity of tumor enzymes and the insensitivity of liver enzymes toward inhibitory compounds may be the main reason behind the potentially selective inhibitory effect of nRNA methylase inhibitors on tumor cells. The desirability of using nRNA methylase inhibitors as antitumor agents is indicated.

**DISCUSSION**

Several factors may be involved in the antitumor effects of poly(I)-poly(C). Considering the inhibitory effect of poly(I)-poly(C) against solid tumors (20, 21), against tumors in immunosuppressed animals (4, 11), and against rapidly proliferating cells in tissues (1, 15, 37), a direct effect of poly(I)-poly(C) or an effect mediated through interferon is likely. A direct inhibition of cell multiplication by interferon has been demonstrated in cultured cells (5). In this case, a prolonged contact for at least 18 hr with interferon was required to produce a significant inhibition of the synthesis of protein and RNA, while the formation of ribosomal subunits was hardly affected. Thus, the action of poly(I)-poly(C) on Novikoff ascites hepatoma is distinctly different from the action of interferon on cultured cells. Inconsistent correlation between the levels of the interferon induced and the antitumor activities of the inducers (4, 35, 43) also suggests that different mechanisms are perhaps involved in the actions of the interferon and the interferon inducers possessing antitumor activity.

The immediate effects detectable within a few hr of exposure of tumor cells to poly(I)-poly(C) correspond remarkably well to the biological effects produced in vitro by poly(I). These are the inhibition of RNA methylation, the selective inhibition of adenine methylase of tRNA, and the stimulation of DNA synthesis (6, 25). The idea that poly(I) is probably the active strand of poly(I)-poly(C) has been suggested on the basis of uptake of poly(I) and the stimulation of DNA synthesis (6, 25). The critical requirement for size of poly(I) but not poly(C) for the demonstration of antitumor and antiviral activities as well as interferon induction (9, 32, 39). The requirement for complex formation with poly(C) for the demonstration of its biological activities in vivo may indicate that poly(C) facilitates the uptake of active poly(I) or its interaction with a receptor site on or within the cell, or that the complex confers resistance to degradation by nucleases (31).

In procaryotic and eucaryotic microorganisms, the rate of synthesis of RNA and the number of ribosomes/genome are proportional to the rate of exponential growth (28, 33), and these characteristics are maintained by several regulatory processes, including methylation of RNA. In mammalian cells, the ability of cells to replicate is specifically correlated to the ability of cells to provide needed ribosomes (33, 38). The formation of ribosomes is greatly affected by the methylation processes. It has been shown that the 45 S pre-rRNA synthesized without methylation under-
The effect of poly(I).poly(C) on the pattern of base methylation of tRNA

Rats bearing Novikoff ascites hepatoma were treated with a single dose of 5 to 6 mg of poly(I).poly(C) per kg for the indicated time intervals. Rats either were labeled with [methyl-3H]methionine for 3 hr, as described in Table 2, or were sacrificed for the preparation of ultracentrifuged cell-soluble extracts ($S_{30}$) as sources of tRNA methylases. In vivo-labeled tRNA was prepared from cytoplasmic fraction, as described in Table 3. The purified tRNA was hydrolyzed in 0.3 M KOH at 37° for 18 hr, and the mononucleotides liberated were separated by paper electrophoresis (26). For the correction of radioactivity incorporated into normal purine bases, radioactivity associated with adenine and guanine nucleotides was recovered from paper by extraction with 0.1 N HCl. The extracts were dried in a vacuum desiccator. Purine bases were liberated from nucleotides by hydrolysis in 1 N HCl at 100° for 1 hr. Adenine and methylated adenine were separated by descending chromatography in butanol/concentrated NH$_4$OH (86/14) for 18 hr, and guanine and methylated guanines were separated by descending chromatography in 2-propanol/12 N HCl/H$_2$O (68/17.6/14.4) for 90 hr. Under the present labeling conditions, 6 to 11% of the radioactivity in the adenine nucleotide and 4 to 8% of the radioactivity in the guanine nucleotide were found to be due to purine ring synthesis, which values were corrected for in the data presented. The incubation of $S_{30}$ extracts with E. coli tRNA and analysis of methylated bases were conducted as previously described (24, 26).

<table>
<thead>
<tr>
<th>Methylated bases</th>
<th>Control</th>
<th>Poly(I).poly(C)</th>
<th>Poly(I).poly(C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2 hr</td>
<td>8 hr</td>
</tr>
<tr>
<td>C</td>
<td>22</td>
<td>24</td>
<td>21</td>
</tr>
<tr>
<td>A</td>
<td>16</td>
<td>12</td>
<td>16</td>
</tr>
<tr>
<td>G</td>
<td>56</td>
<td>59</td>
<td>59</td>
</tr>
<tr>
<td>U</td>
<td>6</td>
<td>6</td>
<td>4</td>
</tr>
</tbody>
</table>

Table 6

Different responses of tumor and liver nucleolar rRNA methylases toward compounds affecting their activities in vitro

Nucleoli were prepared from Novikoff ascites hepatoma cells or host livers as described in "Materials and Methods." The standard assay mixture in a volume of 0.25 ml contained: 0.05 M Tris-chloride (pH 7.8), 0.25 M sucrose, 1 mM MgCl$_2$, 0.2 mM EDTA, 0.04 M NH$_4$F, nucleoli containing approximately 50 µg of DNA, and 1.24 µM [3H]-S-Ado-Met without (control) or with compounds, as listed. The incubation and assay were conducted as described in Table 1. The tumor nucleolar rRNA methylase activity assayed under these conditions was in the range of 85 to 113 pmoles of methyl-[3H] incorporated into RNA per mg DNA, and the liver nucleolar rRNA methylase activity was in the range of 22 to 30 pmoles of methyl-[3H] incorporated into RNA per mg DNA. The data are the average of 2 to 4 determinations.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Tumor</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly(I), 2 µM</td>
<td>57 ± 3.0</td>
<td>139 ± 4.5</td>
</tr>
<tr>
<td>Polynucleotide, 2 µM</td>
<td>67 ± 3.9</td>
<td>144 ± 4.0</td>
</tr>
<tr>
<td>ApA, 2 mM</td>
<td>51 ± 1.6</td>
<td>109 ± 1.6</td>
</tr>
<tr>
<td>2'-O-methylated dinucleotides, 2 mM</td>
<td>55 ± 3.8</td>
<td>105 ± 4.9</td>
</tr>
<tr>
<td>cAMP, 2 mM</td>
<td>62 ± 3.2</td>
<td>118 ± 5.1</td>
</tr>
<tr>
<td>AMP, 4 mM</td>
<td>65 ± 2.5</td>
<td>113 ± 4.7</td>
</tr>
</tbody>
</table>

* Concentration is based on minimum molecular weight.
* Mean ± S.E.
* ApA, adenosyl(3',5'-phospho)adenosine.

most mono-, di-, and oligonucleotides show inhibitory activity against tumor rRNA methylases in isolated nucleoli (23), we postulate that excessive RNA degradation resulting from the initial inhibition of rRNA methylases is probably

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most responsible for the observed inhibition of macromolecular synthesis occurring at later time intervals. The inhibition of macromolecular synthesis is more prominent at a time when the continued existence of poly(I) in its original inhibitory form becomes doubtful. In addition to the likelihood that poly(I) is degraded intracellularly, the specific biological effects manifested by poly(I), such as the inhibition of RNA adenine methylation and stimulation of DNA synthesis, could no longer be demonstrated at the longer time intervals. The argument follows then that an initial inhibition of rRNA methylases is sufficient to trigger the regulatory mechanism of ribosome production operating under normal growth.

The inhibition of tumor mRNA methylases by cAMP (Table 6) is of particular interest, since it has been proposed by Webb et al. (42) that the antitumor effect of synthetic polynucleotides may be mediated by their stimulation of the formation of cAMP.

REFERENCES

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Preferential Inhibition by Homopolyribonucleotides of the Methylation of Ribosomal Ribonucleic Acid and Disruption of the Production of Ribosomes in a Rat Tumor

Ming C. Liau, Don W. Smith and Robert B. Hurlbert


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