

Effects of Partially Thiolated Polycytidylic Acid and Liposomes on *In Vitro* Colony-forming Cells of Leukemic Mice

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ABSTRACT

Partially thiolated polycytidylic acid (MPC), an antileukemic agent, when administered to leukemic RF/UN mice inhibited the clonogenicity of bone marrow progenitor cells in a time- and dose-dependent manner. The effect of a single dose of MPC disappeared within 40 hr due to the rapid degradation of this compound in mice. When MPC was encapsulated in liposomes before injection, its activity at 19 hr after inoculation was similar to that of free MPC. The inhibitory effect of this liposome-MPC complex, however, persisted for at least 40 hr, indicating that the MPC was protected from hydrolysis by the nucleases present in blood. Drug-free liposomes increased the number of clonogenic progenitor cells, whereas a mixture of plain liposomes and MPC decreased the number of clonogenic cells to a greater extent than did MPC alone or MPC within liposomes. A possible explanation for these observations is that the liposomes *per se* altered the clearance function of the reticuloendothelial system and competed with MPC for uptake by the reticuloendothelial system cells, thereby resulting in increased plasma levels of MPC which in turn resulted in greater killing of the target cells.

INTRODUCTION

MPC,⁵ a member of a family of antitemplates, *i.e.*, inhibitory structural analogs of the functional templates of DNA and RNA polymerases (1, 2), is being considered as a candidate drug for clinical trial due to its unusual biochemical and biological activities (3, 5, 9, 10, 13, 16, 19) and because preliminary clinical studies indicated that it may be active against lymphocytic leukemia in humans (6). We have previously shown (10) that MPC decreased the colony-forming ability *in vitro* of bone marrow and spleen cells obtained from myeloid leukemic mice. When injected into leukemic RF/UN mice, MPC significantly inhibited colony production *in vitro*; however, this effect was of short duration, which is not surprising in view of the fact that MPC is rapidly degraded by serum nucleases in mice (14). Entrapment of drugs in liposomes has been shown to protect them from metabolism in the blood and in organs (12). Therefore, it was of interest to study whether liposomes could protect MPC from being hydrolyzed by nucleases, thus extending the duration of its action and increasing the overall effectiveness of the drug when administered to mice. In this communication,

we report the results of our studies of the effects of MPC and liposomes administered separately and in various combinations to leukemic mice.

MATERIALS AND METHODS

Materials. Poly(C) and [³H]poly(C) were purchased from Miles Laboratories, Inc., Elkhart, Ind. Tissue culture media, bovine embryo extract, and bovine plasma were purchased from Grand Island Biological Co., Grand Island, N. Y. Na³⁵S³⁵ was purchased from Amersham-Searle Corp., Arlington Heights, Ill. Cholesterol and β , γ -dipalmitoyl- α -glycerylphosphorylcholine were products of Sigma Chemical Co., St. Louis, Mo.

MPC and [³⁵S]MPC were synthesized as described previously (4, 8, 10). The preparation contained 8.5% 5-mercaptocytidylyl and 91.5% cytidylyl units randomly distributed throughout the polynucleotide chain and was used as the free MPC in the *in vitro* and *in vivo* studies.

Encapsulation of MPC in REV Liposomes. Liposome-encapsulated MPC was prepared using a modification of the REV procedure (23).

An aqueous solution of MPC was mixed with dithiothreitol and [³H]poly(C) (used as a tracer) to a final concentration of 4.4 mg MPC per ml, 0.1 to 0.2 μ Ci [³H]poly(C) per ml and 50 μ mol dithiothreitol per ml. Separately, an equimolar mixture of β , γ -dipalmitoyl- α -glycerylphosphorylcholine and cholesterol at 20 μ mol/ml in chloroform was prepared. The lipid solution was added into a 5- or 15-ml round-bottomed sonicating flask, and an equal volume of freshly distilled isopropyl ether was then added with constant stirring to prevent settling. The polynucleotides were then added, again with constant swirling, to a final ratio of 1 ml MPC stock per 60 μ mol total lipid. Nitrogen was flushed into the flask which was then capped securely. The suspension was sonicated for 5 min at room temperature using a sonicator manufactured by Laboratory Supply Co., Inc., Hicksville, N. Y. The chloroform and isopropyl ether were removed under vacuum using a rotary evaporator. The vacuum was started at 45 to 55 cm, then increased to a maximum as soon as the gel formed. The temperature of the water bath started at room temperature, then gradually warmed to approximately 37° during evaporation and, when all the chloroform and ether were removed, the flask was equilibrated for 30 min. The liposomes were then extruded through a 0.4- μ m nucleopore filter using a Millipore holder under 40 to 60 Psi N₂ pressure. The liposomes have a mean diameter of approximately 0.3 μ m determined as described by Szoka *et al.* (22).

Nonentrapped MPC was removed after dilution of the liposome suspension by centrifugation at 190,000 \times *g* for 30 min at 20° in Sw50 rotor using a Beckman centrifuge. The pellets were combined and rewashed by a further centrifugation as above. Aliquots of the pellets, after resuspension in a small amount of buffer, were taken for radioactivity measurement to determine the amount of MPC captured, using [³H]poly(C) as a tracer. It was shown that [³H]poly(C) could be used as a tracer for MPC by spectroscopic determinations of capture in preliminary experiments. Subsequently, it was also shown that the capture of MPC as determined using [³H]poly(C) was the same as that determined using [³⁵S]MPC. The final capture in liposomes was approximately 1 mg MPC per 20 μ mol lipid. The REV liposome-entrapped MPC preparations were stored at 4° until use; they were diluted immediately before injection. Plain liposomes were prepared in the same manner in the absence of MPC.

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⁵ The abbreviations used are: MPC, partially thiolated polycytidylic acid; poly(C), polycytidylic acid; REV, reverse phase evaporation; CFUC, *in vitro* colony-forming unit of the granulopoietic progenitors; RES, reticuloendothelial system.

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Animals. RF/UN mice were bred in our laboratory, and the leukemic cell line was serially transplanted as described (11, 20). In brief, the RF/UN leukemia was passaged by giving mice i.v. injections of 1×10^6 spleen cells obtained from preterminal leukemic mice with spleens weighing 300 mg or more. Passages were made every 7 to 10 days. All the mice receiving such an inoculation expired within 8 to 10 days.

Stability of Liposome-entrapped MPC in Mouse Serum. Thirty nmol each of [35 S]MPC, liposome-entrapped [35 S]MPC, and [35 S]MPC mixed with liposomes, respectively, were treated with serum (20 or 4 μ l), obtained from RF/UN mice sacrificed 6 days after passage of tumor cells, in 60 μ l of phosphate buffer (30 mM, pH 7.2). After incubation at 37° for various periods of time (0 to 60 min), 200 μ l of 10% trichloroacetic acid were added. The reaction mixture was cooled at 0° for 15 min and centrifuged at $12,000 \times g$ for 15 min, and 100 μ l of the clear supernatant were withdrawn for radioactivity analysis in 5 ml of Ultrafluor. The total radioactivity present in the supernatant was used as a measure of the amount of MPC hydrolyzed by serum nucleases during incubation.

Plasma Clot Colony Techniques. This cloning method was conducted as described previously (10, 15, 17). Briefly, single-cell suspensions in Eagle's minimal essential medium supplemented with Hanks' balanced salt solution (15) were made from femur bone marrow. Bone marrow cells (5×10^4 nucleated cells) were seeded in 35-mm Falcon plastic dishes by the plasma clot method as described (10, 17), using 0.1 ml (10% of the culture medium) of serum from endotoxin-treated mice as a source of colony-stimulating activity. The plates containing the plasma clots were incubated at 37° in 5% CO₂ in a humidified atmosphere for 6 days. The plates were harvested, fixed, and stained with Giemsa and Wright's stains, and the colony number was determined (>40 cells/colony) by viewing the plate under a dissecting microscope. The majority of colonies were estimated to contain >500 cells each. All data were reported as colonies per femur.

Treatment of Mice with Various Agents *In Vivo*. Plain liposomes, MPC (5 to 50 mg/kg), liposome-entrapped MPC, or liposomes mixed with free MPC were injected i.v. into mice 5 to 6 days after passage of tumor cells. At 35 mg per kg, the liposomal lipid dose was 700 μ mol per kg for liposome-entrapped MPC. Seven hundred μ mol lipid per kg were used as the lipid dose in plain liposomes and in MPC plus liposomes studies. The dose of these liposomes lethal to 50% of mice

is greater than 12 mmol/kg (18). Control mice received the same volume (0.35 ml) of 0.9% NaCl solution. The mice were sacrificed 19 or 40 hr after treatment, and the bone marrow cells were collected and prepared as above for plasma clot colony culture.

RESULTS

The kinetics of hydrolysis of MPC alone and its various combinations with liposomes by serum nucleases of the leukemic RF/UN mice is shown in Chart 1. At 7.5 nmol of MPC per μ l of serum, equivalent to an *in vivo* dosage of approximately 100 mg per kg, MPC was hydrolyzed in a linear fashion to 28% at 60 min. When the amount of serum was increased 5 times (1.5 nmol of MPC per μ l of serum), MPC was hydrolyzed much faster to 57% at 60 min when the rate of hydrolysis leveled off. The liposome-entrapped MPC was completely resistant to serum nuclease hydrolysis under these conditions, whereas admixture of liposomes increased the rate of hydrolysis of free MPC. Using RNase A (1 pg/nmol MPC) instead of mouse serum in the hydrolysis, a similar picture was obtained in that MPC was hydrolyzed fast while liposome-entrapped MPC was not sensitive at all to the enzyme (data not shown).

The effect of MPC 24 hr after its administration on the bone marrow CFUC of leukemic mice is summarized in Table 1. MPC reduced the number of CFUCs in a dose-dependent manner, 17% inhibition at a dosage of 5 mg/kg and 68% at 50-mg/kg level. Based on these results, the dose at which MPC inhibited approximately 50% of the CFUC (35 mg/kg) was chosen for the study of the effects of liposomes on the activity of MPC.

As shown in Table 2, when compared to the control group of mice which received the same volume of 0.15 M NaCl, plain liposomes increased the number of marrow CFUCs at 19 and 40 hr after administration. MPC alone inhibited 53% of the CFUC at 19 hr but was without significant effect on the clonogenicity of the bone marrow cells by 40 hr after its administration to the mice. When nonencapsulated MPC was injected into mice together with plain liposomes, the activity of MPC was significantly potentiated to 80% reduction in CFUC numbers at 19 hr. However, the inhibitory effect of MPC decreased with time to 35% at 40 hr. When MPC was encapsulated in liposomes before its administration into the mice, the MPC-liposomes complex reduced CFUC numbers by 30% at 19 hr. The inhibitory effect of the MPC-liposome complex, however, per-

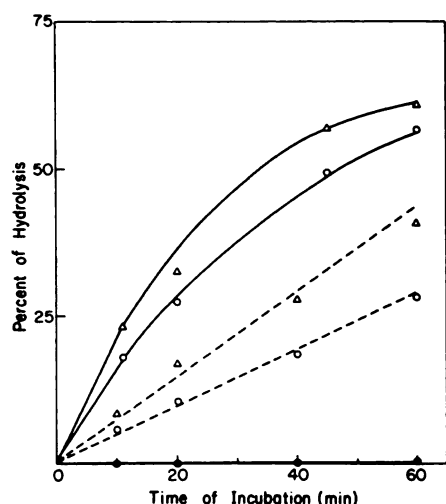


Chart 1. Stability of liposome-entrapped MPC in mouse serum [35 S]MPC samples were incubated with leukemia RF/UN mouse serum at 37° for the indicated periods of time. Trichloroacetic acid was added at 0° to terminate the reaction. Aliquots of the clear supernatant obtained after centrifugation were counted as a measure of percentage of MPC hydrolyzed. — — —, 7.5 nmol of MPC per μ l of serum; —, 7.5 nmol of MPC per 5 μ l of serum ●, liposome-entrapped MPC; ○, free MPC; △, mixture of plain liposomes and MPC. For liposome-entrapped MPC, 100% = 19,000 cpm; for free MPC and its mixture with plain liposomes, 100% = 18,000 cpm.

Table 1
Dose dependence of the effect of MPC on the clonogenicity of the bone marrow cells of leukemic mice

Different doses of MPC were injected into mice which were sacrificed 24 hr later. Their bone marrow cells were assayed for CFUC numbers using the plasma clot method.

Group	MPC administered (mg/kg)	Colonies/femur ^a	% of control
1 ^b	0	16,300 ± 450 ^c	100
2	5	13,600 ± 370	83.6 ± 3.2 ^c
3	10	13,100 ± 1,200	80.7 ± 7.8
4	25	10,900 ± 350	66.7 ± 2.8
5	35	8,800 ± 670	54.3 ± 4.4
6	50	5,200 ± 280	32.0 ± 1.9

^a Colonies/femur = (colonies/5 × 10⁴ nucleated cells) × number of nucleated cells/femur. Average of 2 experiments, 3 mice/group/experiment.

^b Groups 2 to 6 are different from Group 1 at $p = 0.01$ by 2-tailed Student's t test.

^c Mean ± S.E.

Table 2
In vivo effects of liposomes and MPC on the bone marrow CFUC of RF/UN leukemic mice
 Experiments were carried out as described in "Material and Methods."

Group	Agent administered	19 hr		40 hr	
		Colonies/femur ^a	% of control	Colonies/femur ^a	% of control
1 ^b	NaCl	8,130 ± 760 ^{c, d}	100	8,620 ± 820	100
2	Plain liposomes	13,300 ± 1,350	164 ± 23	11,000 ± 860	128 ± 16
3	MPC	3,850 ± 390	47.3 ± 6.5	8,200 ± 1,000	95.1 ± 15
4	MPC + plain liposomes	1,640 ± 200	20.2 ± 3.1	5,600 ± 600	65.3 ± 9.6
5	Liposome-entrapped MPC	5,710 ± 440	70.2 ± 8.5	6,700 ± 800	78.1 ± 12

^a Colonies/femur is calculated as in Table 1. Average of 6 experiments, 3 mice/group/experiment.

^b At 19 hr, Groups 1 to 5 are different from each other at $p = 0.01$. At 40 hr, Group 1 is not different from Group 3 at $p = 0.01$; the rest of the groups are different from each other at $p = 0.01$. All data are analyzed using 2-tailed Student's *t* test.

^c Mean ± S.E.

^d The difference in the number of colonies per femur between the control groups of Tables 1 and 2 is due to different potencies of various batches of colony-stimulating activities used in the culturing experiments.

Table 3
 Reanalysis of the data provided in Table 2 using mice given injections of drug-free liposomes as controls

Group	Agent administered	19 hr		40 hr	
		Colonies/femur ^a	% of control	Colonies/femur ^a	% of control
2 ^b	Plain liposomes	13,300 ± 1350 ^c	100	11,000 ± 862 ^c	100
4	MPC + plain liposomes	1,640 ± 200	12.3 ± 1.9 ^c	5,600 ± 600	51 ± 6.9 ^c
5	Liposome-entrapped MPC	5,710 ± 440	42.8 ± 5.4	6,700 ± 800	61 ± 8.7

^a Colonies/femur is calculated as in Table 1.

^b Groups 2, 4, and 5 are different from each other at both time points at $p = 0.01$; 2-tailed Student's *t* test.

^c Mean ± S.E.

sisted and showed only slow decline over the 40-hr period under study.

Since the administration of liposomes alone increased the number of the colony-forming cells obtained from murine bone marrow, the most appropriate control value for CFUC present in mice which received MPC and liposomes should be the values for mice which received only liposomes. With this in mind, the data provided in Table 2 were reanalyzed as shown in Table 3. Admixture of liposomes significantly potentiated the inhibitory effect of MPC (to 88% at 19 hr) with partial recovery of the CFUC to 49% inhibition at 40 hr. The liposome-entrapped MPC showed similar activity as did MPC alone (58% inhibition of the CFUC at 19 hr, with slow decline to 39% at 40 hr after administration).

DISCUSSION

We previously reported that the inhibition of the clonogenicity of the bone marrow cells by MPC decreased substantially between 19 and 25 hr after its injection into the mice (10). The results from the present study extend the observation to 40 hr post drug administration at which time point MPC practically showed no effect on the colony-forming cells. These data, coupled with the *in vitro* stability study of MPC and liposome-entrapped MPC, indicate that MPC was delivered to the bone marrow and exerted its inhibitory effect on the clonogenic cells at those sites, however, the effects of a single dose of MPC practically vanished within 40 hr due to the rapid metabolism of the polynucleotide in the mice and rapid replacement of damaged CFUCs. When MPC was encapsulated within liposomes before injection into the mice, it inhibited colony production somewhat less than MPC administered alone at 19 hr. However, a reduction in CFUC numbers for MPC-liposomes persisted for at least 40 hr, whereas the effect of MPC alone

had disappeared. These observations are consistent with the concept that MPC encapsulated within liposomes is protected from hydrolysis by nucleases present in blood as shown by the *in vitro* study (Chart 1) and that the MPC-liposomes complex serves as a depot form of the modified polynucleotide, thus extending the duration of its effects in the mice. The decreased activities of MPC-liposomes at 19 hr compared to MPC alone could be related to reduced bioavailability of free MPC entrapped in liposomes shortly after injection due to the slow release of MPC from the relatively impermeable liposomes used (12).

The observation that non-drug-containing liposomes increased the number of marrow colony-forming cells is of interest, but the reasons for this effect are unknown at the present time. It is also of interest that the percentage of CFUC killed was greater in animals which received a mixture of free MPC and liposomes. The reasons for these effects are probably complex, and several explanations are possible. Liposomes and MPC are macromolecules, and it can be expected that after injection they will be cleared from the circulation by cells of the RES. In addition to the evidence (for review, see Ref. 12) that liposomes are cleared by cells of the RES, there is also recent evidence that REV liposomes at the dose used in the studies described in this report can reversibly depress RES clearance function although the depression lasts for 4 to 24 hr (7). It is possible that RES inhibition by liposomes results in decreased clearance of MPC by RES cells, thus causing increased levels of available MPC to be taken up by target cells, or liposomes and MPC compete for uptake by RES cells, which could again result in increased levels of MPC. Alternatively, the change in the RES function caused by liposomes could lead to alterations in the immune status of the animals which could result in an increased effectiveness of MPC; or, as has been suggested (21), liposomes could alter the surface properties of

target cells allowing greater uptake of antitumor agents.

At any rate, the results of the present study indicate that the therapeutic potency of MPC and perhaps other modified polynucleotides can be modulated using liposomes, either by entrapping MPC in the vesicles or by injecting a mixture of both agents into the recipient.

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