

Antitumor Activity of Timed-release Derivative of Mitomycin C, Agarose Bead Conjugate

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A timed-release derivative of mitomycin C (MMC), agarose bead conjugate (MMC-AB), was synthesized using cyanogen bromide method. MMC was released successively for considerably longer period from MMC-AB *in vitro* and *in vivo* after intraperitoneal injection to mice. MMC-AB exhibited almost identical inhibitory effect against growth of Ehrlich ascites carcinoma (EAC) cells to free MMC and less toxicity to ddY mouse than free MMC. Calculated therapeutic index (LD_{10}/ED_{90}) of MMC-AB was two times greater than that of free MMC, suggesting its advantage in cancer chemotherapy. On the life span of EAC bearing ddY mouse, MMC-AB showed almost similar efficiency with that of free MMC but less activity was exhibited against BDF₁ mouse transplanted L1210 leukemia.

Keywords—timed-release delivery system; mitomycin C; agarose bead conjugate; cyanogen bromide method; Ehrlich ascites carcinoma; L1210 leukemia; therapeutic index; growth inhibitory effect on cancer cells; toxicity; cancer chemotherapy

It has been recently one of the most important problems in clinical cancer chemotherapy how the effects of anticancer agents currently available can be enhanced. From this viewpoint, numerous attempts such as structural modification of the drug, alteration in route of administration or dose regimen, and development of a specific drug delivery system have been made to improve chemotherapeutic properties of agents; particularly, much efforts have been directed toward a development of timed-release systems which could be implanted in the closest possible proximity to a malignant neoplasm. Various types of parenteral dosage form, such as emulsions,²⁾ liposome,³⁾ and particulate polymeric matrix⁴⁾ were introduced as a delivery device of satisfying such criteria, and their efficiency was demonstrated.

In previous investigation,⁵⁾ we synthesized mitomycin C-agarose bead conjugate (MMC-AB) by coupling mitomycin C (MMC) to gelled agarose bead (AB) and sustained release of MMC from the conjugate was demonstrated. This article describes detailed characteristics of MMC-AB as a timed-release delivery system and explores its antitumor activity on mouse tumors.

Experimental

Materials—MMC was supplied from Kyowa Hakko Co., Ltd. AB was purchased from Pharmacia Fine Chemicals Co., Sweden (Sephacrose 4B). Other chemicals used were of reagent grade quality and obtained commercially from Nakarai Chemicals Co. Ltd.

Preparation of MMC-AB—MMC-AB was synthesized as described previously,⁵⁾ by modifying the method of Axén, *et al.*⁶⁾ To a stirred suspension of 100 mg AB in 5 ml water, 100 mg of cyanogen bromide

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was added in three portions at intervals of several minutes and pH maintained at 10.7 by addition of 2M sodium hydroxide. After 30 min reaction, the agarose beads were rapidly washed on a glass filter with 300 ml of 0.1M sodium bicarbonate-0.5M sodium chloride solution under suction. The activated beads were resuspended in 10 ml of the same medium dissolving 20 mg of MMC, and then the coupling reaction was allowed to proceed for 24 hr. After completion of the coupling reaction, the products were washed on a glass filter with the coupling solution, and then washed with 0.1M acetate buffer and 0.1M borate buffer alternatively in order to remove unconjugated drug. All the procedures were performed at room temperature. The products were lyophilized and stored at 4°. One milligram conjugate was estimated to contain about 18 µg of MMC by measuring the amount of the unconjugated in the eluate.

In Vitro Release Experiment—Twelve mg of MMC-AB was suspended in 5 ml isotonic phosphate buffer (pH 7.4) maintained at 37° with moderate shaking, and the liberated MMC during 24 hr was determined after separation by filtration. The residual beads were resuspended in a new buffer of the same composition for the following release experiment and these procedures were repeated each time for 40 days.

Animal Experiments—For most of the present experiments, male ddY mice weighing between 20 and 23 g were employed. In all the animal experiments, MMC-AB was administered intraperitoneally as a sterilized saline suspension and MMC was administered intraperitoneally as a sterilized saline solution. The dose of MMC-AB was expressed as an amount corresponding to parent MMC.

For investigating *in vivo* timed-release characteristic, the urinary excretion of MMC in the ddY mouse implanted MMC-AB was determined. After 0.1 mg (MMC equivalent) of MMC-AB or of MMC was injected intraperitoneally, mouse was housed in a metabolic cage and the urine was collected at varying time periods for 5 days.

For studying a growth inhibitory effect of MMC-AB or MMC on Ehrlich ascites carcinoma (EAC), ddY mice were inoculated intraperitoneally with 1.4×10^7 EAC cells on the former day (day 0) and then the drug was administered on the starting day (day 1) or on the five consecutive days starting on day 1. On day 7, all ascitic fluids were removed from the mice and the total number of harvested cells was counted using hemocytometer.

In order to examine an effect of MMC-AB or free MMC on the survival period of tumor bearing mice, the mean life span of each group was calculated and antitumor activity was evaluated by an increase in life span over control. ddY Mice were inoculated intraperitoneally with 1.4×10^7 EAC cells and BDF₁ mice were inoculated intraperitoneally with 10^5 L1210 cells on day 0 respectively, and the drug was injected intraperitoneally on day 1 or on five consecutive days starting on day 1.

Toxicity was evaluated with survivors on the 50th day after the day of administration or the first day of five consecutive daily administration.

Analytical Method—In *in vitro* release experiment, the concentration of MMC in buffer solution was determined spectrophotometrically based on $\epsilon = 22000$, at $\lambda_{\max} = 364$ nm. The antimicrobial activity was determined by the disc-plate method using *Escherichia coli* B, *Staphylococcus aureus* 209P, or *Bacillus subtilis* PCI-219 as test organisms.

Results

In Vitro Sustained Release

Fig. 1(a, b) shows the time course for the release of MMC from MMC-AB *in vitro*. The cumulative amount of MMC released as a function of time is shown in figure (a), and the semilogarithmic plot of calculated percent of drug remained in the conjugate *versus* time is shown in figure (b). As can be seen in Fig. 1, MMC was liberated successively, and the liberation process appears to be biphasic with a half-life of 6 days in the initial log-linear phase and of 12 days in the second phase.

In Table I, the amounts of released MMC during the period of 24 hr of the first and the 16th day determined by spectrophotometric analysis are compared with the values of antimicrobial activity against *E. coli* B, *St. aureus* 209P, and *B. subtilis* PCI-219. Both at the first and the 16th day, the results obtained from these different analyses were almost identical to each other, suggesting that the liberated MMC had retained its original antimicrobial activity. On ascending paper chromatography using a solvent system of 1-propanol-1% ammonium hydroxide, 2:1 (by volume), liberated compound showed a purple major spot with *R_f* value identical to that of original MMC (*R_f* 0.65).

In Vivo Sustained Release of MMC after Administration

Fig. 2 illustrates the urinary excretion rate of MMC after intraperitoneal injection of MMC-AB or free MMC. Urinary concentration of MMC was determined by microbiological

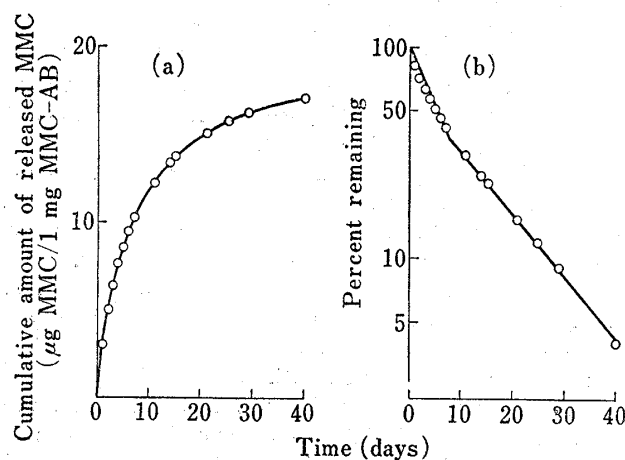


Fig. 1. *In Vitro* Release of MMC from MMC-AB

- (a) cumulative amount of MMC released from 1 mg MMC-AB.
 (b) semilogarithmic plots of percent remaining of MMC in the conjugate.

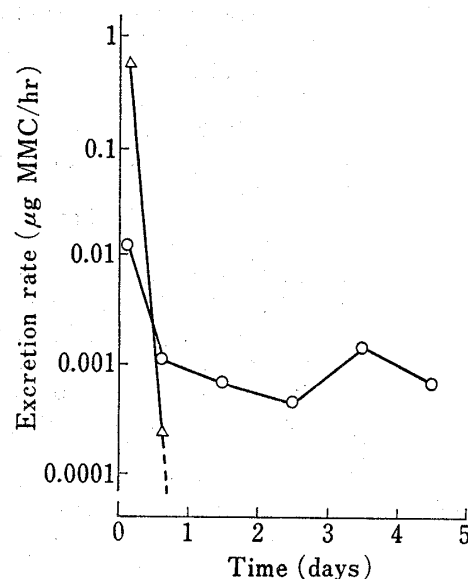


Fig. 2. Urinary Excretion Rate of MMC after Intraperitoneal Injection of MMC-AB or Free MMC

○, MMC-AB; △, free MMC. Dose: 5 mg MMC/kg. Results are expressed as the mean of 2 groups (6 mice).

TABLE I. Amount of MMC released from 1 mg MMC-AB during 24 Hours Incubation *in Vitro*

Incubation period	Amount of released MMC (μg) ^{a)}			
	Spectrophotometric analysis (364 nm)	Microbial analysis		
		<i>E. coli</i> B	<i>S. aureus</i> 209P	<i>B. subtilis</i> PCI-219
First day	3.02±0.004	2.81±0.27	2.88±0.22	2.55±0.31
16 th day ^{b)}	0.33±0.02	0.33±0.04	0.37±0.03	0.28±0.02

a) Results are expressed as the mean ± S.D. of three experiments.

b) This experiment was undertaken following 15 days incubation.

analysis against *E. coli* B. Following injection of free MMC, the drug was excreted with a rate of 0.63 μg/hr in the urine of the first 6 hr, but the excretion rate was reduced rapidly and no antimicrobial activity could be detected in the urine samples after 24 hr. On the contrary, when MMC-AB was administered as a suspension, the excretion rate was 0.012 μg/hr during the first 6 hr and was relatively maintained more than 4.4×10^{-4} μg/hr during 5 days. These results indicate that there occurred the sustained release of MMC from MMC-AB in the peritoneal cavity of mouse.

Growth Inhibitory Effect of MMC-AB or MMC on EAC Cells *in Vivo*

Fig. 3 (a, b, c) shows the growth of EAC cells *in vivo* versus logarithmic dose of MMC-AB or free MMC. MMC-AB was administered in a single injection (a) and free MMC administered in a single dose (b) or in five consecutive daily doses (c). Cell growths are expressed as the percentage of the cell population of the treated group per control group. The control group showed the rapid growth of EAC cells during the course of the experiment and at the end of its experiment the mean total cell number was 8.5×10^8 cells.

As shown in Fig. 3, both MMC-AB and MMC showed dose-dependent growth-inhibitory activity, so the dose of the drug required to inhibit the cell growth by 90% (ED₉₀) was calcu-

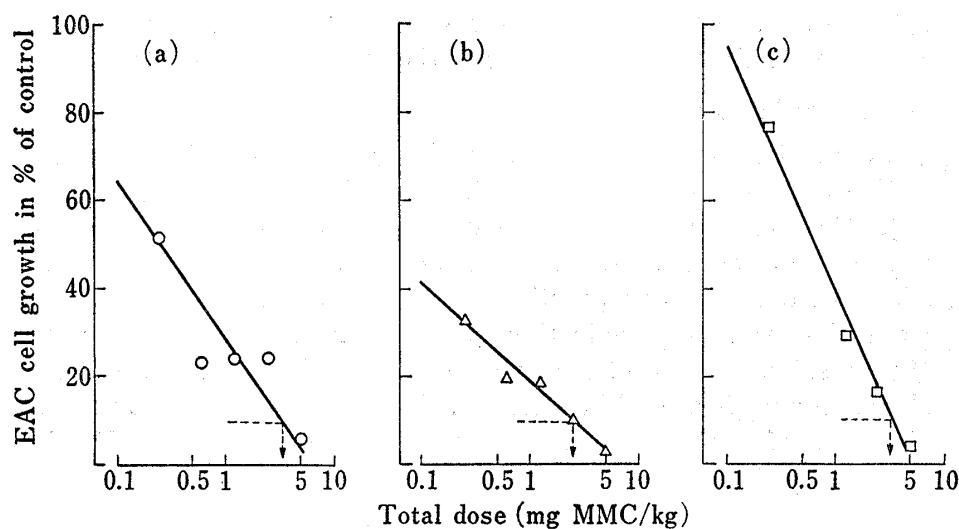


Fig. 3. Effect of MMC-AB and Free MMC on the Growth of EAC Cells

(a) MMC-AB single dose; (b) free MMC single dose; (c) free MMC five consecutive daily doses. Results are expressed as the mean of 5 or 6 mice.

TABLE II. Therapeutic Indices of MMC-AB and Free MMC

Drug	Dosage ^{a)} schedule	ED ₉₀ ^{b)} (mg MMC/kg)	LD ₁₀ ^{c)} (mg MMC/kg)	Therapeutic ^{d)} index
MMC-AB	day 1	3.2	16.0	5.0
free MMC	day 1	2.6	6.8	2.6
free MMC	days 1-5	3.3	6.5	2.0

a) Mice were inoculated intraperitoneally with 1.4×10^7 EAC cells on day 0, and each drug was injected intraperitoneally.

b) the total dose showing 90% inhibition of EAC cell growth.

c) the total dose showing 10% animal death until 50 days.

d) therapeutic index = LD_{10}/ED_{90} .

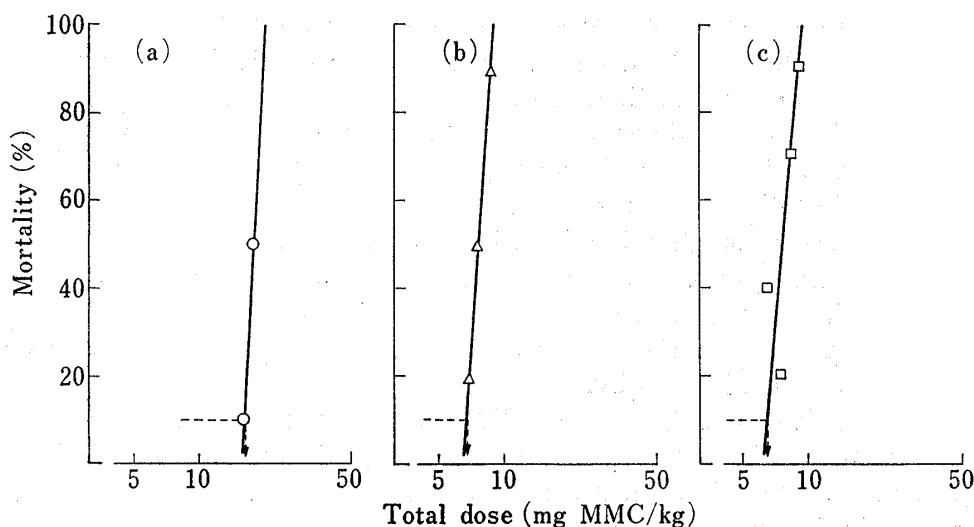


Fig. 4. Lethal Toxicity of MMC-AB and Free MMC on ddY Mouse

(a) MMC-AB single dose, (b) free MMC single dose, (c) free MMC five consecutive daily doses.

Results are expressed as the mortality of 10 mice.

lated (see Fig. 3, dashed lines). The ED_{90} values from three experiments are summarized in Table II, which were relatively similar to each other between 2.6–3.3 mg/kg.

Toxicity of MMC-AB or MMC to ddY Mice

Fig. 4 (a, b, c) illustrates the dose-acute toxicity relationship in normal ddY mice receiving single injection of MMC-AB (a), free MMC (b) or five consecutive daily administration of free MMC (c). Free MMC demonstrated marked toxicity to mice and all the animals did not survive regardless of dosage schedule at the dose of more than 10 mg/kg MMC. On the other hand, MMC-AB did not cause practically any damage to the animals even at the dose of 14 mg/kg (equivalent to MMC), indicating that the conjugate is less toxic than the parent drug. From these results, the dose of the drug which caused 10% of the tested animals death (LD_{10}) was calculated and presented in Table II. The LD_{10} of MMC-AB was more than two times higher than that of single dose of free MMC or multiple dose of free MMC.

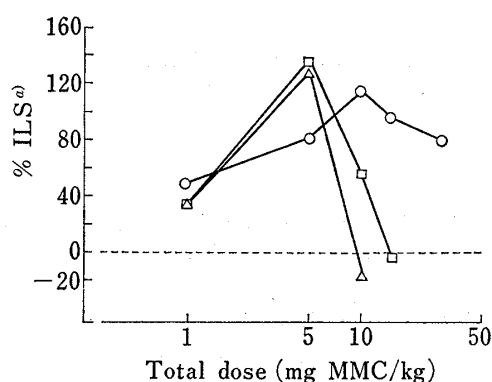


Fig. 5. Effect of MMC-AB and Free MMC on Survival Time of EAC-bearing ddY Mice

○, MMC-AB single dose; △, free MMC single dose; □, free MMC five consecutive daily doses. Results are expressed as the mean of 6 mice.
a) Percentage of increase in life span over controls. The mean survival time of control group is 12.0 days.

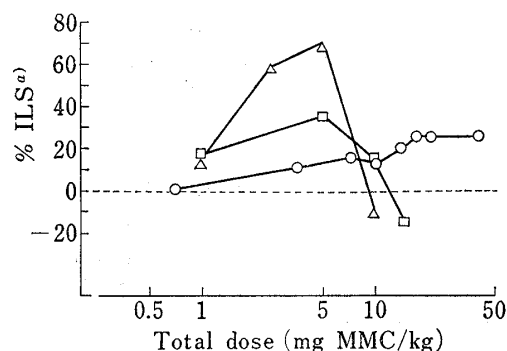


Fig. 6. Effect of MMC-AB and Free MMC on Survival Time of L1210-bearing BDF₁ Mice

○, MMC-AB single dose; △, free MMC single dose; □, free MMC five consecutive daily doses. Results are expressed as the mean of 6 mice.
a) Percentage of increase in life span over controls. The mean survival time of control group is 9.0 days.

Effect of MMC-AB or MMC on the Life Span of Tumor-bearing Mouse

Fig. 5 shows the effect of the drug treatment on the survival time of the ddY mouse bearing EAC. The mean survival time of the untreated group was 12 days. In the case of free MMC of both injection schedules, maximum life span was exhibited at a total dose of 5 mg/kg and percentage of increase in life span (ILS) was 122% on single injection and 136% on five consecutive injection. At a dose of 10 mg/kg, free MMC showed marked toxicity particularly in the case of single administration, in which the mean life span was shorter than that of the control group. On the contrary, survival time in MMC-AB exhibited a gradual increase, which reached to the maximum ILS of 115% at a dose of 10 mg/kg. Over the dose of 10 mg/kg, however, the conjugate showed only slight toxicity, and still showed 81% of ILS even at a dose of 30 mg/kg.

The antitumor activity of MMC-AB and MMC were also tested in L1210 leukemia system and results are illustrated in Fig. 6. The mean life span of control (untreated) group was 9 days. In this tumor system, both drugs showed moderate activity in comparison with EAC system. Free MMC exhibited maximum ILS of 70% following single administration and 35% following multiple administration respectively at a total dose of 5 mg/kg. Marked toxicity was recognized at over dose more than 5 mg/kg. On the contrary, MMC-AB showed lower activity and ILS did not exceed 30%, while ILS increased with an increase of dose until 40

mg/kg. Namely single administration of 40 mg/kg MMC-AB prolonged the life span of L1210 bearing BDF₁ mouse to 26% against control mouse.

Discussion

MMC has been one of the most extensively used anticancer agents both in a clinical treatments and in laboratory investigation since the isolation by Wakaki, *et al.* in 1958.⁷⁾ Its therapeutic responses have been reported in a variety of cancers, but its treatment has been recognized only of palliative benefit since it is always necessary to halt therapy short of a cancer-sterilizing dose because of toxicity. In order to overcome these disadvantages, the prolonged release derivatives of the anticancer agents are required to minimize their toxicity to normal cells. In the present investigation, a timed-release derivative of MMC was prepared using cyanogen bromide method as a drug delivery system which can be directly injected into the target sites, tumor tissues. In the cyanogen bromide procedure, the activated polysaccharide is supposed to be coupled with amino groups bearing the desirable ligand,^{6,8)} although the ligand-polysaccharide bonds formed in these reactions have not been obviously characterized. MMC has three amino groups in its molecular structure, the structure of linkage is, therefore, thought to be more complicated, and detail examination of this problem is beyond the present investigation. Regardless of these difficulties in elucidating the characteristic of the linkage, the release reaction is thought to proceed through hydrolysis and aminolysis as recently described by Schnaar, *et al.*⁹⁾

From our results of *in vitro* 37° release experiment, it is suggested that MMC is slowly and successively released from MMC-AB into buffer solution (pH 7.4) maintaining its original antimicrobial activity. After intraperitoneal injection of MMC-AB, biologically active MMC was excreted successively for 5 days as shown in Fig. 2. Since the beads were considered to be retained in the peritoneal cavity for a considerably long period after injection, it seems reasonable to speculate that the sustained release of MMC occurred in the body cavities prior to excretion. The antimicrobial activity of MMC was proved to be in good correlation with its antitumor activity.¹⁰⁾ Consequently, our synthesized MMC-AB appears to function as a timed-release delivery system of MMC so that malignant cells are exposed to its cytotoxicity for a long period.

In the present investigation we examined growth-inhibitory activity on EAC cells and toxicity of MMC-AB in comparison with free MMC. From these results therapeutic indices (LD₅₀/ED₅₀) of them are calculated for therapeutic evaluation and summarized in Table II. Free MMC showed the therapeutic index of 2.6 on single dose and 2.0 on five daily doses, whereas MMC-AB gave an increased therapeutic index of 5.0. These results suggest the advantage of MMC-AB over the parent drug in cancer chemotherapy.

On the life span of EAC bearing ddY mouse, MMC-AB prolonged it more than two times over controls similar to free MMC. On the other hand, MMC-AB exhibited less activity against the life span of L1210 bearing BDF₁ mouse than free MMC. This difference may be attributed to a different extent of dissemination of growing cells; EAC mainly proliferates in the peritoneum of mice while L1210 proliferates through the system circulation to overall the body of animals. By injection of MMC-AB, sufficient concentration of MMC is supplied for a long period only around the injection site, the peritoneal cavity. A rapid metabolic degradation of MMC in the liver is thought to be also responsible because drug is absorbed from the perito-

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neum through the liver prior to enter the systemic circulation.¹¹⁾ Although in this study the survival time of the mice was not improved over that obtained with equimolar doses of MMC, one should not overlook the possible advantage offered by the reduced toxicity of the MMC-AB to the host.

On the basis of the evidence presented in this investigation, it is concluded that the application of MMC-AB would be advantageous in cancer chemotherapy, because active MMC can be supplied in the injection site for a considerable longer period. Such characteristic would be of value during the surgical operation of tumor or for instillation into body cavities and tissue planes in which drug-sensitive malignant cells are thriving. The marked longer volume doubling time of human cancer as compared with that of experimental mouse tumor¹²⁾ provides encouragement for application of MMC-AB in clinical therapy. In addition, since the present approach can be employed for other anticancer agents of having amino group in its molecular structure, a wide application for clinical use particularly in the case of more timedependent drugs is anticipated.

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