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Regeneration Characteristics of Mitomycin C-Dextran Conjugate in Relation to Its Activity

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The regeneration characteristics and biological activities of mitomycin C-dextran conjugate (MMC-D) were investigated in comparison with those of free mitomycin C(MMC), and the following results were obtained. (1) MMC is coupled at the 1a-nitrogen position *via* an amide linkage to a carboxyl group of ε-aminocaproic acid (6-aminohexanoic acid) which was introduced onto dextran as a spacer. (2) MMC-D was stabler than MMC against acid-catalyzed and metabolic degradation. (3) MMC was liberated from MMC-D with a half-life of about 24 h under physiological conditions (pH 7.4, 37°C) and more rapidly at higher pH. (4) Liver homogenate did not accelerate the liberation of MMC from MMC-D. (5) Antimicrobial activity of MMC-D against *Escherichia coli* B was less than one-eighteenth of that of MMC. (6) MMC-D showed about one-tenth of the antitumor activity of MMC *in vitro* in contact with L1210 leukemia cells, but showed almost equal efficiency in the *i.p.-i.p.* system *in vivo*, suggesting that MMC-D exhibited activity after being regenerated to the parent drug in the body.

Keywords——mitomycin C; high molecular weight prodrug; dextran; linkage structure; stability; regeneration properties; metabolic degradation; antimicrobial activity; *in vitro* antitumor activity; *in vivo* antitumor activity

Although mitomycin C(MMC) is widely used in cancer chemotherapy, its utility is limited by the side effects of severe bone marrow depression and gastrointestinal damage.[1] To overcome these defects, therefore, it seems beneficial to concentrate its cytotoxicity at the tumor site and to minimize the burden to other tissues by modifying the biological and pharmacokinetic properties.[2]

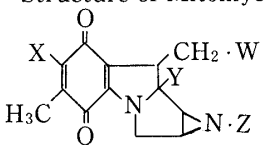
One possible approach to alter the biopharmaceutical behavior of MMC might be its derivatization into a latent form with high molecular weight.[3] In an attempt to realize this idea, we have developed a macromolecular derivatives of MMC, MMC–dextran conjugate (MMC–D), and examined its pharmacodynamic properties.[4,5] We found that MMC–D exhibited improved antitumor activity in the *i.p.-i.p.* system against murine tumors thriving mostly in the peritoneal cavity such as B16 melanoma and Ehrlich ascites carcinoma.[5] Free MMC was detected for several days in plasma and urine of mice given *i.p.* administration of MMC–D, and it was suggested that the persistent retention of this compound in a specific locality as a potential source of free MMC was responsible for its therapeutic efficiency.[4]

In the present study, we investigated in detail the properties of MMC–D in order to clarify the mechanism of the enhanced activity and to develop an effective means of application and a dose regimen for this novel-type prodrug of MMC in clinical therapeutics. The linkage structure, chemical and biological stability, regeneration properties, antimicrobial activity, and *in vitro* and *in vivo* antitumor activities are presented herein.

Experimental

Material——MMC and three kinds of derivatives, mitomycin A (MMA), porfiromycin (PM), and decar-

TABLE I. Structure of Mitomycins



Compound	X	Y	Z	W
Mitomycin A (MMA)	H_3CO-	$-OCH_3$	H	$-OCONH_2$
Mitomycin C (MMC)	H_2N-	$-OCH_3$	H	$-OCONH_2$
Porfiromycin (PM)	H_2N-	$-OCH_3$	$-CH_3$	$-OCONH_2$
Decarbamoyl mitomycin C (DecMMC)	H_2N-	$-OCH_3$	H	-OH

bamoyl mitomycin C (DecMMC), listed in Table I, were supplied by Kyowa Hakko Kogyo Co. Dextran was purchased from Pharmacia Fine Chemicals Co., Sweden (Dextran T-70) and has an average molecular weight of 70000 (elemental analysis; C, 43.15; H, 6.76; N, 0; O, 50.10). All other chemicals were commercial reagent-grade products.

Synthesis of Dextran Conjugates——MMC-D was synthesized as reported previously.[4] In brief, dextran was activated with cyanogen bromide at pH 10.7 according to Axén and Ernback,[6] and ε-aminocaproic acid was coupled to it. The product, spacer-introduced dextran, was washed repeatedly by ultrafiltration (Pellicon PSED 043 10) and precipitated with aceton (elemental analysis; C, 44.60; H, 6.57; N, 4.19; O, 45.02). MMC was conjugated to the spacer-introduced dextran by the use of a carbodiimide. The pH of the mixture of MMC, spacer-introduced dextran, and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride was kept between 5.0 and 6.0 and the reaction was allowed to proceed for 24 h at room temperature. The product was washed, concentrated by ultrafiltration, and precipitated with acetone to yield a purple solid (elemental analysis; C, 44.25; H, 6.39; N, 6.79; O, 40.50).

The conjugates of MMA, PM, and DecMMC were synthesized in the same way as that of MMC. The yield of conjugates was determined by gel filtration chromatography (Sephadex G-75).

Stability Measurement in Aqueous Solutions——All stability experiments were carried out in aqueous buffer solutions at 37 ± 0.2°C. The pH of the solution was maintained at the desired value by using a buffer system consisting of NaH_2PO_4–Na_2HPO_4. The total buffer concentration was 0.1 M except for experiments where buffer effects were studied specifically. The ionic strength of all buffer solutions was adjusted to 0.3 with NaCl, if necessary. Degradation was initiated by the addition of the stock solution to a preheated buffered solution to give a concentration of 3×10^{-3} mg equivalent MMC/ml.

Stability Measurement in Biological Media——The liver, spleen, kidney, and muscle of male Wistar albino rats weighing 240—260 g were each homogenized at 4°C in a glass-Teflon homogenizer, and centrifuged at $600 \times g$ for 10 min, and the supernatant was used for the experiment. An isotonic pH 7.4 phosphate buffer (0.1 M) containing sucrose (0.125 M) and NaCl was used to prepare tissue homogenate. Experiments were initiated by adding the stock solution to give a final concentration of 3×10^{-4} mg equivalent MMC/ml. All incubations were carried out at 37 ± 0.2°C.

Analytical Method——In chemical stability studies, the total degradation of MMC was monitored by direct ultraviolet (UV)-spectrophotometry ($\lambda_{max} = 364$ nm, $\varepsilon = 22000$). For detecting the amount of regenerated MMC, the reaction medium was ultrafiltered in a micropartition system (MPS-1, Amicon) and the filtrate was subjected to high performance liquid chromatography (HPLC). An HPLC system (TRIROTAR, Jasco) equipped with a variable-wavelength UV detector (UVIDEC 100-III, Jasco) was used in a reverse-phase mode with a stationary phase of Coracil $5C_{18}$ (Nakarai Chemicals) and a short guard column (Licosorb RP-2, E. Merck). A mixture of methanol and water was used as the mobile phase at a flow rate of 1.0 ml/min. The standard solutions were chromatographed and calibration lines were constructed on the basis of peak-area measurements. The amount of MMC in conjugated form was estimated by subtracting the amount of free MMC from that of total MMC determined spectrophotometrically.

For monitoring the concentration of MMC-D in a biological medium, aliquots of sample solution were withdrawn at appropriate time intervals, two volumes of pH 8.0 phosphate buffer were added, and the mixture was heated at about 90°C for 5 min. The amount of free MMC in the supernatant (produced by the hydrolysis of MMC-D) was determined by bioassay or by HPLC after centrifugation at 2800 rpm for 10 min. A spiking experiment indicated a constant recovery of MMC from the conjugated form of 70%. The variation coefficient was generally less than 10% for each run. The amount of free MMC in the biological medium was assayed by HPLC or direct bioassay without hydrolysis.

Antimicrobial Activity Studies——Antimicrobial activities of MMC-D and MMC were evaluated by broth dilution and disc-plate methods using *Escherichia coli* B as a test organism. In the broth dilution method, the growth of the bacterium in a medium in which a suitable amount of MMC-D or MMC had been dissolved was monitored by measuring the turbidity at 660 nm during incubation at 37°C. In the disc-plate

method, antimicrobial activity was determined by measuring the diameter of the growth-inhibitory zone after diffusion for 18 h at 4°C and incubation for 24 h at 37°C.

Antitumor Activity Studies——L1210 leukemia was maintained by weekly transplantation of tumor cells into the peritoneal cavity of male DBA/2 mice. Animals used for tests were male hybrid BDF_1 mice (C57Bl/6, female × DBA/2, male). These animals were kept on the breeding diet NMF (Oriental Yeast Co.) with water *ad libitum* in a room maintained at 23±1°C and a relative humidity of 55±5%.

Antitumor activities of MMC-D and MMC were examined in contact with L1210 cells *in vitro*. L1210 cells (5×10^6) were incubated with MMC-D or MMC at various concentrations for 60 min at 37°C in 5 ml of Hanks' solution and then washed three times with the same medium by centrifugation. The cytotoxicity of the drug against the tumor cells was evaluated by recording the survival time of BDF_1 mice inoculated with the drug-treated cells (1×10^5). Results are expressed in $T/C\%$ values, calculated as the mean survival time of mice bearing treated cells divided by that of the control mice.

Results

Structure of Linkage

As reported previously,[4] MMC was coupled to the dextran with high yield (90%) by the present procedure. From the results of spectrophotometric analysis, the content of MMC in MMC–D was calculated to be about 0.3 mmol/g. The pH titration of the spacer-introduced dextran showed that the extent of coupling of the spacer was about 0.8 mmol/g.

Fig. 1 shows the gel filtration patterns of three kinds of MMC analogues and their dextran conjugates on a Sephadex G-75 column. Chromatography was performed using pH 7.4 phosphate buffer as an elution medium. As shown in these figures, the reaction products of MMA and DecMMC were eluted in the macromolecular fractions, like MMC –D.[4] On the other hand, PM was eluted in almost the same fractions as the original drug.

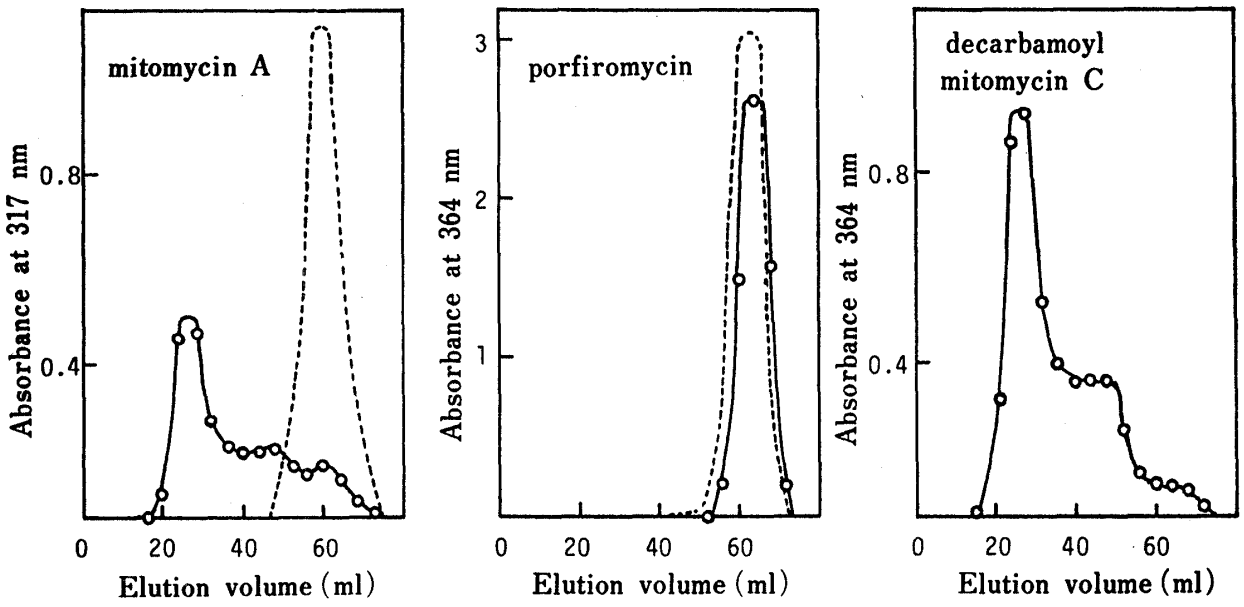


Fig. 1. Gel Filtration Patterns of MMA, PR, and DecMMC in Free (-----) and Dextran-conjugated (—○—) Forms

Chromatography was carried out on a Sephadex G-75 column (1.7×20 cm) with pH 7.4 phosphate buffer at room temperature. Each compound was detected spectrophotometrically. Free DecMMC was adsorbed on the gel and not eluted under these conditions.

On the basis of these results, the structure of MMC–D is proposed to be as shown in Fig. 2, with MMC conjugated through an amide linkage. The degree of substitution with MMC in MMC–D was estimated to be one molecule of MMC per approximately 14—17 glucose units.

Stability in Aqueous Buffer Solutions

The kinetics of decomposition of MMC–D were compared with those of MMC in aqueous

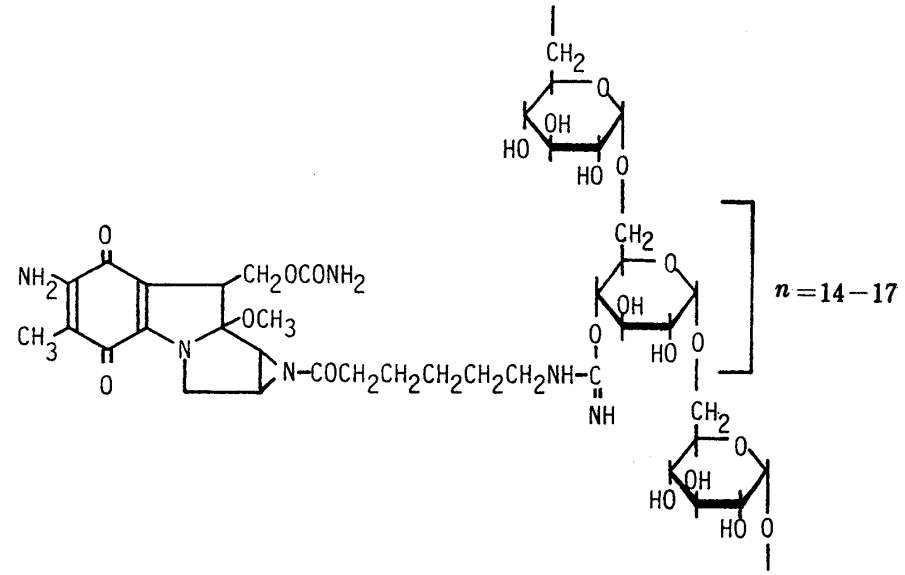


Fig. 2. Proposed Structure of Mitomycin C–Dextran Conjugate

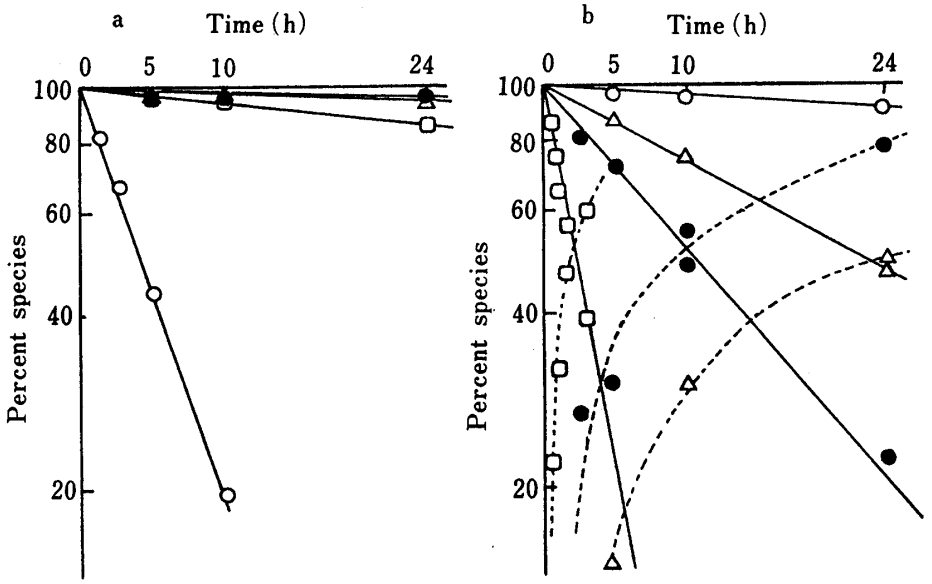


Fig. 3. Stability of MMC(a) and MMC-D(b) in Phosphate Buffer Solutions ($\mu=0.3$) of pH 5.0 (○), 7.4 (△), 8.0 (●), and 9.0 (□) at 37°C

Solid lines show the decomposition of each compound and dotted lines show the regeneration of MMC from MMC-D.

buffer solutions of pH 5.0, 7.4, 8.0, and 9.0 (Fig. 3). The decomposition of both compounds at each pH followed apparent pseudo-first order kinetics with respect to concentration over more than 3 half-lives. Simultaneous appearance of MMC was observed during the decomposition of MMC–D at a pH region higher than 7.4.

On the basis of these findings, the overall reactions may be described roughly by the following scheme of parallel and consecutive reactions for MMC–D:

$$\begin{array}{ccccc} \text{MMC–D} & \xrightarrow{k_1} & \text{MMC} & \xrightarrow{k_3} & \text{decomposition products} \\ \downarrow k_2 & & & & \\ \text{decomposition products} & & & & \end{array}$$

In this scheme k_1, k_2, and k_3 are pseudo-first order rate constants for the depicted chemical

reactions (where $k_1+k_2=k_{obs}$). The corresponding rate equations for this model were integrated with the Laplace transform to give the following equations;

$$[\text{MMC}-\text{D}] = [\text{MMC}-\text{D}]_0 \times e^{-(k_1+k_3)t} \quad (1)$$

$$[\text{MMC}] = \frac{k_1}{k_3-(k_1+k_2)} \times (e^{-(k_1+k_3)t} - e^{-k_3 t}) \quad (2)$$

where $[\text{MMC–D}]_0$ represents the initial concentration of MMC–D. [MMC–D] and [MMC] are the concentrations of MMC–D and MMC at time t, respectively. Based on these equations, curve fitting and parameter estimation were done using a nonlinear least-squares program, MULTI.[7] Concentrations of MMC–D and MMC were fitted simultaneously if possible.

TABLE II. Estimated Conversion Rate Constants of MMC and MMC-D in Various Phosphate Buffer Solutions[a]

Condition (μ=0.3)	Decomposition rate of MMC (k_2 : h^{-1})	Regeneration rate of MMC from MMC-D (k_1 : h^{-1})	Decomposition rate of MMC-D (k_3 : h^{-1})
pH=5.0, $[B]_t$=0.1[b]	0.0194	0.00724 (k_1+k_3)	
pH=7.4, $[B]_t$=0.1	0.00148	0.0295	0.00135
pH=8.0, $[B]_t$=0.1	0.00153	0.0743	0.00383
pH=9.0, $[B]_t$=0.1	0.00664	0.313	0.0831
pH=7.4, $[B]_t$=0.01	0.000907	0.0255	0.00178
pH=7.4, $[B]_t$=0.05	0.00116	0.0284	0.000449

a) Calculated by simultaneous fitting of the results to equations (1) and (2) (see text).
b) Total buffer concentration.

Table II summarizes the results of computer estimation. MMC–D was markedly stabler at pH 5.0 than MMC, which decomposed with a half-life of 4.7 h. In the pH region higher than 7.4 MMC–D was predominantly converted to MMC, and the conversion rate increased as the pH increased. At pH 9.0, MMC was regenerated from the conjugate with a half-life of 123 min.

The effect of buffer concentration on the decomposition kinetics of MMC–D and MMC is also summarized in Table II. The k_{obs} values at pH 7.4 could be expressed as $k_{obs}=2.3\times10^{-2}+8.4\times10^{-2}\times[B]_t$ (h^{-1}, $r=0.96$) for MMC–D and $k_{obs}=8.4\times10^{-4}+6.4\times10^{-4}\times[B]_t$ (h^{-1}, $r=0.99$) for MMC, where $[B]_t$ represents total buffer concentration (M).

Stability in Biological Media

Fig. 4 shows the decomposition of MMC and MMC–D in 5% homogenates of liver, spleen, kidney. Incubations were carried out under a continuous flow of N_2 gas. As reported previously,[8] MMC was degraded rapidly by tissue homogenate under anaerobic conditions. MMC–D decomposed more slowly than MMC in all biological media. Free MMC could not be detected in the medium containing MMC–D throughout the incubation.

The decompositions of MMC and MMC–D in liver homogenate and plasma were also examined under aerobic conditions (without N_2 gas). Both MMC and MMC–D were relatively stable, and about 85% of the activities remained after a 1 h incubation in every samples. The regeneration of MMC from MMC–D was observed in both liver homogenate and plasma but the amount of converted MMC was not significantly different from that obtained in the pH 7.4 buffer solution.

Antimicrobial Activity

Fig. 5 shows the time course of the growth of bacteria in a broth medium containing MMC or MMC–D at various concentrations. MMC completely inhibited the growth at a concentration of 5×10^{-1} μg/ml. MMC–D showed partial inhibition at 5 μg equivalent MMC/ml.

In Fig. 6(a), the extents of growth inhibition are compared (in terms of the ratio of turbidity of the test sample to that of the control at 9 h after the start of incubation). MMC and MMC–D showed almost parallel concentration-activity relationships and the activity of MMC

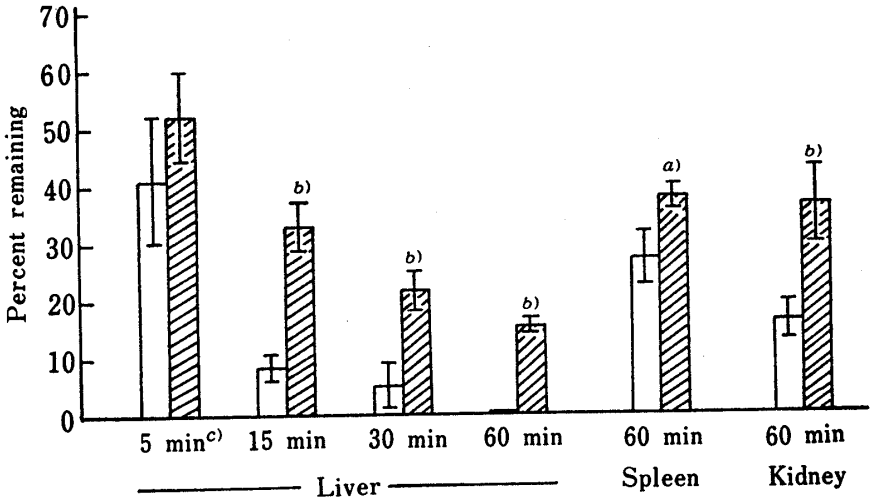


Fig. 4. Decomposition of MMC and MMC-D in 5% Homogenate Supernatant of Liver, Spleen, and Kidney during Incubation at 37°C under N_2 Gas Flow

Results are the means ± S.D. of three experiments.
a) $p<0.05$, *b*) $p<0.01$ (MMC *vs.* MMC-D). *c*) Incubation time.
□, MMC; ▨, MMC-D.

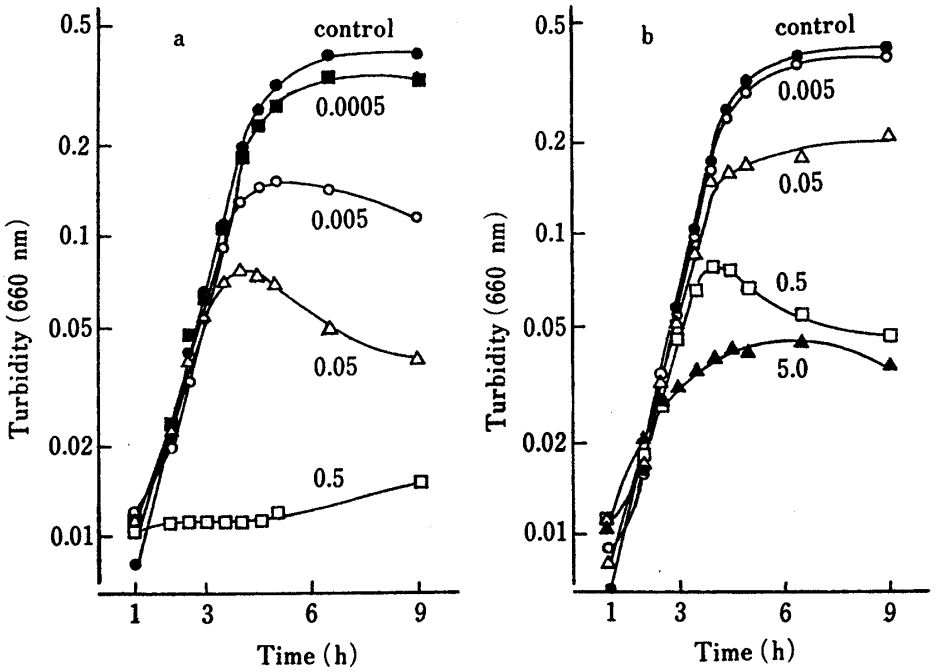


Fig. 5. Effects of MMC(a) and MMC-D(b) on the Growth of *Escherichia coli* B in the Broth Medium

Drugs were added to the broth medium to give various concentrations at time 0 and growth of bacteria was monitored spectrophotometrically. Each result is the mean of three tubes. Each number shows the final concentration of the drug in the medium (μg/ml).

was eighteen times that of the conjugated form.

Fig. 6(b) shows the antimicrobial activities of both compounds determined by the disc-plate method. The diameters of inhibitory zones are plotted against concentrations of test solution. MMC–D dissolved in pH 7.4 buffer and in saline afforded straight lines which were almost parallel to that of MMC. The activity of MMC–D dissolved in saline was slightly less than that of MMC–D dissolved in the buffer solution.

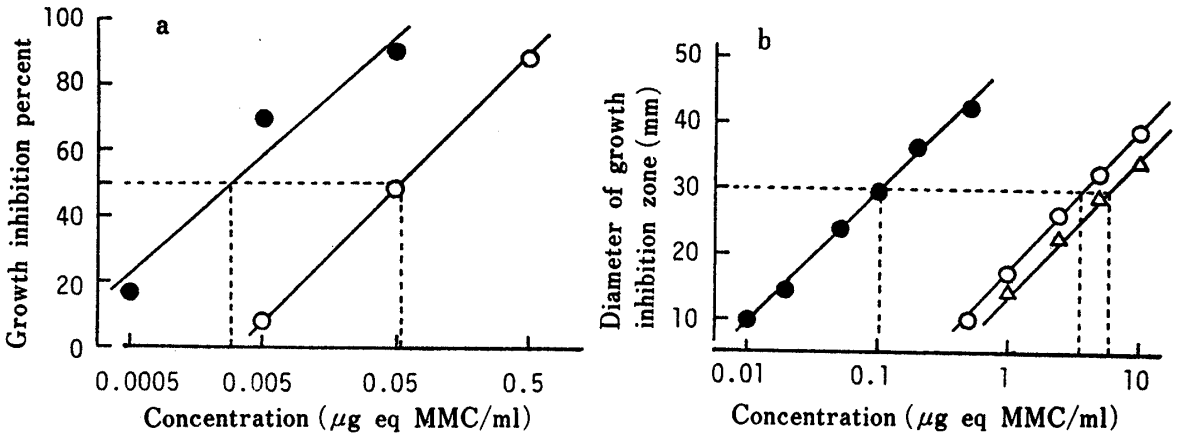


Fig. 6. Antimicrobial Activities of MMC and MMC-D against *Escherichia coli* B Evaluated by the Broth Dilution Method(a) and the Disc-plate Method(b)

a: ●, MMC; ○, MMC-D.
b: ●, MMC dissolved in pH 7.4 phosphate buffer; ○, MMC-D dissolved in pH 7.4 phosphate buffer; △, MMC-D dissolved in saline solution.

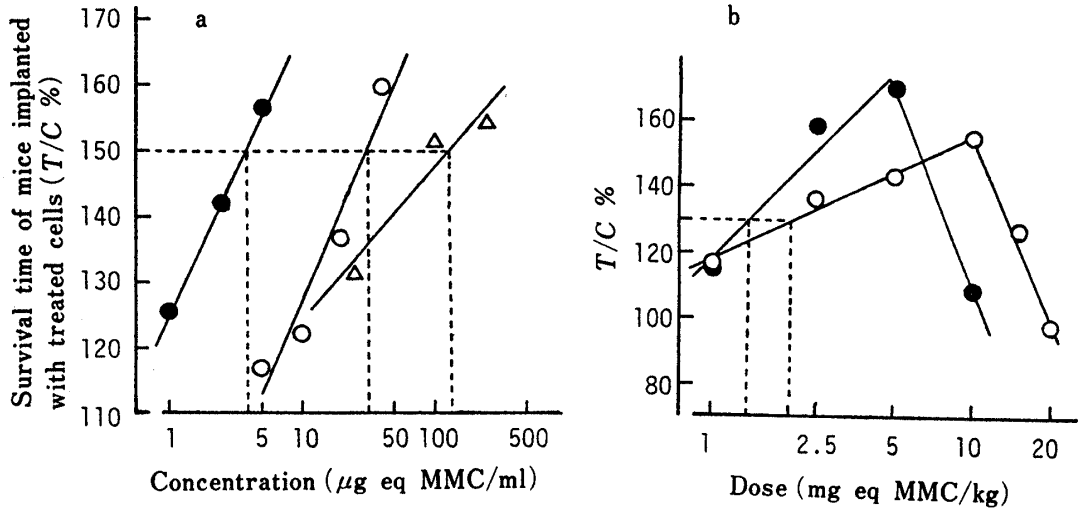


Fig. 7. Antitumor Activities of MMC and MMC-D against L1210 Leukemia Determined by the *in Vitro* Pretreatment Method(a) and the *in Vivo i.p.-i.p.* Method (b)

a: ●, MMC; ○, MMC-D; △, MMC-D with dialysis. Drugs were contacted with L1210 cells for 1 h at 37°C and 1×10^5 treated cells were inoculated into BDF_1 mice after being washed three times. Cytotoxicity was evaluated by comparing the survival time of mice inoculated with treated cells (*T*) with that of control mice given drug-untreated cells (*C*).
b: ●, MMC; MMC-D. Cells (1×10^6) were inoculated and chemotherapy was given at 24 h after inoculation (plotted using the data reported previously).[5]

Antitumor Activity

Fig. 7(a,b) shows the antitumor activities of MMC and MMC–D against L1210 leukemia. Leukemia cells were contacted with the drug in a test tube for 1 h at 37°C (a) or used *in vivo* by the *i.p.-i.p.* system (b).

Pretreatment of leukemia cells with MMC at concentration between 1 and 10 μg/ml caused remarkable prolongation of the survival time of mice inoculated with the treated cells. MMC–D showed somewhat less activity than MMC in this system. In the same experiment, incubation medium containing MMC–D was dialyzed against 500 ml of Hanks' solution during the incubation period in order to remove the liberated MMC from the medium. Dialysis decreased the cytocidal activity of MMC–D to one-fourth of the original level whereas the effect of MMC

was diminished to one-twentieth by the dialysis.

Therapeutic efficiencies of MMC and MMC–D on L1210 leukemia-bearing mice are also shown in Fig. 7(b) for comparison; these data were obtained previously.[5]

Discussion

Recently, the use of macromolecular carriers for the selective targeting of antitumor agents has been advocated with increasing frequency and had led to numerous reports on the conjugation of agents such as anthracyclines,[9-11] alkylating agents,[12] and methotrexate[13,14] with carriers such as DNA,[9] polypeptides[10,12,13] or polysaccharides.[11,14] However, relatively little attention has been paid to the nature of the linkage, the lability problem, and the biopharmaceutical properties of the conjugates.

In our series of investigations, we synthesized the conjugates of MMC with agarose beads,[15] dextran,[4,5,16] and polypeptides,[16] and obtained enhanced activities. In the present work, we examined the regeneration properties and biological activities of MMC–D in order to cast light on the mode of action of this compound.

In the synthesis of MMC–D, MMC was coupled to the carboxyl group of the spacer by carbodiimide-catalyzed condensation. Among four MMC analogues, those without 1a-*N*-substitution could be derivatized to polymeric forms. Furthermore, our previous study revealed that carboxylic acids such as benzoic acid and phenylacetic acid could be conjugated with MMC through an amide linkage at 1a-*N* position.[17] The kinetic data shown in Fig. 3 and Table II show good correspondence with those for phenylacetyl MMC. These results support the linkage structure shown in Fig. 2. Concerning the linkage between spacer and dextran, three structures can be considered.[18] Although pH titration of spacer-introduced dextran showed a cationic charge, probably due to *N*-substituted imidocarbonate or isourea, the details remain obscure. The direct coupling of MMC to the BrCN-activated dextran could not be disregarded, but the MMC content and release characteristics of MMC–D are obviously different from those of the direct conjugate with polysaccharide reported previously.[15]

The kinetic results proved that MMC–D was stabler than MMC against acid-catalyzed or enzymatic degradation and this property in itself should be beneficial for therapeutic efficiency. As shown in Fig. 4, the regeneration of MMC–D to MMC was not accelerated by liver homogenate, while phenylacetyl MMC was converted rapidly under the same conditions.[17] Steric hindrance due to the dextran chain is presumably responsible for this. Consequently the stability characteristics of MMC–D are rather different from those of MMC and its low molecular weight 1a-*N*-acylated derivatives.

TABLE III. Comparison of Biological Activities of MMC and MMC-D

Evaluation method	Concentration or dose of MMC[g]		Activity ratio (MMC/MMC-D)
	Free MMC	MMC-D	
Antimicrobial activity[a]			
Broth dilution method[b]	0.00284	0.0535	18.84
Disc-plate method[c]	0.0986	3.50 (4.96)	35.5 (50.3)
Antitumor activity[d]			
In vitro pretreatment method[e]	3.59	28.99 (122.8)	8.07 (34.2)
In vivo i.p.-i.p. method[f]	1.43	2.18	1.52

a) Antimicrobial activity against *Escherichia coli* B.
b) Concentration (μg MMC/ml) showing 50% inhibition in the broth dilution method (Fig. 6,b).
c) Concentration (μg MMC/ml) giving an inhibitory-zone diameter of 30 mm in the disc-plate method (Fig. 6,b). Parentheses show the value for MMC-D dissolved in saline solution.
d) Antitumor activity against L1210 leukemia.
e) Concentration (μg MMC/ml) showing 50% prolongation of survival time of mice inoculated with treated cells as compared with mice given untreated cells (Fig. 7,a). Parentheses show the value for MMC-D in the case of incubation with dialysis (see text).
f) Dose (mg MMC/kg) giving 30% increase of survival time of mice (Fig. 7,b).
g) Each value was calculated from the regression line obtained by the least-squares method.

In the present study, the pharmacological activities of MMC–D were examined by four methods. Table III summarizes the results; activities are expressed as the concentration or dose necessary to obtain standard efficiency in each system. MMC–D showed less activity against bacteria and tumor cells *in vitro*, but had considerable activity against L1210 leukemia *in vivo*. The 1a-position of MMC appears to be the alkylating site, and its alkylating ability is enhanced when methanol is eliminated from reduced hydroquinone to give the indolohydroquinone.[1] Thus, it is suggested that MMC acted following cleavage from the dextran polymer in the body, but not in the intracellular space.

In the case of polymeric anthracyclines[9–11] and methotrexate,[13] which gave the most successful results among numerous attempts to develop polymeric antineoplastics, drugs are considered to enter the tumor cells in combination with the carrier by endocytosis and to be cleaved to free forms by lysosomal enzymes ("lysosomotropic agent").[19] However, MMC–D showed different properties, as discussed above, *i.e.*, 1) slight cytotoxicity in itself, 2) spontaneous chemical liberation of MMC, 3) no acceleration of regeneration by tissue homogenate, 4) direct inactivation by tissue homogenate. Thus MMC–D acts in a free form following chemical cleavage of MMC from the dextran in the body, including central circulation, body cavities such as the peritoneal cavity, or at the target cell surface, but not in the intracellular lysosomes, and it belongs to another category of polymeric drugs different from lysosomotropic agents.

MMC–D is expected to show activity regardless of animal species, since it is regenerated to the parent drug without any enzyme-mediated process. The activity of a lysosomotropic agent, on the other hand, should depend on the activity of lysosomal enzymes. Thus, MMC–D can be considered to be promising for the treatment of neoplasia especially by local administration. The approach of combining MMC–D with special carriers such as microspheres might offer further advantages.[3,20]

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