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Research Article

Disposition and Tumor Localization of Mitomycin C–Dextran Conjugates in Mice

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Mitomycin C–Dextran conjugates (MMC-D) were intravenously (iv) injected in mice bearing subcutaneous sarcoma 180. The tissue distribution was determined for three ^{14}C-labeled anionic conjugates (MMC-Dan) with molecular weights of 10, 70, and 500 kd and one cationic 70-kd ^{14}C-conjugate (MMC-Dcat). The anionic conjugates were slowly cleared from the plasma, and their elimination rate decreased with increasing molecular weight. Radioactivity accumulated in liver, spleen, lymph nodes, and tumor but not in heart, lung, intestines, kidney, or muscle after iv injection of all types of ^{14}C-MMC-Dan. In contrast, the cationic conjugate was rapidly cleared from the plasma and accumulated mostly in the liver and spleen, while tumor levels remained low. The antitumor effect of the 70-kd MMC-Dan, which afforded the highest tumor concentration, was superior to that of free MMC. Therefore, anionic mitomycin C–dextran conjugates with a high molecular weight may be useful for tumor targeting in cancer chemotherapy.

KEY WORDS: mitomycin C–dextran conjugate; polymeric prodrug; tissue distribution; tumor localization; physicochemical characteristics; antitumor activity.

INTRODUCTION

Recently, various macromolecular drug carrier systems, involving tumor-specific and -nonspecific carriers, have been developed for targeted chemotherapy (1,2). Tumor-specific carriers such as monoclonal antibodies that recognize tumor-associated cell surface antigens seem to be especially promising, and various conjugates with anticancer drugs are currently being investigated. These approaches, however, do not always produce the expected results *in vivo*, because there is little information on the disposition characteristics of the conjugates (3–5). The *in vivo* disposition of macromolecule–drug conjugates, even tumor-specific ones, is basically controlled by a passive mechanism, relying on the physicochemical interactions with the body. Therefore, tumor targeting could be achieved primarily by controlling the physicochemical characteristics of the conjugates to show the appropriate pharmacokinetic behavior regardless of the affinity to the tumor cells.

Previously, we developed polymeric prodrugs of mitomycin C (MMC), mitomycin C–dextran conjugates with a cationic charge (MMC-Dcat), and one with an anionic charge (MMC-Dan) (6–18). The disposition and pharmacokinetics of MMC-D were investigated systematically in normal rats after intravenous (iv) (10,14,18) and intramuscular (11,16,18) injection. Kinetic analysis revealed that MMC-D acts as a reservoir of MMC that behaves characteristically as a macromolecule supplying active MMC in the body (10,16). In local injections, MMC-Dcat showed sustained retention at the injection site and enhanced lymphatic delivery (11,16). A remarkable reduction in tumor size was obtained in a clinical trial involving intratumoral administration (13).

In order to clarify the effect of physicochemical properties of the conjugates such as electric charge and molecular weight on their disposition and to assess the feasibility of using a tumor-nonspecific macromolecule–drug conjugate for targeted cancer chemotherapy, the disposition of MMC-Dan and MMC-Dcat after iv injection was studied in tumor-bearing mice. Unchanged dextran and human serum albumin (HSA) were also tested for comparison.

MATERIALS AND METHODS

Chemicals. MMC was kindly supplied by Kyowa Hakko Kogyo Co., Tokyo. Dextrans with various molecular weights were purchased from Pharmacia, Uppsala, Sweden, and had average molecular weights of about 10 kd (T-10), 70 kd (T-70), and 500 kd (T-500). [$^{14}C(U)$]γ-Aminobutyric acid (2 mCi/mg) and [*carboxyl*-^{14}C]dextran (1 μCi/mg; M_n, 70 kd) were obtained commercially from New England Nuclear, Boston. ^{131}I-labeled human serum albumin (HSA; 100 μCi/mg) was purchased from Daiichi Radioisotopes, Tokyo. All other chemicals were commercial reagent-grade products. MMC-Dan and MMC-Dcat were synthesized as reported previously (6,17). For the synthesis of MMC-Dan, 6-bromohexanoic acid was introduced to dextran as a spacer, and then MMC was coupled by the carbodiimide-catalyzed reac-

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tion. Since not all spacer arms were used for coupling of MMC, the remaining free carboxyl groups give anionic charges in MMC-Dan. For the synthesis of MMC-Dcat, dextran was activated by cyanogen bromide, a spacer, ε-aminocaproic acid, was introduced, and then MMC was coupled as described above. Three structures have been proposed for the linkage between the spacer and the dextran (19), and two of them can give a cationic charge in MMC-Dcat. All conjugates were estimated to contain MMC to almost equal extents of about 8% for MMC-Dan and 10% (w/w) for MMC-Dcat, respectively. Radiolabeled MMC-Dan was synthesized by coupling γ-aminobutyric acid (^{14}C) together with MMC at a molar ratio of 1:2000, and the purified radiolabeled MMC-Dan has a specific activity of 0.02 μCi/mg. Radiolabeled MMC-Dcat was synthesized by coupling γ-aminobutyric acid (^{14}C) together with ε-aminocaproic acid at a molar ratio of 1:1000, and the purified radiolabeled MMC-Dan has a specific activity of 0.05 μCi/mg. MMC was dissolved in saline at a concentration of 0.5 mg/ml for injection. Test macromolecular compounds were prepared in a saline solution at a concentration of 10 mg/ml as macromolecules.

Animals and Tumors. Male ddY mice were obtained from the Shizuoka Agricultural Co-operative Association for Laboratory Animals, Shizuoka. Sarcoma 180 (S180) was maintained in ddY mice by weekly intraperitoneal (ip) transfer of 10^8 cells obtained from ascitic fluid.

Procedure for the Animal Experiment. One-tenth milliliter of ascitic fluid containing 10^7 S180 cells was inoculated subcutaneously into ddY mice, each weighing 22–28 g. At 12 days after inoculation, tumor-bearing mice were injected with saline solutions of MMC or test macromolecular compounds into the tail vein at a dose of 5 or 100 mg/kg (12.5 mg MMC equivalent/kg in the case of MMC-Dan and 10 mg MMC equivalent/kg in the case of MMC-Dcat), respectively, and then housed in metabolic cages for collecting urine samples. At 1, 8, and 24 hr after the injection, blood was collected from the vena cava and the mice were killed. Organs, such as heart, lung, liver, spleen, kidney, intestines, muscle, lymph nodes, and tumor, were excised, weighed, and subjected to the assay.

Analytical Method. The tissue concentration of MMC was determined by microbiological assay using *Escherichia coli* B as a test organism (20). The excised tissues were homogenized in saline and centrifuged at 3000 rpm. The supernatants were subjected to assay. Blood was centrifuged at 3000 rpm to obtain a plasma sample. Urine was assayed without any special procedure. The procedure for the determination of ^{14}C and radioactivity was a modification of the method of Mahin and Loftberg (21). Weighed tissues were solubilized in 1 *N* NaOH solution by shaking overnight at 37°C and diluted to an appropriate volume with the same medium. Then, 0.2 ml of sample solution was put into a counting vial, and equal volumes of perchloric acid (60%) and hydrogen peroxide (35%) were added. The resulting mixture was heated at 70°C for 90 min with agitation. After cooling to room temperature, 10 ml of scintillation medium (Univer-gel, Nakarai Chemicals, Kyoto) was added and the radioactivity was determined in a liquid scintillation system. The lymph nodes, plasma, and urine were assayed without the solubilization with NaOH. The ^{131}I radioactivity in the tissue was determined with a well NaI-scintillation counter without any special procedure.

Pharmacokinetic Analysis. The change in the amount of drug in a tissue with time can be described as follows:

$$dT(t)dt = CL_{in} \times C(t) - K_{out} \times T(t) \quad (1)$$

where $T(t)$ is the amount of drug in the tissue, $C(t)$ is the plasma concentration, CL_{in} is the uptake (influx) clearance from the plasma to the tissue, and K_{out} is the efflux rate constant. The efflux is assumed to be negligible at early time points, and Eq. (1) integrates to

$$CL_{in} = T(t_1)/\int_0^{t_1} C(t_1)dt = T(t_1)/AUC_{0-t_1} \quad (2)$$

According to Eq. (2), the influx clearance can be calculated using the amount of drug in the tissue and the area under the plasma concentration–time curve (AUC). The values at 24 hr after injection were used for the calculation of the influx rate clearances of MMC or macromolecular compounds.

Inhibitory Effect on the Growth of S180. S180 cells (1×10^8) were inoculated into the subcutaneous tissue of the axillary region of ddY mice. Four days after inoculation, the saline solution of the test compound was intravenously injected into the tumor-bearing mice. The tumor size was measured with calipers, and the ratio of the tumor volume at each measurement time to the volume at 4 days (initial volume) was calculated by the following equation:

$$V = (a \times b^2)/2 \quad (3)$$

where a is the length (mm), b is the width (mm), and V is the volume (mm^3) of the tumor.

RESULTS

Tissue Distribution

After intravenous injection, free MMC was rapidly cleared from plasma following first-order kinetics, with a half-life of 10 min (data not shown). No biological activity was detected in any tissue 1 hr after injection.

On the other hand, MMC-D in the tissue was detected by radioactivity counting. Since less than 3% of the ^{14}C radioactivity was liberated from MMC-Dan and MMC-Dcat during a 48-hr incubation in tissue homogenates of liver and plasma, the ^{14}C label in MMC-D was considered to be stable and show the pharmacokinetic behavior of the carrier dextran. Figures 1, 2, and 3 show the tissue distribution of radioactivity after iv injection of ^{14}C-MMC-Dan (T-10), ^{14}C-MMC-Dan (T-70), and ^{14}C-MMC-Dan (T-500), respectively. Plasma clearances of MMC-Dan were slow and dependent on their molecular weights. Radioactivity gradually accumulated in the reticuloendothelial organs such as the liver, spleen, and lymph nodes and the tumor tissue but not in the heart, lung, kidney, intestines, or muscle.

On the contrary, ^{14}C-MMC-Dcat (T-70) was rapidly cleared from plasma and a marked accumulation of radioactivity was observed even 1 hr after injection in liver, spleen, and lymph nodes, while the tumor level was low, as shown in Fig. 4. Figure 5 shows the tissue distribution of neutral ^{14}C-dextran with a molecular weight of 70 kg after iv injection. It was eliminated relatively rapidly from the circula-

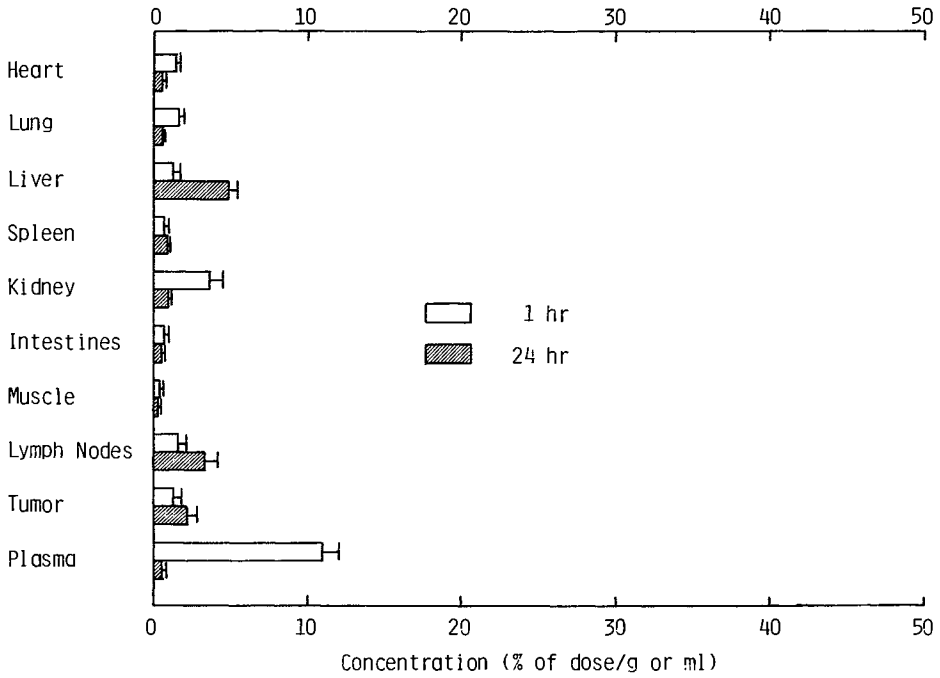

Fig. 1. Tissue distribution of ^{14}C-MMC-Dan (T-10) after iv injection to mice bearing S180 subcutaneously. Results are expressed as the mean ± SD of at least four mice.

tion and accumulated in liver, lymph node, and tumor. As shown in Fig. 6, ^{131}I-HSA showed a consistent plasma concentration, and the radioactivity accumulated only in the tumor.

Urinary Excretion

Table I summarizes the cumulative amount excreted in urine up to 24 hr after iv injection of MMC, the three ^{14}C-

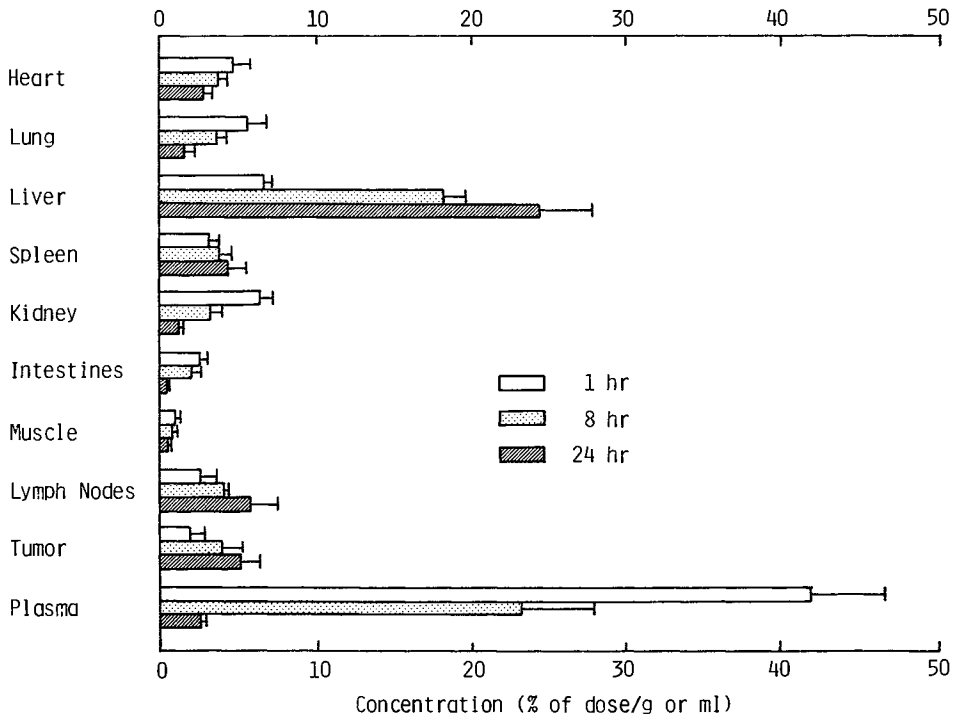

Fig. 2. Tissue distribution of ^{14}C-MMC-Dan (T-70) after iv injection to mice bearing S180 subcutaneously. Results are expressed as the mean ± SD of at least four mice.

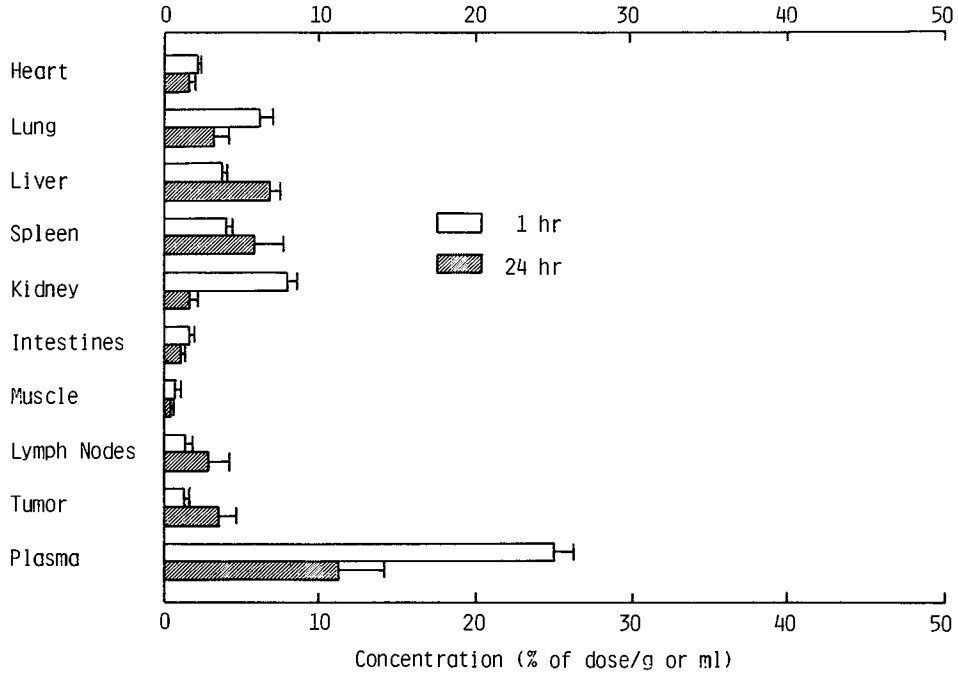

Fig. 3. Tissue distribution of ^{14}C-MMC-Dan (T-500) after iv injection to mice bearing S180 subcutaneously. Results are expressed as the mean ± SD of at least four mice.

MMC-Dan, ^{14}C-MMC-Dcat (T-70), ^{14}C-dextran, and ^{131}I-HSA. Approximately 30% of the dose was recovered in the urine after the injection of free MMC. Urinary recovery was increased with the decrease in molecular weight of ^{14}C-MMC-Dan. In the case of ^{14}C-MMC-Dan (T-70), the recovery percentage was higher than that of ^{14}C-MMC-Dcat (T-70) and lower than that of neutral ^{14}C-dextran with the same molecular weight. The recovery of ^{131}I-HSA was almost the same as that of ^{14}C-MMC-Dan (T-70).

Inhibitory Effect on the Growth of S180

Figure 7 shows the growth inhibition curve of saline (A), free MMC (B), and MMC-Dan (T-70) (C) against S180

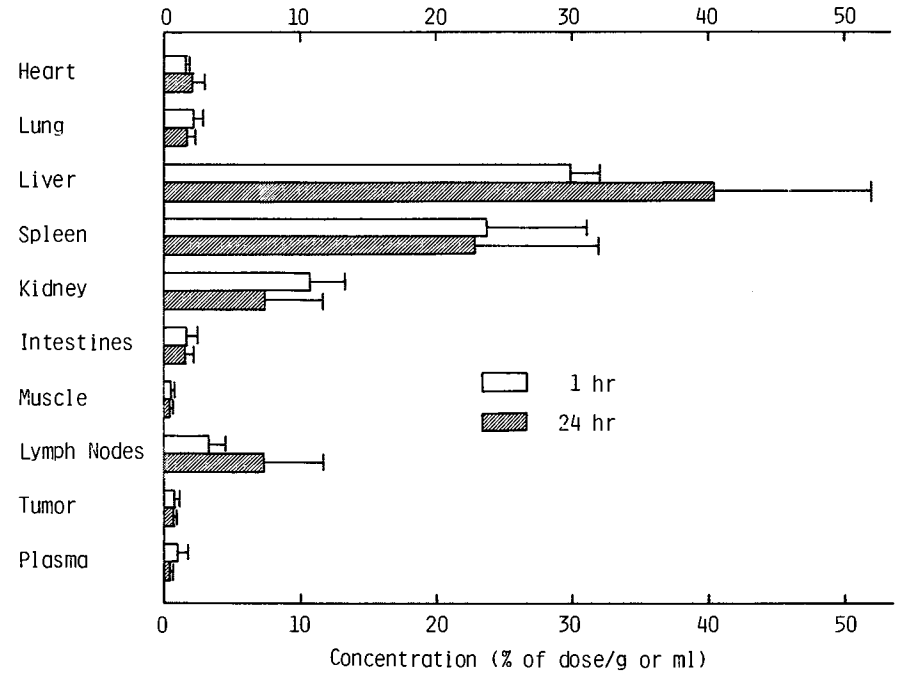

Fig. 4. Tissue distribution of ^{14}C-MMC-Dcat (T-70) after iv injection to mice bearing S180 subcutaneously. Results are expressed as the mean ± SD of at least four mice.

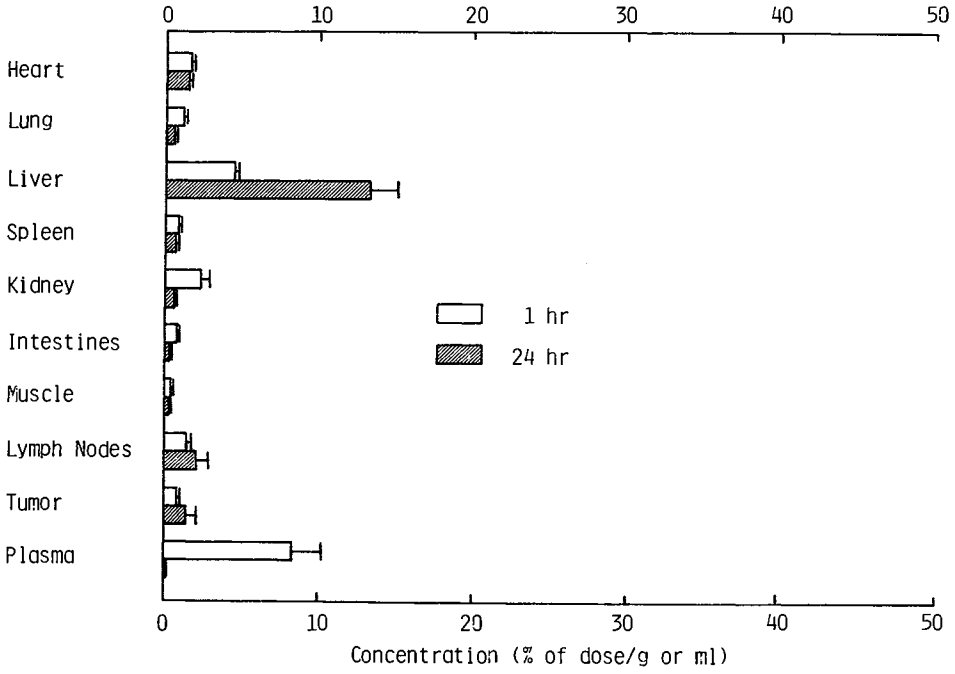

Fig. 5. Tissue distribution of ^{14}C-dextran (M_r, 70 kd) after iv injection to mice bearing S180 subcutaneously. Results are expressed as the mean ± SD of at least four mice.

inoculated subcutaneously. Each curve represents the ratio of tumor volume in a different mouse. The doses of free MMC and MMC-Dan (T-70) were 5 mg/kg and 30 mg MMC equivalent/kg, respectively, which are the maximum tolerable doses for mice (approximately three-fourths of the LD_{50}). In the control group, remarkable tumor growth was observed, and the ratio of the tumor volume at 30 days to the initial volume was more than 10. A single iv injection of free MMC suppressed the tumor growth in three of six mice, while an apparent increase in tumor volume was observed in three mice. On the other hand, marked growth inhibition was obtained with a single dose of MMC-Dan (T-70), and the ratio of tumor volume was less than 1.0 at 30 days after inoculation for all mice. The survival of the mice also confirmed

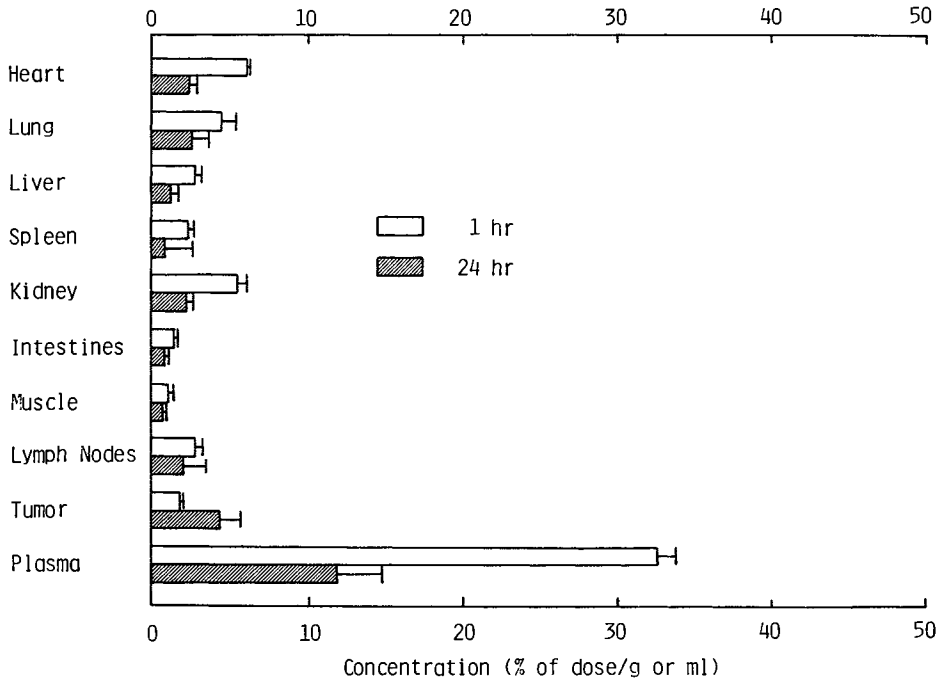

Fig. 6. Tissue distribution of ^{131}I-HSA after iv injection to mice bearing S180 subcutaneously. Results are expressed as the mean ± SD of at least four mice.

Table I. Urinary Recovery of Radioactivity After iv Injection of Three Types of ^{14}C-MMC-Dan, ^{14}C-MMC-Dcat (T-70), ^{14}C-Dextran, ^{131}I-HSA, and MMC to Mice Bearing S180

Compound	% of dose recovered in urine (24 hr)
^{14}C-MMC-Dan (T-10)	85.71 ± 2.58 (4)[a]
^{14}C-MMC-Dan (T-70)	25.69 ± 3.00 (3)
^{14}C-MMC-Dan (T-500)	18.05 ± 2.97 (5)
^{14}C-MMC-Dcat (T-70)	17.54 ± 1.07 (5)
^{14}C-Dextran	45.43 ± 13.15 (4)
^{131}I-HSA	25.08 ± 4.01 (5)
MMC	10.87 ± 4.17 (3)

[a] Results are expressed as the mean ± SD, with the number of mice in parentheses.

the effectiveness of MMC-Dan (T-70). Within 90 days after inoculation, all mice in the control group and four of the six mice in the free MMC-treated group died. On the other hand, five of the seven mice given MMC-Dan (T-70) treatment survived for more than 90 days.

DISCUSSION

MMC is an antitumor antibiotic that has antitumor activity against a number of human neoplasms (22). Plasma clearance of MMC is very rapid, and metabolism is a major route of the elimination (23). Therefore, radioisotope tracer experiments cannot serve to study free MMC. The tissue distribution of MMC was determined in guinea pigs at a dose of 5 mg/kg using the microbial assay reported by Fujita (20). Biological activity was detected in muscle, heart, and lung but not in liver and spleen even at 5 minutes after injection. These reports correspond well to the fact that MMC could not be detected in any tissues we tested.

The fate of carrier dextran in MMC-D was traced by radioactivity counting. MMC is released from MMC-D by hydrolysis with half-lives of 24–35 hr in the body (10). Since MMC is more resistant to enzymatic metabolism in the conjugated form than in the free form (9), the radioactivity can be considered as an indication of the dextran–drug conjugate. The present study demonstrated that ^{14}C-MMC-Dan was slowly cleared from plasma and gradually accumulated in liver, spleen, lymph nodes, and tumor. In contrast, ^{14}C-MMC-Dcat (T-70) was rapidly removed from the circulation by reticuloendothelial organs such as liver, spleen, and lymph nodes. Similar distribution patterns were observed for ^{14}C-MMC-Dan and ^{14}C-MMC-Dcat in normal rats (10,18). Unchanged dextran showed a behavior similar to that of MMC-Dan, but its plasma clearance was more rapid than that of MMC-Dan (T-70), which had the same molecular weight. Restricted urinary excretion of MMC-Dan should account for this difference (24). The clearance of HSA seemed to be faster than that of a homogeneous albumin (25) but almost similar to that reported for a heterogeneous albumin (26).

To evaluate quantitatively the tissue distribution observed in this study, tissue uptake rates were calculated in terms of clearance by dividing the tissue level by the area under the plasma concentration–time curve (AUC). Table II shows the parameters for the uptake of ^{14}C-MMC-D by liver, spleen, tumor, kidney, and urine, together with those of ^{14}C-dextran, ^{131}I-HSA, and MMC. Parameters for ^{14}C-MMC-Dcat with molecular weights of 10, 70, and 500 kd were also calculated from our previous data obtained in normal rats

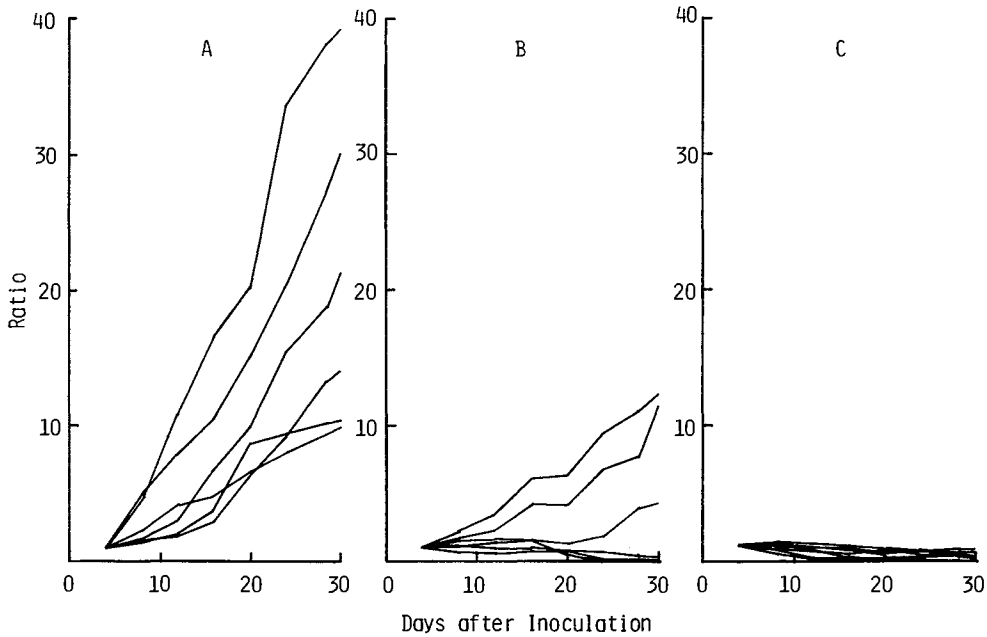

Fig. 7. Inhibitory effect of free MMC and MMC-Dan (T-70) on the growth of sarcoma 180 implanted subcutaneously. Each curve represents the ratio of tumor volume in a seprate mouse. (A) Control; (B) free MMC (5 mg/kg); (C) MMC-Dan (T-70) (30 mg MMC equivalent/kg). Mice were inoculated with S180 cells (1×10^8) subcutaneously. At 4 days after inoculation, a saline solution of test substances was injected intravenously. Tumor size was measured periodically and the ratio of the tumor volume to that at 4 days after inoculation in each mouse was calculated.

Table II. AUC and Tissue Uptake Rates for ^{14}C-MMC-Dan, ^{14}C-MMC-Dcat, ^{14}C-Dextran, ^{131}I-HSA, and MMC in Tumor-Bearing Mice and Normal Rats

Compound	AUC[a]	CL_{liver}[b]	CL_{spleen}[b]	CL_{tumor}[b]	CL_{kidney}[b]	CL_{urine}[c]
MMC-Dan (T-10)[d]	94.1	47.6	7.8	21.2	10.1	910
MMC-Dan (T-70)[d]	408	54.8	10.2	13.2	2.8	62.9
MMC-Dan (T-500)[d]	422	17.1	13.6	8.1	3.7	42.7
MMC-Dcat (T-70)[d]	8.1	3460	2190	26.6	532	2167
Dextran[d]	43.4	306	16.0	33.1	14.3	1045
HSA[d]	479	2.5	1.8	9.4	5.3	52.3
MMC[d]	0.8	0	0	0	0	13600
MMC-Dcat (T-10)[e]	3.3	1727	646	—	2897	8463
MMC-Dcat (T-70)[e]	2.7	1805	660	—	1438	1435
MMC-Dcat (T-500)[e]	2.5	2003	526	—	243	936

[a] Area under the plasma concentration–time curve (% of dose · hr/ml).
[b] Uptake rate expressed in terms of clearance (μl/g/hr).
[c] Clearance of urinary excretion (μl/hr).
[d] Values were calculated from the data 24 hr after injection in tumor-bearing mice.
[e] Values were calculated from the data 8 hr after injection in normal rats.

(10), for comparison. Clearance values of MMC-Dan by both liver and spleen were almost the same, regardless of its molecular weight. The clearance of MMC-Dcat (T-70) by liver and spleen was two orders of magnitude higher than that of MMC-Dan, which suggests that the electrical charge plays an important role in the uptake by reticuloendothelial systems. Neutral dextran had a clearance value intermediate between that of MMC-Dcat and that of MMC-Dan. The low values of ^{131}I-HSA suggest metabolic degradation in the organ, followed by reflux into the blood circulation and urinary excretion of degradation products.

Our *in vitro* study revealed that MMC-Dcat was adsorbed on rat liver parenchymal and nonparenchymal cells (unpublished data). This interaction based on the electrostatic force may account for the specific *in vivo* distribution of MMC-Dcat to liver and spleen. MMC-Dcat in the blood would easily make contact with the parenchymal cells because these reticuloendothelial organs have discontinuous capillaries.

Remarkable effects of electrical charge and molecular weight were observed in the kidney uptake and urinary clearance. Urinary clearance of MMC-Dcat (T-70) was much higher than that of MMC-Dan (T-70), and that of unchanged dextran was intermediate. On the other hand, the values increased with increasing molecular weights in both series of MMC-Dan (mice) and MMC-Dcat (rats). These results conform well to the fact that the glomerular capillary wall functions as a sieving filter with a negative charge to macromolecules (24).

Tumor tissues are known to have a capillary with enhanced permeability and to lack lymphatics. Macromolecules such as albumin (27) and globulin (28) are reported to be accumulated in the tumor tissues. Corresponding to these findings, all types of macromolecules showed accumulation in the tumor tissue in the present study. Macromolecules with large AUC such as large MMC-Dan and HSA have a high tumor concentration. However, tumor clearance of the three types of MMC-Dan, MMC-Dcat, unchanged dextran, and HSA are similar (Table II), suggesting that the rates of leakage of macromolecules through the capillary wall of S180 tumor are roughly the same in spite of the difference in their physicochemical properties. Accordingly, the residence time in the blood circulation greatly influenced the tumor level of macromolecular prodrugs. MMC-Dcat, which has a small AUC because it is rapidly removed by liver and spleen, did not localize in the tumor. In the present study, MMC-Dan (T-70) afforded the highest tumor level among the macromolecular prodrugs of MMC tested. Therefore, this compound was selected for the antitumor activity study.

MMC-D is inert in itself but supplies active MMC by chemical hydrolysis with half-lives of 24 hr (MMC-Dcat) and 35 hr (MMC-Dan) under physiological conditions (pH 7.4, 37°C), and enzymes do not participate in the conversion (10,18). In general, polymeric antitumor drugs are considered to enter the tumor cells by endocytosis and to be cleaved to free forms by lysosomal enzymes (lysosomotropic agents) (29,30). In contrast to this mechanism, MMC-D can supply active MMC not only in the tumor cells but also in the extracellular space of the tumor mass because the conversion process proceeds only by chemical hydrolysis (15). Remarkable accumulation of radioactivity in the tumor tissue observed after iv injection of ^{14}C-MMC-Dan (T-70) should result in a gradual supply of active MMC and a superior therapeutic effect.

The toxicity of free MMC was high (LD_{50} = 7.5 mg/kg, ip) in spite of its rapid clearance. On the other hand, the LD_{50} of MMC-Dan (T-70) was about six times higher (LD_{50} = 45 mg/kg, ip) than that of free MMC because the rate of active MMC supply was very low. Therefore, the growth-inhibitory effect of MMC-Dan (T-70) and that of free MMC were compared at an equivalent toxic dose. At these doses, the growth-inhibitory effect of MMC-Dan (T-70) against the subcutaneous tumor was superior to that of free MMC. The cytotoxicity of MMC was reported to be in proportion to the given exposure dose (product of the concentration multiplied by the contact time) (31). Furthermore, tumor cells are more sensitive to a relatively low concentration of MMC under hypoxic conditions, e.g., tumor cells in a solid tumor (32). These findings should account for the excellent efficacy of MMC-Dan (T-70) compared with free MMC. A consistent

supply of free MMC from the conjugate in the central circulation may partially contribute to the growth inhibition of the tumor implanted subcutaneously.

In conclusion, MMC-Dan (T-70) is suitable for tumor targeting since it has a long circulating life and accumulates in the tumor according to a passive mechanism based on the physiological and anatomical characteristics of the solid tumor. On the other hand, MMC-Dcat is cleared rapidly by the reticuloendothelial systems and fails to supply a sufficient amount of the drug to the tumor tissue in systemic administration. However, MMC-Dcat is considered to be suitable for local injection since it remains at the injection site and had lymphotropicity (11,16). The present study thus suggests that even a nonspecific macromolecular carrier system can be useful in targeting antitumor drugs.

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