URINARY EXCRETION CHARACTERISTICS OF A POLYMERIC PRO- DRUG OF MITOMYCIN C, MITOMYCIN C-DEXTRAN CONJUGATE

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Urinary excretion characteristics of a polymeric prodrug of mitomycin C (MMC), mitomycin C-dextran conjugate (MMC-D), following intravenous administration was studied in rats. Three types of MMC-D, conjugates with dextrans of molecular weights of 10000, 70000 and 500000, were tested and urine concentration of MMC, dextran and spacer were determined by three analytical methods, *i.e.*, bioassay, anthrone method and radioactivity counting. MMC was assayed separately as a free form and conjugated form based on antimicrobiological activity. MMC administered as a free form was excreted rapidly into urine but only a small amount of MMC was excreted following the administration of MMC-D. The excreted amount of MMC in a conjugated form varied with the size of carrier dextran while similar sustained excretion was observed regardless of the carrier size. The excretion of carrier dextran determined by anthrone method was confined as the molecular weight was increased. The effect of molecular weight was also observed in the case of spacer-introduced dextran (dextran-C₆ spacer) and original dextran. Compared with neutral dextran, cationic MMC-D and anionic dextran-C₆ spacer exhibited diminished excretion, indicating the effect of charge on urinary excretion. The urinary recovery of radioactivity was almost in accordance with that of carrier dextran. However, the urinary recovery of MMC based on biological activity was considerably lower than that of carrier dextran. It was suggested that MMC-D underwent inactivation to a great extent before releasing active MMC in the body. The effect of physicochemical properties such as molecular weight and electric charge on the urinary excretion of the polymeric prodrug was thus elucidated.

Keywords —mitomycin C; dextran; mitomycin C-dextran conjugate; urinary excretion; physicochemical property; molecular weight; electric charge; bioassay; anthrone method; radioactivity counting

INTRODUCTION

In cancer chemotherapy, an application of antitumor agents is often limited on account of their indiscriminate effect on both tumor and normal cells. Therefore an approach to modify their biological properties for concentrating their cytotoxicity at the target cells would be a promising one. Various kinds of carrier systems have been recently developed in an attempt to deliver antitumor agents into tumor site.¹⁾ Especially, macromolecular compounds such as proteins,²⁾ polysaccharides,³⁾ and synthetic polymers,⁴⁾

including biospecific ligands like antibodies⁵⁾ and lectins,⁶⁾ have been widely utilized as carriers which can control the pharmacokinetic behavior of parent drugs. In spite of numerous reports on the macromolecular derivatives of antitumor agents, however, little is known about pharmacokinetic fate of these conjugates.

In previous studies, we have developed a polymeric prodrug of mitomycin C (MMC), mitomycin C-dextran conjugate (MMC-D), by coupling MMC to dextran employing ϵ -aminocaproic acid as a spacer.⁷⁾ MMC-D showed

increased activities against various murine tumors⁸⁾ and a size of carrier dextran was proved to affect their efficiency.⁹⁾ *In vitro* stability experiment revealed that MMC-D was converted to MMC by base-catalyzed hydrolysis but the conversion was not accelerated by the addition of tissue homogenate.¹⁰⁾ In local injection, MMC-D exhibited slow elimination from the injection site and remarkable accumulation in the lymph node, suggesting its own usefulness for a direct intratumoral injection and prevention of the lymph node metastasis.¹¹⁾

In the present study, we investigated the urinary excretion characteristics of MMC-D with various molecular sizes in rats after intravenous injection, in order to clarify the *in vivo* fate of MMC-D in detail and evaluate the possible effect of MMC-D on the kidney.

MATERIALS AND METHODS

Material—MMC was kindly supplied by Kyowa Hakko Kogyo Co. Dextran of various molecular weights were purchased from Pharmacia Fine Chemicals Co., Sweden and had average molecular weights of about 10000 (T-10), 70000 (T-70) and 500000 (T-500). Radiolabeled γ -aminobutyric acid [¹⁴C(U)] was purchased from New England Nuclear, Mass. with a specific radioactivity of 2 mCi/mg. All other chemicals were reagent grade products obtained commercially.

Preparation of MMC-D—MMC-D was synthesized as reported previously.⁷⁾ In brief, dextran was activated with cyanogen bromide at pH 10.7 and ϵ -aminocaproic acid was coupled covalently to a glucose chain as a spacer. The product, spacer introduced dextran (dextran-C₆ spacer), was washed with water by ultrafiltration and precipitated by acetone. MMC was conjugated to the dextran-C₆ spacer by a carbodiimide-catalyzed reaction. The conjugate was purified by the same manner as dextran-C₆ spacer. All MMC-Ds were estimated to contain MMC to an equal extent of about 10% (w/w) and a degree of substitution of dextran was one molecule per 14–17 glucose units. Radiolabeled MMC-D was synthesized by

coupling ¹⁴C- γ -aminobutyric acid to dextran together with ϵ -aminocaproic acid at a molar ratio of 1 : 1000. The purified radiolabeled MMC-D had a specific activity of 0.05 μ Ci/mg. The doses reported for MMC-D refer to the quantity of MMC contained in the conjugate. Both MMC and MMC-D were prepared in saline solution with a concentration of 2 mg eq MMC/ml for injection. Both dextran and dextran-C₆ spacer were dissolved in saline solution at a concentration of 20 mg/ml.

Molecular Charge Estimation—The electric charge of MMC-D and dextran-C₆ spacer were estimated using DEAE-Sephadex A-50 anion exchanger and CM-Sephadex C-50 cation exchanger (Pharmacia Fine Chemicals Co., Sweden). Ion exchanger (25 mg) was suspended in 5 ml of pH 7.2 buffered saline with 20 mM *N*-2-hydroxyethylpiperazine *N'*-2-ethanesulfonic acid (HEPES) and shaken with 0.5 ml of sample solution. After centrifugation, the amount of compound in the supernatant was measured spectrophotometrically at 364 nm for MMC-D and by anthrone method¹²⁾ for dextran-C₆ spacer.

Procedure of Animal Experiment—Male Wistar albino rats weighing between 200 and 250 g were used in all animal experiments. The animals, anesthetized with ether, were injected with the drug solution into the femoral vein and then housed in metabolic cages. Urine samples were collected at adequate intervals in cooled test-tubes and stored in a freezer until assay.

Analytical Method—The concentration of MMC was determined by microbiological assay. Assay was performed by a disk-plate method using *E. coli* B as a test organism. The urine containing MMC and MMC-D was divided into two groups and each sample was diluted with saline and pH 8.0 phosphate buffer. The former was directly subjected to bioassay in which free MMC was detected predominantly. The latter sample was heated at 90 °C for 5 min in order to hydrolyze MMC-D to MMC, followed by bioassay. Under this condition, approximately 70% of MMC was constantly recovered from MMC-D and total amount of initial and regener-

ated MMC was determined from this sample. In order to make accurate estimation, urine sample was spiked with various amount of MMC and MMC-D and assayed by the same procedure in each run. Calculation was generally carried out in triplicate and variation coefficient was less than 10% for each sample. The amount of dextran was measured by anthrone method.¹²⁾ The urine sample was deproteinized with 5% trichloroacetic acid and filtered if protein was present. Filtrate (1 ml) was diluted with 5 ml of 95% ethyl alcohol to precipitate dextran. After centrifugation at 3000 rpm for 5 min, the supernatant was removed. The precipitated dextran was resolved in distilled water and subjected to assay.¹³⁾ The procedure employed for the determination of ¹⁴C-radioactivity was a modification of the method of Mahin and Loftberg.¹⁴⁾ Urine sample (0.2 ml) was put into a counting vial and 0.2 ml of perchloric acid (60%) and 0.2 ml of hydrogen peroxide 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) was added and

radioactivities were determined in a liquid scintillation system. The counts obtained were corrected with the aid of external standards.

RESULTS

Urinary Excretion of MMC after Intravenous Injection of Free MMC and MMC-D

Fig. 1 shows the cumulative amount of excreted MMC in the urine as free and conjugated forms after intravenous injection of MMC (A) and MMC-D with various molecular sizes (B–D) at a dose of 5 mg/kg. Free MMC was excreted rapidly and 26% of the dose was recovered in the urine sample of initial 2 h. No biological activity was detected in the urine at 12 h after administration of MMC. On the other hand, only a small amount of MMC was excreted in the urine following the injection of MMC-D. The excreted amount of MMC in the conjugated form depended on the molecular weight, namely 1.9% and 0.2% of dose were excreted within 6 h after injection of MMC-D (T-10) and MMC-D (T-70), respectively. MMC-D (T-500) was not excreted in the urine by itself. However, free

FIG. 1. Cumulative Amount of MMC and MMC-D Excreted in the Urine after Intravenous Injection of Free MMC (A), MMC-D (T-10) (B), MMC-D (T-70) (C), and MMC-D (T-500) (D)
 ● ■ ▲, MMC; ○ □ △, MMC-D. Results are expressed as the mean of at least four rats and vertical bars indicate S.E.

MMC was slowly but successively detected in the urine during the period studied following injection of MMC-D irrespective of the molecular weight.

Electric Charge of MMC-D and Dextran-C₆ Spacer

Table I summarizes the results of the adsorption experiments of MMC-D and dextran-C₆ spacer by an ion exchanger. All types of MMC-D were adsorbed by the cation exchanger CM-Sephadex at pH 7.2 to an equal extent, indicating MMC-D acted as positively charged molecules.

On the contrary, dextran-C₆ spacer (T-70) was adsorbed by the anion exchanger DEAE-Sephadex and proved to possess negative charge. These results elucidated that MMC-D behaves as a polycation and dextran-C₆ spacer as a polyanion at a neutral pH range.

Urinary Excretion of Dextran after Intravenous Injection of MMC-D, Dextran and Dextran-C₆ Spacer

Fig. 2 illustrates the cumulative amount of excreted dextran in the urine after intravenous

TABLE I. *Adsorption of MMC-D and Dextran-C₆ Spacer to CM- and DEAE-Sephadex at pH 7.2*

Compound	% adsorbed ^{a)}	
	CM-Sephadex	DEAE-Sephadex
MMC-D (T-10)	66.6	0
MMC-D (T-70)	64.0	0
MMC-D (T-500)	70.8	0
Dextran-C ₆ spacer (T-70)	0	73.1

a) % adsorbed to an ion exchanger was calculated from the amount remaining in the supernatant.

FIG. 2. *Cumulative Amount of Dextran Excreted in the Urine after Intravenous Injection of MMC-D (A), Dextran (B), and Dextran-C₆ Spacer (C) of Various Molecular Size*

○, T-10; □, T-70; △, T-500. Results are expressed as the mean of at least four rats and vertical bars indicate S.E.

injection of MMC-D (A), dextran (B) and dextran- C_6 spacer (C). The effect of molecular weight was obvious since the excreted amount was decreased as the molecular weight of the polymer was increased in each compound. In the case of MMC-D, 25.0%, 12.6% and 2.8% of dose were excreted in the urine up to 48 h after injection of MMC-D (T-10), MMC-D (T-70) and MMC-D (T-500), respectively. These values were considerably higher than the amount of excreted MMC as a conjugated form determined by bioassay. Less urinary recovery of dextran was observed following the injection of cationic MMC-D and anionic dextran- C_6 spacer compared with the injection of neutral dextran of the same molecular weight except T-500.

Urinary Excretion of ^{14}C -Labeled MMC-D

Fig. 3 shows the cumulative amount of radioactivity excreted in the urine after intravenous injection of ^{14}C -MMC-D (T-10) (A), ^{14}C -MMC-D (T-70) (B) and ^{14}C -MMC-D (T-500) (C), respectively.

The results are accompanied by the plasma and kidney concentration of radioactivity¹⁵⁾ in order to compare the pharmacokinetic behavior of ^{14}C -MMC-D simultaneously. As shown in Fig. 3 (A), radioactivity distributed rapidly to the kidney and high concentration was maintained up to 8 h after injection of ^{14}C -MMC-D (T-10) and 28% of the dose was excreted in the urine. In the case of ^{14}C -MMC-D (T-70) (Fig. 3 (B)), relatively high radioactivity was observed in the kidney immediately after administration but it declined to a lower level which persisted for 8 h. Urinary recovery was 9% of dose in this case. Following the injection of ^{14}C -MMC-D (T-500) (Fig. 3 (C)), distribution to the kidney was extremely restricted and only 2% of dose was recovered in the urine.

DISCUSSION

The biopharmaceutical characteristics such as distribution, metabolism and excretion of

FIG. 3. Cumulative Amount of Radioactivity Excreted in the Urine (○), and Plasma (●) and Kidney (△) Concentration after Intravenous Injection of ^{14}C -Labeled MMC-D (T-10) (A), ^{14}C -Labeled MMC-D (T-70) (B), and ^{14}C -Labeled MMC-D (T-500) (C)

Results are expressed as the mean of at least four rats and vertical bars indicate S.E. Plasma and kidney data are published.¹⁵⁾

polymeric prodrugs of antitumor agents seem to be the most important factors which determine the therapeutic potency of the prodrugs. Based on this idea, we studied the disposition and pharmacokinetics of MMC-D after systemic administration in rats.¹⁵⁾ Accumulation of radioactivity was observed in the reticuloendothelial system such as liver, spleen and lymph nodes after injection of three types of ¹⁴C-MMC-D. The kinetical analysis revealed that MMC-D can act as a reservoir of MMC which behaves characteristically as a macromolecule while supplying active MMC in the body. However, detailed information on urinary excretion of MMC-D has not been obtained, whereas urinary excretion is the major route of elimination of dextran and furthermore, MMC is known to cause some toxic effect on the kidney.

In our present study, therefore, the urinary excretion of MMC-D after bolus intravenous injection was evaluated by means of three kinds of analytical methods, that is, biological assay which can determine the biologically active com-

ponent in the conjugate, anthrone method for measuring the carrier portion and radioactivity counting of spacer-labeled MMC-D.

As shown in Fig. 1, 28% of the biological activity was rapidly recovered in the urine after injection of free MMC, suggesting that metabolism was a major route of elimination of MMC. In contrast, total recovery was only 2.2%, 0.8% and 0.4% of dose even at 72 h after the same dosage of MMC conjugated with dextran (T-10), dextran (T-70) and dextran (T-500), respectively. These results revealed that polymeric modification decreases and retards the urinary excretion of the drug. Thus the exposure of the renal cells to MMC is expected to be considerably restricted in the case of MMC-D. Although the excretion of the conjugated MMC depended on the carrier size, all types of MMC-D gave similar sustained excretion of MMC in a free form. This corresponds to the fact that sustained plasma concentration of free MMC was obtained after intravenous injection of MMC-D regardless of the molecular

FIG. 4. Excretion Rates of Conjugated MMC (Δ), Dextran ($\circ$), and Radioactivity ($\bullet$) after Intravenous Injection of MMC-D (T-10) (A), MMC-D (T-70) (B), and MMC-D (T-500) (C)
Results are expressed as the mean of at least four rats.

weight in our previous study.¹⁵⁾ It can be concluded, from these results, that most part of free MMC excreted into the urine was liberated from MMC-D during its circulation in the body.

In order to compare the urinary excretion characteristics of three analytical methods, excretion rates of conjugated MMC, dextran and radioactivity of initial 8 h were calculated as shown in Fig. 4. Excretion rate of radioactivity almost coincided with those of carrier dextran and it was confirmed that linkage between spacer and dextran was stable in the body. However, apparent difference was observed between the excretion rate of biologically active MMC as the conjugated form and that of carrier dextran, which could not be explained by the presumed hydrolytic liberation of free MMC. Therefore, this result implies that MMC-D underwent metabolic degradation before liberating active MMC or MMC was inactivated immediately after liberation. The dominant organ for inactivation of MMC-D seems to be the liver since selective accumulation¹⁵⁾ and significant metabolizing capacity¹⁰⁾ were observed in this organ.

Dextran of molecular weight of 40000 and 70000 have been used as plasma expanders to maintain or replace blood volume in clinical field. Dextran is mainly eliminated by the renal excretion, especially by glomerular filtration. It has been known that glomerular capillary wall has a barrier based on discrimination of molecular size and charge.¹⁶⁾ Clearance measurement study using inulin as a reference revealed that neutral dextran with molecular radii less than 20 Å cross the glomerular wall without measurable restriction, whereas dextran with radii greater than 42 Å are almost completely barred. Negatively charged dextran sulfate is restricted to a greater extent, while positively charged DEAE-dextran is enhanced in its ability to cross the glomerular wall compared with neutral dextran with similar size. Charge selectivity of capillary is considered to originate from fixed negative charge on the glomerular capillary wall, such as glycoproteins.

The effect of molecular size on the urinary excretion was clarified from our results in Fig. 2

about each polymer, MMC-D, dextran-C₆ spacer and original dextran. Comparing the similar size of polymer except T-500 which was hardly excreted in each case, neutral dextran exhibited the highest excretion among them. Taking into account that the effective sizes of the derivatives were only slightly larger than those of original dextran, effect of electric charge was suggested. There was not, however, significant difference between cationic MMC-D and anionic dextran-C₆ spacer. The discordance between our results and those obtained clearance study seems to be due to the difference of marker compounds. Furthermore our results could be considered to be influenced by the plasma concentration of each compound, because we did not evaluate the clearance but the urinary recovery of the polymer. Details remained obscure about the effect of charge.

The present investigation has thus shown that physicochemical properties, especially molecular size and charge affect the urinary excretion of the polymeric prodrug. Further, MMC-D, especially having molecular weight of 500000, was proved to have desirable urinary excretion characteristics from the viewpoint of the prevention of the side effect on the kidney, because MMC-D (T-500) gave the lowest radioactivity and no biological activity of MMC in the kidney after intravenous injection (unpublished data). These information seem to be useful for the development of new macromolecular prodrugs and cancer drug delivery systems.¹⁷⁾

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