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# The Accumulation Mechanism of Cationic Mitomycin C-dextran Conjugates in the Liver: In-vivo Cellular Localization and In-vitro Interaction with Hepatocytes

SACHI NAKANE, SATOSHI MATSUMOTO, YOSHINOBU TAKAKURA, MITSURU HASHIDA AND HITOSHI SEZAKI

*Department of Basic Pharmaceutics, Faculty of Pharmaceutical Sciences, Kyoto University, Kyoto 606, Japan*

**Abstract**—To elucidate the mechanism of the accumulation of mitomycin C-dextran conjugate (MMC-D) in the liver, in-vivo cellular uptake and in-vitro cellular interaction of MMC-D have been studied. Localization of cationic and anionic MMC-D (MMC-Dcat. and MMC-Dan.) in different liver cell types following i.v. administration was examined in rats and the significant contribution of parenchymal cells demonstrated. In-vitro cellular interaction was determined by measuring the drug concentration in the medium after incubation with rat isolated hepatocytes. MMC-Dcat. was highly adsorbed on the surface of hepatocytes at pH 7·2, while the interaction between MMC-Dan. and hepatocytes was negligible. The percentage association of MMC-Dcat. with hepatocytes remained almost constant during the course of incubation and no significant difference was observed between the incubation at 4 and 37 °C. The adsorption phenomenon was shown to conform to Langmuir's adsorption isotherm. The amount of MMC-Dcat. associated with hepatocytes increased as the molecular weight of the dextran chain increased. These results showed that MMC-Dcat. was adsorbed on the surface of hepatocytes by an electrostatic force and this binding was responsible for its remarkable accumulation in the liver in-vivo. Thus some physicochemical properties of the MMC-D conjugates are thought to play an important role in the disposition characteristics of the conjugates.

Mitomycin C (MMC)'s use in cancer chemotherapy is limited by side effects (Crooke 1979). These could be overcome and therapeutic efficacy enhanced if the cytotoxicity was localized at the tumour site and the burden on other tissues could be minimized by improving the pharmacokinetic properties and cellular access of MMC (Sezaki et al 1982). One possible approach to accomplish these goals might be conjugation of MMC with a high molecular weight compound (Poznansky & Cleland 1980; Sezaki & Hashida 1984a). Therefore, we have developed a macromolecular derivative of MMC (Kojima et al 1980), MMC-dextran conjugate (MMC-D), with a cationic (Fig. 1A) and an anionic charge (Fig. 1B), and examined their physicochemical, pharmacodynamic, and pharmacokinetic characteristics (Kojima et al 1980; Kato et al 1982; Hashida et al 1981, 1983, 1984; Sezaki & Hashida 1984b; Matsumoto et al 1985).

A disposition study of MMC-Dcat. in rats demonstrated that radiolabelled MMC-Dcat. accumulated in the liver, spleen, and lymph node, the amount in liver being about 40% of dose at 8 h after i.v. administration of the drug (Hashida et al 1984). Furthermore, we have also observed the rapid accumulation of MMC-Dcat. in the liver in a single pass perfusion experiment (unpublished data). An in-vitro cellular interaction study with tumour cells showed that the MMC-Dcat. was adsorbed to the cell surface by an electrostatic force, resulting in a larger antitumour effect than MMC (Matsumoto et al 1986).

The present study was undertaken to clarify the mechanism of the large accumulation of MMC-Dcat. in the liver from the viewpoint of cellular interaction. The relationship between the physicochemical characteristics and the hepatic uptake of macromolecular prodrugs is also discussed.

Correspondence to: H. Sezaki, Dept of Basic Pharmaceutics, Faculty of Pharmaceutical Sciences, Kyoto University, Kyoto 606, Japan.

## Materials and Methods

*Chemicals*

MMC was kindly supplied by Kyowa Hakko Kogyo Co., Tokyo, Japan. Dextrans with average molecular weights of 10 000 (T-10), 70 000 (T-70), and 500 000 (T-500) were obtained from Pharmacia, Uppsala, Sweden. $\gamma$-Amino-[U-$^{14}$C]butyric acid with a specific radioactivity of 2 mCi $mg^{-1}$ was purchased from New-England Nuclear, Boston, MA. Collagenase (type I) and bovine serum albumin (fraction V) were obtained from Sigma Chemical Co., St Louis, Mo. and Amersham, UK, respectively. All other chemicals were of finest grade available. MMC-Dcat. and MMC-Dan. were synthesized as reported previously (Kojima et al 1980; Matsumoto et al 1986). Radiolabelled MMC-Dcat. was synthesized by coupling $\gamma$-amino[U-$^{14}$C]butyric acid to dextran with $\varepsilon$-aminocaproic acid at molar ratio of 1:1000. Radiolabelled MMC-Dan. was synthesized by coupling $\gamma$-amino-[U-$^{14}$C]butyric acid together with MMC to dextran at molar ratio of 1:2000. The products were washed and concentrated by ultrafiltration. All conjugates contained almost equal amounts of MMC, about 10% (w/w), and the degree of substitution of dextran by MMC was 1 molecule MMC/14–17 glucose units. The radiolabelled MMC-Dcat. and MMC-Dan. had specific radioactivities of 0·05 and 0·02 $\mu$Ci $mg^{-1}$, repectively. The doses reported for MMC-D refer to the quantity of MMC in the conjugate.

*Molecular charge*

The net charge of MMC-D was estimated by the batch

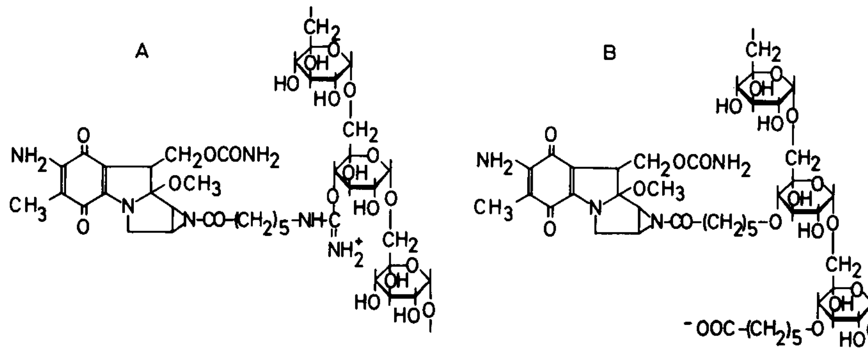

FIG. 1. Representative chemical structures of mitomycin C-dextran conjugates. (A), MMC-Dcat.; (B), MMC-Dan.

method using the DEAE-Sephadex A-50 anion exchanger and CM-Sephadex C-50 cation exchanger, as described by Roos et al (1984).

*Isolation and purification of liver cells*
Parenchymal cells from male Wistar rats (180–240 g) were isolated according to Seglen (1976) with minor modifications. In brief, liver was perfused with $Ca^{2+}$,$Mg^{2+}$-free Hanks' balanced salt solution (HBSS) for 10 min and then with HBSS containing 5 mM $CaCl_2$ and 0·05% (w/v) collagenase for 10–20 min. The perfusion rate was ca 30 mL $min^{-1}$. Parenchymal cells were separated from non-parenchymal cells by differential centrifugation. Non-parenchymal cells were prepared from total cell suspension as described by Horiuchi et al (1985). The viability of cells was checked by a trypan blue exclusion method.

*In-vivo localization of MMC-D in different liver cell types*
Male Wistar rats (210–240 g) were anaesthetized with pentobarbitone and injected with [$^{14}$C]MMC-D solution in the femoral vein (50 mg $kg^{-1}$, 0·05–0·1 μCi/rat). Their body temperature was kept at 37 °C by a heat lamp during the experiment. To determine plasma radioactivity, blood samples (0·15 mL) were withdrawn under anaesthesia from the jugular vein over 8 h and centrifuged at 3000 rev $min^{-1}$ for 2 min. At 8 h after the administration, the liver was perfused with collagenase as above to isolate the liver cells, just after the animal was killed. Parenchymal and non-parenchymal cell fractions were obtained by differential centrifugation. The radioactivities of plasma and each cell fractions were determined by liquid scintillation counting. Area under the plasma concentration–time curves (AUC) over 8 h was estimated using the kinetic parameters calculated by the non-linear least squares method (MULTI) (Yamaoka et al 1981) for MMC-D.

Cellular uptake was evaluated by three indices: (i) uptake amount, (ii) endocytic index and (iii) contribution of each cell type. The amount of the uptake was expressed as % of dose of radioactivity recovered in $10^8$ cells. The endocytic index was calculated by dividing the uptake amount by AUC for 8 h. The contribution of each cell type was calculated by multiplying the uptake amount by the number of each cell type contained in 1 g liver ($1·25 \times 10^8$ cells $g^{-1}$ liver for parenchymal cells; $6·5 \times 10^7$ cells $g^{-1}$ liver for non-parenchymal cells, Blomhoff et al 1985).

*In-vitro cellular association of MMC-D*
Isolated liver cells ($10^6$ cells $mL^{-1}$) were incubated in 10 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid-buffered HBSS (pH 7·2) containing MMC-D (10 μg equivalent MMC $mL^{-1}$). The incubation was carried out in test tubes in a total volume of 3 mL. After 15 min incubation at 37 °C, cells were separated from the medium by centrifugation at 3000 rev $min^{-1}$ for 10 min. Drug concentration remaining in the supernatant was determined spectroscopically by measuring the absorbance at 365 nm, and the percentage of association was calculated. The experiments were made in triplicate. The effects of incubation temperature, drug concentration, and medium pH on cellular association were examined. Bis(2-hydroxyethyl)-2,2′,2″-nitrilotriethanol (pH 5·5–6·5) and N-2-hydroxyethylpiperazine-N′-3-propanesulphonic acid (pH 8·0–8·5) were used at a concentration of 20 mM. When the effect of albumin on cellular association was examined, bovine serum albumin was added at various concentrations (1–5% (w/w)) to the incubation medium. The interaction of MMC-D with erythrocytes was also examined. Fresh blood of rats was centrifuged at 3000 rev $min^{-1}$ for 10 min and sedimentary erythrocytes were washed with HBSS several times. The cellular association of MMC-D was then investigated in a similar manner to the case of the liver cells, except for cell density in the incubation medium ($10^8$ cells $mL^{-1}$).

## Results

*Molecular charge of MMC-D*
MMC-Dcat. was adsorbed on CM-Sephadex but not on DEAE-Sephadex, regardless of molecular weight in the range of pH 5·5–10·0. On the other hand, MMC-Dan. interacted with DEAE-Sephadex but not with CM-Sephadex. These results indicated that the MMC-Dcat. molecule had a cationic charge while MMC-Dan. molecule had an anionic charge.

*In-vivo localization of MMC-D in different liver cell types*
Table 1 shows the distribution of radiolabelled MMC-D between parenchymal and non-parenchymal liver cells at 8 h after their i.v. administration. The uptake amount (% of dose per $10^8$ cells) of MMC-Dcat. (T-70) was some 10 times as large as that of MMC-Dan. (T-70) in both cell

Table 1. Distribution of [$^{14}$C]MMC-D in rat liver parenchymal and non-parenchymal cells at 8 h after intravenous administration.

| Compounds | Cell type | Uptake amount (%/10$^8$ cells) | Endocytic index (μL plasma h$^{-1}$/ 10$^8$ cells) | Contribution (% g$^{-1}$ liver) |
|---|---|---|---|---|
| MMC-Dcat. (T-70) | PC[a] | 2·57 ± 0·54 | 440 ± 125 | 3·21 ± 0·68 |
| | NPC[b] | 1·85 ± 0·36 | 336 ± 90 | 1·14 ± 0·24 |
| MMC-Dan. (T-70) | PC | 0·20 ± 0·05 | 3·1 ± 0·5 | 0·26 ± 0·06 |
| | NPC | 0·20 ± 0·05 | 2·6 ± 0·8 | 0·13 ± 0·03 |

[a] Parenchymal cells; [b] Non-parenchymal cells. Distribution of [$^{14}$C]MMC-D in each cell type was estimated at 8 h after a bolus intravenous injection (50 mg kg$^{-1}$). Each index was calculated as described in Materials and methods. Results are expressed as the mean ± s.d. of at least three experiments.

types. In the endocytic index (μL plasma h$^{-1}$ per 10$^8$ cells), the value of MMC-Dcat. was about 100 times greater than that of MMC-Dan. The calculated total contribution of each cell type suggested that the parenchymal cells took the larger part in the uptake of MMC-D by the whole liver.

3A. There was a linear relation for the reciprocal of the amount of the drug bound to the cells and that of the drug concentration remaining in the incubation medium. This suggested that the cellular binding of MMC-Dcat. conformed to Langmuir's adsorption isotherm (equations A and B).

$$X = \frac{X_\infty KC}{1 + KC} \quad (A)$$

$$\frac{1}{X} = \frac{1}{X_\infty K}\frac{1}{C} + \frac{1}{X_\infty} \quad (B)$$

where X represents the adsorbed amount of drug, $X_\infty$ is the maximum adsorption amount of drug, K is the constant which expresses strength of the binding, and C is the drug concentration in the medium after incubation. $X_\infty$ and K were calculated for each MMC-Dcat. from the solid lines in Fig. 3B obtained by the least squares method (Table 2).

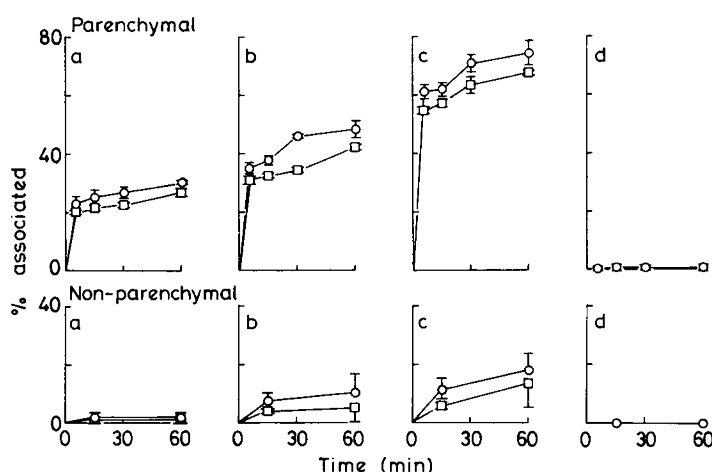


FIG. 2. In-vitro association time courses of MMC-Dcat. with parenchymal or non-parenchymal cells at 37 °C (○) and 4 °C (□). (a), MMC-Dcat. (T-10); (b), MMC-Dcat. (T-70); (c), MMC-Dcat. (T-500); (d), MMC-Dan. (T-70). Each result is expressed as the mean ± s.d.

*In-vitro cellular interaction of MMC-D*

Fig. 2 shows the cellular association-time profiles of MMC-D at 4 and 37 °C with the parenchymal cells. MMC-Dcat. was rapidly associated with the parenchymal cells to an extent that was almost constant during the course of incubation. No significant difference was observed between incubation at 4 or 37 °C. Greater interaction was observed with increase in the molecular weight of dextran. In contrast, MMC-Dan., which has an anionic charge, did not intract with the parenchymal cells.

Fig. 2 also shows the cellular association-time profiles of MMC-D with the non-parenchymal cells. The extent of association amount was less than that of parenchymal cells, but the interaction also increased with an increase in the molecular weight of dextran.

Fig. 3A shows the effect of concentration of MMC-Dcat. on the association with the parenchymal cells. The correlation of molecular weight and cellular association was observed at all concentrations. At low concentrations, cellular uptake increased in proportion to drug concentration, but it was saturated at higher concentrations. Fig. 3B shows a double reciprocal plot of the data shown in Fig.

The maximum amount of MMC-Dcat. adsorbed, expressed in MMC equivalent amounts (x), increased with increase in molecular size of dextran as well as K values. In

Table 2. Parameters of Langmuir's adsorption isotherm for the interaction of MMC-Dcat. to parenchymal cells.

| Compounds | $x_\infty$[a] (μg equiv MMC/ 10$^6$ cells) | k[b] (mL μg$^{-1}$ equiv MMC) | $X_\infty$[a] 10$^{10}$(mol/ 10$^6$ cells) | K[b] 10$^{-8}$ mL mol$^{-1}$) |
|---|---|---|---|---|
| MMC-Dcat. (T-10) | 5·11 | 0·117 | 46·45 | 1·29 |
| MMC-Dcat. (T-70) | 5·69 | 0·356 | 7·95 | 25·47 |
| MMC-Dcat. (T-500) | 8·89 | 1·560 | 1·64 | 844·13 |

Parameters were obtained from Fig. 4B.
[a] Calculated maximum amount of the drug adsorbed to parenchymal cells is expressed as the amount of MMC conjugated ($x_\infty$) or mole numbers of MMC-Dcat. molecules ($X_\infty$).
[b] k and K, constant of Langumuir's adsorption isotherm, were calculated as MMC equivalent and mole numbers of MMC-Dcat., respectively.

contrast the maximum amount of adsorption expressed as moles of the total conjugate (X), decreased as the molecular weight of the dextran increased.

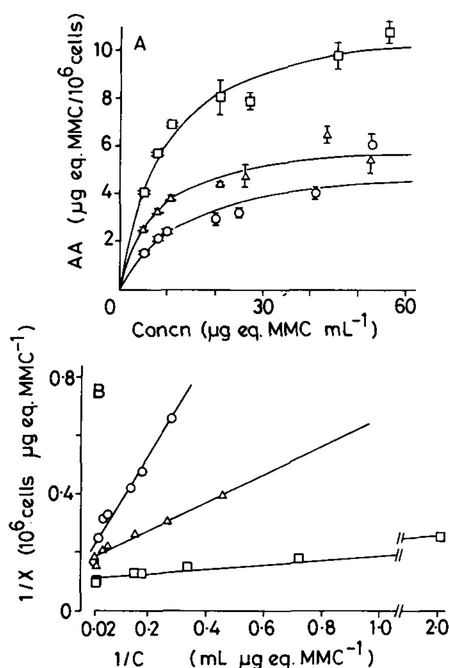


FIG. 3. Effect of concentration of MMC-Dcat. on its association with parenchymal cells. ○, MMC-Dcat. (T-10); △, MMC-Dcat. (T-70); □, MMC-Dcat. (T-500). The incubation was carried out at 37 °C for 15 min. (A) Association amount (AA) at various concentrations. The results are expressed as the mean ± s.d. The curves were computer-generated for each set of data. (B) Double reciprocal plot of the data shown in Fig. 3A. Lines were calculated by the least squares methods. Correlation coefficients were 0·975 for MMC-Dcat. (T-10), 0·974 for MMC-Dcat. (T-70), and 0·969 for MMC-Dcat. (T-500), respectively.

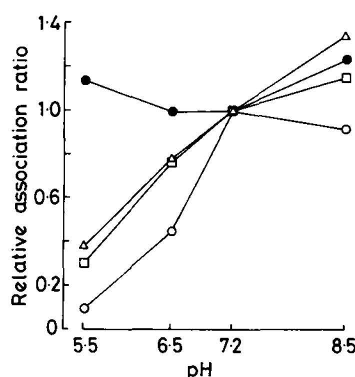


FIG. 4. Effect of the pH of the medium on association of MMC and MMC-Dcat. with parenchymal cells. ●, MMC; ○, MMC-Dcat. (T-10); △, MMC-Dcat. (T-70); □, MMC-Dcat. (T-500). The incubation was carried out at 37 °C for 15 min. The amounts of the drug associated with cells at various pH values were compared as the amount associated at pH 7·2 was standardized to be 1·0 (ordinate).

Fig. 4 shows the effect of pH of the medium on the extent of cellular association of MMC and MMC-Dcat. The amount of MMC-Dcat. associated decreased with fall in pH, while for MMC it was almost constant in the pH range tested.

In Table 3, the amounts of cationic and anionic MMC-D associated with parenchymal and non-parenchymal liver cells and erythrocytes of rats are compared. Considering that the number of erythrocytes in the medium was 100 times greater than that of liver cells, the interaction of MMC-Dcat. with erythrocytes was not large.

Table 3. Association of MMC-D with liver parenchymal and non-parenchymal cells and red blood cells of rats.

| | % Association | | |
|---|---|---|---|
| Compounds | PC[a] ($10^6$ cells mL$^{-1}$)[d] | NPC[b] ($10^6$ cells mL$^{-1}$) | RBC[c] ($10^8$ cells mL$^{-1}$) |
| MMC-Dcat. (T-10) | 25·40 ± 2·04[e] | 2·12 ± 1·61 | 2·60 ± 2·23 |
| MMC-Dcat. (T-70) | 37·43 ± 1·93 | 7·67 ± 2·78 | 1·52 ± 1·65 |
| MMC-Dcat. (T-500) | 61·53 ± 2·31 | 11·36 ± 2·97 | 11·88 ± 1·21 |
| MMC-Dan. (T-70) | 0·56 ± 1·21 | N.D.[f] | 0·29 ± 0·41 |

The incubation was carried out at 37 °C for 15 min.
[a] Parenchymal cell; [b] Non-parenchymal cell; [c] Red blood cell.
[d] Values in parentheses are the cell density of incubation medium for each cell type.
[e] Each value is expressed in % of association, and expressed as the mean ± s.d.
[f] Not detectable.

The effect of albumin on the adsorption of MMC-Dcat. (T-500) to the parenchymal cells was examined by adding different concentrations of albumin to the incubation medium. In 15 min, 77·1, 79·6 and 74·9% of the initial amount of MMC-Dcat. disappeared from the medium containing albumin at 1, 3 and 5%, respectively. No significant difference was observed between these values and the control value without albumin.

## Discussion

MMC-Dcat. may be considered as having three kinds of chemical structure for the linkage between the spacer and dextran (Schnaar et al 1977). The cationic charge is probably due to structures such as isourea and *N*-substituted imidocarbonate, although a carbamate linkage is also possible. For MMC-Dan., the spacer, 6-bromohexanoic acid, was introduced to the dextran through an ether linkage with no electric charge, but MMC was not coupled with all spacer arms; the remaining free carboxyl groups may give the anionic charge. In both cationic and anionic MMC-D, MMC is coupled to the spacer arm at 1a-*N*-position through the amide linkage. At pH 7·2, MMC is liberated by chemical hydrolysis from dextran backbone with half-lives of about 24 h (MMC-Dcat.) and 36 h (MMC-Dan.), and this slow release may result in a superior therapeutic effect for MMC. For short experiments, therefore, the release of MMC-D may be neglected in analysis.

In general, i.v. administered microparticles such as liposomes, microspheres, and colloids are readily taken up by the reticuloendothelial system of the liver, i.e. Kupffer cells (Kao & Juliano 1981; Roerdink et al 1981; Bradfield 1984; Lenaerts et al 1984). Macromolecules with molecular size larger than the glomerular pore are localized in the intravascular space and circulated in the body with blood. If there is no special interaction between tissues and these macromolecules, they would be gradually cleared from blood with a fluid-phase endocytosis mechanism. On the contrary, macromolecules having specific affinity for tis-

sues like the reticuloendothelial system would accumulate in them. In the present investigation, the distribution mechanisms of macromolecular prodrugs were investigated from a view point of cellular interaction.

After i.v. administration, MMC-Dcat. disappeared from the circulating blood faster than MMC-Dan. and gave an AUC about one-tenth as small as that of MMC-Dan. MMC-Dan. gave the endocytic indices of 0·64 and 5·5 mL plasma $day^{-1}$ (g $protein)^{-1}$ for parenchymal and non-parenchymal cells, respectively, when expressed on the basis of the protein content of each cell type. These values are broadly in agreement with those reported for polyvinylpyrrolidone (PVP) (0·95 and 9·95 mL $day^{-1}$ (g $protein)^{-1}$ for parenchymal and non-parenchymal cells) which is taken up by the liver through fluid-phase endocytosis (Munniksma et al 1980). Therefore, MMC-Dan. may be considered to be taken up by fluid-phase endocytosis. On the other hand, MMC-Dcat. had an endocytic index 100-times higher than that of MMC-Dan. This value also is not compatible with those of liposomes (Roerdink et al 1981), colloidal albumin, and antimony sulphur colloid (Praaning-Van Dalen et al 1981) which are taken up by adsorptive endocytosis mainly by Kupffer cells. These facts suggested that neither the fluid-phase endocytosis nor the phagocytic uptake by the Kupffer cells plays a major role in the cellular accumulation of MMC-Dcat.

Since the molecular sizes of all kinds of MMC-D tested in this investigation are smaller than that of fenestrae of the sinusoidal lumen (ca. 0·1 m), they can be considered to freely enter the space of Disse and interact with parenchymal cells. In our investigation, therefore, interaction between MMC-D and liver cells was examined using isolated parenchymal and non-parenchymal cells.

As illustrated in Fig. 2, more MMC-Dcat. than MMC-Dan. was associated with both parenchymal and non-parenchymal cells, which indicates that electric charge to be a factor in their interaction. The slow uptake of MMC-Dan. by a fluid-phase endocytosis probably could not be detected in this experimental system. Compared with parenchymal cells, less MMC-Dcat. was associated with non-parenchymal cells, reflecting the smaller surface area of non-parenchymal cells. The parenchymal cells occupy 73% in the surface area of total liver plasma membrane (Praaning-Van Dalen et al 1981), and this value is closely consistent with that of in-vivo cellular distribution of MMC-Dcat. (Table 1). The significant contribution of interaction of MMC-Dcat. with parenchymal cells to its characteristic disposition in-vivo was suggested in these results, and further investigation was focused on this point.

Several aspects of the mode of cellular interaction of MMC-Dcat. was investigated. The findings that cellular uptake of MMC-Dcat. at 4 °C was similar to that at 37 °C (Fig. 2), and that MMC-Dcat. was associated rapidly with both cell types, suggested that MMC-Dcat. is physically adsorbed on the cell surface. When the concentration of the drug in the medium was changed, the association phenomenon was seen to conform to Langmuir's adsorption isotherm (Fig. 3). A lowering of the pH of the medium resulted in a decrease in the cellular adsorption of MMC-Dcat. (Fig. 4), indicating that an electrostatic force between the anionic charges on the cell surface and the cationic charges of MMC-Dcat. plays an important role in the cellular interaction of MMC-Dcat.

The cellular interaction of MMC-Dcat. differed with the molecular weight of dextran (Figs 2, 3). The maximum numbers of MMC-Dcat. molecules adsorbed by parenchymal cells were $2{\cdot}80 \times 10^{9}$ molecules for MMC-Dcat. (T-10), $4{\cdot}79 \times 10^{-8}$ molecules for MMC-Dcat. (T-70), and $9{\cdot}87 \times 10^{7}$ molecules for MMC-Dcat. (T-500) estimated from Langmuir's adsorption isotherm.

The interaction of MMC-Dcat. with rat erythrocytes was examined but adsorption to erythrocytes was not considerable (Table 3). The difference in the properties and the density of the surface charge and the cellular size between erythrocytes and liver cells might be responsible for these findings. No significant difference was observed in the extent of the adsorption in the presence or absence of albumin in the incubation medium. These results show that interaction between MMC-Dcat. and erythrocytes or plasma protein in-vivo is a minor one and suggest that the results of the present in-vitro experiments are applicable to the in-vivo phenomenon.

From the results obtained in this investigation it may be concluded that MMC-Dcat. circulating in the blood rapidly accumulates in the liver through adsorption to the surface of hepatocytes in the space of Disse. This may suggest a possibility of utilizing MMC-Dcat. as a targeting device to the liver. On the other hand, our previous studies showed that MMC-Dcat. is suitable for intratumoural injection in clinical use, because it may act as a depot which gradually supplies active MMC on the surface of the tumour cells (Takakura et al 1984, 1986; Satomura et al 1985; Honda et al 1985). In this circumstance, small amounts of MMC-Dcat. leaking from the injection site to the circulatory system might be rapidly cleared by the liver as elucidated herein, thereby reducing drug's side effects. MMC-Dan. circulates in the body without any significant interaction with tissues and slowly accumulates in the liver probably by a fluid-phase endocytosis. However, further investigation is necessary to elucidate a detailed mechanism for the hepatic uptake of MMC-Dan.

In conclusion, the physicochemical characteristics of MMC-Ds have been shown to play a role in the disposition of the drug. Although the results for cationic and anionic MMC-D in this work are only examples, these disposition characteristics should be exploited in the development of any macromolecular prodrugs.

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