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SYNTHESIS AND CYTOTOXIC ACTIVITY OF DEXTRAN-IMMOBILIZING PLATINUM(II) COMPLEX THROUGH CHELATE-TYPE COORDINATION BOND

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ABSTRACT

cis-Dichloro(cyclohexane-*trans-l*-1,2-diamine)platinum(II):Dach-Pt-(chlorato) is a platinum complex which is expected to exhibit higher antitumor activity than cisplatin and which shows no cross-resistance with cisplatin. However, its strong side-effects and low water solubility have been noted. We have reported that polymer/antitumor drug conjugates show reduced side-effects and high antitumor activity. In order to provide a macromolecular prodrug of Dach-Pt having reduced side-effects and high water solubility, we synthesized dextran derivatives containing dicarboxylic acid groups to immobilize Dach-Pt via a chelate-type coordination bond, dicarboxymethyl-dextran(DCM-Dex)/Dach-Pt conjugate. We investigated the release behavior of the platinum complex from the carrier polymer and the cytotoxic activity of the conjugate against p388D$_1$ *lymphocytic leukemia* cells in vitro compared with the carboxymethyl-dextran (CM-Dex)/Dach-Pt conjugate. The DCM-Dex/Dach-Pt conjugate showed almost the same level of cytotoxic activity as free Dach-Pt(chlorato) or Dach-Pt(malonato). Although the cytotoxic activity of free Dach-Pt(chlorato) was decreased by incubation in a medium with serum, the DCM-dextran/Dach-Pt conjugate kept its higher level of cytotoxic activity after incubation in a medium with serum. These results suggested that the stability of the Dach-Pt moiety in the

medium was increased and cytotoxic activity of Dach-Pt was not decreased by fixation to dextran through a chelate-type coordination bond.

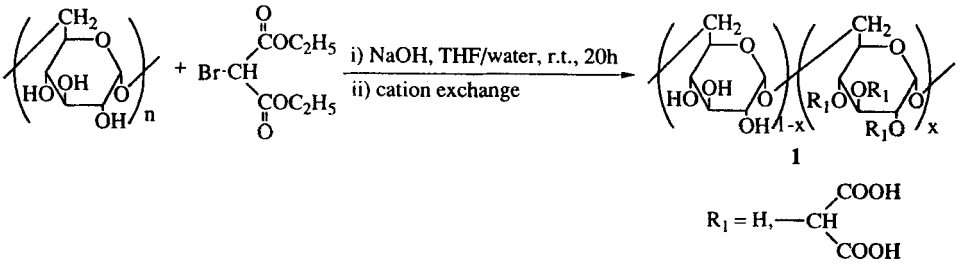
INTRODUCTION

Recently, drug delivery systems (DDS) using polymers as drug carriers have been investigated to achieve efficient delivery of anticancer agents to tumor cells. In comparison with a low-molecular-weight prodrug, a macromolecular prodrug can be expected to overcome the problem of side-effects by improving the body distribution of drugs and prolongation of their activities [1]. In recent years, macromolecular prodrug systems using polymers, such as poly[*N*-(2-hydroxypropyl)methacrylamide] (HPMA) [2], poly(L-glutamic acid) [3], poly(ethylene glycol)-*block*-poly(aspartic acid) [4], oxidized dextran [5], copolymer of divinylether and maleic anhydride (DIVEMA) [6], poly(α-malic acid) [7], and chitin [8, 9], as drug carriers have been reported.

cis-Dichlorodiammineplatinum(II) [10] (cisplatin) has been widely used for clinical cancer therapy in spite of its severe renal toxicity. Many approaches have been attempted to synthesize new platinum complexes having a broader spectrum of antitumor activity, reduced side-effects, greater solubility, and lack of cross-resistance with cisplatin [11–14]. Some platinum(II) complexes having 1,2-cyclohexanediamine(Dach) as a ligand were reported to exhibit high antitumor activity [12, 14]. Among them, *cis*-dichloro(cyclohexane-*trans*-*l*-1,2-diamine)platinum(II):Dach-Pt-(chlorato) is one of the platinum complexes expected to exhibit higher antitumor activity than cisplatin and show no cross-resistance with cisplatin. However, its strong side-effects and low water solubility have also been cited.

Schechter et al. reported the usage of water-soluble polymers, such as poly(L-glutamic acid), DIVEMA, and carboxymethyl(CM)-dextran, as carriers of cisplatin to reduce its side-effects [15]. They also reported that the increase in water solubility of cisplatin was achieved by complex formation with the water-soluble polymers.

Dach-Pt(chlorato) has lower water solubility than cisplatin and its potential for antitumor activity is higher. The merits of conjugation of Dach-Pt with water-soluble polymers are larger than of cisplatin to achieve water solubility and reduce the side-effects. In this paper we report on the synthesis of polymer complexes of Dach-Pt and dextran derivatives having dicarboxylic acid groups, the DCM-Dex/Dach-Pt conjugate, to achieve reduced side-effects and high water solubility of Dach-Pt. We investigated the release behavior of the platinum complex from the conjugate in phosphate buffer solution (PBS) (pH 7.4) and the cytotoxic activity of the conjugate against p388D_1 *lymphocytic leukemia* cells in vitro. On the other hand, it is known that the cytotoxic activity of the platinum complex gradually decreases in the bloodstream because of ligand exchange reactions with substances having amino groups in the serum, such as proteins, amino acids, and so on. Polymer/Dach-Pt complexes are expected to keep the platinum complex away from these deactivating factors and retain its cytotoxic activity during circulation in the bloodstream. Therefore, we investigated the residual cytotoxic activity of DCM-Dex/Dach-Pt conjugate and low-molecular-weight platinum complexes against tumor cells after preincubation in the medium with serum.



SCHEME 1.

EXPERIMENTAL

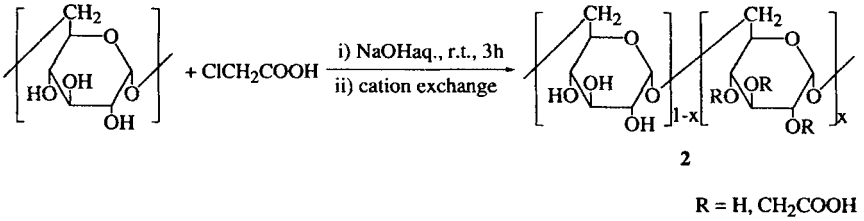
Materials

Dach-Pt(chlorato) was obtained from Sumitomo Pharmaceuticals Co. Dextran (MW = 0.6×10^5) was purchased from Wako Pure Chemical Industry. The organic solvents were purified by the usual distillation methods. The other material were of commercial grade and used without further purification.

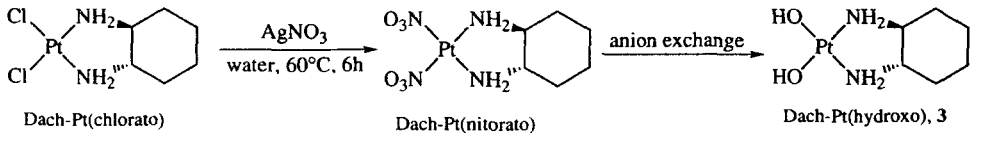
Preparation of Dextran Derivatives

The preparation of DCM-Dex was carried out according to Scheme 1. Dextran (MW = 0.6×10^5, 500 mg) was dissolved in 8.8 M NaOH(aq) (10 mL). Bromodiethylmalonate (10 g) dissolved in 10 mL tetrahydrofuran was added to the solution at 0°C. The solution was stirred at room temperature for 20 hours. The reaction mixture was dialyzed in distilled water using cellulose tubing for 7 days. The solution obtained was passed through a column packed with a cation-exchange resin (Amberlite 120B H^+ type) and freeze dried to give DCM-Dex **1** in 292 mg yield (91.4%) as a white powder. The degree of introduction of the carboxylic acid group (DCA) as measured by a neutralization titration method [16] was 23.4 mol% per sugar unit. The number-average molecular weight (M_n) of the **1** was determined to be 1.0×10^4, and the ratio of weight-average molecular weight (M_w) to M_n was determined to be 2.03 by gel-permeation chromatography (GPC). The following spectral data of **1** confirmed the structure. IR (KBr): 3284 (OH), 2928 (CH_2), 1723 (COOH), 1013 cm^{-1} (C—O—C). ^{13}C NMR (DMSO): δ 63.6 (CH_2), 72.7–76.5 (CHOH, *C*H-(CH_2O)), 88.0 (O*C*H($COOH)_2$), 100.8 (OCHO), 175.0–177.0 ppm (COOH).

The preparation of CM-Dex **2** was carried out according to Scheme 2. Dextran (M_w = 0.6×10^5, 500 mg) was dissolved in 8.8 M NaOH(aq) (10 mL). Aqueous



SCHEME 2.

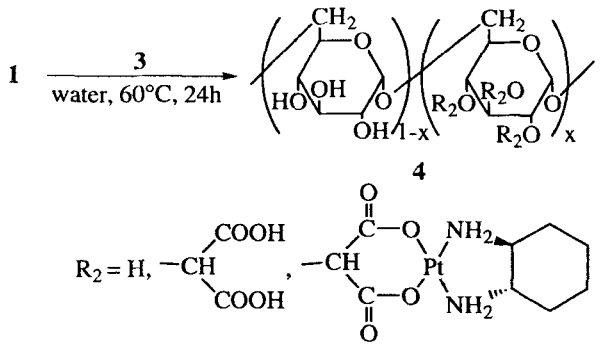


SCHEME 3.

chloroacetic acid (7.4 M, 10 mL) solution was added dropwise to the solution at 0°C. The solution was stirred at room temperature for 3 hours. The reaction mixture was dialyzed in distilled water using cellulose tubing for 7 days. The solution obtained was passed through a column packed with a cation-exchange resin (Amberlite 120B H^+ type) and freeze dried to give **2** in 292 mg yield (91.4%) as a white powder. The M_n and DCA for the **2** were determined to be 1.2×10^4 and 58.2 mol% per sugar unit, respectively. The following spectral data of **2** confirmed the structure. IR (KBr): 3356 (OH), 2936 (CH_2), 1730 (COOH), 1014 cm^{-1} (C—O—C). ^{13}C NMR (D_2O): δ 66.2 (CH_2), 70.2-73.3 (CHOH, $CH(CH_2O)$), 103.0-105.5 (OCHO), 66.9 (CH_2COOH), 172.2 ppm (COOH).

Synthesis of Dextran Derivative/Dach-Pt Conjugates

Synthesis of DCM-Dex/Dach-Pt conjugate was carried out according to Schemes 3 and 4. Dach-Pt(chlorato) (50 mg) was dissolved in 30 mL water and stirred at 60°C for 3 hours. Aqueous silver nitrate solution (0.1 M, 0.22 mL) was added to the solution. The reaction mixture was stirred at 60°C for 6 hours. The silver chloride precipitate was removed by filtration. The filtrate containing Dach-Pt(nitrato) was passed through a column packed with an anion-exchange resin (Diaion SA-10A OH^- type) to convert to Dach-Pt(hydroxo). The solution obtained was added to **1** (226 mg) in water. The reaction mixture was stirred at 60°C for 24 hours. The product was purified by gel-filtration chromatography (Sephadex G-25, eluent: water). The high-molecular-weight fraction was corrected and freeze dried to give DCM-Dex/Dach-Pt conjugate **4** in 210 mg yield (76.0%) as a white powder. The degree of introduction of Dach-Pt (DPt) per sugar unit was determined by



SCHEME 4.

atomic absorption spectrometry. The following spectral data of **4** confirmed the structure. IR (KBr): 3356 (OH), 2936 (CH_2), 1725 (COOH), 1654 (COO^-), 1016 cm^{-1} (C—O—C). ^1H NMR (DMSO): δ 1.2-2.1 (CH_2, cyclohexane ring), 2.4 (CH—NH_2), 3.0-4.0 ($CHOH$, $CH(CH_2O)$), 5.0 ppm (OCHO). ^{13}C NMR (DMSO): δ 25.0 (CH_2CH_2), 40.4 (CH_2CH), 66.9 (CH—NH_2), 66.2 (CH_2), 70.2-73.3 (CHOH, $CH(CH_2O)$), 88.2 ($OCH(COOH)_2$), 103.0-105.5 (OCHO), 177.0-178.0 ppm (COO).

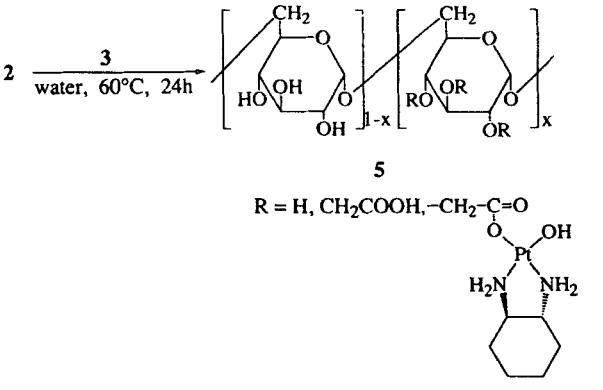
CM-Dex/Dach-Pt conjugate was synthesized using **2** (226 mg) and Dach-Pt(chlorato) (50 mg) by the same procedures described above (Scheme 5). CM-dextran/Dach-Pt conjugate **5** was obtained in 230 mg yield (76.0%) as a white powder. The DPt for **5** was determined by atomic absorption spectrometry. The following spectral data of **5** confirmed the structure. IR (KBr): 3317 (OH), 2934 (CH_2), 1730 (COOH), 1648 (COO^-), 1014 cm^{-1} (C—O—C). ^1H NMR (DMSO): δ 1.2-2.1 (CH_2, cyclohexane), 2.4 ($CHNH_2$), 3.0-4.0 ($CHOH$, $CH(CH_2O)$), 5.0 ppm (OCHO). ^{13}C NMR (DMSO): δ 25.0 (CH_2CH_2), 40.2 (CH_2CH), 66.7 ($CHNH_2$), 66.2 (CH_2), 70.1-73.0 (CHOH, $CH(CH_2O)$), 102.8-105.5 (OCHO), 177.0-178.0 ppm (COO).

Determination of Release of Platinum Complex from the Conjugates

Release behaviors of platinum complex from **4** and **5** were investigated in PBS in vitro. The conjugates (10 mg) were dialyzed in PBS (pH 7.4, 20 mL) containing 1 M NaCl using cellulose tubing (cut-off MW = 1.0×10^4). The solution outside the cellulose tubing was replaced with fresh PBS containing 1 M NaCl at certain time intervals. The amount of platinum complex released to the medium from the carrier polymer was estimated by a method reported previously [15] using complex formation reaction with *o*-phenylenediamine (OPDA).

Measurement of Cytotoxic Activity of the Conjugates

The in vitro cytotoxic activities of the conjugates against $p388D_1$ *lymphocytic leukemia* cells were measured according to methods described previously [8]. The cell line of $p388D_1$ *lymphocytic leukemia* was maintained in RPMI-1640 medium



SCHEME 5.

containing 10% heat inactivated fetal calf serum (FCS), 2 mmol/L L-glutamine, 18 mmol/L sodium bicarbonate, and 60 mg/L kanamycin. The tumor cell suspension (10 mL) containing 1.0×10^6 cells in a culture medium containing 10% FCS were distributed in a 96-well multiplate (Corning 25860MP) and incubated with conjugates or free platinum complexes in a humidified atmosphere containing 5% CO_2 at 37°C for 24 hours. A low-molecular-weight chelate-type platinum complex, Dach-Pt(malonato), was prepared according to a method reported previously [17] and used as a reference drug in these experiments. The number of viable cells was determined by means of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] enzyme assay using a microplate reader (MPT-120, Corona Electric Co.). The cytotoxic activity was calculated by the following equation:

$$\text{Cytotoxic activity (\%)} = (C - T)/C \times 100$$

where C = number of viable cells after 24 hours incubation without drug
T = number of viable cells after 24 hours incubation with drug

The residual cytotoxic activity of the conjugates was also measured against p388D_1 *lymphocytic leukemia* cells in vitro. The conjugates or free platinum complexes (Pt concentration = 2.0×10^{-5} mol/L) were preincubated in RPMI-1640 medium containing 10% FCS at 37°C for a certain time period before measurement of cytotoxic activity. The cytotoxic activities of the samples were measured by the same method described above. The residual activity was calculated by the following equation:

$$\text{Residual cytotoxic activity (\%)} = (C_0 - C_t)/C_0 \times 100$$

where C_t = cytotoxic activity of the drug after preincubation in medium at 37°C
C_0 = cytotoxic activity of the drug without preincubation

Measurement

The molecular weights of the polymers were measured by gel-permeation chromatography (GPC) (column: Shodex OHpack KB-803, Showa Denko K.K.; eluent: 1/15 M phosphate buffer; standard: pullulan). IR spectra were measured on a Perkin-Elmer 1600 Series FT-IR spectrometer. ^{1}H-NMR and ^{13}C-NMR spectra were measured with a Jeol GSX-400 spectrometer using DSS or TMS as the reference. Atomic absorption spectra were measured on a Nippon Jarrell Ash AA-855.

RESULTS AND DISCUSSION

Preparation of the Conjugates

1 was prepared by conventional desalting and alkali saponification reactions using bromodiethylmalonate. **2** was also prepared by the conventional method using chloroacetic acid. The synthesis of dextran derivative/Dach-Pt conjugates was carried out by the ligand exchange reaction of dextran derivatives (**1**, **2**) with **3** in water. The conjugates were separated from the reaction mixture by gel-filtrate chromatography. A typical elution profile of gel-filtrate chromatography (Sephadex G-25, eluent: water) monitored by UV at 200 nm of the reaction mixture for the prepara-

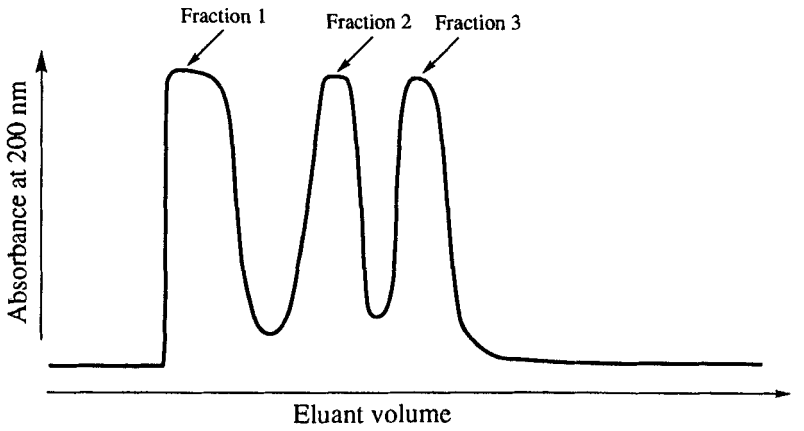


FIG. 1. Elution profiles of gel-filtration chromatography (Sephadex G-25, eluant: water) for the reaction mixture of DCM-Dex and Dach-Pt(hydroxo).

tion of **4** is shown in Fig. 1. Fractions 1-3 showed the specific atomic absorption spectra of platinum. Fraction 1, the high-molecular-weight fraction, is **4**. Fractions 2 and 3 are low-molecular-weight platinum complexes not bound to the polymer, such as unreacted Dach-Pt(hydroxo) or Dach-Pt(aquo) as by-products (not identified). The IR spectra of **1** showed absorption at 1723 cm^{-1} assigned to the COOH group, while this peak was decreased and a new absorption band at 1654 cm^{-1}, assigned to carboxylate (COO^-), appeared in the IR spectra of **4**. A similar result was observed in the preparation of **5**. There were no cation species in the reaction system which could form carboxylate except for platinum. These results mean that the Dach-Pt moiety was immobilized to the dextran derivatives having carboxylic acid groups as a carboxylato complex via a coordination bond (COO-Pt). In the case of **4**, the Dach-Pt moiety could be immobilized to a DCM-Dex chelate-type coordination bond to form a stable six-membered ring in the same way as Dach-Pt(malonato). The DCA values and number-average molecular weights (M_n) of **1** and **2**, and the DPt values of the conjugates obtained, are summarized in Table 1.

TABLE 1. Preparation of Dextran Derivatives/Dach-Pt Conjugates

	M_n of dextran derivative	DCA,[a] mol%	DPt, mol%[b] Per sugar unit	DPt, mol%[b] Per carboxy acid group
DCM-Dex/Dach-Pt conjugate **4**	1.0×10^4	23.4	2.4	20.5
CM-Dex/Dach-Pt conjugate **5**	1.2×10^4	58.2	16.4	27.3

[a]Degree of introduction of carboxy acid group per sugar unit.
[b]Degree of introduction of platinum complex.

The DCA values in Table 1 show the degree of introduction of carboxylic acid per sugar unit for dextran derivatives before immobilization of the Dach-Pt moiety. So the DCA value for the DCM-Dex/Dach-Pt conjugate (23.4 mol%) means that 11.7 mol% of the sugar unit was modified with the malonic acid group. DCM-Dex has a lower DCA value (23.5%) than does CM-Dex. This is because the reactivity and stability under the basic condition of bromodiethyl malonate were lower than those of chloroacetic acid. **4** showed high water-solubility; **5** didn't show high water-solubility but did swell only after freeze drying. This phenomenon suggests that crosslinking of CM-Dex by the platinum complex partially occurred during drying of **5**. **5** dissolved completely after 6 hours of incubation in distilled water. Figure 2(A) shows the elution profiles of GPC for **1** and **4** are almost the same. A change of molecular weight was not observed before or after immobilization of the Dach-Pt moiety to **1**. These results mean that neither a crosslinking reaction nor hydrolysis of the polymer occurs in the preparation of **4**.

Release Behavior of Platinum Complex from the Conjugates

Figure 2(B) shows the elution profiles of GPC for Dach-Pt(chlorato) and **4** before and after incubation in PBS (pH 7.0) at room temperature for 4 days. **4** showed only one peak in the high-molecular-weight fraction; however, after 4 days of incubation in PBS the conjugate showed the other sharp peak which has a molecular weight similar to Dach-Pt(chlorato). These results mean that **4** can release a low-molecular-weight platinum complex, such as Dach-Pt(aquo), in aqueous media. Determinations of the extent of release of a platinum complex from the conju-

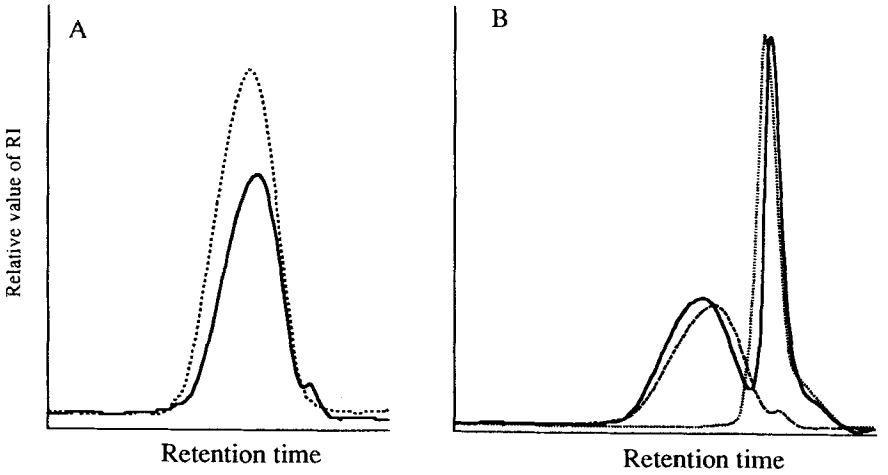


FIG. 2. Elution profiles of gel-permeation chromatography (GPC) (column: Shodex OHpack KB-803, eluent 1/15 M phosphate buffer) for DCM-Dex **1** and DCM-Dex/Dach-Pt conjugate **4** (A), and DCM-Dex/Dach-Pt conjugate **4** before and after incubation in PBS (pH 7.0) at RT for 4 days (B). A: (---) DCM-Dex **1**, (—) DCM-Dex/Dach-Pt conjugate **4**. B: (---) DCM-Dex/Dach-Pt conjugate **4** before incubation, (—) DCM-Dex/Dach-Pt conjugate **4** after 4 days incubation, (· · ·) free Dach-Pt(chlorato).

gates were carried out in PBS (pH 7.4) containing 1 M NaCl in vitro. The results are shown in Fig. 3. It took about 5 days to release all of the Dach-Pt immobilized in **4**. A sustained release of a platinum complex from the carrier polymers was achieved. The release rate from **5** was faster than that from **4**. These results mean that the coordination bonds between Dach-Pt moieties and the carrier polymer for **4** were more stable than those of **5**. This fact also supports the view that chelate-type coordination bonds formed in **4**. From these results, **4** is expected to show the slow release of a platinum complex in the bloodstream after intravenous injection in vivo.

Cytotoxic Activity of the Conjugates *in Vitro*

The cytotoxic activities of the DCM-Dex/Dach-Pt conjugates and CM-dextran/Dach-Pt conjugate were investigated against p388D$_1$ *lymphocytic leukemia* cells in vitro compared with low-molecular-weight platinum complexes. The results are shown in Fig. 4. The IC_{50} values for the conjugates and low-molecular-weight platinum complexes are summarized in Table 2. The cytotoxic activity of **5** was very low compared with that of free Dach-Pt(chlorato). On the other hand, **4** showed almost the same level of cytotoxic activity as free Dach-Pt(chlorato) and Dach-Pt(malonato). These results mean that the immobilization of Dach-Pt to DCM-Dex didn't have a fatal effect on the cytotoxic activity of Dach-Pt. **4** is also expected to exhibit the same level of cytotoxic activity against tumor cells in vivo.

It is well known that the cytotoxic activity of platinum complexes gradually decreases in the bloodstream because of ligand exchange reactions with compounds having amino groups in the serum, such as proteins, amino acids, and so on. The DCM-Dex/Dach-Pt conjugates are expected to keep the platinum complex away from these deactivating factors and retain its cytotoxic activity during circulation in the bloodstream due to the immobilization of the Dach-Pt moiety to polymer by a

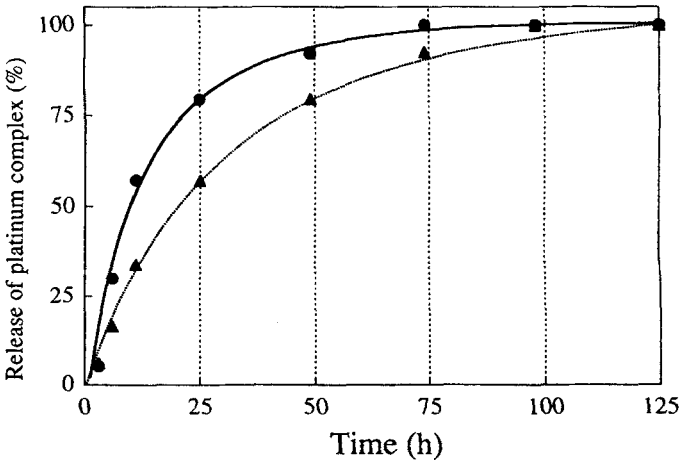


FIG. 3. Release behavior of platinum complex from DCM-Dex/Dach-Pt conjugate **4** and CM-Dex/Dach-Pt conjugate **5** in PBS (pH 7.4) containing NaCl (0.1 M) at 37°C. (▲) DCM-Dex/Dach-Pt conjugate **4**, (●) CM-Dex/Dach-Pt conjugate **5**.

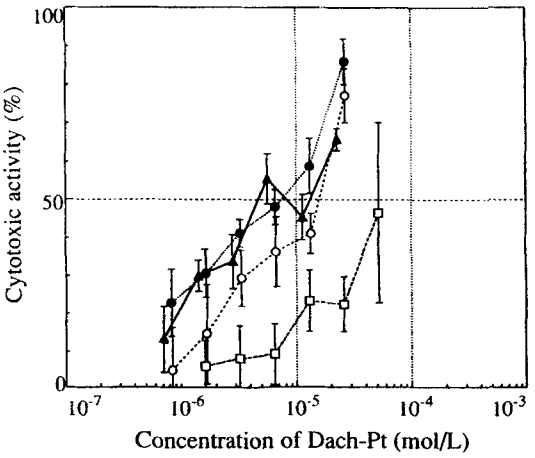


FIG. 4. Cytotoxic activity of the conjugates and free platinum complexes against p388D$_1$ *lymphocytic leukemia* cells for 24 hours in vitro. (▲) DCM-Dex/Dach-Pt conjugate **4**, (□) CM-Dex/Dach-Pt conjugate **5**, (●) Dach-Pt(chlorato), (○) Dach-Pt(malonato).

stable, chelate-type coordination bond. Therefore we investigated the residual cytotoxic activities of **4**, **5**, and free platinum complexes against tumor cells after a certain preincubation period in the medium containing FCS. The results are shown in Fig. 5. While the cytotoxic activity of Dach-Pt(chlorato) decreased gradually with preincubation time in the medium, **4** kept its residual cytotoxic activity at a higher level than did Dach-Pt(chlorato) or Dach-Pt(malonato), especially for the initial 12-hour period. On the other hand, the cytotoxic activity of **5** rapidly decreased. Dach-Pt(malonato) showed a slightly higher stability than did Dach-Pt(chlorato). These results suggest that the single coordination bond in **5** is not stable enough to keep its cytotoxic activity. The higher maintenance of cytotoxic activity of **4** is due to both of the stable immobilization of the Dach-Pt moiety by the chelate-type coordination bond and excluded volume effect of the carrier polymer which keeps off the deactivating factors.

TABLE 2. Cytotoxic Activity of Dextran Derivatives/Dach-Pt Conjugates and Free Platinum Complexes against p388D$_1$ *Lymphocytic Leukemia* Cells in Vitro

	IC_{50}, mol/L[a]
DCM-Dex/Dach-Pt conjugate **4**	8.0×10^{-6}
CM-Dex/Dach-Pt conjugate **5**	5.0×10^{-5}
Dach-Pt(chlorato)	7.2×10^{-6}
Dach-Pt(malonato)	1.6×10^{-5}

[a]IC_{50} = concentration of platinum complex at which the cytotoxic activity reaches 50%.

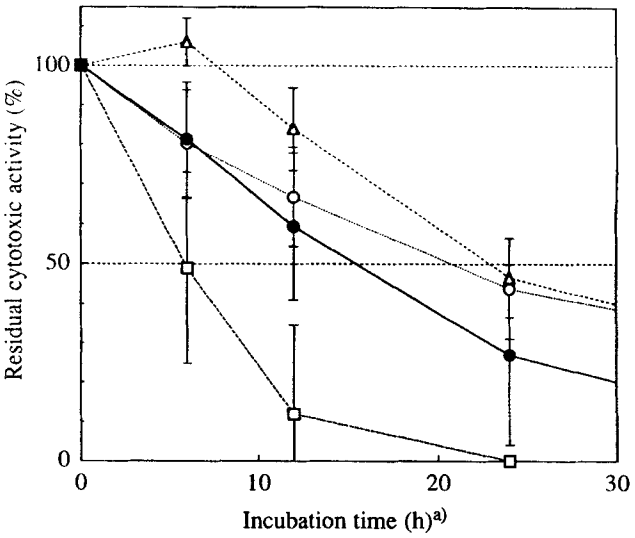


FIG. 5. Residual cytotoxic activity of the conjugates and free platinum complexes after preincubation in the medium containing fetal calf serum (FCS) against p388D$_1$ *lymphocytic leukemia* cells for 24 hours in vitro. (△) DCM-Dex/Dach-Pt conjugate **4**, (□) CM-Dex/Dach-Pt conjugate **5**, (●) Dach-Pt(chlorato), (○) Dach-Pt(malonato). a) Preincubation time of conjugates or free platinum complexes in RPMI-1640 medium containing 10% FCS at 37°C.

CONCLUSIONS

Based on these results, the DCM-Dex/Dach-Pt conjugate is expected to show a longer antitumor activity by maintaining the cytotoxic activity of Dach-Pt. Moreover, since the DCM-Dex/Dach-Pt conjugate has a large molecular size compared with typical low-molecular-weight anticancer platinum complexes, it should show a longer half-life in the body and a larger accumulation at the inflammatory tumor site than do low-molecular-weight platinum complexes [1]. The DCM-Dex/Dach-Pt conjugate should have a good therapeutic index against tumors in vivo. We will report on the in vivo antitumor activity of the conjugate in our next paper.

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