Characterization and Anticancer Activity of the Micelle-forming Polymeric Anticancer Drug Adriamycin-conjugated Poly(ethylene glycol)-Poly(aspartic acid) Block Copolymer

Masayuki Yokoyama,1 Mizue Miyachi, Noriko Yamada, Teruo Okano, Yasuhisa Sakurai, Kazunori Kataoka, and Shohei Inoue

Institute of Biomedical Engineering, Tokyo Women's Medical College, Kawada-cho, Shinjuku-ku, Tokyo 162, Japan; M. Y. M., M. N., T. O., Y. S.; Department of Materials Science and Technology and Research Institute for Biosciences, Science University of Tokyo, Yamazaki 2641, Noda-shi, Chiba 278, Japan; and Department of Synthetic Chemistry, Faculty of Engineering, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan.

ABSTRACT

Adriamycin (ADR), an anthracycline anticancer drug, was bound to the poly(aspartic acid) chain of poly(ethylene glycol)-poly(aspartic acid) block copolymer by amide bond formation between an amino group of Adriamycin and the carboxyl groups of the poly(aspartic acid) chain. The polymeric drug thus obtained was observed to form a micelle structure possessing diameter of approximately 50 nm, with a narrow distribution, in phosphate-buffered saline and to show excellent water solubility despite a large amount of ADR introduction. Further, it was able to be stored in lyophilized form without losing its water solubility in the redissolving procedure. Increased stability of the bound Adriamycin molecules in phosphate-buffered saline and elimination of binding affinity for bovine serum albumin due to the micelle formation were further advantages of this polymeric drug. In vivo high anticancer activity of this micelle-forming polymeric drug against P388 mouse leukemia was obtained with less body weight loss than that seen with free ADR, due to low toxicity as compared with free ADR.

INTRODUCTION

It is well known that the utility of cancer chemotherapy is considerably restricted by toxic side effects of anticancer drugs. This restriction results from the fact that the anticancer drugs used in the present chemotherapy lack efficient selectivity for malignant cells. To suppress the toxic side effects of the anticancer drugs to normal cells and to improve their efficiency toward malignant cells, studies of conjugating anticancer drugs to polymeric carriers have been carried out as one promising approach. This drug-polymer conjugate is called a "polymeric drug." Expected advantageous features of polymeric drugs are preferable tissue distribution of drug given by the character of the polymeric carrier, prolonged half-life of drug in plasma, and controlled drug release from the polymeric carrier by adjustment of the chemical properties of the bond between the drug and the carrier. Several kinds of polymers, naturally occurring and synthetic polymers, have been examined as carriers of anticancer drugs. Among naturally occurring polymers, immunoglobulins are most widely used as the carrier, due to their high specificity and wide applicability to many kinds of malignant cells. Utility of immunoglobulin as the polymeric carrier is, however, restricted by its chemical and physical properties. For example, modification of immunoglobulins by anticancer drugs often leads to precipitation due to hydrophobicity of the drug. Furthermore, modification procedures are limited to ones performed in mild conditions to avoid denaturation of the immunoglobulins during the modification. Alternatively, the polymeric carrier of the drug can be freely designed using many kinds of synthetic polymers available today, and various organic reactions can be used to introduce drug to the synthetic polymeric carrier. From this point of view, several kinds of synthetic polymers have been investigated, such as poly[N-2-(hydroxypropyl)methacrylamide] (1), poly(divinyl ether-co-maleic anhydride) (2), poly(styrene-co-maleic anhydride) (3), dextran (4, 5), poly(ethylene glycol) (6), poly(l-glutamic acid) (7, 8), poly(l-aspartic acid) (9), and poly(l-lysine) (10, 11). All these examples are in the category of homopolymers or alternating copolymers. Although considerable improvements in anticancer activity have been obtained, the polymeric drugs using homopolymer or alternating copolymer often face a problem of water solubility. Introduction of a large quantity of drug to the polymers leads to precipitation of the polymeric drug, since most drugs have a hydrophobic character. One promising approach to overcome this difficulty is utilization of micelle-forming polymeric drug. A hydrophilic outer shell surrounds a drug-conjugated hydrophobic inner core, and this outer shell is considered to help prevent the conjugate from precipitation.

The basic concept of this paper is the utilization of the micelle-forming polymeric drug as a novel anticancer agent. The polymeric drug possessing a micellar structure is expected to have a large diameter, as compared with unbound drug, which is a small molecule. The polymeric drugs with a micellar structure of the ideal diameter are expected to circulate in the blood stream without emobilization at capillaries, to escape from excretion in kidney, and to permeate into the target cells through blood vessels. And this micellar form is expected to help protect the conjugated drug from enzymatic attack in plasma by concealing the conjugated drug in the hydrophobic core of the micelles. Furthermore, nonselective uptake of the polymeric drug by the reticuloendothelial system is thought to be suppressed by choosing the appropriate character of the outer shell.

This paper deals with the anticancer activity as well as several chemical and physical properties of a newly designed micelle-forming polymeric drug, poly(ethylene glycol)-poly(aspartic acid) block copolymer conjugated with Adriamycin. Adriamycin is one of the most powerful and widely used anticancer drugs. Poly(ethylene glycol) is known to be a nontoxic and nonimmunogenic water-soluble polymer, which is expected to be the outer shell of the micelle. Poly(aspartic acid) is a synthetic poly(amino acid) and, therefore, possible hydrolysis of its main chain may play a role in releasing Adriamycin quickly from the conjugate after the uptake by the target cells. And it is noted that the substance resulting from the main chain hydrolysis of the poly(aspartic acid) is a naturally occurring amino acid.

MATERIALS AND METHODS

Chemicals

α-Methyl-ω-aminopoly(oxyethylene) (1) [CH3-poly(ethylene glycol)-NH2, Mn = 4300] was kindly supplied by Nippon Oil & Fats Co., Ltd.,
MICELLE-FORMING POLYMERIC ANTICANCER DRUG

Synthesis of Polymeric Drug

The synthetic procedure for the polymeric drug, Adriamycin-bound poly(ethylene glycol)-poly(aspartic acid) block copolymer, was reported elsewhere (12, 13). Here, the procedure is stated briefly. The synthetic route to the polymeric drug is shown in Fig. 1.

The starting material, α-methyl-ω-aminopoly(oxyethylene) (1) [(CH₂-poly(ethylene glycol)-NH₂, Mr = 4300), is poly(ethylene glycol) with a methoxy group at one terminal and a primary amino group at the other terminal. β-Benzyl l-aspartate N-carboxy anhydride (2) was polymerized from the terminal amino group of 1 to obtain poly(ethylene glycol)-poly(β-benzyl l-aspartate) block copolymer (3). β-Benzyl l-aspartate N-carboxy anhydride (2) (7.21 g) was dissolved in 12 ml of doubly distilled N,N-dimethylformamide followed by an addition of 60 ml of distilled chloroform. α-Methyl-ω-aminopoly(oxyethylene) (1) (6.00 g) was added, after vigorous stirring at 0°C for 10 min, the reaction mixture was poured into 2000 ml of diethyl ether, and precipitate was collected by filtration, dissolved in distilled water, and dialyzed against distilled water with Spectrapor 7 dialysis membrane (molecular weight cut-off = 1000), followed by lyophilization. Yield was 10.09 g (84.5%). The number of β-benzyl l-aspartate units of 3 was found to be 17 by a 1H NMR spectrum of 3.

Protective benzyl groups of the β-benzyl l-aspartate units were removed by alkaline hydrolysis to obtain PEG-P(Asp)(4). Poly(ethylene glycol)-poly(β-benzyl l-aspartate) block copolymer (3) (10.03 g) was dissolved in 100 ml of chloroform. Then, 78 ml of a solution of NaOH (0.43 M) in a mixture of water, methanol, and 2-propanol (volume ratio, 1:2:2) were added. After vigorous stirring at 0°C for 10 min, the reaction mixture was poured into 2000 ml of diethyl ether, and precipitate was collected by filtration, dissolved in distilled water, and dialyzed against distilled water with Spectrapor 7 dialysis membrane (molecular weight cut-off = 1000), followed by lyophilization. Yield was 3.94 g (84%). The content of Adriamycin in the polymeric drug was determined by measuring absorbance at 485 nm; on the assumption that t₄₈₅ of the Adriamycin residue bound to the polymeric drug was 1694 unit. ADR was introduced into the PEG-P(Asp) (4) by amide bond formation between an amino group of the Adriamycin molecule and a carboxyl group of an aspartic acid residue in the poly(aspartic acid) chain, using EDC as a coupling agent. ADR.HCl was dissolved in N,N-diethyl-3-(3-dimethylaminopropyl)carbodiimide; BSA, bovine serum albumin; ADR, Adriamycin; PBS, sodium phosphate-buffered solution; PEG, poly(ethylene glycol); P(Asp), poly(aspartic acid); PEG-P(Asp), poly(ethylene glycol)-poly(aspartic acid) block copolymer; PEG-P(Asp)(ADR), Adriamycin-bound poly(ethylene glycol)-poly(aspartic acid) block copolymer; SDS, sodium dodecyl sulfate; T/C, treated/control.

1 The abbreviations used are: ADR·HCl, Adriamycin hydrochloride; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; BSA, bovine serum albumin; ADR, Adriamycin; PBS, sodium phosphate-buffered solution; PEG, poly(ethylene glycol); P(Asp), poly(aspartic acid); PEG-P(Asp), poly(ethylene glycol)-poly(aspartic acid) block copolymer; PEG-P(Asp)(ADR), Adriamycin-bound poly(ethylene glycol)-poly(aspartic acid) block copolymer; SDS, sodium dodecyl sulfate; T/C, treated/control.

Fluorescence Measurement

Fluorescence was measured using a Hitachi Fluorescence Spectrophotometer 650-60, with excitation at 471 nm, in PBS (pH 7.4, 0.155 M), at room temperature.

Albumin Binding

Binding affinity of ADR and PEG-P(Asp)(ADR) to BSA was investigated using ultrafiltration for ADR and gel filtration for PEG-P(Asp)(ADR).

Binding Assay of Adriamycin to BSA by Ultrafiltration. Two ml of a solution of ADR (7–8 × 10⁻⁵ M) and BSA (5 and 40 mg/ml) in 0.1 M PBS (pH 7.4) were prepared. This mixed solution was incubated for 10 min and then was ultrafiltered with a Centricon-30 tube (equipped with a YM-30 ultrafiltration membrane), followed by washing twice with 1.0 ml of PBS. [Blank tests with a BSA solution (1 mg/ml) and an ADR solution (7.1 × 10⁻⁵ M) revealed that 95% of BSA was filtered out and 91% of ADR was recovered in the filtrate.] Amounts of ADR in the residue on the ultrafiltration membrane and in the filtrate were determined by measuring absorbance at 485 nm.

Binding Assay of PEG-P(Asp)(ADR) to BSA by Gel Filtration. Thirty μl of solutions of PEG-P(Asp)(ADR) (5) (ADR = 1.83 × 10⁻⁴ M) and of BSA (20 mg/ml) in 0.1 M PBS (pH 7.4) were mixed. The mixture was analyzed by high performance liquid chromatography (gel filtration type) using a Toyo Soda model HLC-803 in 0.1 M PBS (pH 7.4, containing 0.3 M NaCl) at a flow rate of 1.0 ml/min, equipped with an Asahipak GS-520 column. Detection was performed at 280 nm (JACSO Uvidec 100) and 470 nm (ISCO UA-5).

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In Vitro Stability

Samples were incubated in PBSa (pH 7.4, 0.155 M) at 37°C in the dark. Absorbance of the samples at 485 nm was measured at appropriate time intervals.

In Vitro Cytotoxicity

P 388 mouse leukemia cells (1 × 10^6 cells/ml) were incubated in RPMI 1640 medium (containing 10% fetal calf serum and 5000 units/liter penicillin and streptomycin), in a Multiwell tissue culture plate (24-well) (Falcon 3047) for 24 h in 5% CO2 at 37°C. Drug was added to the cells, and the number of cells was counted with a Coulter Counter ZB-1 after 24 and 48 h of incubation.

In Vivo Anticancer Activity

P 388 mouse leukemia cells were maintained by i.p. passage in DBA/2 mice every week. Female CDF1 mice were i.p. inoculated with P 388 mouse leukemia cells (1 × 10^6 cells in 0.1 ml) on day 0. The mice were i.p. inoculated with drug dissolved in a 0.9% NaCl solution on day 1, in a volume of 0.1 ml/10 g of body weight (the average body weight is approximately 21 g). The controls were i.p. inoculated with a 0.9% NaCl solution on day 1, in a volume of 0.1 ml/10 g of body weight. Six mice were included in each group (with some exceptions). The mortality was monitored daily (until day 60), and body weights were measured at intervals of a few days.

RESULTS

Synthesis of Polymeric Drug. The polymeric drug PEG-P(Asp(ADR)) (5) was successfully synthesized without any precipitation in various molar ratios of ADR-HCl:Asp unit in the reactants, as shown in Table 1. Comparing runs 3–5, the content of ADR bound to the block copolymer was observed to increase with an increase in a ratio of ADR-HCl:Asp unit in the reactants. As for the purification method, the runs purified by ultrafiltration afforded higher ADR content than the runs purified by gel filtration, comparing the ADR content at the same molar ratio of ADR-HCl:Asp unit in the reactants. Ultrafiltration-purified run 4 (Asp unit:ADR-HCl = 1:0.52) afforded 22 mol % of the bound ADR with respect to the aspartic acid residue of the reactant, whereas run 1, done at almost the same molar ratio as that of run 4 but purified by gel filtration, had a smaller value (18 mol %) for the ADR content. Comparing the ADR content of the runs done at Asp unit:ADR-HCl = 1:1, the difference becomes clearer. Run 5–7 afforded 1.5–1.7 times as high a content of the conjugated ADR as run 2. These differences in the ADR content due to the purification method indicate that the synthesized PEG-P(Asp(ADR)) was adsorbed on Sephadex G-25 gel to some extent in 0.1 M acetate buffer.

The water solubility of PEG-P(Asp(ADR)) was maintained even after the lyophilization (for runs 3, 4, and 5). Its aqueous solution was able to be concentrated by ultrafiltration, without any precipitate, up to 20 mg equivalent of ADR-HCl/ml (for runs 6 and 7). An addition of 0.9% (w/v) NaCl to this concentrated solution did not lead to precipitation nor gelation of the solution.

As shown in runs 6 and 7, PEG-P(Asp(ADR)) containing approximately 30 mol % bound ADR was synthesized reproducibly and was used for assays of in vitro cytotoxicity and in vivo anticancer activity. In these runs, the aqueous solutions were concentrated to 20 mg ADR-HCl equivalent/ml and washed with distilled water by repeated ultrafiltration, until the ADR concentration of the filtrate was reduced to under 1/100 of the ADR concentration of the residual solution on PM 30 membrane. Finally, 0.9% (w/v) NaCl was added to these solutions.

Laser Scattering. PEG-(P(Asp(ADR))) was analyzed by laser scattering in PBSa. Results are shown in Fig. 2 and Table 2. PEG-P(Asp(ADR)), run 5 of Table 1, at a concentration of 0.1 mg equivalent ADR-HCl/ml, was found to show unimodal distribution in size, and its mean diameter (number average) was found to be 48.5 nm. This value was much larger than that expected from its molecular weight (approximately 9000). This result indicates that PEG-P(Asp(ADR)) in run 5 formed a micellar structure in PBSa. An addition of 1% (w/v) SDS brought about a complete shift of the peak to a smaller size and, accordingly, the mean diameter was reduced to 2.7 nm. This decrease in mean diameter with the SDS addition indicates disruption of these micelles due to the interaction with the surfactant. PEG-P(Asp(ADR)) in run 5 formed almost the same size of micelles in water and at higher (0.4 mg equivalent ADR-HCl/ml) and lower (0.025 mg equivalent ADR-HCl/ml) concentrations, as shown in runs 6 and 7.

**Table 1 Reaction conditions and results of introduction of ADR into PEG-P(Asp) with amide bond formation between the amino group of ADR and the carboxyl group of Asp units**

<table>
<thead>
<tr>
<th>Run</th>
<th>ADR-HCl (mg)/ DMP* (ml)</th>
<th>PEG-P(Asp) (mg)</th>
<th>Asp:ADR-HCl mol ratio</th>
<th>EDC (µl), reaction time (h)*</th>
<th>Purification**</th>
<th>ADR conc (mol %)**</th>
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<tr>
<td>1</td>
<td>20.7/15</td>
<td>27.3</td>
<td>1:0:49</td>
<td>1 100, 4</td>
<td>Gel</td>
<td>18</td>
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<td>19.9/15</td>
<td>12.7</td>
<td>1:1:02</td>
<td>1 50, 4</td>
<td>Gel</td>
<td>20</td>
</tr>
<tr>
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<td>10.6/8</td>
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<td>15</td>
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<td>22</td>
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<tr>
<td>5</td>
<td>20.4/15</td>
<td>13.2</td>
<td>1:1:00</td>
<td>1 50, 4</td>
<td>Ultra</td>
<td>33</td>
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<tr>
<td>6</td>
<td>349.2/260</td>
<td>230.3</td>
<td>1:0:98</td>
<td>1 886, 4</td>
<td>Ultra</td>
<td>31</td>
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<tr>
<td>7</td>
<td>604.6/450</td>
<td>391.8</td>
<td>1:1:00</td>
<td>1 1500, 4</td>
<td>Ultra</td>
<td>29</td>
</tr>
</tbody>
</table>

* DMF, N,N-dimethylformamide.
* Mol ratio of aspartic acid residue to ADR:HCl in the reactants.
* First addition of EDC at 0°C; second addition at room temperature.
** Purification procedure to remove unbound ADR. Gel, gel filtration with Sephadex G-25 (medium) gel; Ultra, ultrafiltration with Micon PM-30 membrane.
** ADR content is shown in mol % with respect to aspartic acid residues of PEG-P(Asp) of the reactant.
concentration of the surfactant, SDS. PEG-P(Asp(ADR)) in destruction of the micellar structure was dependent on the with 1% (w/v) SDS. This fact suggests that the degree of addition was found to be a little larger than that concentrations in PBSa. The mean diameter of run 5 with 0.1% (w/v) SDS addition was found to be almost identical but showed a little difference.

Fluorescence Measurement. Fluorescence measurements supported the formation of a micellar structure of PEG-P(Asp(ADR)). Fluorescence of the ADR bound to PEG-P(Asp(ADR)) in run 4 was found to form almost the same size of micelles as run 4, irrespective of the difference in the ADR content. The fluorescence intensity for PEG-P(Asp(ADR)) than for ADR supports the formation of a micellar structure of PEG-P(Asp(ADR)). Fluorescence of the ADR bound to PEG-P(Asp(ADR)) in run 4 showed 14.9-fold quenching of the fluorescence intensity between PEG-P(Asp(ADR)) in run 4 and ADR suggests the enhanced quenching is due to the micelle formation for run 4. PEG-P(Asp(ADR)) in run 4 showed 14.9-fold quenching of the fluorescence intensity (without SDS addition) at an ADR concentration of $6.0 \times 10^{-4}$ M, as compared with the intensity with SDS addition, whereas ADR showed only 3.5-fold quenching at $5.7 \times 10^{-4}$ M. More significant quenching of the fluorescence intensity for PEG-P(Asp(ADR)) than for ADR supports the micelle formation of PEG-P(Asp(ADR)).

Plots of fluorescence intensity against concentration of the bound ADR of runs 3–5 with and without the addition of SDS were found to be almost identical but showed a little difference. A run with a lower content of ADR showed a little higher fluorescence intensity. This fact indicates that the content of the bound ADR reflected the packing degree of the bound ADR molecules in the micelles as well as the degree of resistance to the destruction of the micellar structure by the SDS addition.

Albumin Binding. Binding affinity of ADR and PEG-P(Asp(ADR)) (ADR content = 18 mol %; run 1 in Table 1) to bovine serum albumin, which plays a central role in drug binding in plasma, was measured by two different methods, ultratillation for ADR and gel filtration for PEG-P(Asp(ADR)). ADR was observed to bind to BSA. The binding affinity was dependent on the concentration of BSA; 45% of ADR (at $8.45 \times 10^{-4}$ M) was bound to BSA at a concentration of 40 mg/ml, and 8% of ADR (at $7.38 \times 10^{-4}$ M) was bound to BSA at a concentration of 5 mg/ml.

In contrast, the Adriamycin introduced into PEG-P(Asp) did not bind to BSA at all, as shown in Fig. 4. PEG-P(Asp(ADR)) (30 µl; [ADR] = $1.83 \times 10^{-4}$ M) was eluted at the gel exclusion volume (8.4 ml), due to the micelle formation of this block copolymer. BSA (30 µl; [BSA] = 20 mg/ml) was eluted at 13.8 ml. A 1:1 mixture (60 µl) of PEG-P(Asp(ADR)) and BSA solutions had elution peaks at both 8.4 and 13.8 ml, and the intensities of these two peaks were quantitatively the same as those obtained from the separate injections.

In Vitro Stability. The change in absorbance at 485 nm for 4.5 (13.) For Adriamycin itself, the same quenching behavior, which was reduced by the SDS addition, was also observed. Martin (14) reported dimerization of daunomycin (an analogue of Adriamycin) by circular dichroism measurements. Considering his result, the quenching of the fluorescence intensity of Adriamycin itself results from the dimerization or oligomerization of ADR molecules by hydrophobic interaction. The difference in the degree of quenching of the fluorescence intensity between PEG-P(Asp(ADR)) in run 4 and ADR suggests the enhanced quenching is due to the micelle formation for run 4. PEG-P(Asp(ADR)) in run 4 showed 14.9-fold quenching of the fluorescence intensity (without SDS addition) at an ADR concentration of $6.0 \times 10^{-4}$ M, as compared with the intensity with SDS addition, whereas ADR showed only 3.5-fold quenching at $5.7 \times 10^{-4}$ M. More significant quenching of the fluorescence intensity for PEG-P(Asp(ADR)) than for ADR supports the micelle formation of PEG-P(Asp(ADR)).

Plots of fluorescence intensity against concentration of the bound ADR of runs 3–5 with and without the addition of SDS were found to be almost identical but showed a little difference. A run with a lower content of ADR showed a little higher fluorescence intensity. This fact indicates that the content of the bound ADR reflected the packing degree of the bound ADR molecules in the micelles as well as the degree of resistance to the destruction of the micellar structure by the SDS addition.
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PEG-P(Asp(ADR)) (ADR content = 18 mol %; run 1 in Table 1) significantly increased the stability of ADR in PBSa at 37°C, as shown in Fig. 5. The absorbance at 485 nm decreased as the samples were incubated in PBSa. These decreases were greater than those observed for ADR alone or for a mixture of ADR and PEG-P(Asp). As shown in Table 1, the absorbance at 485 nm for these three samples was found to decrease as the samples were incubated in PBSa. The decrease in absorbance at 485 nm for the ADR mixture was almost the same as that for ADR alone, whereas the decrease for the mixture of ADR and PEG-P(Asp) was smaller. These decreases mean destruction of an anthracycline ring of an Adriamycin molecule, possibly by nucleophilic attack of hydroxyl ion, since shift of the peak at 485 nm for all three samples was not observed. Stabilization of Adriamycin by covalent bonding to PEG-P(Asp) was remarkable. For example, ADR retained only 42% of its initial absorbance at 485 nm after a 168-h incubation, whereas the ADR introduce into the block copolymer with covalent bonding [PEG-P(Asp(ADR))] maintained 91% of its initial absorbance after a 168-h incubation. A mixture of ADR and the block copolymer [in the same proportion as that of PEG-P(Asp(ADR))] was also found to improve its stability, as compared with ADR. This stabilization in the mixture is considered to result from localization of ADR molecules around the block copolymer by ionic interaction between amino groups of Adriamycin molecules and carboxyl groups of the block copolymer. Localized hydrophobic ADR molecules are expected to restrict the access of hydrophilic hydroxyl ion. ADR molecules in the mixture, however, were still less stable than the bound ADR of PEG-P(Asp(ADR)).

In Vitro Cytotoxicity. Results are shown in Fig. 6. ADR and a mixture of ADR and PEG-P(Asp) [mixed in the same ratio as that of PEG-P(Asp(ADR))] showed high cytotoxicity, and under these experimental conditions there was no concentration dependence on ADR and the mixture, since even concentrations of these two samples of 0.1 μg ADR-HCl/ml attained almost the maximum cytotoxicity (approximately 50% of the cell number of the control in a 24-h incubation, approximately 20% in a 48-h incubation).

PEG-P(Asp(ADR)) (ADR content = 31 mol %; run 6 in Table 1) showed concentration-dependent cytotoxicity, and the concentration required for maximum cytotoxicity was found to be higher than that for ADR, for both 24-h and 48-h incubations. Cytotoxicity of PEG-P(Asp) is shown in the same figure where the scale of PEG-P(Asp) concentration is adjusted to the ADR concentration in the composition of PEG-P(Asp(ADR)). The cytotoxicity of PEG-P(Asp) itself was negligible for 24 h of incubation and quite low even for 48 h of incubation. This fact indicates that the cytotoxicity of PEG-P(Asp(ADR)) obtained was not based on that of PEG-P(Asp) but based on the cytotoxicity of the bound ADR.

In Vivo Anticancer Activity. Results are summarized in Table 2.
3. Fig. 7 shows plots of $T/C$ versus dose. For Adriamycin, prolonged survival was observed in doses from 1 to 15 mg ADR·HCl/kg, and the maximum value of $T/C$ was obtained at a dose of 15 mg/kg. A dose of 30 mg/kg resulted in a shorter survival time than that of the control because of its acute toxicity. All three runs at 15 mg/kg afforded very high anticancer activity with values of $T/C$ over 300%. These three runs, however, showed a remarkable decrease in body weight on day 5, over 12% of the initial body weights of the mice. This fact indicates that very high anticancer activity against P 388 leukemia was obtained only in the dose range with severe side effects, judged from a significant decrease in body weight.

For PEG-P(Asp(ADR)), doses are expressed in mg of ADR·HCl equivalent contained in PEG-P(Asp(ADR))/kg of mouse body weight. A dose of 15 mg/kg, which brought about the maximum value of $T/C$ for ADR, brought about only 141% $T/C$. However, the value of $T/C$ increased with an increase in dose up to 200 mg/kg. Although a dose of 400 mg/kg showed a slightly smaller value of $T/C$ than that of 200 mg/kg, it still held very high anticancer activity, over 300% $T/C$. A dose of 600 mg/kg resulted in toxic deaths, which were represented in a value of $T/C$ under 100%. These results indicate that PEG-P(Asp(ADR)) expressed very high anticancer activity, similar to ADR, at higher doses than those of ADR. As for the side effects judged from body weight change, PEG-P(Asp(ADR)) gave fewer side effects than ADR. Comparing the result of PEG-P(Asp(ADR)) at 200 mg/kg with that of ADR at 15 mg/kg (both are the optimum doses concerning $T/C$), PEG-P(Asp(ADR)) showed a smaller decrease in body weight (7.4%) than ADR at 15 mg/kg (over 12%). This difference in body weight change becomes clearer when the body weight change is monitored at an interval of a few days. Fig. 8 shows the time course of the average body weight of the mice for PEG-P(Asp(ADR)) at 200 mg/kg and ADR at 15 mg/kg (of control B).

For ADR, body weight showed a minimum on day 5, and it then recovered a little to −7.3% on day 9. After day 9, however, body weight decreased to around 15% and never recovered to the initial value. PEG-P(Asp(ADR)) first showed a decrease, with a minimum body weight on day 2, and recovered to the initial weight on day 9. Then, the body weights of mice treated with PEG-P(Asp(ADR)) never decreased after day 9. This difference concerning body weight change proved that PEG-P(Asp(ADR)) could afford very high anticancer activity with much smaller side effects than ADR.

Table 4 shows a comparison of in vivo anticancer activity between PEG-P(Asp), a mixture of ADR and PEG-P(Asp), and PEG-P(Asp(ADR)). The dose of PEG-P(Asp(ADR)) was 200 mg equivalent ADR·HCl/kg, which afforded the maximum anticancer activity as shown in Table 3. Doses of PEG-P(Asp) and a mixture of PEG-P(Asp) and ADR were adjusted to the equivalent quantities of the components of PEG-P(Asp(ADR)) at 200 mg/kg. Both PEG-P(Asp) and the mixture brought about values of $T/C$ under 100%. This fact indicates that the high anticancer activity obtained in PEG-P(Asp(ADR)) resulted from the anticancer activity of the bound ADR, not from the activity of PEG-P(Asp) or the combined activity of PEG-P(Asp) and unbound ADR.

**DISCUSSION**

The maximum content of bound ADR in PEG-P(Asp(ADR)) was obtained in run 5, where 33 mol % of carboxyl groups in the poly(aspartic acid) chain were substituted with Adriamycin molecules. The value of 33 mol % is remarkably large, considering the strongly hydrophobic character of ADR. Hoes et al. (15) reported a synthesis of ADR-conjugated poly(glutamic acid) homopolymer with maximum substitution of 10 mol % by ADR with respect to glutamic acid units. Tsukada et al. (8) also reported 10 mol % substitution of poly(l-glutamic acid) homopolymer by daunomycin (an analogue of Adriamycin) as a maximum value. PEG-P(Asp(ADR)) synthesized here exceeds the maximum substitution percentages of these two studies. PEG-P(Asp(ADR)) of runs 6 and 7 with approximately 30 mol % substitution by ADR remained water soluble even after concentrating these solutions by ultrafiltration to 20 mg equivalent ADR·HCl/ml. Duncan et al. (16) reported that 2 mg equivalent daunomycin/ml was the critical concentration for precipitation in their conjugate of daunomycin and poly[N-(2-hydroxypropyl)methacrylamide]. On the other hand, an aqueous solution of ADR·HCl at 20 mg/ml became a solid gel after the addition of 0.9% (w/v) NaCl. This gelation was con-

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**Table 3 In vivo anticancer activity against P 388 mouse leukemia of ADR and PEG-P(Asp(ADR))**

Female CDF, mice were inoculated with $1 \times 10^6$ cells i.p. and inoculated with drug [ADR or PEG-P(Asp(ADR))] on day 1. Six mice were included in each group.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dose (mg/kg)*</th>
<th>Control*</th>
<th>Median survival (days)</th>
<th>$T/C$ (%)†</th>
<th>Long survivors‡</th>
<th>Body weight change at day 5 (%)§</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADR</td>
<td>1</td>
<td>E</td>
<td>15.5</td>
<td>178</td>
<td>0/6</td>
<td>−0.2 ± 1.4</td>
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<tr>
<td>ADR</td>
<td>2</td>
<td>D</td>
<td>14.0</td>
<td>163</td>
<td>0/6</td>
<td>+2.9 ± 0.8</td>
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<td>ADR</td>
<td>4</td>
<td>D</td>
<td>14.8</td>
<td>175</td>
<td>0/6</td>
<td>+4.8 ± 1.1</td>
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<tr>
<td>ADR</td>
<td>4</td>
<td>E</td>
<td>15.5</td>
<td>178</td>
<td>0/5</td>
<td>−2.3 ± 0.9</td>
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<tr>
<td>ADR</td>
<td>7.5</td>
<td>A</td>
<td>15.3</td>
<td>191</td>
<td>0/7</td>
<td>+4.4 ± 0.8</td>
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<tr>
<td>ADR</td>
<td>15</td>
<td>A</td>
<td>30.5</td>
<td>381</td>
<td>1/7</td>
<td>−12.5 ± 2.1</td>
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<tr>
<td>ADR</td>
<td>15</td>
<td>B</td>
<td>&gt;59.4</td>
<td>&gt;691</td>
<td>5/7</td>
<td>−16.4 ± 1.4</td>
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<tr>
<td>ADR</td>
<td>15</td>
<td>C</td>
<td>27.5</td>
<td>306</td>
<td>0/6</td>
<td>−15.2 ± 1.3</td>
</tr>
<tr>
<td>ADR</td>
<td>30</td>
<td>A</td>
<td>6.5</td>
<td>82</td>
<td>0/6</td>
<td>−18.7 ± 0.4</td>
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<td>PEG-P(Asp(ADR))</td>
<td>7.5</td>
<td>E</td>
<td>11.7</td>
<td>135</td>
<td>0/6</td>
<td>+1.9 ± 1.0</td>
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<tr>
<td>PEG-P(Asp(ADR))</td>
<td>15</td>
<td>A</td>
<td>11.3</td>
<td>141</td>
<td>0/7</td>
<td>+8.9 ± 1.4</td>
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<tr>
<td>PEG-P(Asp(ADR))</td>
<td>30</td>
<td>A</td>
<td>13.4</td>
<td>168</td>
<td>0/6</td>
<td>+6.2 ± 1.1</td>
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<tr>
<td>PEG-P(Asp(ADR))</td>
<td>80</td>
<td>A</td>
<td>18.0</td>
<td>225</td>
<td>0/6</td>
<td>+6.2 ± 1.2</td>
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<td>PEG-P(Asp(ADR))</td>
<td>120</td>
<td>B</td>
<td>32.5</td>
<td>378</td>
<td>2/6</td>
<td>−5.6 ± 0.8</td>
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<td>PEG-P(Asp(ADR))</td>
<td>200</td>
<td>B</td>
<td>&gt;42.0</td>
<td>&gt;488</td>
<td>3/6</td>
<td>−7.5 ± 0.9</td>
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<td>PEG-P(Asp(ADR))</td>
<td>400</td>
<td>C</td>
<td>33.0</td>
<td>367</td>
<td>1/6</td>
<td>−4.6 ± 1.5</td>
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<td>PEG-P(Asp(ADR))</td>
<td>600</td>
<td>C</td>
<td>7.5</td>
<td>83</td>
<td>0/6</td>
<td>−17.5 ± 1.0</td>
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</table>

* Expressed in ADR·HCl equivalent/kg of body weight.
† Median survival: A, 8.0; B, 8.6; C, 9.0; D, 8.6; E, 8.7 days.
‡ Median survival day of sample × 100 (%).
§ Median survival day of control.
" Long survivors are included as long survivors.
* Expressed in the mean ± SE; initial mean body weight = 20-21 g.
runs, shown in Table 3, are plotted as the mean value of all the runs. In ADR-HCI equivalents. G, ADR; •¿, PEG-P(Asp(ADR)). Doses with multiple solution, on day 1, in a volume of 0.1 ml/10 g of body weight. Doses are plotted (mL) on day 0. The mice were i.p. inoculated with drug dissolved in a 0.9cc NaCI solution, approximately 50 nm) corresponds to a range of the size of single unilamellar vesicles of liposomes. Although liposomes have been studied as a drug carrier for many years, most examinations of targeting using liposomes have been not very successful, owing to the structural fragility of liposomes (especially for single unilamellar vesicles) and nonselective capture of liposomes by the reticuloendothelial cells. Because polymeric micelles are considered to be structurally stronger than liposomes, PEG-P(Asp(ADR)) can be a promising alternative candidate for the carrier used in drug targeting. The unimodal distribution of diameter of the micelles indicates that the micelles of PEG-P(Asp(ADR)) were free from intermicellar aggregation, and this distribution is very narrow, possibly owing to the use of the well designed block copolymer. Furthermore, linking of the PEG chain is a promising way to inhibit the nonselective uptake by the reticuloendothelial cells, as speculated from the study on PEG-modified enzymes (18).

The micelle-forming character of PEG-P(Asp(ADR)) is considered to contribute to the drastic changes in binding affinity for BSA as well as in stability in PBSa, since the micelle structure is considered to inhibit the access of BSA (complete inhibition) and OH ion (partial inhibition) to the bound ADR molecules by packing the ADR molecules tightly inside a hydrophobic core of the micelles. Considering the importance of albumin binding for pharmacokinetics of drugs, it is very interesting to diminish the binding affinity of ADR for BSA drastically by binding ADR to the block copolymer. And this is the first example of ADR stabilization through binding to polymeric carriers. Stability of drugs in plasma is an important factor for effective chemotherapy, especially for Adriamycin. The stability is a critical problem for ADR since Adriamycin was reported (19) to be metabolized very rapidly to inactive derivatives mainly by liver after an injection into the blood stream. Lisi et al. (18) reported that accumulation of β-glucuronidase in hepatic tissues was reduced by PEG modification. Therefore, the micellar structure of PEG-P(Asp(ADR)) with the PEG chain in the outer shell is a promising form to bring about resistance of Adriamycin to metabolism.

The results of in vitro cytotoxicity and in vivo anticancer activity against P 388 mouse leukemia showed that the polymeric drug required a greater amount of ADR molecules than intact ADR to obtain the activity equal to intact ADR and that in vivo very high anticancer activity was obtained for PEG-P(Asp(ADR)) with a smaller body weight loss than that of free ADR. The release rate of ADR from the micelle drug is expected to be very slow, because there is no spacer such as

Table 4 Comparison of in vivo anticancer activity against P 388 mouse leukemia of PEG-P(Asp), PEG-P(Asp) plus ADR (mixture of block copolymer and drug), and PEG-P(Asp(ADR)) (drug-conjugated block copolymer)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Median survival (days)</th>
<th>T/C (%)</th>
<th>Long survivors</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG-P(Asp)</td>
<td>7.7</td>
<td>90</td>
<td>0/6</td>
</tr>
<tr>
<td>PEG-P(Asp) + ADR</td>
<td>2.7</td>
<td>31</td>
<td>0/6</td>
</tr>
<tr>
<td>PEG-P(Asp(ADR))</td>
<td>&gt;47.0</td>
<td>&gt;547</td>
<td>3/6</td>
</tr>
</tbody>
</table>

*Control, 8.6 days.
† Median survival day of sample.
‡ Median survival day of control.
§ 60-day survivors are included as long survivors.
δ ADR was dissolved in H2O.

considered to result from self-association of Adriamycin molecules under a high ionic strength, as reported for daunomycin by Martin (14). No such gelation was observed for our PEG-P(Asp(ADR)) system even at the ADR concentration of 20 mg ADR-HCl equivalents/ml. Furthermore, runs 3-5 were able to be redissolved in water after lyophilization. All these results indicate that PEG-P(Asp(ADR)) has excellent water solubility and stability against precipitation. These properties were provided by the poly(ethylene glycol) chain linked to the poly(aspartic acid) chain, since a precipitate was produced during the introduction reaction of ADR when poly(aspartic acid) homopolymer was used instead of PEG-P(Asp) under the same reaction conditions as those of a synthesis of PEG-P(Asp(ADR)). These facts demonstrate one of the advantageous features of the block copolymer drug carrier. And this excellent water solubility and stability against precipitation are thought not to result only from the increased hydrophilicity by the linked PEG chain but to result from the micelle formation. The PEG chain is expected to exist as the outer shell of the micelle, and this hydrophilic outer shell is supposed to suppress the intermicellar aggregation of the hydrophobic P(Asp(ADR)) chain.

Bader et al. (17) proposed a micelle-forming polymeric drug composed of cyclophosphamide-conjugated poly(ethylene glycol)-poly(L-lysine) block copolymer derivative. They obtained sustained release of cyclophosphamide from the block copolymer. In their system, however, introduction of long alkyl chains to the poly(L-lysine) chain was needed to add hydrophobicity for micelle formation. Although they suggested possible micelle formation, there was no direct evidence for micelle formation. Our study is the first example of a micelle-forming polymeric drug with definite diameter. The diameter of the micelle (approximately 50 nm) corresponds to a range of the size of single unilamellar vesicles of liposomes. Although liposomes have been studied as a drug carrier for many years, most examinations of targeting using liposomes have been not very successful, owing to the structural fragility of liposomes (especially for single unilamellar vesicles) and nonselective capture of liposomes by the reticuloendothelial cells. Because polymeric micelles are considered to be structurally stronger than liposomes, PEG-P(Asp(ADR)) can be a promising alternative candidate for the carrier used in drug targeting. The unimodal distribution of diameter of the micelles indicates that the micelles of PEG-P(Asp(ADR)) were free from intermicellar aggregation, and this distribution is very narrow, possibly owing to the use of the well designed block copolymer. Furthermore, linking of the PEG chain is a promising way to inhibit the nonselective uptake by the reticuloendothelial cells, as speculated from the study on PEG-modified enzymes (18).

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oligopeptides (20) between ADR and the poly(aspartic acid) chain and because main chain cleavage of poly(aspartic acid) by proteolytic enzymes is thought to be very slow, as reported (21, 22). There is a possibility that the requirement for a large quantity of bound ADR molecules reflected the slow action of the polymeric drug due to slow release of ADR molecules. On the other hand, Wingard et al. (23) reported that ADR chemically bound to an insoluble gel could exhibit cytotoxicity by directly interacting with cell membranes without being taken up by the cells and, therefore, there is another possibility, that the polymeric drug PEG-P(Asp(ADR)) showed cytotoxic activity to P 388 without releasing intact ADR molecules.

Although the mechanism of action of the polymeric drug is still unknown, this paper reports the important example of the polymeric drug showing high in vivo anticancer activity with greatly improved body weight loss, as compared with intact ADR.

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REFERENCES

Characterization and Anticancer Activity of the Micelle-forming Polymeric Anticancer Drug Adriamycin-conjugated Poly(ethylene glycol)-Poly(aspartic acid) Block Copolymer

Masayuki Yokoyama, Mizue Miyauchi, Noriko Yamada, et al.


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