J.Pharm.Dyn., **7**, 688–698 (1984)

ANTITUMOR AGENT POLY (AMINO ACID) CONJUGATES AS A DRUG CARRIER IN CANCER CHEMOTHERAPY

YASUNORI MORIMOTO,* KENJI SUGIBAYASHI, SATOSHI SUGIHARA, KEN-ICHI HOSOYA, SUKEKATSU NOZAKI AND YOSHIHIRO OGAWA**

*Faculty of Pharmaceutical Sciences, Josai University, 1-1 Keyakidai, Sakado, Saitama, 350-02, Japan and Department of Neuropathology, Tokyo Metropolitan Institute for Neurosciences,** 2-6 Musashidai, Fuchu, Tokyo, 183, Japan*

(Received April 20, 1984)

Antitumor agent melphalan was conjugated through a carbodiimide-catalyzed reaction to poly (L-lysine) (71.3K and 2K) and poly (L-glutamic acid) (60K and 14K) at a ratio of approximately one molecule per 7-lysyl and 23-glutamate residues, respectively. These conjugates had 40–70% of alkylating activities by themselves *in vitro* as compared with free melphalan. Poly (glutamic acid) conjugates showed the antitumor activity *in vivo* against Yoshida sarcoma in rats. In addition, poly (glutamic acid) conjugates containing ^{3}H-phenylalanine which was used as a model compound instead of melphalan had a sustained release property of radioactivity and the release rates could be regulated by exopeptidases. After subcutaneous injection as the first choice of routes of administration, moreover, the conjugates were found to be absorbed through the lymphatic transport system, probably due to the macromolecularity. These results suggest that the melphalan-poly (amino acid) conjugates are one of good candidates to attain the sustained release and targeting of drugs in cancer chemotherapy.

Keywords —melphalan; poly (lysine); poly (glutamic acid); poly (amino acid); drug-carrier conjugate; release; antitumor activity; lymph concentration; drug delivery system

INTRODUCTION

In order to attain the maximum antitumor effect of chemotherapeutic agents with less side effects, drug carrier complexes may be a good drug delivery system which can deliver the containing agents into a specific target site and sustain the drug release over a predetermined term in cancer chemotherapy. There are two kinds of approaches for preparing the drug carriers; one is physical and pharmacuetical modifications such as albumin microspheres,[1-3] liposomes,[4, 5] desoxyribonucleic acid (DNA)-complexes[6] and emulsions[7, 8] and the another is chemical modifications between antitumor agents and macromolecules such as melanotropin,[9] concanavalin A,[10] antibody[11] and other natural and synthetic polymers.[12,13] Most of investigations regarding such drug carriers, however, are still under preliminary study especially for chemical drug carriers.

In this paper, we selected melphalan (MPL) and poly (amino acid) as a model antitumor agent and biodegradable polymer to study several physicochemicl properties and drug carrier properties of drug-polymer conjugates. Poly (L-lysine) and poly (L-glutamic acid) were selected as typical cationic and anionic poly (amino acid), respectively.

MATERIALS AND METHODS

Chemicals —MPL was kindly supplied from Nippon Wellcome Co., Ltd. (Minoo, Osaka,

* To whom correspondence should be addressed.

Japan). Poly (L-lysine) (weight average molecular weight, 71.3K, 2K) and poly (L-glutamic acid) (60K, 14K) were purchased from Sigma Chemical Co. (St. Louis, MO). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride [water soluble carbodiimide, EDC] (Nakarai Chemicals, Ltd., Kyoto, Japan), 1-hydroxybenzotriazol [HOBT] (Protein Research Foundation, Minoo) and 2-*tert*-butoxycarbonyloxyimino-2-phenyl-acetonitrile [Boc-ON] (Protein Research Foundation) were used as reagents for the conjugation. γ-(*p*-Nitrobenzyl) pyridine [NBP] (Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan), dimethylsulfoxide (Wako Pure Chemical Industries, Ltd., Osaka), ninhydrin mixed reagent [0.5 ml of 1% ninhydrin solution in 0.5 M citrate buffer (pH 5.5), 1.2 ml of glycerol and 0.2 ml of 0.5 M citrate buffer (pH 5.5)][14] and phenol-sodium nitroprusside solution [1 g of phenol and 5 mg of sodium nitroprusside in 100 ml of water] were used for determination of substitution ratio of MPL onto poly (amino acid) residues. L-[ring-2,6-^{3}H (N)]-Phenylalanine [^{3}H-Phe] (specific activity 48.3 Ci/mmol) (New England Nuclear, Boston, MS) was selected as a model compound instead of radiolabeled MPL because of its difficult availability. Exopeptidases, carboxypeptidase A (E.C. 3,4,12,2, from bovine pancreas) and leusine aminopeptidase, cytosol (E.C. 3,4,11,1, from porcine kidney) were purchased from Sigma Chemical Co. All other chemicals were reagent grade quality and obtained commercially.

Preparation MPL-Poly (Amino Acid) Conjugates — First step for preparation of MPL-poly (L-lysine) conjugates was the protection of amino-group of MPL with Boc-group. The methods are as follows. To a solution of MPL powder (2.5 mmol) and triethylamine (3.75 mmol) in N, N-dimethylformamide [DMF]-water (1:1, 2.5 ml), Boc-ON (2.75 mmol) was added and the mixture was stirred with a magnetic stirrer (200—300 rpm) at room temperature over night. The reaction mixture was diluted with 5 ml of water and was washed 2—3 times with 2.5 ml of ethyl acetate. The pH of the aqueous layer was adjusted to 2 with 0.1 N HCl to give oily drops, which was extracted by ethyl acetate and washed with water. Then, the organic layer was dried with a rotary evaporator (Type N-1, Tokyo Rikakikai Co., Ltd).

Second step for preparation of MPL-poly (lysine) conjugates was the coupling reaction between Boc-MPL and poly (L-lysine). Boc-MPL (0.25 mmol) as prepared above, HOBT (0.25 mmol), EDC (0.25 mmol) and poly (L-lysine) (32 mg) were mixed in 2.5 ml of DMF-water (1:1) solution and agitated with a magnetic stirrer over night. After evaporation of DMF under reduced pressure at 40°C, 25 μl of 12 N HCl was added and the reaction vial was kept in ice for 5 min. After addition of 300 μl of 1 N $NaHCO_3$, the resultant precipitation was purified by dialysis in water for 3 d.

We modified the preparation method of *p*-phenylenediamine mustard-poly (glutamic acid) by Rowland *et al.*[11] for synthesis of MPL-poly (glutamic acid) conjugates. Four ml of mixed solution containing MPL (66 μ mol), poly (L-glutamic acid) (50 mg) and EDC (125 mg) were stirred over night. Then the conjugates were isolated by dialysis. MPL-poly (amino acid) conjugates prepared as mentioned above were kept as a powder at −15°C.

Determination of Substitution Ratio of MPL onto Poly (Amino Acid) Conjugates — Two mg of conjugates were hydrolyzed to free MPL and amino acids with 6N HCl at 110°C for 24 h in the usual way. After removal of the acid, the amount of hydrolysate was reconstituted with 2 ml of water, and then MPL, total amino acid and impurity ammonia were determined by NBP method,[15] ninhydrin reaction[14] and indophenol method,[16] respectively. Since ninhydrin reaction was sensitive against MPL and ammonia also, the amount of amino acid residue unreacted was estimated from the value for the ninhydrin reaction (MPL+amino acid+ammonia) minus the values for NBP method (MPL) and indophenol method (ammonia).

Estimation of Alkylating Activity of MPL-Poly (Amino Acid) — Alkylating activity due to the

conjugates themselves was estimated by the modified method of Skibba *et al.*[15] using unhydrolyzed MPL-poly (amino acid).

Measurement of the in Vitro Radioactivity Release from ^{3}H-Phenylalanine-Poly (Amino Acid) Conjugates — *In vitro* release of radioactivity was determined by an equilibrium dialysis method with a cellulose tubing (24/36, Visking Cellulose Tubing, Union Carbide Corp., Chicago, IL). ^{3}H-Phe-poly (L-glutamic acid) conjugates were prepared with ^{3}H-Phe instead of MPL by the method as shown above. About 1 μCi of free or poly (glutamic acid) conjugated ^{3}H-Phe was dissolved in 2 ml of phosphate buffer saline (pH 7.4) and put into a cellulose tubing. The tubing containing the conjugate solution was placed in a 20 ml vial containing 10 ml of the same buffer. The effects of exopeptidases on the *in vitro* release were determined by adding 50 units of carboxypeptidase A or leusine aminopeptidase to the inner solution. The whole system was shaken by an incubator at a rate of 100 strokes/min at 37°C. At appropriate intervals, aliquots of 1 ml were withdrawn from the outer solution and 1 ml of the same buffer was added to keep the volume constant. The radioactivity was measured by a liquid scintillation counter (LSC-700, Aloka Co., Ltd., Mitaka, Tokyo).

Measurement of Antitumor Effect of MPL-Poly (Amino Acid) Conjugates — Male Donryu rats, weighing about 130 g, and Yoshida sarcoma were selected in the experiments to measure the antitumor effects of the drug-carrier conjugates. Yoshida sarcoma cells (1×10^6 /1.0 ml) were implanted subcutaneously into the scapular region of rats.

In order to measure the antitumor effect, 0.1 ml of free or poly (glutamic acid) conjugated (60K, 14K) MPL (containing 50 μg MPL each) or same volume of saline were injected directly into the tumor site on day 4 after tumor inoculation (10 rats for each group). Daily measurement of tumor size was made with callipers and the volume of the tumor was evaluated by the method of Geran *et al.*[17] Antitumor effects after multiple-shot administration were measured with the same method except that the treatments were carried out three times on day 4, 5 and 6.

Determination of Absorption Profiles after Subcutaneous Administration of ^{3}H-Phe-Poly (Amino Acid) Conjugates — Male Wistar rats, weighing between 250 and 280 g, were used in this experiments. After each rat was anesthetized with sodium pentobarbital at a dose of 60 mg/kg *i.p.*, 10 μCi of free or poly (glutamic acid) conjugated (60K, 14K) ^{3}H-Phe were injected subcutaneously into the left femur of rats. Dose for free ^{3}H-Phe, 60K and 14K poly (glutamic acid) conjugates was calculated as 0.342, 4.83 and 5.88 mg, respectively, from each specific radioactivity and total radioactivity for dosage form. Each rat was sacrified at appropriate intervals, and the injection site (skin and hypodermis having a diameter of about 2 cm), left (regional) and right (non-regional) inguinal lymph nodes and blood were collected. In addition the thoracic lymph was collected by the method of Bollman *et al.*[18] Radioactivities in the biological samples were measured by a liquid scintillation counter after combustion with a sample oxidizer (ASC-111, Aloka).

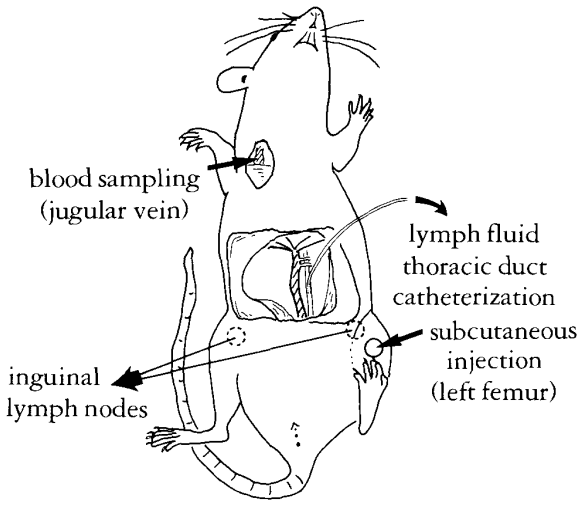

FIG. 1. *Schematic Diagram of Injection Site and Sampling Parts for in Vivo Absorption Experiments of ^{3}H-Phe-Poly(Glutamic Acid) Conjugates*

Schematic diagram of the injection site, left and right inguinal nodes and blood sampling part (jugalar vein) in the rat was shown in Fig. 1. Fig. 1 also shows the part for the thoracic duct catheterization.

RESULTS

MPL Ratio and Alkylating Activity of Conjugated MPL

Table I summarized the substitution ratio of MPL onto poly (amino acid) conjugates and

TABLE I. *MPL Ratio and Alkylating Activity of Conjugated MPL*

	MPL-poly(lysine) conjugates		MPL-poly(glutamic acid) conjugates	
	71.3K	2K	60K	14K
Number of amino acid residue for substitution of a molecule of MPL[a)]	6.7	6.9	23.0	23.1
Number of MPL molecule substituted on a poly (amino acid) molecule[b)]	83.0	2.3	20.2	4.7
% MPL in weight[c)]	25.1	24.6	9.3	9.3
% alkylating activity	69.4	64.0	42.0	48.7

a) Number of amino acid residue required for conjugation of a molecule of MPL. These numbers were measured after hydrolysis of MPL-poly(amino acid) conjugates.

b) For the case of poly(lysine);

$$\frac{(MW_{pl}-18)}{(MW_{l}-18)\cdot A}$$

For the case of poly(glutamic acid);

$$\frac{(MW_{pg}-18)}{(MW_{g}-18)\cdot A}$$

where MW_{pl}, MW_{pg} MW_{l} and MW_{g} mean molecular weight of poly(lysine), poly(glutamic acid), lysine and glutamic acid, respectively. "A" means number of amino acid residue for substitution of a MPL molecule as shown in upper column. "18" is the molecular weight of water.

c) For the case of MPL-poly(lysine) conjugates;

$$\frac{(MW_{mpl}-17)\cdot N}{(MW_{pl}+MW_{mpl}\cdot N)-18\cdot N}\times 100$$

For the case of MPL-poly(glutamic acid) conjugates;

$$\frac{(MW_{mpl}-1)\cdot N}{(MW_{pg}+MW_{mpl}\cdot N)-18\cdot N}\times 100$$

where MW_{mpl} means the molecular weight of MPL, and "17" and "1" mean loss in weight of MPL molecule during dehydration and condensation between MPL and amino acid residue (OH and H, respectively).

their alkylating activity *in vitro*. Each number of amino acid residues for substitution of one molecule of MPL was determined as 7 and 23 for poly (lysine) and poly (glutamic acid) conjugates, respectively, from MPL, total amino acid and ammonia concentrations measured by NBP, ninhydrin and indophenol methods. From those values, on the other hand, numbers of MPL molecules substituted on one molecule of poly (amino acid) were calculated as 83.0, 2.3, 20.2 and 4.7 for 71.3K and 2K poly (lysine) and 60K and 14K poly (glutamic acid) conjugates, respectively (see calculation method in Table I). Percent MPL in weight was also calculated (Table I) and the numbers were about 25 and 10% for poly (lysine) and poly (glutamic acid), respectively. There was no effect of the molecular weight of poly (amino acid) on the % MPL in weight and number of amino acid residue for substitution of a molecule of MPL.

Alkylating activity of the conjugates was measured by NBP reaction *in vitro* and the values were 40—70% of free MPL. The activity of poly (lysine) conjugates was slightly bigger than that of poly (glutamic acid). Since MPL-poly (lysine) conjugates were practically insoluble in water, however, only poly (glutamic acid) conjugates were used for the following experiments.

In Vitro Release of Radioactivity from ^{3}*H-Phe-*

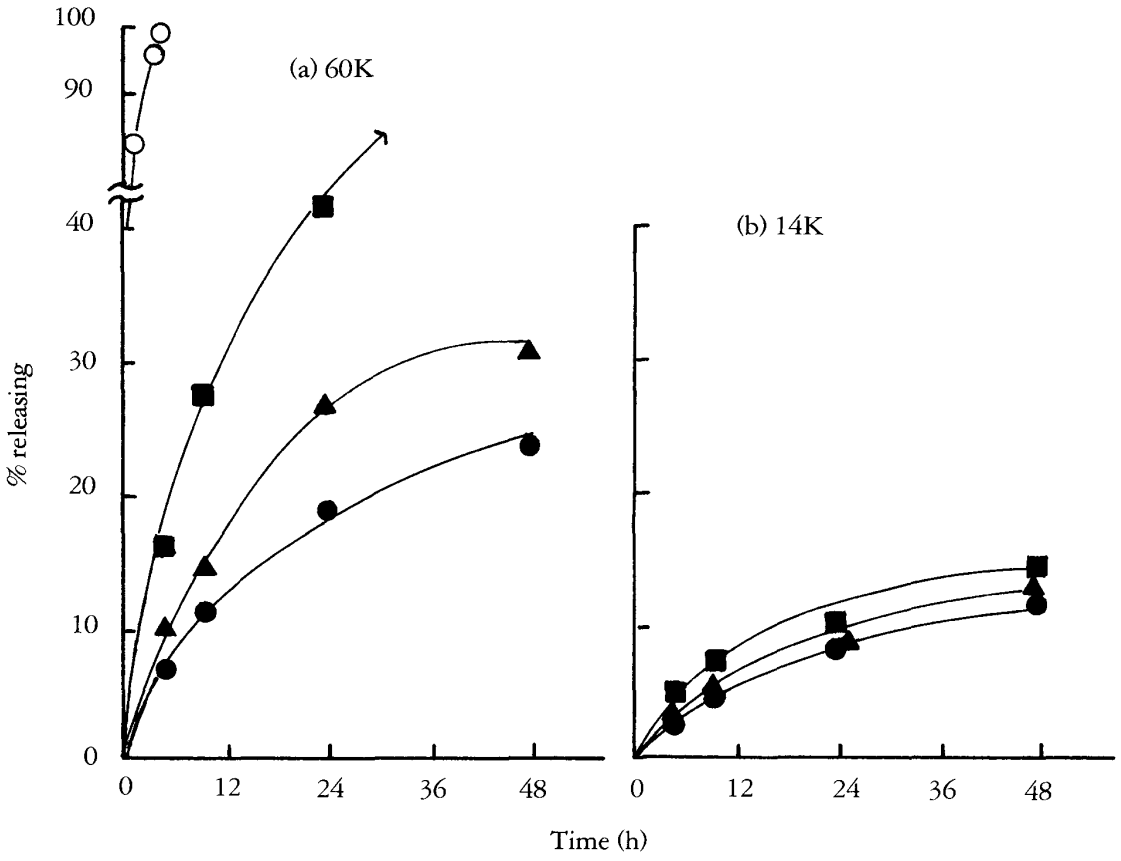

FIG. 2. *In Vitro Release of Radioactivity from* ^{3}*H-Phe-Poly (L-Glutamic Acid) Conjugates with and without Exopeptidases*

(○) free ^{3}*H-Phe; (●)* ^{3}*H-Phe-poly (L-glutamic acid) alone; (■) with carboxypeptidase A; (▲) with leusine aminopeptidase.*

Each point represents the mean of three experiments.

Poly (Amino Acid) Conjugates

MPL-poly (amino acid) are thought to degrade to smaller peptides in the aqueous solution and also in the human bodies. The smaller peptides would become oligomers, tri- and di-peptides and finally free drug. It is too complicated to separate and determine the each level of several components such as free MPL, and MPL-mono-, -di-, -tri-, and -oligo-amino acid conjugates. Since it is also difficult to get radiolabeled MPL easily and cheaply, we use ^{3}H-Phe because of its similar molecular structure to MPL.

Fig. 2 shows time course of the cumulative amount of radioactivity passed through a cellulose membrane after ^{3}H-Phe-poly (glutamic acid) conjugates were put into the inside of the membrane tubing with and without exopeptidases. Free Phe was permeated through the membrane and reached equilibrium within 5 h. In contrast, about 20 and 10% of radioactivities passed through the membrane at 24 h for 60 and 14K of ^{3}H-Phe-poly (glutamic acid) conjugates, respectively. Although both conjugates showed the sustained release (degradation), the release rate of 60K conjugates was about 2 times higher than 14K. Carboxypeptidase, which can successively hydrolyze off carboxy-terminal residues from peptides, promoted the release (digestion) rate about 2 times. Aminopeptidase, which can remove amino-terminal residues successively, also promoted the release rates, but the effects were smaller than the carboxypeptidase.

Antitumor Effect of MPL-Poly (L-Glutamic Acid) Conjugates on Yoshida Sarcoma

Fig. 3 shows the effects of single-shot or multiple-shot administration of free and poly (L-glutamic acid)-conjugated MPL on the growth of Yoshida sarcoma. Treatments of free MPL and co-administration of free MPL and poly (glutamic acid) had no suppression effect as compared with control groups. On the other

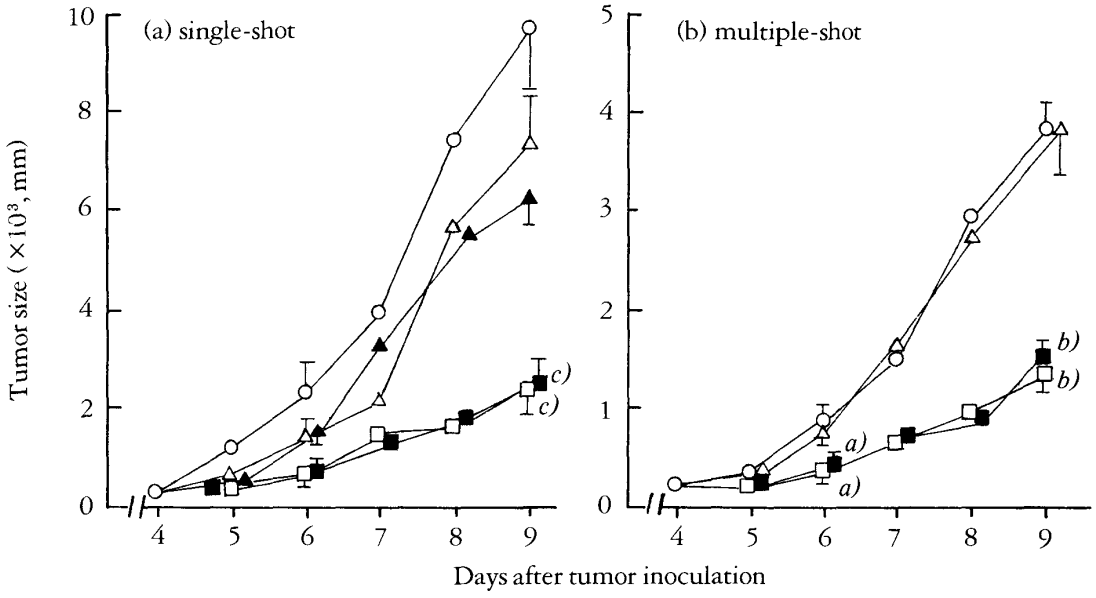

FIG. 3. *Effect of Single-Shot and Multiple-Shot Administrations of Free or Conjugated MPL on the Growth of Yoshida Sarcoma*

Each rat was subcutaneously injected with 0.1 ml of free MPL (△), poly(glutamic acid) conjugated [60K (□), 14K (■)] MPL, mixture of free MPL and poly(glutamic acid) (14K) (▲), or saline (○) into the tumor site on day 4 after inoculation. Dose for MPL was 50 μg/rat.
Student's t-test: a) $p < 0.05$; b) $p < 0.02$; c) $p < 0.001$.
Each point represents the mean (± S.E.) of ten experiments.

hand, 14 K and 60 K of MPL-poly (glutamic acid) conjugates had suppression effects on the growth of Yoshida sarcoma and the volumes of the tumor on day 9 were about one third (multiple-shot) or one fourth (single-shot) as compared with each control group. In addition, these antitumor effects on Day 9 were significant from each control (Student's t-test).

Fig. 4 is a photograph of the tumor section on day 6 after tumor inoculation. The rat was administered with 14K of MPL-poly (glutamic acid) on day 4, 5 and 6. Left side of the photo shows the proliferous tumor tissues in the subcutaneous part. On the other hand, the right side shows vacuolation of the cytoplasm and aggregations of the chromatin in the nuclei of tumor cells (the boundaries of the nuclei are obscure), which suggests the disintegration of tumor cells and the resulting antitumor potential of MPL-poly (glutamic acid) conjugates. But such vacuolation of the cytoplasm and aggregations of the chromatin were also observed in the tumor sections after administration of free MPL. From this histological examinations, therefore, quantitative differences in the antitumor effects between free and conjugated MPL were not found.

Absorption Profiles after Subcutaneous Administration of ^{3}H-Phe-Poly (Glutamic Acid) Conjugates

Since the results as shown above suggest that MPL-poly (glutamic acid) conjugates would have the possibility for sustained release of MPL and antitumor effects, absorption profiles after subcutaneous administration into the left femur were measured in order to determine the lymph transport of the conjugates for a next step. In this experiments, we used ^{3}H-Phe also instead of MPL.

Fig. 5 shows the clearance of ^{3}H-radioactivities from the injection site following administration of free and conjugated (14K, 60K) ^{3}H-Phe into the left femur in rats. As shown in the figure, 2—6 times differences of radioactivity were found between the free and conjugated groups in the initial stage after injection. The dif-

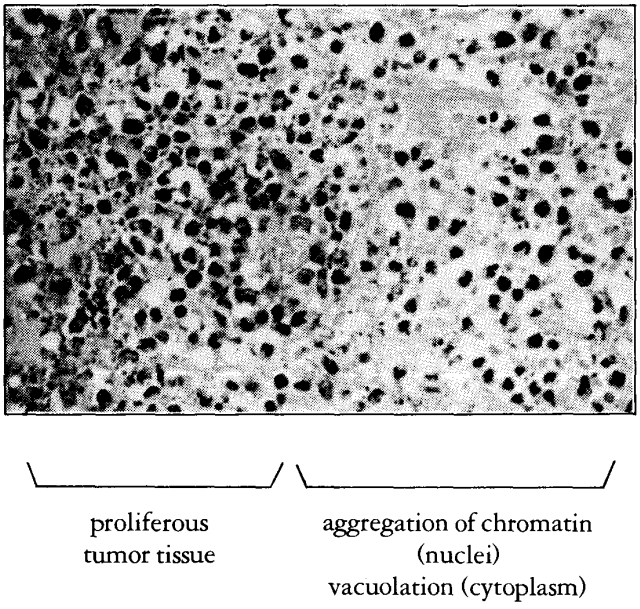

FIG. 4. *Microphotograph of Yoshida Sarcoma in the Scapular Region*
The exised tumor tissues were fixed in 10% formalin and stained with hematoxylin and eosin. × 400.

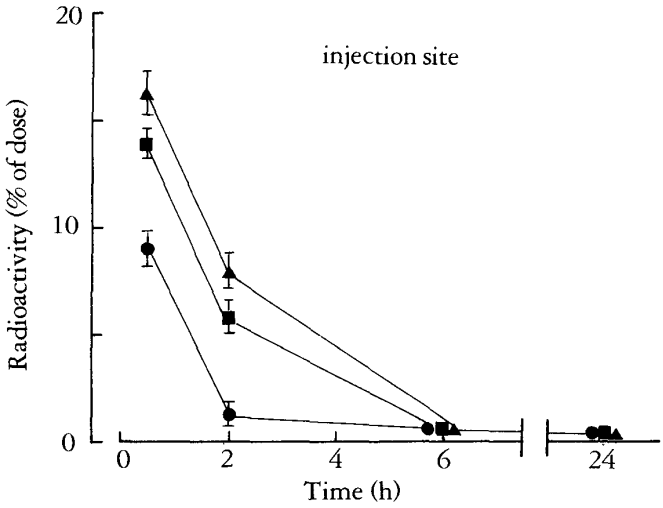

FIG. 5. *Clearance of Free and Conjugated ^{3}H-Phe from the Subcutaneous Injection Site*
(●) free ^{3}H-Phe; (■) ^{3}H-Phe-poly (L-glutamic acid) conjugates (60K); (▲) ^{3}H-Phe-poly (L-glutamic acid) conjugates (14K). Each point represents the mean ± S.E. of 3—5 experiments.

ference, however, became negligibly small after 6 h. The comparably rapid disappearance of radioactivity from the site of administration may be due to the rapid distribution of the macromolecule antitumor agents and/or degradation of the conjugates to the smaller peptides.

Fig. 6a and b show the time course of radioactivity in the left (regional) and right (non-regional) inguinal lymph nodes. When free ^{3}H-Phe was injected, radioactivity in the regional node decreased slowly with time; whereas the level in the non-regional node increased slowly and reached the same value as in the regional node at 24 h. On the other hand, 5—20 times differences between the radioactivities in the right and left inguinal lymph nodes were observed for 0—6 h after injection of conjugated ^{3}H-Phe. These results suggest the existence of the lymph transport of poly (glutamic acid) conjugates.

Fig. 7 shows the blood concentration of radioactivity after subcutaneous injection of free or conjugates ^{3}H-Phe in rats. The radioactivity after injection of free ^{3}H-Phe was much higher than that of both (14K, 60K) conjugates, which suggests the importance of lymphatic transport for the poly (glutamic acid) absorption from the hypodermis.

Time course of radioactivity in the thoracic duct lymph in rats following subcutaneous injection is shown in Fig. 8. The concentration of radioactivity reached the plateau after 2 h following injection of both conjugates. Radioactivity in the thoracic duct lymph following ^{3}H-Phe injection was smaller than that after injection of both conjugates (14K, 60K). But the difference became smaller with time.

These results as shown in Figs. 5—8 suggest that the drug-poly (glutamic acid) conjugates would have a tendency to be transported through the lymphatic system. This finding was probably explained by the macromolecularity of the conjugates, but there was no significant difference between 14K and 60K conjugates.

DISCUSSION

There are two kinds of polymeric (macromolecular) antitumor agents. Polymers which have antitumor effects by themselves come under one category. Another is polymer-antitumor agents which have therapeutic effects after digestion of polymers and/or release of the antitumor agents. MPL-poly (amino acid) had 40—70% of alkylating activities by itself and surely has antitumor effects also after polymer-digestion and

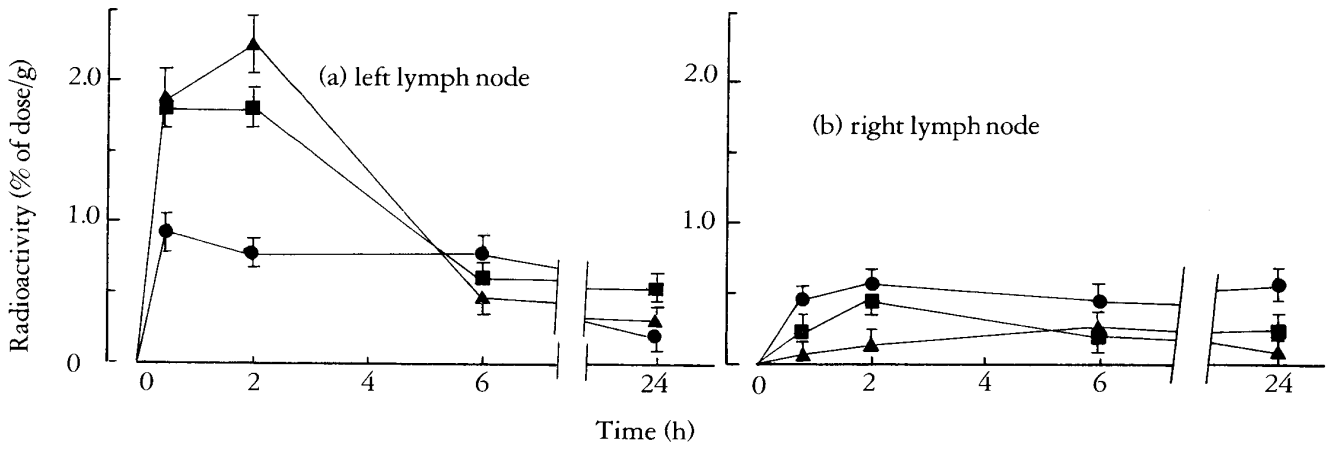

FIG. 6. *Left (Regional) and Right (Non-Regional) Lymph Node Concentrations of Radioactivity after Injection of Free and Conjugated ^{3}H-Phe*
Symbols: same in Fig. 5.
Each point represents the mean ± S.E. of 3—5 experiments.

MPL-release. In addition, some poly (cation)s and poly (anion)s have been found to show some extents of antitumor effects,[19, 20] especially poly (L-lysine).[21] In this respect, MPL-poly (glutamic acid) and -poly-(lysine) may come under the third category of polymeric antitumor agents.

Table I shows the large differences in the substitution ratios of MPL and its weight ratios between MPL-poly (lysine) and -poly(glutamic acid) conjugates. This difference may be due to the protection procedure of amino group of MPL with Boc-group during the preparation of MPL-poly (lysine). Therefore, the low MPL ratios in poly (glutamic acid) may be improved by the modification of synthesis method, *i.e.* protection of carboxylic acid of MPL before peptide synthesis. Since alkylating activity of MPL-poly (amino acid) conjugates would be dependent on the bis (2-chloroethyl) animo group of MPL,[22] hinder effects (stereochemical obstructions of the antitumor moiety) and ionic environments between the antitumor active moiety and alkyl-groups might affect the alkylating activities and antitumor effects of these conjugates. For the quantitative discussion, however, we should wait to get more experimental evidences.

When MPL- and ^{3}H-Phe-poly (lysine) conjugates were dried once by an evaporator, they became very difficult to solubilize in aqueous solutions because of their low wetting property. Same phenomenon was found in ^{3}H-Phe-14K poly (glutamic acid) conjugates although their solubility was much higher than ^{3}H-Phe-poly (lysine) conjugates. Slow release rate of radioactivity from the 14K conjugates, as shown in Fig. 2, might be explained by this occurrence. In contrast, 60K poly (glutamic acid) conjugates shows 25% release over 48 h and the release rate was 2—3 times higher than 14K conjugates. γ-Carboxylic acids of glutamate residues and a terminal α-carboxylic acid of poly (glutamic acid) are supposed for the binding sites of poly (glutamic acid) with MPL. Effects of carboxypeptidase should be larger than aminopeptidase which can remove amino-terminal residues successively, since carboxypeptidase which can hydrolyze successively carboxy-terminal off could release MPL (Phe) with only one cutting of peptide bonds. In contrast, aminopeptidase needs several cuttings of peptide bonds to release MPL

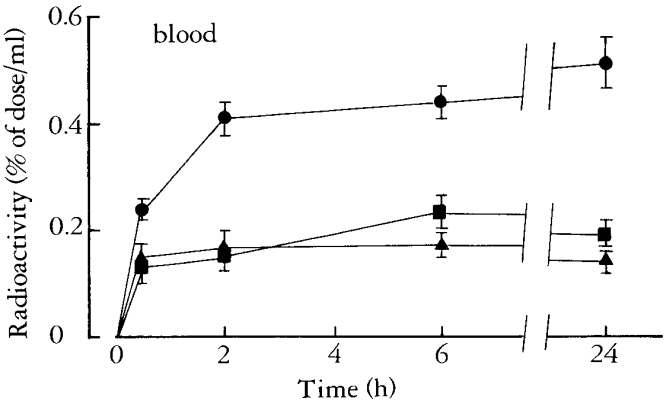

FIG. 7. *Blood Concentration of Radioactivity after Injection of Free and Conjugated ^{3}H-Phe*
Symbols: same in Fig. 5.
Each point represents the mean ± S.E. of 3—5 experiments.

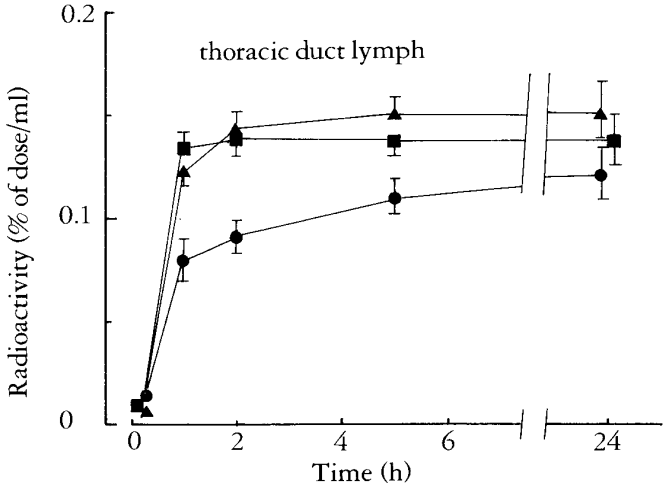

FIG. 8. *Thoracic Duct Concentration of Radioactivity after Injection of Free and Conjugated ^{3}H-Phe*
Symbols: same in Fig. 5.
Each point represents the mean ± S.E. of 3—5 experiments.

(Phe). *In vitro* release profiles with the exopeptidases were consistent with the predicted order of the release rates. These experimental findings suggest that poly (glutamic acid) conjugates would have a potential to sustain the binging antitumor agent MPL and that the release rate would be regulated with some exopeptidases.

Alkylating activity (Table I) and antitumor activity against Yoshida experimental tumor (Figs. 3 and 4) are promissing future developments of antitumor agents-poly (amino acid) conjugates as one of the chemical drug delivery systems although many improvements should be needed for the preparation method and their structures.

MPL is now used as an injective form and a tablet for intravenous injection and oral administration, respectively, in hospitals. But MPL is unstable in aqeous solutions and its injection should be used immediately after dissolving MPL powder in the fitting alcohol-acid solution. In addition, the absorption of MPL after oral administration is variable and in some patients it is poorly absorbed. Therefore dosage may need to be cautiously increased until myelosuppression is seen, in order to ensure that potentially therapeutic levels have been reached. Since MPL should need several pharmaceutical modification as mentioned above, poly (amino acid) conjugates might take a role instead of current preparations.

In this experiment, we measured the lymphatic transport of poly (glutamic acid) conjugates after subcutaneous injection as the first choice among several routes of administration. As shown in Figs. 5—8, there were no significant differences between 14K and 60K poly (glutamic acid) conjugates. But both ^{3}H-Phe-poly (glutamic acid) conjugates had a tendency to be absorbed through the lymphatic routes compared to free ^{3}H-Phe. Higher values in the regional lymph node and thoracic duct lymph and lower levels in the blood after subcutaneous injection of the conjugates than that of free Phe strongly suggest that the lymphatic transport would be important for the absorption of the polymer conjugates. These results also suggest that the conjugates might be effective against the lymph node metastasis.

For the next step, we plan further several experiments; (i) use of copolymer of neutral amino acids (*e.g.* alanine) and acidic amino acids (*e.g.* glutamic acid) or basic amino acids (*e.g.* lysine), (ii) use of time-dependent antitumor agents such as arabinofuranosyl cytosine, (iii) measurement of the absorption and elimination behaviors after intravenous injection and oral administration, and (iv) preparation of "homing device"[23] containing tumor specific antibody or monoclonal antibody.

Acknowledgements This work was supported in part by a Grant-in-Aid for Cancer Research (58-15) from the Ministry of Health and Welfare.

REFERENCES

1) K. Sugibayashi, Y. Morimoto, T. Nadai and Y. Kato: Drug-carrier property of albumin microspheres in chemotherapy. I. Tissue distribution of microsphere-entrapped 5-fluorouracil in mice, *Chem. Pharm. Bull.*, **25**, 3433—3434 (1977).
2) K. Sugibayashi, M. Akimoto, Y. Morimoto, T. Nadai and Y. Kato: Drug-carrier property of albumin microspheres in chemotherapy. III. Effect of microsphere-entrapped 5-fluorouracil on Ehrlich ascites carcinoma in mice, *J. Pharm. Dyn.*, **2**, 350—355 (1979).
3) Y. Morimoto, K. Sugibayashi and Y. Kato, Drug-carrier property of albumin microspheres in chemotherapy. V. Antitumor effect of microsphere-entrapped adriamycin on liver metastasis of AH 7974 cells in rats, *Chem. Pharm. Bull.*, **29**, 1433—1438 (1981).
4) G. Gregoriadis and A.C. Allison (eds), "Lipsome in Biological Systems," Wiley-Interscience Pub., New York, 1980.
5) R. L. Magin and J. N. Weinsten: Delivery of drugs in temperature-sensitive liposomes, "Targeting of Drugs," eds. by G. Gregoriadis, J. Senior and A. Trouet, Plenum Press, New York, 1981, pp. 203—221.
6) A. Trouet, D. D. Campeneere and C. De Duve: Chemotherapy through lysosomes with a DNA-daunorubicin complex, *Nature* (London), **239**, 110—112 (1972).
7) T. Takahashi, S. Ueda, K. Kono and S. Majima: Attempt at local administration of antitumor agents in the form of fat emulsion, *Cancer*, **38**, 1507—1514 (1976).

8) M. Hashida, S. Muranishi, H. Sezaki, N. Tanigawa, K. Satomura and Y. Hikasa: Increased lymphatic delivery of bleomycin by microsphere in oil emulsion and its effect on lymph node matastasis, *Int. J. Pharm.* **2,** 245–256 (1979).
9) J. M. Varga, N. Asato, S. Lande and A. B. Lerner: Melanotropin-daunomycin conjugate shows receptor-mediated cytotoxicity in cultured murine melanoma cells, *Nature* (London), **267,** 56–58 (1977).
10) K. Kitao and K. Hattori: Concanavalin A as a carrier of daunomycin, *Nature* (London), **265,** 81–82 (1977).
11) G. F. Rowland, G. J. O'Neill and D. A. L. Davies: Suppression of tumour growth in mice by a drug-antibody conjugate using a novel approach to linkage, *Nature* (London), **255,** 487–488 (1977).
12) M. Szekerke and J. S. Driscoll: The use of macromolecules as carriers of antitumor drugs, *Eur. J. Cancer,* **13,** 529–537 (1977).
13) P. Molz, H. Rinsdorf, B. Abel and P. J. Cox: Synthesis and first *in vitro* cytotoxicity studies of bis (2-chloroethyl) amino group containing polymers. Pharmacologically active polymers. 22, *Int. J. Biol. Macromol.,* **2,** 245–250 (1980).
14) Y. P. Lee and T. Takahashi: An improved colorimetric determination of amino acids with the use of ninhydrin, *Anal. biochem.,* **14,** 71–77 (1966).
15) J. L. Skibba and F. G. Collins: Modification of the 4- (*p*-nitrobenzyl) pyridine assay for measurement of melphalan and mechlorethamine in plasma, *J. Pharmcol. Methods,* **4,** 155–163 (1980).
16) S. Kanno, S. Fukui, S. Naito and M. Kaneko: Hygienic chemical studies on public nuisance by injurious gasses. XIII. Studies on the method for determination of ammonia in air, *Eisei Kagaku,* **14,** 280–284 (1968) (in Japanese).
17) R. I. Geran, N. H. Greenberg, M. M. Macdonald, A. M. Schumacher and N. H. Abbott: Protocols for screening chemical agents and natural products against animal tumors and other biological systems. *Cancer Chemother. Rep.,* **3,** 103 (1972).
18) J. L. Bollman, J. C. Cain and J. H. Grindlay: Techniques for the collection of lymph from the liver, small intestine, or thoracic duct of the rat, *J. Clin. Med.,* **33,** 1349–1352 (1948).
19) T. Richardson, J. Hodgett, A. Lindner and M. A. Stahmann: Action of polylysine on some ascites tumors in mice. *Proc. Soc. Exp. Biol. Med.,* **101,** 382–386 (1959).
20) W. Regelson, S. Kuhar, M. Tunis, J. Fields, J. Johnson and E. Gluesenkamp: Synthetic polyelectrolytes as tumour inhibitions, *Nature* (London), **186,** 778–780 (1960).
21) L. J. Arnold, Jr., A. Dagan, J. Gutheil and N. O. Kaplan: Antineoplasmic activity of poly (L-lysine) with some ascites tumor cells, *Proc. Natl. Acad. Sci. U.S.A.,* **76,** 3246–3250 (1979).
22) F. Bergel and J. A. Stock: Cytotoxic alpha-amino acids and peptides, *Brit. Empire Cancer Campaign Ann. Rep.,* **31,** 6–7 (1953).
23) G. Gregoriadis and E. D. Neerunjun: Homing of liposomes to targeting cells, *Biochem. Biophys. Res. Commun.,* **65,** 537–544 (1975).