Reduced Hepatic Accumulation of Radiolabeled Monoclonal Antibodies with Indium-111-Thioether-Poly-L-Lysine-DTPA-Monoclonal Antibody-TP41.2F(ab')$_2$

Theodore S.T. Wang, Rashid A. Fawwaz, and Philip O. Alderson

Columbia-Presbyterian Medical Center, Columbia University, New York, New York

In an attempt to improve bifunctional chelate labeling of Mab, we investigated the use of a polyamino acid backbone for multiple DTPA substitutions. Poly-L-lysine (PL) (3.8 Kd, n = 25) was partially acetylated with MADTPA to yield 11 moles of DTPA per mole of PL. The average numbers of DTPA on PL were directly quantified with MADTPA-C-14. The remaining epsilon amino groups on PL-DTPA (I) were measured with TNBS reagent. A selective maleimide derivatization of (I) with S-SMPB yielded (II), which contains 2.3 moles of meleimido groups per mole of (I). The sulfhydryl activation of Mab-TP41.2F(ab')$_2$ with 2-Iminothiolane hydrochloride produced (III), containing 1.3 moles of sulfhydryl groups per mole of Mab. Compounds (II) and (III) were combined to form a single thioether-spaced chain linkage of Mab-PL-DTPA (IV), which was subsequently chelated with ^{111}In to yield (V), which was the compound of interest. Indium-111-PL-DTPA (VI) and ^{111}In-DTPA-MabTP41.2F(ab')$_2$ (VII) also were prepared for control studies. Direct cell binding assay revealed the mean immunoreactivity of (V) to be 79.4% and that of (VII) to be 39.5%. In a biodistribution study on melanoma tumor-bearing athymic mice at 4, 24, and 48 hr postinjection, the tumor/blood and tumor/liver ratios at 48 hr were 11.6 and 1.2 for (V), compared to 3.7 and 0.13, respectively, with (VII). Thus, the PL configuration for radiolabeled antibodies seems to result in decreased hepatic accumulation and retained tumor avidity. The findings suggest that further studies of this new compound are warranted.

J Nucl Med 1992; 33:570–574

The bicyclic anhydride of DTPA (BADTPA) and mixed anhydride DTPA (MADTPA) are currently the most commonly used bifunctional chelating agents for ^{111}In-monoclonal antibody (Mab) labeling (*1,2*). BADTPA is especially convenient to use and is commercially available. The current direct methods of attaching the DTPA and Mab molecules, however, can cause decreased immunoreactivity due to the intra- or inter-molecular cross-linking of Mab, even at low levels of DTPA substitution (*1–3*). Therefore, the number of DTPA molecules and the amount of ^{111}In added have to be severely limited in order to preserve full immunoreactivity of the Mab. In addition, the accumulation of these cross-linked radiotracers in the liver is high, resulting in a higher total radiation dose and diminished clinical usefulness of ^{111}In-DTPA labeled Mab (*4–6*) because hepatic activity decreases detectability of hepatic metastases.

In this paper, we have examined the use of a polyamino acid as a backbone for multiple DTPA substitutions. Consequently, a high degree of radiolabeling with ^{111}In can be achieved. We also used a single-chain linker to conjugate the polychelates to this Mab. This was done to minimize structural modifications of the Mab and thereby preserve immunoreactivity. We chose poly-L-lysine (PL) for this purpose because PL is a well-known chemotherapy conjugate drug (*7–9*) and a macromolecular carrier (*10,11*) with very low toxicity in vivo (*12*). PL is also an excellent substrate for intracellular proteolytic enzymes, as it is broken down by trypsin and reutilized in the cell (*7*).

MATERIALS AND METHODS

All reagents were purchased from commercial suppliers and used as received, unless otherwise specified. MabTP41.2F(ab')$_2$ is an IgG$_2$a that recognizes a human high molecular weight melanoma-associated antigen (*13*). Molecular porous membrane for dialysis was made from regenerated cellulose with a molecular weight cutoff (MWCO) of 2–10 K (Spectra/Pro 2–10 K). The membrane was treated with EDTA to remove heavy metals and then stored at 4°C in a 0.05% sodium azide solution. Chemical reagents used are abbreviated as follows: BADTPA: bicyclic anhydride of diethylenetriaminepentaacetic acid; DMSO: dimethyl sulfoxide; IBCF: isobutyl chloroformate; 2-IT: 2-iminothiolane hydrochloride; HEPES: N-(2-Hydroxyethyl)piperazine-N'-ethanesulfonic acid; MADTPA: Mixed carboxycarbonic anhydride of DTPA; PL: Poly-L-lysine (3.8 Kd, n = 25); PNPD: Bis(p-nitrophenyl)disulfide; S-SMPB: sulfosuccinimidyl-4-(p-maleidophenyl)butyrate; TNBS: trinitrobenzene-sulfonic acid.

Received Jun. 28, 1991; revision accepted Nov. 12, 1991.

For reprints contact: Theodore S. T. Wang, PhD, Department of Radiology, Division of Nuclear Medicine, Columbia-Presbyterian Medical Center, 622 West 168th St., New York, NY 10032.

Preparation of PL-DTPA (I) (Fig. 1)

MADTPA (*14*), 12.6 g (14 mmol) in 30 ml of acetonitrile was added dropwise into a solution of 3.8 g (1.0 mmol) of PL (3.8 Kd, n = 25) in 80 ml of 0.1 N $NaHCO_3$, pH 9.0, at 0°C. The reaction mixture was stirred at 0°C for 1 hr and then at room temperature for 2 hr. The acetonitrile was evaporated and the product was concentrated in vacuo and dialyzed in a 2 K MWCO molecularporous membrane against 0.1 *M* PBS, pH 8.4, at 4°C for 24 hr and then against 0.1 *M* PBS, pH 7.4, at 4°C for another 24 hr. The buffer solutions were changed repeatedly during dialysis. The product was precipitated by adding a large amount of absolute ethanol. The product was collected, filtered and washed with absolute ethanol, then washed with ether and dried in vacuo. The purity of the product was identified by TLC-SG with a single compound: mp > 300°C.

Determination of Average Number of DTPA Linked on PL

A mixture of DTPA-1-C-14 (55.7 mg, 14 μmol, 100 μCi) in 5 ml of acetonitrile, and triethylamine (6 mg, 8.4 ml, 60 μmol) in 5 ml of acetonitrile was stirred magnetically and kept at 68°–70°C for 2 hr under an atmosphere of nitrogen until a clear solution was formed. The reaction mixture was cooled to −30°C by means of acetone-dry ice and was added dropwise to 20.4 mg (15 μmol) of IBCF in 1 ml of acetonitrile. The resultant mixture was stirred and kept at −30°C for 30 min until a white precipitate formed out of solution. The reaction mixture was warmed to 0°C, and 3.8 mg (1.0 μmol) of PL in 5 ml of 0.1 N $NaHCO_3$ was added dropwise at 0°C, kept at 0°C for 1 hr, and then at room temperature for 2 hr. The resultant solution was concentrated in vacuo and dialyzed in a 2K MWCO molecularporous membrane against 0.1 *M* PBS, pH 8.4, and 0.1 *M* PBS, pH 7.4, at 4°C for 24 hr. Quantification of the average DTPA-1-C-14 ratio was accomplished by liquid scintillation counting of the DTPA-1-C-14 (15 μl sample, n = 3). Measurement of PL concentration was obtained by the method of Lowry et al. (*15*). The absorbance was measured at 660 nm. A serial sample of known amounts of PL was used to construct a standard curve. The ratio of DTPA-C-14 concentration to PL concentration provided the number of DTPAs per PL molecule (*16*).

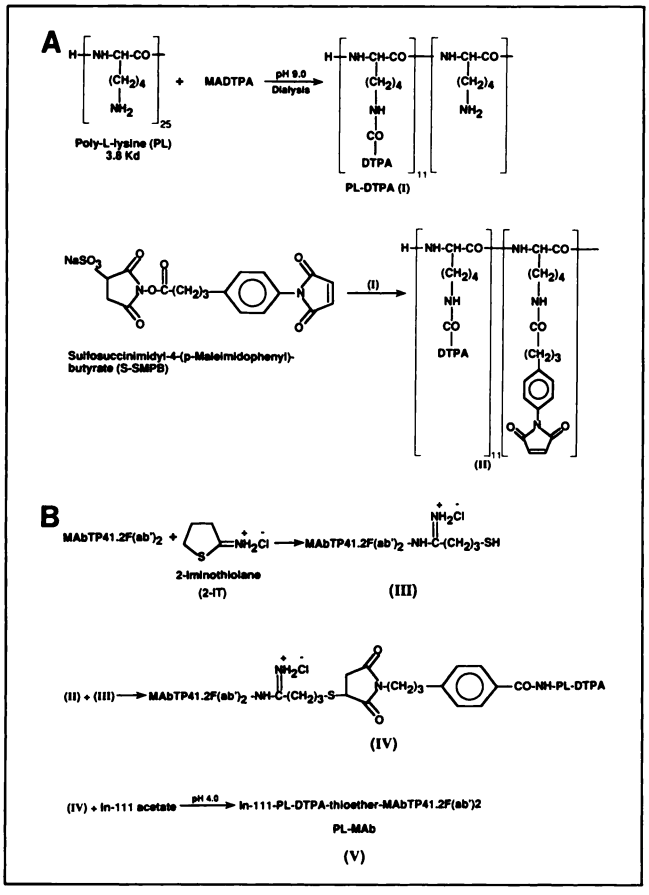

FIGURE 1. Schematic representation of the synthesis of ^{111}In-PL-DTPA-thioether-MabTP41.2F(ab')$_2$(PL-Mab)(V).

Assessment of Epsilon Amino Groups in PL-DTPA

The free epsilon amino groups of lysines were identified by the method of Fields and co-workers (*17*). The sample of (I) (0.1 ml) was mixed with 0.5 ml of 0.1 *M* sodium borate buffer in 0.1 N NaOH solution, a sufficient quantity of distilled water was added to a total of 1.0 ml. Next, 0.02 ml of 1.1 *M* TNBS solution was added rapidly and the solution was mixed. After 5 min, the reaction was stopped by adding 2 ml of 0.1 *M* PBS containing 1.5 *M* sodium sulfite, and the absorbance was determined by spectrophotometry at 420 nm (molar extinction coefficient at 420 nm of 19,200 $M^{-1}cm^{-1}$). A serial sample of known amounts of PL was used as a standard.

Preparation of Maleimide Derivative of PL-DTPA (II)

Compound (I), 1.32 mg (2×10^{-5} mmol), was dissolved in 0.5 ml of 0.05 *M* HEPES, pH 8.0, and then S-SMPB 0.046 mg (1×10^{-4} mmol) in 0.1 ml of DMSO was added. The reaction mixture was kept at room temperature for 30 min and then dialyzed at 4°C overnight against deionized water, with several changes of water.

SDS-PAGE Analysis

Compounds (I), (II), and two controls (PL, 3.8 Kd; and BSA) were analyzed by electrophoresis. This was performed with 15% polyacrylamide gel (PAGE) in presence of 2% sodium dodecyl sulfate (SDS) and 6 N urea. Following completion of electrophoresis, the gel was stained with 0.025% Coomassie brilliant blue in 25% methanol and 10% acetic acid.

Determination of the Maleimide Groups on PL-DTPA

The maleimide groups on (II) were determined by the method of Ellman (*18*). Briefly, a small amount of sample (0.1 ml) was diluted with 3.9 ml of distilled water, and 1.0 ml of 0.1 *M* PBS, pH 8.0, was added. In addition, 4 ml of acetone, a known amount of 2-mercaptoethanol, and 1.0 ml of 1.0 m*M* of PNPD in acetone were added to the resultant mixture. After 5 min of incubation at room temperature, the remaining sulfhydryl groups were measured by a UV spectrophotometer at an absorbance of 412 nm (molar extinction coefficient at 412 nm of 13,600 $M^{-1}cm^{-1}$).

Preparation of Sulfhydryl-amidinium Derivative of MabTP41.2F(ab')$_2$ (III)

A 0.05-ml aliquot of 7.9 μg (6×10^{-5} mmol) of 2-IT in 25 m*M* sodium borate was added at room temperature to a 0.5-ml of solution of 2 mg (2×10^{-5} mmol) MabTP41.2F(ab')$_2$ in the same buffer at pH 9.0.

The reaction mixture was kept at 4°C for 3 hr and then was dialyzed at 4°C overnight with 0.1 *M* PBS, pH 6.4, with several changes of buffer. The sulfhydryl groups present in the Mab were determined by Ellman's method (*18*).

Preparation of PL-DTPA-thioether-MabTP41.2F(ab')$_2$ Conjugate (IV)

Equal millimol amounts (2×10^{-5} mmol) of (II) and (III) were mixed in 1.0 ml of 0.1 *M* PBS, pH 6.0, at room temperature. The reaction mixture was kept at 4°C for 20 hr (no sulfhydryl residue was detected in the mixture at this point). The product was again dialyzed in a 10K MWCO molecularporous membrane against 0.1 *M* PBS, pH 7.4, at 4°C overnight, with a few changes of buffer.

Preparation of ^{111}In-PL-DTPA-thioether-MabTP41.2F(ab')$_2$, (PL-Mab), (V)

Sodium acetate (1 *M*) was added dropwise into a solution of 50 μl 2 N HCl and ^{111}In-chloride (1 mCi) to adjust the solution to pH 4. The resultant solution was immediately added to compound (IV) and the reaction mixture was kept at room temperature for 30 min. The product was isolated by passage through a Sephadex G-75 column, with identification by UV spectrophotometer monitor at 280 nm, and was eluted with 0.1 *M* PBS, pH 7.4. The Mab peak fractions were collected and combined. The product was further purified by extensive dialysis at 4°C against 0.1 *M* PBS, pH 7.4. The concentration of Mab was assayed by the Bio-Rad Protein Assay method (*19*) and the absorbance was determined by spectrophotometer at 595 nm. A serial of known amount of bovine gamma globulin was used as standard. The ^{111}In-radioactivity that remained was used to calculate the number of ^{111}In-moles bound per mole of MabTP41.2F(ab')$_2$ (*1*).

Preparation of ^{111}In-PL-DTPA (PL-DTPA) (VI) and ^{111}In-DTPA-MabF(ab')$_2$ (DTPA-Mab) (VII) for Control Study

The compounds (VI) and (VII) were prepared for control studies. To prepare compound (VI), compound (I) was reacted with ^{111}In-acetate at pH 4.0. The product was extensively dialyzed in a 2K MWCO molecularporous membrane against 0.1 *M* PBS pH 7.4 at 4°C for 2 days. Compound (VII) was prepared by a conventional method (*20*). Briefly, MADTPA (21 μg) was mixed with MabTP41.2F(ab')$_2$ (2 mg) in 1 ml of 0.5 *M* HEPES solution, pH 8.6, at room temperature for 1 hr. The reaction mixture was dialyzed in 0.1 *M* PBS, pH 7.4, at 4°C overnight. Indium-111-acetate then was added at pH 4.0, and the mixture was incubated at room temperature for 1 hr. The product was isolated by passage through a Sephadex G-50 column and eluted with 0.1 *M* PBS, pH 7.4. The product was further purified by extensive dialysis at 4°C against 0.1 *M* PBS, pH 7.4.

Immunoreactivity Assays

Melanoma cells (human COLO-38, 1×10^6) or B-lymphoblastoid cells (human Wil-2, 1×10^6) (*21*) were incubated with (PL-Mab) (V) and (DTPA-Mab) (VII) in a 96-well polyvinyl chloride microliter plate. After 1 hr of incubation, the cells were washed three times and radioactivity bound to the cells was determined in triplicate in the region of antigen excess (plateau region).

Animal Studies

PL-Mab and two control radiolabeled compounds were injected into the tail vein of athymic mice (n = 4–6 per group) bearing subcutaneous melanoma tumors measuring 3–7 mm in diameter. The animals were killed at 4, 24, and 48 hr postinjection. Tissue was removed, weighed, and the radioactivity determined in a well-type scintillation counter. Statistical analyses were performed using an unpaired Student's t-test. Results are presented as mean ± s.d.

RESULTS

Elemental and Chemical Analyses

The elemental analyses of (I) revealed that C = 46.21%, H = 9.04%, N = 14.01%. The structure of (I) was calculated for the theoretical values: (C = 47.60%, H = 9.52%, N = 14.52). The molecular weight of (I) was assigned to be 6,600. The molar ratio of DTPA of (I) was also determined by direct quantification with MADTPA-C-14. It was found that there was an average of eleven molecules of DTPA in each molecule of PL. The remaining free epsilon amino residues of lysine were identified by TNBS reagent, which indicated that at least six epsilon amino groups were measurable. A selective maleimide derivatization of (I) yielded 2.3 moles of maleimide groups per mole of (I). Electrophoresis results revealed that Compounds (I) and (II) yielded similar profiles. The absence of a higher molecular band in compound (II) indicated that there was no cross-linking (Fig. 2). A control sulfhydration of MabTP41.2F(ab')$_2$ yielded 1.3 moles of sulfhydryl groups per mole of MabTP41.2F(ab')$_2$.(II). A quantitative yield of compound (IV) was obtained by combination of compounds (II) and (III). Indium-111 labeling of (IV) yielded about eleven molecules of ^{111}In per molecule of (V). Specific activity was 0.92 μCi/mg. Labeling efficiency was 92%.

Immunoreactivity Assay

Direct cell binding immunoreactivity assays revealed that in the region of antigen excess (plateau region) 79.4% ± 5.3% of (V) and 39.5% ± 3.6% of (VII) bound to melanoma cells ($p < 0.001$). Only 3.1% ± 0.4% of (V) and (VII) bound to lymphoma cells. The results are summarized in Table 1.

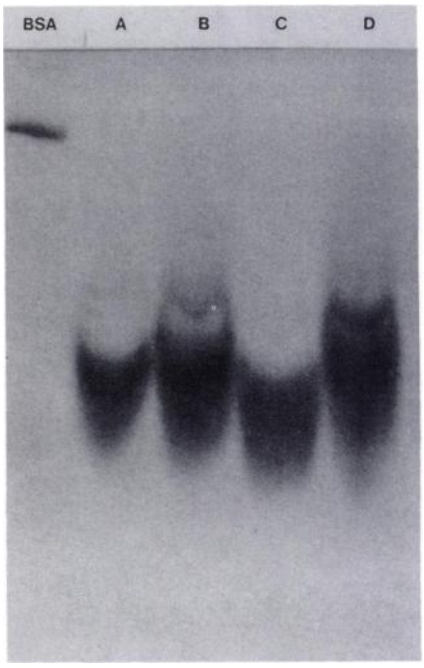

FIGURE 2. SDS-PAGE electrophoresis: A & C represent PL (3.8 Kd); B represents Compound (I); and D represents Compound (II).

TABLE 1
Immunoreactivity Assay

Cells	Compound	Percent of binding
Melanoma	PL-Mab (V)	79.4 ± 5.3
	DTPA-Mab (VII)	39.5 ± 3.6
Lymphoma	PL-Mab	3.1 ± 0.4
	DTPA-Mab (VII)	3.1 ± 0.4

TABLE 2
The Tumor/Blood and Tumor/Liver Ratios of PL-Mab (V) and DTPA-Mab (VII) at 4, 24, and 48 Hours Postinjection

	PL-Mab (V)			DTPA-Mab (VII)		
	4	24	48	4	24	48
Tumor/Blood	0.03	6.80	11.6	0.02	1.9	3.7
Tumor/Liver	0.10	0.63	1.2	0.02	0.13	0.13

Biodistribution Studies

The results of biodistribution in melanoma tumor-bearing athymic mice for (V), (VI), and (VII) are summarized in Figure 3. At 48 hr postinjection, the blood and liver concentrations of PL-Mab(V) were found to be significantly lower as compared with the standard DTPA-Mab(VII) ($p < 0.001$). The tumor concentration, on the contrary, was found to be significantly ($p < 0.001$) higher for PL-Mab (1.63% ± 0.13%), as compared with the DTPA-Mab (0.86% ± 0.07%). Furthermore, the tumor:blood and tumor:liver ratios of PL-DTPA(V) were found to be about ten times greater than those achieved with the DTPA-Mab(VII) (Table 2).

DISCUSSION

The use of PL-DTPA as a polychelator to achieve high specific activity radiolabeling of antibodies for diagnostic imaging has been reported by other authors (*22–24*). These studies have emphasized the need for maximum substitution of epsilon amino groups with DTPA in order to achieve maximum radiometallic labeling. Such an approach, however, results in the reduction of the cationic property of PL, which has been reported to be essential for enhancing the tumor uptake of macromolecules (*10,25–27*). The purpose of this study was to develop a method for partial substitution of DTPA on PL that would allow for a relatively high degree of radiometallic labeling but at the same time conserve the polycationic property of PL. The bifunctional linkers 2-IT and S-SMPB were chosen because: (1) they form a noncleavable thioether bond (compounds II and III) that is stable in vivo (*28*); and (2) the single amino group of the Mab used for binding with the PL to form a thioether chain results in a lower probability of loss of immunoreactivity. Manabe et al. (*22*) achieved high immunoreactivity using SPDP to serve as a linker between Mab and PL-DTPA. The disulfide bond, however, proved labile and unstable in circulation (*29–31*). Our results indicate that partial substitution of DTPA in PL using the 2-IT and S-SMPB combination resulted in an in vivo stable compound as shown by differences in biodistribution between compounds (V) and (VI). The low blood and high kidney concentration found in our studies are in contrast to those reported for the high negatively charged compound ^{111}In-antimyosin-Fab-succinimidyl-PL-DTPA (*24*), which demonstrated high blood but low kidney concentration. These differences in biodistribution may be related to electron charge. Further studies utilizing PL with different molecular weights and therefore different numbers of electron charges may be warranted. The low blood and liver concentrations achieved with the bifunctional reagents 2-IT and S-SMPB combined with preservation of immunoreactivity may significantly improve the

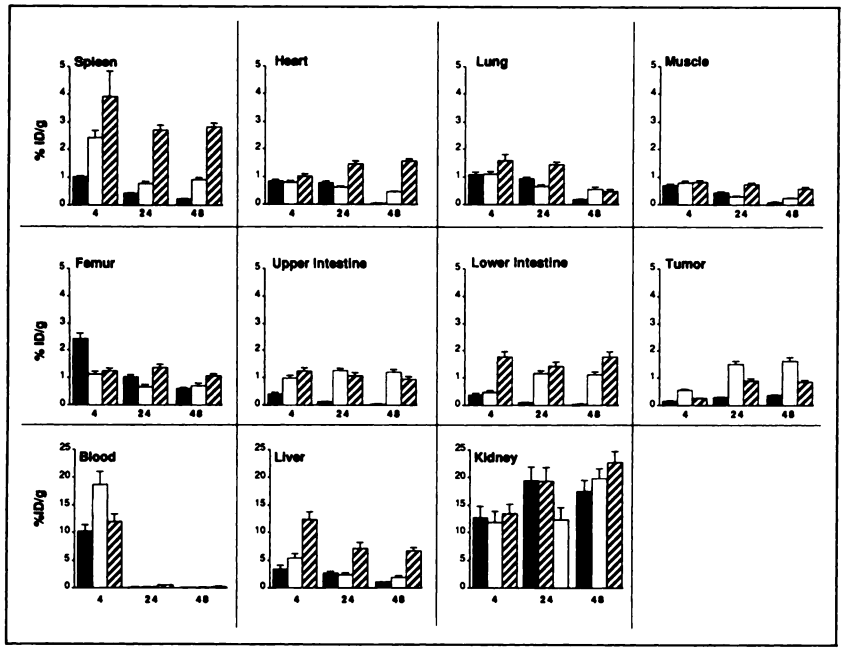

FIGURE 3. Biodistribution of PL-Mab (V), (□), PL-DTPA (VI), (■), and DTPA-Mab (VII), (▨) in athymic mice bearing human melanoma tumor (%ID/g n = 4–6 per group).

sensitivity of tumor detection in radioimmunoscintigraphy.

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