Reduction of Background Activities by Introduction of a Diester Linkage Between Antibody and a Chelate in Radioimmunodetection of Tumor

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A diester linkage was added between monoclonal anti-melanoma antibody 96.5 and a diethylenetriaminepentaacetic acid derivative to test if a tumor-to-blood and -to-organ ratio of the injected antibody in nude mice with human melanoma FEM XII xenografts could be increased by the addition of the readily cleavable linkage. Compared to the ^{111}In-labeled antibody DTPA with a peptide linkage, the diester conjugate cleared much faster from the blood and was retained much less in muscle and normal organs such as liver, spleen and kidney over a 48-hr period. On the other hand, the activity retained in the tumor was larger than or similar to that of the peptide conjugate for this time period. This resulted in a 2.5, 2.1, and 2.6 fold increase in a tumor to blood, to liver and to kidney ratio at 48 hr for the diester conjugate as compared to the peptide conjugate. The whole-body biologic half life of the antibody was 36 hr, three times shorter than the peptide conjugate. The external imaging demonstrated a clearly visible tumor at 4 hr and a lower pool activity at 72 hr for the diester conjugate. The peptide conjugate, however, showed a persistant blood-pool activity at 72 hr. The addition of the diester linkage, therefore, may be beneficial for imaging tumors in patients at early time intervals after injection.

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A bifunctional chelate approach using acylation agents such as cyclic diethylenetriaminepentaacetic acid (DTPA) dianhydride and DTPA carboxycarbonic mixed anhydride has been widely used for radiolabeling of antibodies with indium-111 (^{111}In) for scintigraphic detection of tumors. The acylating agents conjugate DTPA to antibody through an amide or peptide bond. The acylation methods have been thoroughly optimized for a high conjugation yield and the integrity of antibody immunoreactivity (*1–6*). A drawback for the use of the acylation methods to radiolabel antibodies with ^{111}In is that the labeled antibody produces a high ^{111}In activity in normal organs such as liver and kidneys (*7–19*).

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One approach in reducing the normal organ activities is to add a readily metabolizable chemical linkage between antibody and a DTPA chelate, thereby accelerating the cleavage of the chelate from the antibody in normal organs. The metabolites containing radioactivity are polar and are expected to clear rapidly from the circulation through renal excretion, thereby reducing blood and normal organ activities. Quadri et al. (*20*) and Paik et al. (*21,22*) introduced readily cleavable linkages such as diester and disulfide, and stable linkages such as thioether and hydrocarbon between antibody and an [^{111}In]DTPA analog. The biodistributions of these antibody DTPA conjugates were then compared with that of antibody DTPA conjugate containing an amide linkage in rats with antigen target models localized in lungs. It was reported that the antibody conjugates with the readily metabolizable linkages cleared from blood much faster than the conjugates with stable linkages whereas all of the conjugates were

quite stable in the target once the conjugates were bound to the target. Thus, a significant increase in the target to blood ratio was obtained for the diester and disulfide conjugates. The diester and the disulfide conjugates also substantially increased the target to liver ratio. Haseman et al. (*23*) reported that the introduction of the diester linkage between antibody and an EDTA analogue reduced blood activity, thereby increasing the tumor to blood ratio in nude mice with a B-cell lymphoma. However, the diester conjugate did not improve the tumor to liver and to kidney ratios as compared to the control conjugate. Yokoyama et al. (*24*) compared the biodistribution of ^{111}In labeled monoclonal anti-melanoma antibody 96.5-DTPA conjugate with the diester linkage to that of the antibody conjugate with a peptide linkage in nude mice with human melanoma FEM XII xenografts. The diester conjugate was reported to improve the target to blood, liver and kidney ratios substantially. We have now extended this work and report in this paper the effects of the diester linkage between monoclonal anti-melanoma antibody 96.5 and an [^{111}In]DTPA derivative on the biodistribution and the imaging of the melanoma tumor in nude mice.

MATERIALS AND METHODS

DTPA and 2-(4-aminophenyl)-ethylamine, and ethylene glycolbis(succinimidyl succinate) were obtained from Aldrich Chemical Company, Milwaukee, WI and Pierce Chemical Company, Rockford, IL, respectively. Monoclonal anti-melanoma antibody 96.5 (Ab) is an IgG_{2a} isotype which is directed against human melanoma antigen epitope p 97 (*25*) and obtained from Oncogen, Seattle, WA. Nonspecific monoclonal antibody (nAb) used for the biodistribution studies is antiidiotype antibody 2-135 of an IgG_{2a} isotype. It was purchased from Damon Biotech, Needham Heights, MA. Human melanoma FEM XII cells were obtained from Frederick Cancer Research Center, Frederick, MD. The cells were grown in RPMI 1640 medium at Meloy Laboratories, Annandale, VA.

Synthesis

Antibody DTPA conjugate with a peptide linkage. A peptide bond was introduced between Ab and DTPA by an acylation reaction using cyclic DTPA dianhydride (Fig. 1). Briefly, Cyclic DTPA dianhydride (cDTPAA) was reacted with Ab (15 mg/ml, 1.0×10^{-4} *M*) at a cDTPAA/Ab molar ratio ranging from 2 to 32 at pH 8.2 (*4*). The conjugation yield was calculated based on the percent no-carrier-added ^{111}In distributed between the Ab DTPA conjugate and free DTPA on a thin layer chromatographic (TLC) plate after the product mixture was radiolabeled with ^{111}In and TLC of the product mixture was developed as described previously (*4,26*). The Ab-Peptide-DTPA produced from the reaction at a cDTPAA/Ab molar ratio of 2 was radiolabeled with ^{111}In and was purified by Sephadex G-50 molecular permeation chromatography as described previously (*4*). Molecular permeation high performance liquid chromatography (HPLC) (a Bio-Sil TSK 250 7.5 × 600 mm column) of the antibody conjugate showed a single peak with a retention time of 19 min when eluted with a solvent mixture containing 0.02*M* sodium phosphate at pH 6.8 and 0.2*M* sodium sulfate at a flow rate of 1 ml per min. The retention times of the standard IgM, monoclonal anti-melanoma antibody 96.5 and human serum albumin samples were 11, 19, and 23 min under the same HPLC condition.

Antibody DTPA conjugate with diester linkage. The synthesis of DTPA-p-(aminoethyl)anilide has been reported (*22*). 2-(4-aminophenyl)ethylamine (70 mg, 0.51 mmol) dissolved in 5 ml of 0.1*M* citrate buffer was added dropwise through a 23-gauge needle into 20 ml of 0.1*M* citrate buffer at pH 5 in a 50-ml round bottom flask and at the same time cDTPAA (700 mg, 1.96 mmol) was added in small portions over a period of 30 min. The concentration of 2-(4-aminophenyl)ethylamine in the reaction flask was kept below 10^{-4} *M* throughout the reaction to avoid the formation of the diamide of DTPA. At pH 5 the aliphatic amino group (pKa = 10) is completely protonated and protected from the acylation reaction. On the other hand, ~50% of the aromatic amino group (pKa = 5) is unprotonated and is expected to react selectively with cDTPAA. The reaction solution gave a major spot (95%) and a minor spot (5%) at a R_f value of 0.7 and 0.55, respectively, when a silica gel TLC (Macherey-Nagel, Germany) was developed with 2:2:1 10% ammonium formate in water:methanol:0.2*M* citric acid. These spots were positive on fluorescamine test indicating the presence of primary amines (*27*). The product solution was negative on an azo coupling test with resorcinol after a reaction with nitrous acid (*28*). This indicates that the product does not contain an aromatic amine. HPLC of the product mixture gave a minor product peak and a major product peak at a retention time of 7 and 20 min, respectively, when the product mixture was eluted through a μBondapak NH_2 column (0.8 × 10 cm Radial-Pak cartridge, Waters, Millipore Corporation) with a solvent mixture containing 2:98% acetonitrile:0.01*M* phos-

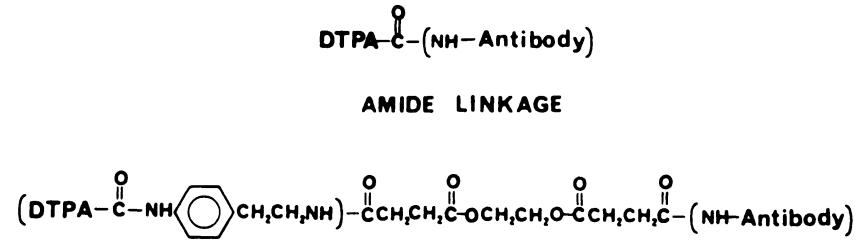

FIGURE 1
Simplified structures of Ab DTPA conjugates.

phoric acid at pH 5.0 at a flow rate of 1 ml per min. The minor and the major product peak corresponds to the TLC spot at a R_f of 0.55 and 0.7, respectively, on the TLC plate. The product was purified from free DTPA by precipitating DTPA repeatedly at pH 2.0. Although the final product was not completely free of DTPA, the product was used for the conjugation of the DTPA derivative to Ab through a diester linkage because DTPA does not interfere with the reactions.

Introduction of diester bonds. Ethylene glycolbis(succinimidyl succinate) (EGS) was used as a cross linking agent (*29*). 0.1 ml of dimethyl sulfoxide solution of EGS at a concentration ranging from 2×10^{-2} to 1.2*M* was mixed rapidly with DTPA-p-(aminoethyl)anilide dissolved in 0.9 ml of 0.1*M* phosphate buffer at pH 7.0. The reaction solution was stirred gently for 5 min at room temperature. The molar ratio of two reactants in each reaction was 1 to substitute no more than 1 DTPA to EGS. To this solution 1 ml of Ab solution (1×10^{-4} *M*) in the phosphate buffer was added rapidly with a gentle stirring. The conjugation reaction was continued for 2 hr at room temperature. The conjugation reaction was performed at several different EGS (or the amino DTPA) to Ab molar ratios to investigate the effect of EGS (or the amino DTPA) concentration on the conjugation yield (Table 1). The stepwise reactions resulted in the conjugation of DTPA to Ab presumably through a diester linkage (Fig. 1). The conjugation yield of DTPA molecule to Ab was determined by the TLC method described for the Ab-Peptide-DTPA. The Ab-Diester-DTPA containing 1 DTPA molecule per Ab was radiolabeled with ^{111}In and purified by Sephadex G-50 chromatography for the biodistribution studies. The molecular permeation HPLC of the antibody conjugate also showed a single peak at a retention time of 19 min.

To substantiate that the Ab-Diester-DTPA-^{111}In contained a chemically labile diester linkage, the antibody conjugate was subjected to hydrolysis reactions in buffer solutions at three different pH values; 0.1*M* bicarbonate buffer at pH 8.5, 0.1*M* phosphate buffer at pH 7.5 and pH 7.0. The percent cleavage of the linkage was determined by the TLC method using the solvent mixture described for the analysis of DTPA-(p-aminoethyl)anilide. Likewise, the stability of the antibody-Peptide-DTPA-^{111}In was tested in the same buffer solutions.

TABLE 1
Effects of Reagent Concentrations on the Number of DTPA Molecules Conjugated per Ab

Ab-Peptide-DTPA reaction		AB-Diester-DTPA reaction	
[cDTPAA]/[Ab]	DTPA molecule [Ab]	[EGS]/[Ab]	DTPA molecule [Ab]
2	1.0	10	0.9
4	2.2	20	1.7
8	5.7	30	3.0
16	8.9	40	4.2
32	15.6	80	7.6
		120	11.0

The data are averages of duplicate experiments. Ab is monoclonal anti-melanoma antibody 96.5.

Immunoreactivity Determination

Competitive cell binding assay. Fresh FEM XII cells were washed three times with phosphate buffered saline (PBS, 0.02*M* phosphate buffer and 0.16*M* NaCl) at pH 7.4 containing 1% bovine serum albumin (BSA). The tumor cells (1×10^6) in 125 μl of the medium was then mixed with 75μl of the medium containing 5 ng of ^{125}I Ab and unlabeled Ab DTPA conjugate at an amount ranging from 0 to 250 ng. The assay solution was incubated at 10°C for 1 hr with gentle shaking. The cells were washed twice with 3 ml of the cold medium and centrifuged at 1,500 *g* for 5 min. The supernatant was pipetted off and the activity bound to the tumor cells was counted in a gamma counter. The total activity was measured separately by counting 75 μl of the medium containing 5 ng of ^{125}I Ab. For the determination of the immunologic activity, the percent ^{125}I Ab bound to the cells was plotted against the logarithm of the concentration of Ab DTPA conjugate. The immunologic activity of Ab DTPA conjugate was assessed at 50% inhibition of ^{125}I Ab bound to the cells (Fig. 2).

Direct cell binding assay. Two point binding assays were performed using a condition where the tumor cell antigens are in excess to the antibody concentration. The cell binding assay was reported previously (*30*). Briefly, 5 ng of ^{111}In-labeled Ab DTPA conjugate in 75 μl of the medium was incubated with 4 and 8 million tumor cells in 125 μl of the medium at 10°C for 1 hr with gentle shaking. The tumor cells were separated from the supernatant as described above. The labeled antibody bound to the tumor cells was counted in a gamma counter. For the determination of a percent nonspecific binding to the tumor cells, 25 μg of Ab was added to the assay solution and ^{111}In-Ab-DTPA conjugate bound nonspecifically to the tumor cells was counted in a gamma counter. The percent specific binding was obtained by subtracting the percent nonspecific binding from the percent total binding.

Biodistribution and Imaging Studies

Nude mice (20–25 g male, Harlan Sprague Dawley, Inc., Indianapolis, In 46229) were injected with FEM XII cells (5

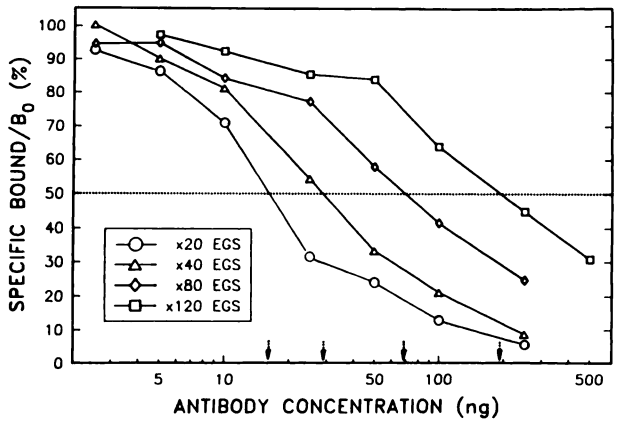

FIGURE 2
Effect of DTPA conjugation through a diester linkage on immunologic activity of Ab as determined by a competitive cell binding assay. (○), (△), (◇), and (□): the Ab-Diester-DTPA conjugate from the conjugation reaction with 20, 40, 80, and 120 times excess ethylene glycolbis(succinimidylsuccinate), respectively. B_0 is the radioactivity of ^{125}I Ab specifically bound to FEM XII cells in the absence of the Ab conjugate.

$\times 10^6$) subcutaneously in the right flank. The tumors were grown for 2 wk until they became 0.2–0.5 cm in diameter. The nude mice were then injected i.v. with the ^{111}In-labeled Ab DTPA conjugates (5–13 μg for biodistribution and 32–40 μg for imaging, a specific activity of 1–2 μCi/μg). The mice were killed 6, 12, 24, and 48 hr after injection. Organs were excised, cleaned from blood, weighed promptly and counted in a gamma counter. The biodistribution data are reported in % injected dose (ID) per g and normalized to 20 g mice (Table 2). Most of the tumors larger than 0.2 g had a lytic necrotic center which was thoroughly removed from solid tumors by tissue papers and counted separately. For control biodistribution experiments, antiidiotype antibody 2–135 (nAb) of IgG_{2a} isotype was conjugated with DTPA through the diester and the peptide linkage the same as described for the specific antibody. All of the experimental procedures used for the biodistribution studies of the nonspecific antibody were identical to those of the specific antibody except that the animals were killed at 12, 24, and 48 hr postinjection. The percent ^{111}In-labeled nAb DTPA conjugates bound to the tumor cells in vitro was <10%.

For imaging studies, nude mice were anesthetized with a 0.1 ml mixture of Ketamine.HCl (700 μg, Ketaset, Bristol Lab., Syracuse, NY) and Xylazine (60 μg, Rompun, Miles Lab., Shawnee, KS). Imaging was performed with a scintillation camera equipped with a 0.25-in. aperture pinhole collimater positioned 5 cm above the animals. The camera was set to image both 172 and 247 keV energy peaks, and a total of 100,000 counts was accumulated for each image. Images were obtained at 4, 16, 48, and 72 hr postinjection (Fig. 3). The whole-body activity of the animals was measured with a dosimeter (Radioisotope Calibrator CRC-2N) (Fig. 4).

RESULTS

Chemistry of Conjugation

cDTPAA can react with hydroxy groups, imidazole groups and sulfhydryl groups in addition to reacting with amino groups of proteins. However, the resulting products from the reactions with the hydroxy, imidazole and sulfhydryl groups are known to be unstable at the basic pH where the conjugation reaction was performed (*31*). We, therefore, think that the acylation reaction at the amino groups only gave rise to a stable antibody DTPA conjugate with an amide or peptide bond (*3*). The percent DTPA conjugation yield from the reaction of cDTPAA with Ab was ~50% based on the concentration of cDTPAA (Table 1).

DTPA-p-(aminoethyl)anilide was used as a bifunctional chelator to conjugate to Ab through a diester linkage. For the introduction of a diester linkage, EGS was reacted stepwise with the amino group of DTPA-p-(aminoethyl)anilide and then with an amino group of Ab. EGS and the bifunctional amino DTPA were reacted at a molar ratio of 1 to obtain no more than 1 DTPA substitution to the carbonyl groups of the two N-hydroxy succinimidyl ester moieties of the cross link-

TABLE 2
Biodistribution (% ID/g) of ^{111}In-labeled Ab DTPA Conjugates in Nude Mice with FEM XII Human Melanoma Xenografts

	Ab†-diester-DTPA				Ab-peptide-DTPA			
	6 hr	12 hr	24 hr	48 hr	6 hr	12 hr	24 hr	48 hr
Blood	21.21	14.59	8.74	2.75	25.65	13.60	12.65	7.35
	0.88	1.20	0.98	0.18	1.80	2.63	2.23	1.16
Liver	8.36	6.62	6.75	5.03	10.62	10.64	12.78	11.42
	0.39	2.21	1.19	0.62	1.05	2.81	2.52	2.26
Spleen	6.94	3.34	4.22	3.25	9.28	10.93	13.91	7.95
	0.56	0.40	0.65	0.75	0.62	2.93	6.56	2.14
Kidney	4.89	4.81	4.38	3.30	8.19	8.09	10.00	9.74
	0.43	0.63	0.29	0.37	0.89	1.10	1.02	2.13
Intestine	1.77	1.73	1.36	1.13	2.36	2.41	2.88	2.24
	0.18	0.06	0.10	0.19	0.18	0.56	0.72	0.41
Muscle	0.96	0.84	0.60	0.38	1.38	1.35	1.39	0.86
	0.13	0.13	0.03	0.07	0.15	0.17	0.39	0.14
Bone	2.38	2.17	2.70	1.18	3.62	4.17	3.84	3.52
	0.42	0.13	1.17	0.01	0.37	1.09	0.80	0.51
S‡ tumor	10.91	32.09	26.20	14.93	11.28	13.88	16.82	16.36
	2.34	6.73	5.80	2.22	1.80	3.36	4.29	4.64
N* tumor§	4.00	4.92	4.75	3.43	5.88	4.85	6.62	5.81
	1.95	1.80	2.61	1.58	1.59	1.65	2.93	2.66

The data are averages and standard deviations, and are normalized to 20 g body weight.
* N = 4 – 7.
† Ab = monoclonal anti-melanoma antibody 96.5.
‡ S tumor = solid tumor.
§ N tumor = necrotic tumor.

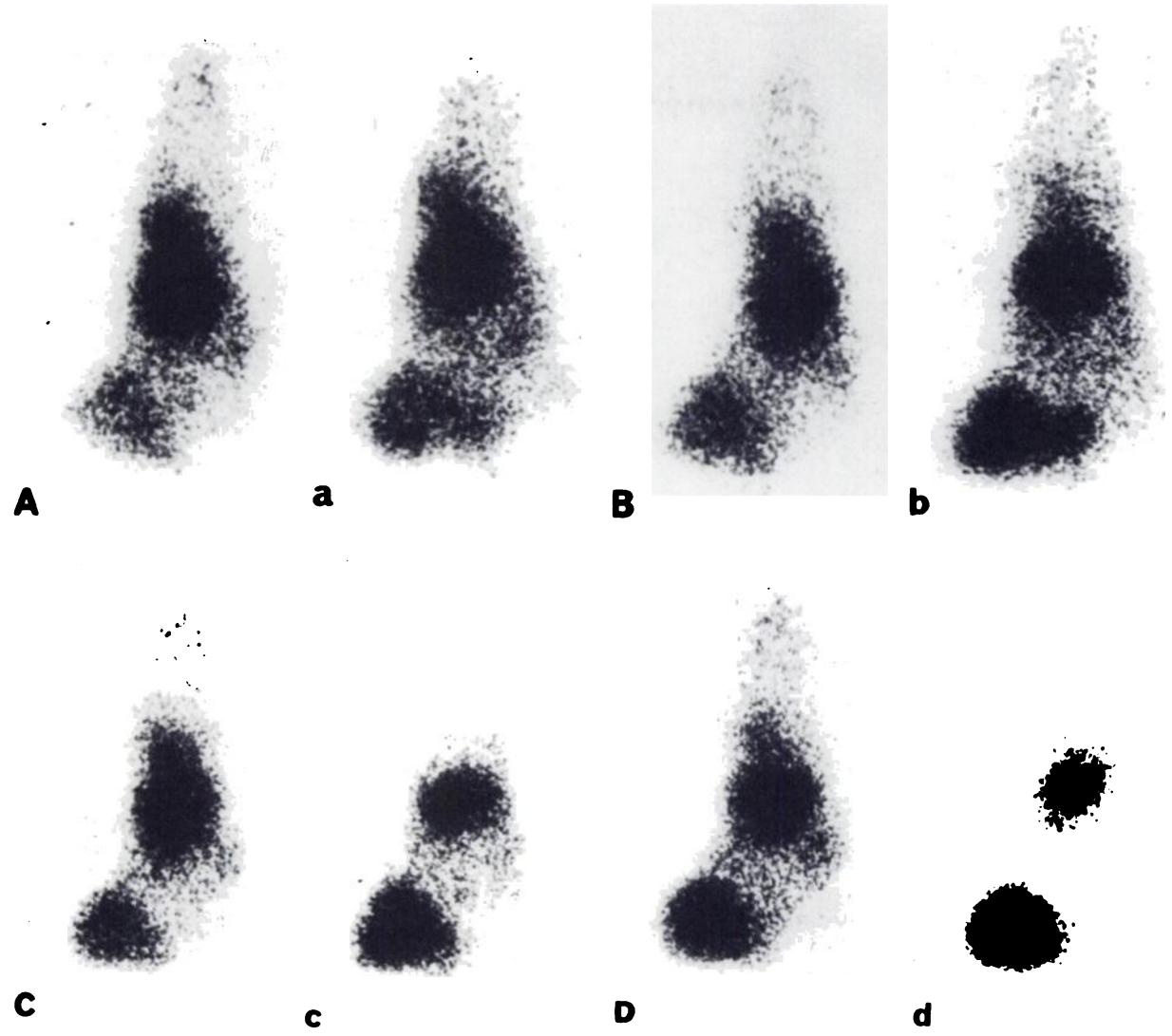

FIGURE 3
Images of nude mice with human melanoma FEM XII xenograft after the injection of the ^{111}In-labeled Ab DTPA conjugates. Upper case and lower case is for the peptide and the diester conjugate, respectively. A and a: 4-hr images. B and b: 16-hr images. C and c: 48-hr images. D and d: 72-hr images.

ing agent. The reaction was continued for 5 min to minimize the hydrolysis of the remaining N-hydroxy succinimidyl ester of the cross linking agent which was essential for the conjugation of DTPA to Ab through a diester linkage. The overall reaction yield for conjugating DTPA-p-(aminoethyl)anilide to Ab through a diester linkage was 10% based on the concentration of the cross linking agent or the bifunctional chelating agent (Table 1).

The diester conjugate was less stable than the peptide conjugate to the hydrolysis reactions. The percent formation of a small [^{111}In]DTPA chelate from the cleavage of the Ab-Diester-DTPA-^{111}In was 96, 12, and 5.1%, respectively when incubated in a buffer medium at a pH value of 8.5, 7.5, and 7.0 at 37°C for 24 hr. The formation of a small ^{111}In chelate from the Ab-Peptide-DTPA-^{111}In under the identical hydrolysis reaction conditions was 21, 2.4, and 1.1%, respectively. The results of the hydrolysis reactions support indirectly that the stepwise reactions of EGS with the bifunctional chelator and then with Ab conjugated the chelator to Ab through the readily cleavable diester linkage. A reaction of the amino group of the bifunctional chelating agent with a carbonyl group of the N-hydroxy succinimidyl ester moieties of EGS and a reaction of Ab with a carbonyl group of the glycol ester moieties of EGS would conjugate the bifunctional chelator to antibody through two amide linkages. The cleavage rate of these amide linkages, however, would be somewhat similar to that of the Ab-Peptide-DTPA.

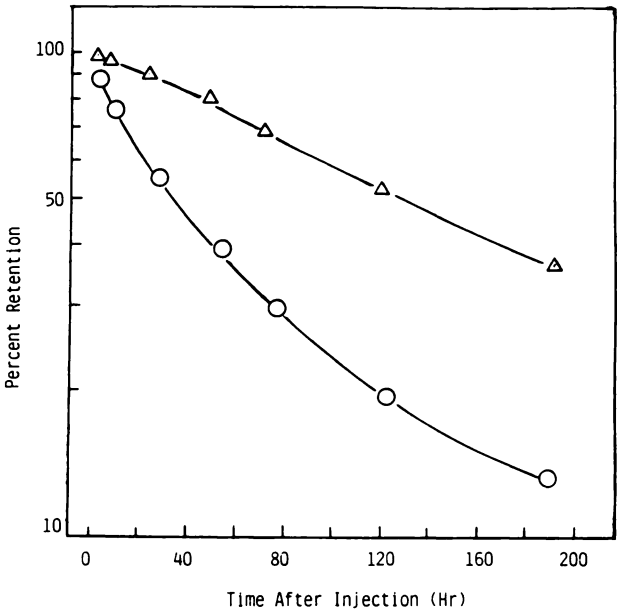

FIGURE 4
Percent whole-body retention of the ^{111}In-labeled Ab DTPA conjugates in nude mice with human melanoma FEM XII xenograft. Δ and O is for the peptide and the diester conjugate, respectively.

Relationship Between DTPA Conjugation and Immunologic Activity

The effect of DTPA conjugation on the immunologic activity of Ab was investigated in detail for the Ab-Diester-DTPA preparation. As assessed by 50% inhibition of the ^{125}I Ab binding to the tumor cells, the immunological activity of the Ab-Diester-DTPA decreased as the number of DTPA molecules conjugated per Ab was increased. The immunologic activity of the antibody conjugate containing 4.2, 7.6, and 11 DTPA molecules per Ab was 55, 24, and 8%, respectively of the immunological activity of the antibody conjugate containing 1.7 DTPA molecules (Fig. 2). It was determined in a separate experiment that the immunologic activity of the antibody conjugate containing 1.7 DTPA molecules was almost identical to the native Ab.

Biodistribution Studies

The Ab-Diester-DTPA and the Ab-Peptide-DTPA containing 1 DTPA molecule per Ab were radiolabeled with ^{111}In for the biodistribution studies. The immunoreactivity of the ^{111}In labeled Ab DTPA conjugates ranged from 50 to 60% as assessed by the direct cell-binding assay.

Most of the tumors larger than 200 mg had a lytic necrotic center which was thoroughly removed from a solid tumor and counted separately from the solid tumor. The activities (% ID/g) in the solid tumors did not change depending upon the size of the tumors but they were 2 to 6 times higher than those in the necrotic tumors for both the diester and the peptide conjugates. This indicates that a larger tumor with a bigger necrotic center accumulates less activity in % ID/g than a small and completely viable tumor.

The biodistribution of the diester conjugate was compared with that of the peptide conjugate at 6, 12, 24, and 48 hr postinjection. The Ab-Peptide-DTPA-^{111}In produced the maximum activities in tumor and organs such as liver, spleen, kidney, intestinal contents, muscle, and bone at 24 hr (Table 2). The tumor and normal organ activities, however, did not change significantly whereas the activity cleared steadily from blood over a period of 48 hr. The Ab-Diester-DTPA-^{111}In, on the other hand, produced the maximum tumor activity at 12 hr but the maximum organ activities at 6 hr. Thereafter, the tumor and the normal organ activities decreased slowly whereas the blood activity cleared much rapidly.

Comparing the solid tumor activities, the diester conjugate produced higher activities (32 and 26%) than those (14 and 17%) of the peptide conjugate at 12 and 24 hr ($p < 0.005$). The solid tumor activities (11 and 15%) of the diester conjugate were similar to those (11 and 16%) of the peptide conjugate at 6 and 48 hr ($p > 0.25$). Comparing the activities in organs, the diester conjugate produced lower activities in all of the organs than the peptide conjugate did. The liver activities for the diester conjugate were 8.4, 6.6, 6.8, and 5.0% at 6, 12, 24, and 48 hr whereas the corresponding activities for the peptide conjugate ranged from 11 to 13%. The liver activities of the diester conjugate at 12, 24, and 48 hr are much lower ($p < 0.020$) than the corresponding activities of the peptide conjugate. At the same time intervals, the kidney activities were 4.9, 4.8, 4.4, and 3.3% for the diester conjugate. These activities are much lower ($p < 0.005$) than the corresponding activities of the peptide conjugate which ranged from 8.2 to 10%. Comparing the blood clearances of both antibody conjugates, the diester conjugate cleared from blood much faster than the peptide so that the blood activity (2.8%) for the diester was almost three times lower than that (7.4%) of the peptide at 48 hr. The faster blood clearance for the diester conjugate was also supported by the imaging studies. The diester produced a clearly visible tumor image at 4 hr and a much lower blood activity at 72 hr as compared to the blood activity of the peptide conjugate.

For the peptide conjugate, the solid tumor to liver and kidney ratios did not change appreciably for a 48 hr period (Table 3). The clearance of the activity from blood was, however, much faster than those from tumor, liver and kidney so that the tumor to blood and the organ to blood ratios increased with respect to time (Table 4). Unlike the peptide conjugate, the diester conjugate produced maximum tumor to liver, spleen, and kidney ratios at 12 hr. Moreover, there was a big increase in the tumor to the organ ratios of the diester

TABLE 3
Solid Tumor to Organ Ratios of ^{111}In-Labeled Ab DTPA Conjugates in Nude Mice with FEM XII Human Melanoma Xenografts

	Ab*-Diester-DTPA				Ab-Peptide-DTPA			
	6 hr	12 hr	24 hr	48 hr	6 hr	12 hr	24 hr	48 hr
Blood	0.5	2.2	3.0	5.4	0.4	1.0	1.3	2.2
Liver	1.6	4.9	3.9	3.0	1.2	1.3	1.3	1.4
Spleen	1.7	9.6	6.2	4.6	1.6	1.3	1.3	1.4
Kidney	2.2	6.7	6.0	4.5	1.4	1.7	1.7	1.7
Intestine	6.2	18.5	19.2	13.2	4.8	5.8	5.8	7.3
Muscle	11.3	38.2	43.4	39.6	8.2	10.3	12.1	19.0
Bone	4.6	14.8	9.7	12.6	3.1	3.3	4.4	4.6

* Ab = monoclonal anti-melanoma antibody 96.5.

conjugate at 12 hr as compared to those at 6 hr. Thereafter, the tumor to normal organ ratios of the diester conjugate decreased with respect to time. On the other hand, the tumor to blood ratio of the diester conjugate increased with respect to time for a 48-hr period. The tumor to blood and normal organ ratios of the diester conjugate were similar to those of the peptide conjugate at 6 hr but the ratios of the diester conjugate became 2 to 7 times larger than those of the peptide conjugate at 12 hr.

The biodistribution of ^{111}In-labeled nonspecific antibody DTPA conjugates was similar to that of the corresponding specific antibody DTPA conjugates except that the solid tumor activities were two to eight times smaller than those of the corresponding specific antibody conjugates and that the kidney activities were somewhat larger than those of the specific antibody conjugates (Table 5). Two to eight times larger solid tumor activities appear to indicate that the specific antibody conjugates accumulated in the tumors primarily by the specific binding to the tumor antigen.

TABLE 4
Organ to Blood Ratios of ^{111}In-Labeled Ab DTPA Conjugates in Nude Mice with FEM XII Human Melanoma Xenografts

	Ab*-Diester-DTPA				Ab-Peptide-DTPA			
	6 hr	12 hr	24 hr	48 hr	6 hr	12 hr	24 hr	48 hr
Liver	0.3	0.4	0.8	1.8	0.4	0.8	1.0	1.6
Spleen	0.3	0.2	0.5	1.2	0.3	0.8	1.1	1.1
Kidney	0.2	0.3	0.5	1.2	0.3	0.6	0.8	1.3
Intestine	0.08	0.1	0.2	0.4	0.09	0.2	0.2	0.3
Muscle	0.05	0.06	0.07	0.1	0.05	0.1	0.1	0.03
Bone	0.1	0.2	0.3	0.4	0.1	0.3	0.3	0.5
S tumor†	0.5	2.2	3.0	5.4	0.4	1.0	1.3	2.2

* Ab = monoclonal anti-melanoma antibody 96.5.
† S tumor = solid tumor.

DISCUSSION

An ideal radioimmunoimaging agent would be one that accumulates in tumor in a high concentration but clears from blood and normal organs rapidly, thereby resulting in high tumor to normal organ ratios at an early time interval after injection. Our strategy to achieve this goal was to add a labile chemical linkage between antibody and an [^{111}In]DTPA derivative, thereby enhancing the cleavage of the linkage between antibody and the chelate in normal organs. The [^{111}In]DTPA metabolite is expected to enter into the circulation and clears rapidly from the circulation by renal excretion.

The purpose of this study was to investigate if the addition of the diester linkage between monoclonal anti-melanoma antibody 96.5 and [^{111}In]DTPA increases tumor to organ ratios in nude mice with a human melanoma tumor xenograft as compared to the antibody conjugate with a peptide linkage. We have selected a diester linkage for this study because liver is rich in carboxyesterases which rapidly metabolize ester linkages (*32*). Our preliminary investigation using rats with target antigen models localized in lungs indicated that the diester linked antibody conjugate was quite stable over a 48hr period once bound to the target but cleared rapidly from blood, giving rise to a high target

TABLE 5
Biodistribution (% ID/g) of ^{111}In Labeled nAb DTPA Conjugates in Nude Mice with FEM XII Human Melanoma Xenograft

	nAb†-Diester-DTPA			nAb-Peptide-DTPA		
	12 hr	24 hr	48 hr	12 hr	24 hr	48 hr
Blood	10.58	8.06	2.40	13.50	14.00	6.48
	1.47	0.58	1.72	4.55	0.34	1.81
Liver	8.83	7.16	5.74	13.69	13.28	15.17
	2.42	1.22	0.39	1.94	1.33	1.88
Spleen	8.41	6.21	5.64	10.26	9.67	13.30
	3.90	2.63	3.24	6.63	4.03	4.28
Kidney	8.97	8.15	7.67	14.88	14.76	14.95
	0.76	0.87	1.13	1.52	1.17	1.38
Intestine	2.39	2.05	1.64	2.69	3.00	3.13
	0.87	0.69	0.29	0.64	0.63	0.71
Muscle	0.80	0.76	0.44	1.26	1.70	1.14
	0.16	0.10	0.07	0.31	0.73	0.30
Bone	2.83	2.98	3.08	4.97	5.20	5.27
	0.90	2.04	1.07	1.63	1.72	1.19
S tumor‡	4.81	3.72	3.08	6.54	6.67	5.79
	1.52	0.69	0.97	1.24	1.75	0.76
N* tumor§	2.63	2.62	1.79	4.82	4.61	4.18
	1.43	0.69	0.36	1.62	1.04	0.62

The data are averages and standard deviations, and normalized to 20 g body weight.
* N = 6.
† nAb = Monoclonal antiidiotype antibody 2-135 of IgG_{2a}.
‡ S tumor = solid tumor.
§ N tumor = necrotic tumor.

to blood ratio (*22*). We used the peptide linkage as a control linkage because it has been the most common linkage connecting antibody to [^{111}In]DTPA for biodistribution and imaging studies. The antibody DTPA conjugates used for this study gave a single HPLC peak with a retention time identical to that of the native antibody. The ^{111}In-labeled antibody conjugates used for the biodistribution studies had similar immunoreactivities. We think that the antibody injectates were different primarily in their chemical linkages.

The diester conjugate cleared from blood much faster and was retained in organs much lower than the peptide conjugate. Moreover, the diester conjugate produced the maximum activities in organs such as liver, spleen, and kidney at 6 hr but the maximum tumor activity at 12 hr whereas the peptide conjugate produced the maximum organ activities and the maximum tumor activity at the same time at 24 hr. These differences in the pharmacokinetics resulted in two to seven times higher tumor to blood and tumor to organ ratios for the diester conjugate for the time intervals between 12 to 48 hr.

The diester conjugate cleared from whole body three times faster, with a biological half life of 36 hr, than the peptide conjugate (Fig. 4). The activity mainly excreted through kidneys and the activity in intestinal contents was 2 to 3% ID/g over a 48-hr period for both conjugates. The whole-body biologic half life of the diester conjugate is similar to the result (43% of the injected dose cleared from whole body of the tumor bearing nude mice in 24 hr) of Haseman et al. (*23*) but is different from the result of Meares et al. (*33*). Haseman et al. and Meares et al. used a bifunctional chelator, p-aminobenzyl EDTA but conjugated it to two different monoclonal antibodies through the diester linkage. Another difference in their experiments is that Haseman et al. used mice bearing B-cell lymphoma and Meares et al. used normal mice. Meares et al. hypothesized that the faster whole-body clearance reported by Haseman et al. as compared to their result might have been caused by a presence of active esterases in the B-cell lymphoma. Our result does not support a view that esterases in FEM XII melanoma xenograft in our animal model are primarily responsible for the three times faster clearance of the diester conjugate from the whole-body because the tumor activities of the diester conjugate were either larger than or similar to those of the peptide conjugate for a 48-hr period. The major site of metabolism of the diester linkage is not yet known. However, blood does not appear to be the major site of metabolism because the diester linkage cleaves at a rate of 10% per day in serum in vitro at 37°C. This is similar to the in vitro cleavage rate (12% per day) of the diester linkage between anti-human serum albumin antibody and the [^{111}In]DTPA chelate in serum at 37°C (*22*).

This study supports a hypothesis that tumor to organ and blood ratios can be optimized by the addition of a metabolizable linkage between antibody and a chelate. The diester linked conjugate clears from blood much faster and is retained much less in normal organs than the peptide linked conjugate. The addition of the diester linkage, therefore, may be beneficial for imaging tumors in patients at early time intervals after injection.

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