Effect of DTPA Conjugation on the Antigen Binding Activity and Biodistribution of Monoclonal Antibodies Against α-Fetoprotein

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Indium-111- (^{111}In) labeled monoclonal antibodies (Ab) prepared with a bifunctional chelating agent, diethylenetriaminepentaacetic acid (DTPA), have been used for the radioimmunoimaging of cancer. In the present experiment, using monoclonal Ab against human α-fetoprotein (AFP) as a model, we have studied the effect of DTPA conjugation on the antigen binding activity and the biodistribution in nude mice transplanted with AFP-producing human testicular tumor. In Ab heavily conjugated with DTPA, the Scatchard plot analysis demonstrated that the maximum binding capacity rather than the affinity constant was affected. Under selected conditions, ^{111}In-labeled Ab were made available with almost full retention of the antigen binding activity and scintigrams of nude mice clearly delineated the site of the tumor. However, the number of DTPA molecules incorporated per Ab molecule markedly influenced the in vivo biodistribution as well as the in vitro antigen binding activity.

J Nucl Med 26:750–755, 1985

With the advent of hybridoma technology (*1*), large quantities of monoclonal antibodies (Ab) specific for tumor associated antigens have been provided and used for the radioimmunoimaging of cancer (*2–4*).

In many studies, Ab were labeled with iodine-131 (^{131}I) but radioiodinated Ab have been proved to be unstable in vivo (*5,6*). Improvements of labeling techniques permit proteins to be labeled with metallic radionuclides, such as indium-111 (^{111}In) or gallium-67, using bifunctional chelating agents and obtained materials have shown good in vitro and in vivo stability (*7–10*). Among various applicable bifunctional chelating agents, diethylenetriaminepentaacetic acid (DTPA) has been most widely studied (*7,8,10–12*).

Using monoclonal Ab against human α-fetoprotein (AFP) as a model (*3*), we have evaluated the effect of DTPA conjugation on the Ab activity and the in vivo biodistribution.

Received Oct. 6, 1984; revision accepted Mar. 19, 1985.

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MATERIALS AND METHODS

The following reagents were procured: monoclonal Ab against human AFP (AFY1, for details see Ref. *13*), AFP*, $Na^{125}I$, $Na^{131}I$, indium metal† and $^{111}InCl_3$‡.

Cyclic DTPA anhydride was prepared according to the method of Eckelman et al. (*8,14*). The [^{111}In]acetate solution was obtained by adding an equal volume of 1*M* sodium acetate to the [^{111}In]chloride solution and the final pH and acetate concentration were 5.5–6.0 and 0.5*M*, respectively (*8*). Radioiodination of Ab was performed by the chloramine-T method and iodinated Ab were confirmed to retain their Ab activity (*15*).

Conjugation of DTPA to monoclonal Ab against AFP

To the Ab solution (0.05–5 mg/ml in 0.1*M* $NaHCO_3$) was added solid cyclic DTPA anhydride (cyclic DTPA anhydride to Ab molar ratio of 33–250). The solution was mixed vigorously and was left to stand for 1 hr at room temperature. The reaction mixture was concentrated§ to the protein concentration of ~2.0 mg/

ml. Then, unconjugated DTPA was separated from DTPA conjugated Ab by Sephadex G-50 column chromatography (1 × 26 cm) eluted with 0.01*M* acetate buffer, pH 6.0. Half of the purified solution was immediately neutralized by addition of 1 N NaOH to be used for the evaluation of the Ab activity.

Determination of the number of DTPA molecules incorporated per Ab molecule

The number of attached DPTA molecules per Ab was determined by the following two methods before and after the purification from free DTPA.

Direct method: Before removing unconjugated DTPA, a small aliquot of the reaction mixture was acidified with 1 N HCl to pH 6.0 and reacted with tracer amount of [^{111}In]acetate for 30 min at room temperature. The mixture was then put on Sephadex G-50 column (0.7 cm × 20 cm) and eluted with 0.01*M* acetate buffer, pH 6.0. The percentage of radioactivity present at the protein fraction, that is, the coupling efficiency was determined. The number of DTPA molecules incorporated per Ab molecule was calculated from the coupling efficiency and the molar ratio of added cyclic DTPA anhydride to Ab.

Tracer method: A small aliquot of purified DTPA conjugated Ab was incubated with tracer amount of ^{111}In and stable indium in 0.02 N HCl, which contained two- to fivefold molar excess of the attached DTPA estimated by the above method. Unconsumed indium ion was then complexed with an excess of ethylenediaminetetraacetic acid to prevent the formation of insoluble indium hydroxide and the nonspecific attachment of indium to the protein. The solution was applied to Sephadex G-50 column (0.7 cm × 20 cm) and eluted with 0.01*M* acetate buffer pH 6.0. The number of DTPA molecules attached per Ab molecule was estimated from the labeling efficiency and the molar ratio of protein to indium ion added into the solution (*12,16*).

Determination of the antigen binding activity of DTPA conjugated Ab

Purified and neutralized DTPA conjugated Ab were diluted in the range of 100 to 0.01 μg/ml with 0.05*M* phosphate buffered saline, pH 7.5, containing 0.25% bovine serum albumin. One hundred microliters of the diluted Ab solution, 100 μl of ^{125}I-labeled AFP (15,000 cpm) and 100 μl of human γ-globulin solution (2 mg/ml in 0.05*M* phosphate buffered saline, pH 7.5) were incubated for 3 hr at room temperature. One milliliter of 21% polyethylene glycol was added and mixed. After the centrifugation (3,000 rpm, 20 min), supernatants were aspirated and radioactivity of precipitates was measured.

The affinity constant and the maximum binding capacity of Ab were determined by the Scatchard analysis (*17,18*). The amount of Ab giving 20–25% bound radioactivity, various concentrations of unlabeled AFP (10–10,000 ng/ml) and ^{125}I-labeled AFP were incubated as mentioned above. The amount of bound radiolabel (in cpm) divided by the unbound "free" radiolabel (in cpm) was plotted against the amount of bound AFP (radiolabeled plus unlabeled AFP), expressed as μg of AFP per mg of Ab. The linear plot gave the affinity constant and the maximum binding capacity of Ab from its slope and x-intercept, respectively.

Indium-111 labeling of DTPA conjugated Ab and radioimmunochemical purity

DTPA conjugated Ab in 0.01*M* acetate buffer, pH 6.0, was simply mixed with an equal volume of [^{111}In]acetate (0.1–1.0 mCi for 1 mg of Ab) and let stand for 30 min at room temperature. The labeling efficiency determined by Sephadex G-50 column chromatography yielded values as high as 95–100%. The obtained ^{111}In-labeled Ab were used for in vivo studies without further purification.

Radioimmunochemical purity was examined to know what percentage of the radiolabeled compound is specifically immunoreactive in the final product (*19*). Obtained ^{111}In-labeled Ab or radioiodinated ones were incubated with AFP-coated polystyrene beads (0.25 in. in diameter) (*10*). After the exhaustive absorption (three times, each for 2 hr at room temperature), the percentage of radioactivity bound to AFP-coated beads was determined (*20*).

In vivo studies

The AFP-producing human testicular tumor has been maintained in nude mice by serial transplantation (*21*). The basic histological pattern of the tumor was embryonal carcinoma. The tumor was inoculated subcutaneously in the back of nude mice and serum AFP levels in mice were elevated up to 3,500 ng/ml with the tumor growth. One hundred micrograms (50 μCi) of ^{111}In-labeled Ab or 100 μg (150 μCi) of ^{131}I-labeled Ab were injected by way of tail vein. The size of tumors was about 2.0 cm in diameter. Scintigrams were taken 4 days after the injection by a gamma camera with a pinhole collimator. Nude mice were then killed and organs and tumors were removed, weighed, and counted. Nonradioactive iodine was administered into mice from 3 days before the injection and throughout the experiment. Statistical analysis was performed using Student's t-test.

RESULTS

DTPA conjugation to monoclonal Ab

The number of attached DTPA molecules per Ab molecule was determined before and after the purification from free DTPA and similar results were obtained. It was largely dependent on both the Ab concentration

TABLE 1
Effect of Antibody Concentration and Added Cyclic DTPA Anhydride to Antibody Molar Ratio on DTPA Conjugation

Concentration of Ab (mg/ml)	Added cyclic DTPA anhydride to Ab molar ratio	Number of DTPA molecules attached per Ab molecule*	
		Direct method	Tracer method
0.1	250	1.9	2.4
0.2	250	2.6	3.2
0.4	250	4.4	5.5
0.2	50	0.9	0.9
0.5	33	1.0	1.0
5	33	11.8	13.6

* Mean of duplicate experiments.

and the added cyclic DTPA anhydride to Ab molar ratio (Table 1). When the concentration of the Ab solution was 0.2 to 0.5 mg/ml and the cyclic DTPA anhydride to Ab molar ratio was 50 to 33, obtained Ab contained approximately one molecule of DTPA per Ab molecule.

Antigen binding activity of DTPA conjugated Ab and radioimmunochemical purity

Conjugation of DTPA remarkably influenced the antigen binding activity of monoclonal Ab against AFP (Fig. 1). As demonstrated by Scatchard plots of original (Fig. 2A) and of DTPA conjugated Ab (Fig. 2 B, C, D), the loss of Ab activity was considered to be a result of the decrease of the maximum binding capacity rather than the affinity constant (Table 2). At a conjugated DTPA to Ab molar ratio of 1.0, minimum decrease of the maximum binding capacity was observed. When more than 2.4 DTPA molecules were incorporated per Ab molecule, the maximum binding capacity was significantly decreased, although the affinity constant was less affected. The maximum binding capacity of Ab containing 5.5 DTPA molecules per Ab molecule was only about one hundredth of original Ab, but column chromatography of Sephadex G-150 (1.6 cm × 90 cm) indicated the absence of dimer or polymer formation (data not shown).

About 90% of radioiodinated Ab showed the cumulative binding to AFP-coated beads and radioimmunochemical purity was considered to be about 90%. By the same approach, radioimmunochemical purity of ^{111}In-labeled Ab containing 1.0 DTPA molecule was estimated to be 70–80%, whereas that of heavily conjugated ones containing 5.5 DTPA molecules was only 10–20%.

In vivo studies

Scintigrams of nude mice injected with ^{111}In-labeled Ab clearly delineated the site of the tumor (Fig. 3) and the localization of ^{111}In-labeled Ab was significantly

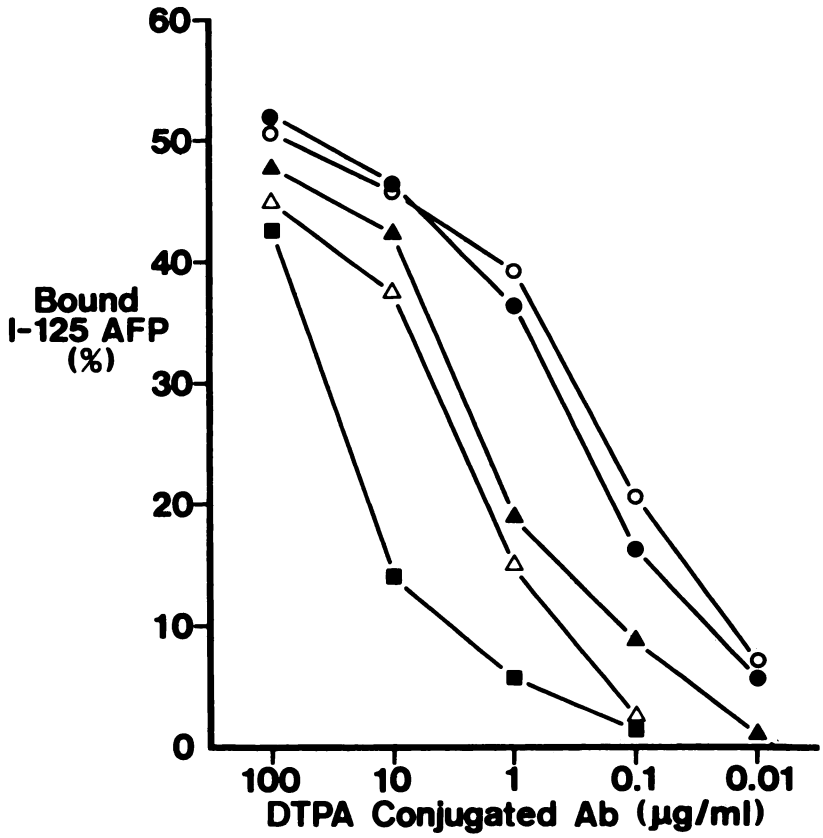

FIGURE 1
Dose-response curves obtained by plotting dilution of DTPA conjugated Ab against percentage of bound ^{125}I-labeled AFP. Original or DTPA conjugated Ab were diluted to range of 100 to 0.01 μg/ml. (O) = Original Ab; (●) = Ab containing 1.0 DTPA molecule per Ab molecule; (▲) = Ab containing 2.4 DTPA molecules per Ab molecule; (△) = Ab containing 3.2 DTPA molecules per Ab molecule; and (■) = Ab containing 5.5 DTPA molecules per Ab molecule, respectively. Ab conjugated with more than 2.4 DTPA molecules per Ab molecule remarkably lost their antigen binding activity

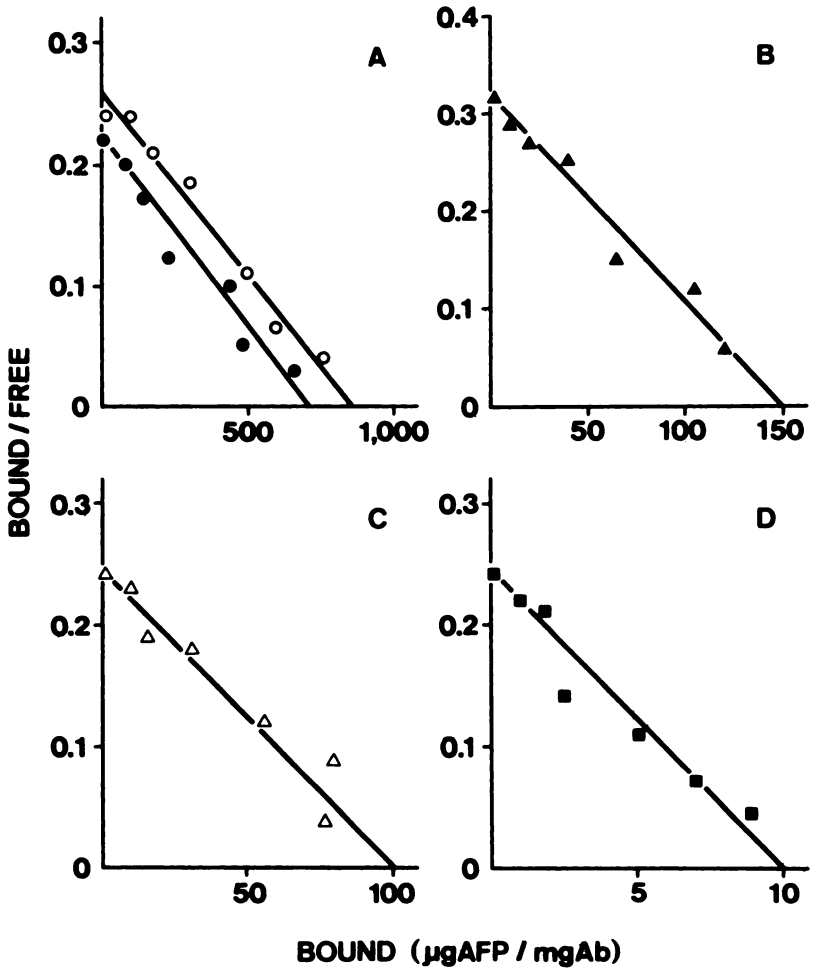

FIGURE 2
Typical Scatchard plots of monoclonal Ab against human AFP. Ratio of bound to free ^{125}I-labeled AFP plotted against amount of bound AFP (radiolabeled plus unlabeled AFP) expressed as μg of AFP per mg of Ab. The affinity constant and the maximum binding capacity were determined by slope and x-intercept, respectively (*17,18*). Amount of Ab showing 20–25% bound radioactivity in Fig. 1 was used for this analysis. A: Original Ab (0.1 μg/ml, O) and Ab containing 1.0 DTPA molecule per Ab molecule (0.1 μg/ml, ●); B: Ab containing 2.4 DTPA molecules per Ab molecule (1.0 μg/ml); C: Ab containing 3.2 DTPA molecules per Ab molecule (1.0 μg/ml); D: Ab containing 5.5 DTPA molecules per Ab molecule (10 μg/ml). Note difference of amount of bound AFP per mg Ab after coupling of DTPA with Ab

higher than that of ^{131}In-labeled Ab (Table 3). Furthermore, ^{111}In-labeled Ab containing 5.5 DTPA molecules per Ab molecule showed lower tumor accumulation but higher liver uptake than Ab conjugated with 1.0 DTPA molecule per Ab molecule.

TABLE 2
Affinity Constant and Maximum Binding Capacity of DTPA Conjugated Antibodies

Number of DTPA molecules incorporated per Ab molecule*	Affinity constant† (X 10^8 M^{-1})	Maximum binding capacity† (μg AFP/mg IgG)
Original	6.0–8.5	700–1020‡
1.0	6.0–8.3	580–820
2.4	5.0–7.7	140–170
3.2	4.9–7.2	90–100
5.5	2.8–4.9	10–30

* Determined by the tracer method.
†Range of triplicate experiments.
‡Assuming that valency of antigen binding of Ab is two, monoclonal Ab (AFY1) used in present study are calculated to have 80–105% of immunoreactivity (purity), which corresponds well to radioimmunochemical purity of radioiodinated Ab.

DISCUSSION

The radioimmunoimaging of cancer is based on the binding of radiolabeled Ab to the corresponding antigen present in the tumor and monoclonal Ab used for this approach have their own affinity constant and maximum binding capacity. In the present study, a quantitative measurement of the effect of DTPA conjugation on the immunoreactivity of Ab was examined by the Scatchard plot analysis, using monoclonal Ab against human AFP as a model. As previously described (*12*), the conjugation of DTPA with monoclonal Ab affects the Ab activity and the number of DTPA molecules attached per Ab molecule is critical. Our data demonstrated that the inactivation of immunoreactivity by DTPA conjugation was due to the decrease of the maximum binding capacity rather than the affinity constant. Ab containing 1.0 DTPA molecule per Ab molecule showed a minimum change of Ab activity. However, with the increase of the number of DTPA molecules incorporated, the maximum binding capacity was remarkably influenced. The reaction of DTPA conjugation is dependent on pH, protein concentration, and added cyclic DTPA anhydride to Ab molar ratio (*8,12*). Ab conjugated at various DTPA to Ab

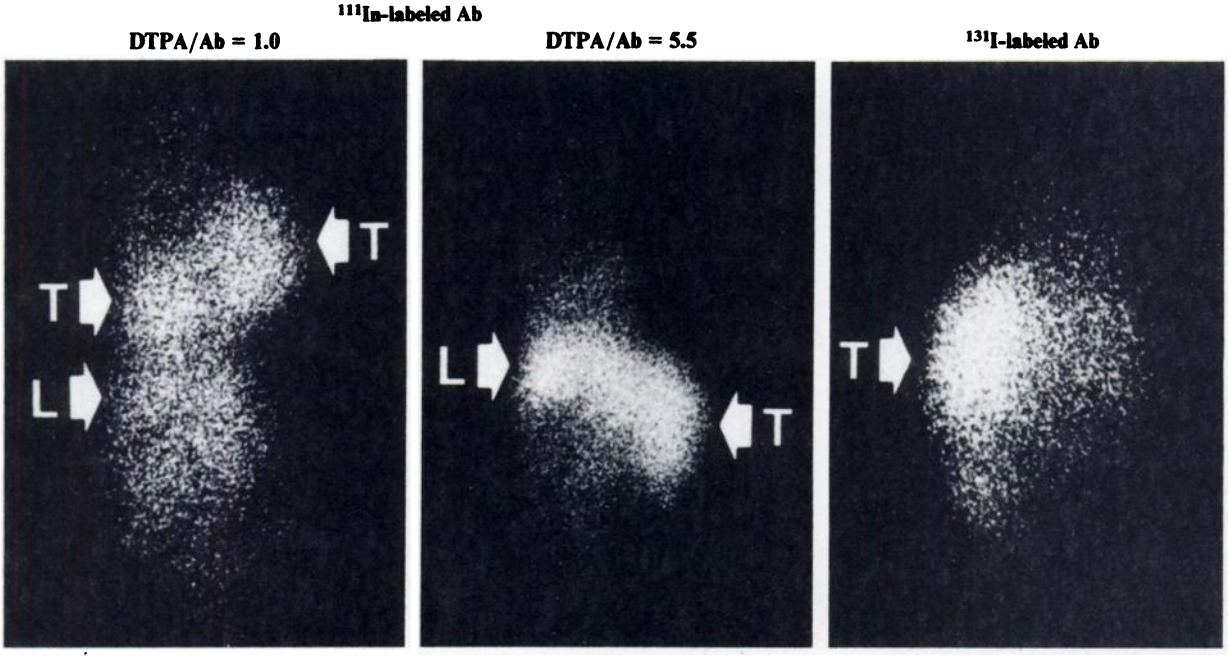

FIGURE 3
Scintigrams of nude mice bearing human testicular tumor at 4 days after injection of ^{111}In-labeled Ab containing 1.0 DTPA molecule per Ab molecule (left) and containing 5.5 DTPA molecules per Ab molecule (middle) and ^{131}In-labeled Ab (right), respectively. (T) Tumor; (L) Liver

molar ratios, by varying either the concentration of Ab or the amount of cyclic DTPA anhydride, showed similar results to those of Hnatowich (*22*) and Paik (*12*), although lower Ab concentrations and higher molar ratio were used than in the former study.

The ^{111}In-labeling of DTPA conjugated Ab was an easy procedure accomplished by simple addition of [^{111}In]acetate, reaching labeling efficiency of 95 to 100% through the chelation with DTPA. Therefore, further purification was not required as frequent steps in radioiodination. ^{111}In-labeled Ab containing 1.0 DTPA molecule per Ab molecule with almost full retention of Ab activity, showed higher tumor accumulation than that obtained with ^{131}I-labeled Ab. Dehalogenation in the tumor is considered to be a cause for low tumor accumulation of radioiodinated Ab (*10*). In addition, physical properties of ^{111}In makes it a more desirable agent. These results indicated the potential usefulness of ^{111}In-labeled Ab for the radioimmunoimaging of cancer. However, the inverse correlation

TABLE 3
Biodistribution of Radiolabeled Antibodies in Nude Mice Bearing Tumor at 4 Days After Injection

	% Injected dose/g tissue (mean ± s.d. or range)		
	^{111}In-labeled Ab		^{131}I-labeled Ab
Tissue	DTPA/Ab* = 1.0 (n = 5)	DTPA/Ab* = 5.5 (n = 5)	(n = 3)
Blood	4.54 ± 0.74	3.85 ± 1.77	4.82 (4.22–5.54)
Tumor	10.93 ± 2.40†§	7.05 ± 2.16§	4.34 (3.92–4.79)†
Liver	6.64 ± 1.30‡	11.34 ± 3.20‡	1.18 (0.86–1.43)
Kidney	11.55 ± 1.19	6.16 ± 1.01	1.21 (1.09–1.33)
Intestine	1.43 ± 0.35	1.55 ± 0.57	0.30 (0.26–0.35)
Stomach	0.94 ± 0.40	0.92 ± 0.37	1.01 (0.53–1.48)
Spleen	4.63 ± 1.82	4.30 ± 1.25	0.69 (0.58–0.85)
Lung	3.16 ± 0.72	3.12 ± 0.83	1.72 (1.54–2.06)
Muscle	1.14 ± 0.45	0.54 ± 0.16	0.34 (0.28–0.42)
Bone	5.60 ± 2.19	2.27 ± 0.51	0.50 (0.35–0.67)

* Conjugated DTPA to Ab molar ratio. Determined by tracer method.
† $p < 0.01$.
‡ $p < 0.02$.
§ $p < 0.05$.

between the number of DTPA molecules attached per Ab and the immunoreactivity was also demonstrated in the in vivo biodistribution studies. Indium-111-labeled Ab containing 5.5 DTPA molecules per Ab molecule showed low tumor accumulation but high liver uptake. This probably reflected the denaturation of Ab by the heavy conjugation of DTPA, although the formation of dimer or polymer complexes was not confirmed by the column chromatography technique.

In conclusion, the Scatchard plot analysis provided a very good basis for understanding the effect of DTPA conjugation on the immunoreactivity of monoclonal Ab. The number of DTPA molecules incorporated per Ab molecule markedly influenced the antigen binding activity especially the maximum binding capacity and the in vivo biodistribution of ^{111}In-labeled Ab. Under selected conditions, ^{111}In-labeled Ab showed almost full retention of the Ab activity and scans of tumor bearing nude mice clearly delineated the site of the tumor.

FOOTNOTES

* Green Cross Corp., Osaka, Japan.
† Nakarai Chemicals, LTD., Kyoto, Japan.
‡ Nihon Medi-physics Corp., Takarazuka, Japan.
§ Minicon B concentrator, Amicon Corp., Danvers, MA.

ACKNOWLEDGMENT

This work was supported in part by a grant from the Ministry of Health and Welfare, Japan.

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