Technetium-99m-Labeled Monoclonal Antibody with Preserved Immunoreactivity and High In Vivo Stability

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Recent availability of monoclonal antibodies (MoAb) and their radiolabeling through the use of the bifunctional chelating agents (BCA) have become an alternative procedure for in vivo radioimmunodetection. Using a newly synthesized BCA, a p-carboxyethylphenylglyoxal-di(N-methylthiosemicarbazone) (CE-DTS), the coupling and technetium-99m (^{99m}Tc) labeling of monoclonal IgG against hCG were carried out. In the system presented, factors affecting stability and immunoreactivity were examined. Immunoreactivity of the original IgG (56C) was preserved by conjugating one CE-DTS molecule per molecule of IgG (56C) using the phosphorylazide method, however, ^{99m}Tc labeling pH affected the immunoreactivity and limited the ^{99m}Tc labeling reaction between pH 4.5 and 6.2. A screening of labeling conditions, such as pH, reaction time, and reducing agent system were then carried out. Technetium-99m-labeled IgG (56C), [^{99m}Tc]CE-DTS-IgG (56C), showed good stability upon incubation with mice sera and comparable mice biodistribution to that of indium-111 (^{111}In) DTPA-IgG (56C). Thus, these results indicate the excellent potential of CE-DTS as a BCA for labeling MoAb with ^{99m}Tc.

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In recent years, radioimmunodetection of tumor using monoclonal antibodies (MoAb) has been extensively researched by many investigators (*1–11*). Since the coupling methodology of diethylenetriaminepentaacetic acid (DTPA) to macromolecules was established by Krejcarek and Hnatowich et al. (*12,13*), indium-111- (^{111}In) labeling of MoAb through the use of DTPA as a bifunctional chelating agent (BCA) has become one of the most used methods (*5–11*).

Despite many attempts (*14–18*), procurement of technetium-99m- (^{99m}Tc) labeled MoAb that satisfy the need for both high in vivo stability and preserved immunoreactivity has been difficult. Labeling of MoAb with ^{99m}Tc using DTPA has been reported to provide good in vivo stability (*16–18*); biodistribution studies, however, have revealed lower in vivo stability than the [^{111}In]DTPA-MoAb, even at high DTPA conjugation levels (*18*).

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In previous studies, suitable properties of p-carboxyethylphenylglyoxal - di(N - methylthiosemicarbazone) (CE-DTS) (Fig. 1) as a BCA for ^{99m}Tc labeling of proteins has been reported (*19,20*), and it appears to have high potentiality to label other biomolecules. Thus, in the present report, the ^{99m}Tc-labeling of MoAb using CE-DTS was carried out. Using monoclonal IgG against hCG [IgG (56C)] as a model, and after determining the conditions for the CE-DTS conjugation and ^{99m}Tc labeling, a screening on the immunoreactivity and in vivo stability of the [^{99m}Tc]CE-DTS-IgG (56C) was conducted.

MATERIALS AND METHODS

The bifunctional chelating agent, CE-DTS, was synthesized according to procedures previously reported (*19*). Monoclonal IgG against hCG [(IgG (56C)] was obtained according to the standard procedure (*21*). Iodine-125-labeled hCG ([^{125}I]hCG) was prepared by the chloramine-T method. Indium-111-labeled DTPA-IgG (56c) (<1 In atom/IgG) was prepared by coupling cyclic DTPA anhydride with IgG (56C), followed by

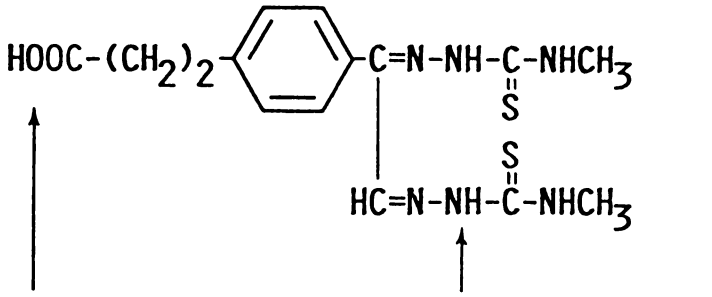

FIGURE 1
Chemical structure of p-carboxyethylphenylglyoxal-di(N-methylthiosemicarbazone) (CE-DTS).

^{111}In labeling with [^{111}In]acetate, according to the procedure by Hnatowich et al. (*13*), with slight modification (*11*).

Preparation of CE-DTS-IgG (56C)

CE-DTS was coupled to monoclonal IgG (56C) at 1:1 molar ratio [(CE-DTS-IgG (56C)] by the phosphorylazide method as previously reported (*19*). To a chilled solution (−5 to −10°C) of CE-DTS (5×10^{-2} mmol) prepared in 0.5 ml of dimethylformamide was added equimolar amount of triethylamine and diphenylphosphoryl azide.* After stirring at the same temperature for another hour, 20 μl of this solution was added to a cold (0°C) solution of IgG (56C) (3×10^{-4} mmol) in 10 ml of 0.05*M* borate buffer containing 0.05*M* NaCl (pH 9.0) and stirred gently for an hour. After overnight dialysis against a large excess of 0.02*M* acetate buffer (pH 5.0) at 4°C, this solution was applied on CM-23† column chromatography (0.8 × 20 cm) and washed with the same buffer until no uv absorbance at 320 nm (free CE-DTS) was detected; then eluted with 0.2*M* acetate buffer (pH 6.2). Following the concentration by ultrafiltration, a CE-DTS-IgG (56C) solution (6 mg/ml) was prepared with appropriate 0.2*M* acetate buffer. The number of CE-DTS molecule coupled to IgG (56C) was determined by the spectrophotometric measurement at 280 nm and 320 nm, using the absorption coefficient of CE-DTS of $E_{280}^{0.1\%} = 25$ and $E_{320}^{0.1\%} = 75$, and that of IgG of $E_{280}^{0.1\%} = 1.4$, respectively, as described (*19*).

Technetium-99m Labeling

To 100 μl solution of CE-DTS-IgG (56C) (6 mg/ml) in 0.2*M* acetate buffer with varying pH (2.0–6.2) was added 40 μl of $10^{-4}M$ $SnCl_2$‡ prepared in N_2 purged $10^{-2}M$ ascorbic acid (Sn/Asc) or $2 \times 10^{-3}M$ tartrate buffer (pH 3.0) (Sn/Tart). Then, 60 μl of [^{99m}Tc]pertechnetate eluate (0.5–2.5 mCi) was added. After appropriate incubation time at 37°C under N_2 atmosphere, this solution was passed through 0.22 μm Millipore filter.

Competitive Binding Studies

A mixture of 50 μl of uncoupled IgG (56C) (12 mg/ml) and 50 μl of buffer solution containing varying amount of free CE-DTS was labeled with ^{99m}Tc according to the same procedure as described above.

Immunoreactivity Measurement

Immunoreactivity of [^{99m}Tc]CE-DTS-IgG (56C) was measured by radioimmunoassay using [^{125}I]hCG (70 μCi/μg, 5×10^{-8} μg hCG/μl). The affinity constant and the maximum binding capacity of [^{99m}Tc]CE-DTS-IgG (56C) were determined by the Scatchard analysis (*11,12*). Technetium-99m-CE-DTS-IgG (56C) or original IgG (56C) was diluted to 50 ng/ml with 0.05*M* phosphate buffered saline (pH 7.5). Then, 100 μl of these solutions, 100 μl each of [^{125}I]hCG containing varying amount of hCG (0–3,200 mU/ml), and 200 μl of normal mice serum were incubated at 4°C overnight. Then, 100 μl of anti-mice IgG rabbit antibody was added and incubated for 3 hr at room temperature. After centrifugation (3,000 rpm, 20 min), supernatant were aspirated and radioactivity of the precipitates was counted. The amount of bound radioactivity divided by the unbound "free" radioactivity was plotted against diluted hCG (mU/ml) or the amount of μg of hCG per mg of IgG (56C).

In Vitro Analysis

The labeling efficiency of ^{99m}Tc was determined by cellulose acetate electrophoresis in veronal buffer ($\mu = 0.05$, pH 8.6) at the electrostatic field of 0.8 mA/cm for 15 min and 45 min. The electrophoresis carried out for 15 min was used for the detection of free pertechnetate registered at 5 cm toward the anode, and the electrophoresis for 45 min was used for the analysis of protein-bound and nonprotein bound radioactivity. Position of the protein was determined by Ponceau 3R staining.

In vitro stability of [^{99m}Tc]CE-DTS-IgG (56C) was determined by HPLC using TSK G4000SW§ column eluted with 0.02*M* phosphate buffer (pH 7.0) containing 0.1*M* NaCl. After labeling, 50 μl of the preparation was added to 500 μl of fresh mice serum and incubated at 37°C. Then, 50 μl aliquots were removed at 2 and 6 hr for HPLC analysis.

In Vivo Studies

An amount of 50 μl of [^{99m}Tc]CE-DTS-IgG (56C) (150 μg, 15 μCi) or [^{111}In]DTPA-IgG (56C) (150 μg, 5 μCi) was injected into male ddY mice (weighing 25–30 g) through a lateral tail vein. After desired time interval, each mouse was killed and organs of interest were removed, weighed, and counted in a well counter.

RESULTS

Conjugation of CE-DTS with IgG (56C) at 1:1 molar ratio was easily carried out by the phosphorylazide

method with 16.5% yield after some modification of the buffer components, pH and the conjugation or purification system employed for the CE-DTS-IgG (56C) conjugation (described under method). The conjugation of one CE-DTS molecule per molecule of IgG (56C) displayed an alike immunoreactivity to the original IgG (56C), as shown in Figure 2 and Table 1.

The major factor affecting the immunoreactivity of [^{99m}Tc]CE-DTS-IgG (56C) was the ^{99m}Tc labeling pH (Fig. 2); the original immunoreactivity of IgG (56C) was preserved between pH 4.5 and 6.2. At pH below 4.0, immunoreactivity of [^{99m}Tc]CE-DTS-IgG (56C) decreased along with a decrease of labeling pH. As summarized in Table 1, the loss of immunoreactivity was due to the decrease of maximum binding capacity rather than the affinity constant. Consequently, following a survey of the optimal conditions, ^{99m}Tc labeling was carried out between pH 4.5 and 6.2.

In this pH range, using the Sn/Asc procedure, previously estimated as optimal for the ^{99m}Tc labeling of CE-DTS coupled HSA (*20*), the presence of a single peak was registered (15 min electrophoresis profile), only if pH was lowered to pH 4.5, although no free pertechnetate was detected at either pH (Fig. 3). In the 45 min electrophoresis, however, a ^{99m}Tc radioactivity peak other than that related to the protein fraction was detected; in the pH 6.2 labeling, a third fraction was registered. Under these circumstances, another procedure, the Sn/Tart system was tested. As shown in Fig. 4, a single ^{99m}Tc radioactivity peak was detected at the protein fraction at every pH in a 15 min electrophoresis.

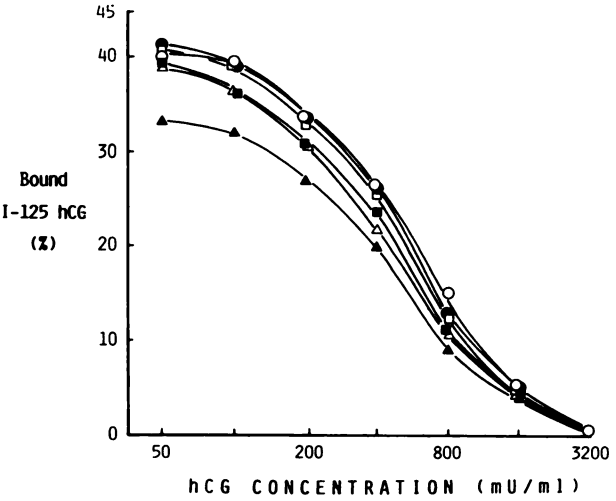

FIGURE 2
Immunoreactivity of [^{99m}Tc]CE-DTS-IgG (56C) at various labeling pH. Dose-response curve obtained by plotting diluted hCG against bound/total ^{125}I-labeled hCG. Original IgG (56C) at pH 6.2 (O), CE-DTS-IgG (56C) (1:1 molar ratio) labeled with ^{99m}Tc at pH 6.2 (●), at pH 4.5 (□), at pH 4.0 (■), at pH 3.0 (△) and at pH 2.0 (▲). Means for triplicate experiments.

TABLE 1
Immunoreactivity of [^{99m}Tc]CE-DTS-IgG (56C) at Various pH

	^{99m}Tc-labeling pH	Immunoreactivity: Affinity constant [M^{-1}]	Immunoreactivity: Maximum binding capacity [μg hCG/mg IgG (56C)*]
CE-DTS-IgG (56C)‡	6.2	5.5×10^9	167
	5.0	5.6×10^9	169
	4.5	5.5×10^9	167
	4.0	5.6×10^9	155
	3.0	5.7×10^9	147
	2.0	5.8×10^9	122
Original IgG (56C)	6.2	5.5×10^9	160

* 1 mg hCG = 12,000 U.
‡ CE-DTS was coupled to IgG (56C) at 1:1 molar ratio.

In the 45 min electrophoresis, however, increasing labeling efficiency was observed with a rise of the labeling pH from 4.5 to 6.2. ^{99m}Tc labeling at pH 6.2 afforded about 90% radioactivity at the protein fraction (Fig. 4).

The specificity of the Sn/Tart system was then screened. Under set reaction conditions, the labeling of uncoupled IgG (56C) in the presence of free CE-DTS at 1:1 and 1:10 molar ratio or in the absence of free CE-DTS was carried out (Fig. 5). When the uncoupled IgG (56C) was labeled with ^{99m}Tc, 40–45% radioactivity was detected by a 45 min electrophoresis at the fraction corresponding to the protein, and remained unchanged up to 24 hr. In the labeling of uncoupled IgG (56C) in the presence of free CE-DTS, ^{99m}Tc radioactivity at the protein fraction decreased with the increase of CE-DTS molar ratio; at the equimolar ratio, 15–20% radioactivity was found at the protein fraction, but at the tenfold excess of free CE-DTS, only 3–5% radioactivity was found at the protein fraction. These values were unchanged even after the reaction was extended for 24 hr. In the labeling of CE-DTS-IgG (56C) conjugate, however, ^{99m}Tc labeling was time dependent and more than 3 hr was required to reach at 87–90% labeling efficiency with no further labeling yield decrease.

The in vitro stability of [^{99m}Tc]CE-DTS-IgG (56C) was tested by incubation with mice sera. As shown in Figure 6, the HPLC analysis of [^{99m}T]CE-DTS-IgG (56C) at 2 hr (B) and 6 hr (C) postincubation with mouse serum was nearly identical with that of the original [^{99m}Tc]CE-DTS-IgG (56C) alone in the absence of serum (A). The radioactivity elution profile of the radiolabeled conjugate in the presence or absence of serum coincided and no other ^{99m}Tc species of larger or smaller molecular weight were detected (Fig. 6A–C). The uv profile of the conjugate coincided with the fraction corresponding to albumin, the major component of the serum (Fig. 6D). No absorption at other fractions of larger molecular size was detected, an in-

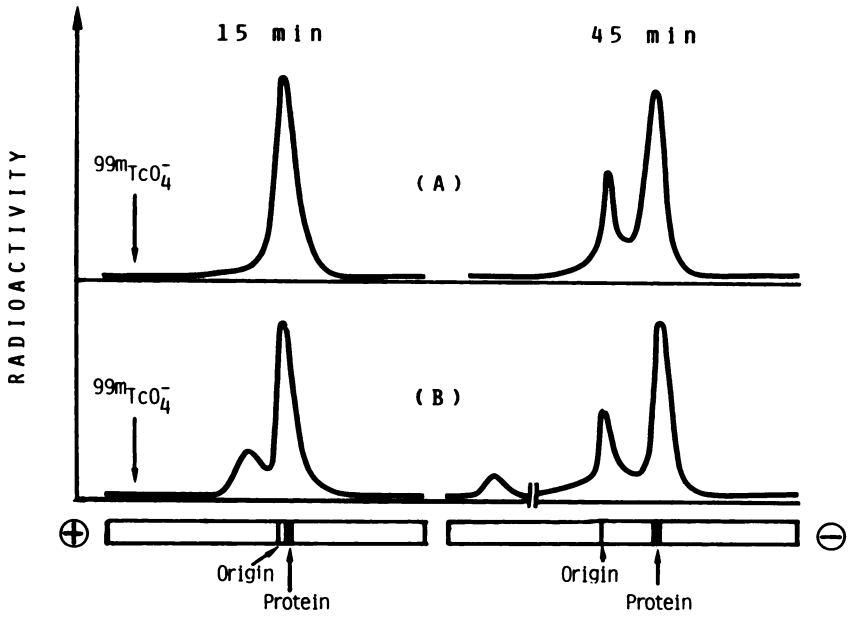

FIGURE 3
Cellulose acetate electrophoresis profile of [^{99m}Tc]CE-DTS-IgG (56C). Technetium-99m labeling of CE-DTS-IgG (56C) was carried out at pH of (A) 4.5, and (B) 6.2 for 3 hr in the presence of stannous chloride prepared in ascorbic acid (Sn/Asc). Electrophoresis was carried out in veronal buffer pH 8.6 (μ = 0.05) with the electrostatic field of 0.8 mA/cm for 15 min and 45 min. Position of the protein was determined by the Ponceau 3R staining.

dication of the good stability of the [^{99m}Tc]CE-DTS-IgG (56C).

Mouse biodistribution of this [^{99m}Tc]CE-DTS-IgG (56C) was compared with [^{111}In]DTPA-IgG (56C). As shown in Table 2, these two compounds showed almost the same percent injected radioactivity in blood up to 24 hr postinjection. The greatest differences were observed in their organ distribution; while the ^{99m}Tc radioactivity in the liver and kidney decreased between 6 hr and 24 hr postinjection, the ^{111}In radioactivity in these organs were unchanged up to 24 hr.

DISCUSSION

Radioimmunodetection is based on the in vivo binding of the administered radiolabeled antibodies to the corresponding antigen present on the target tissues. Consequently, procurement of ^{99m}Tc-labeled MoAb with preserved original immunoreactivity as well as good in vivo stability are required for successful radioimmunodetection. As for the procurement of a stable ^{99m}Tc-labeled MoAb, selective binding of ^{99m}Tc to the CE-DTS part is necessary. The coupling of a large

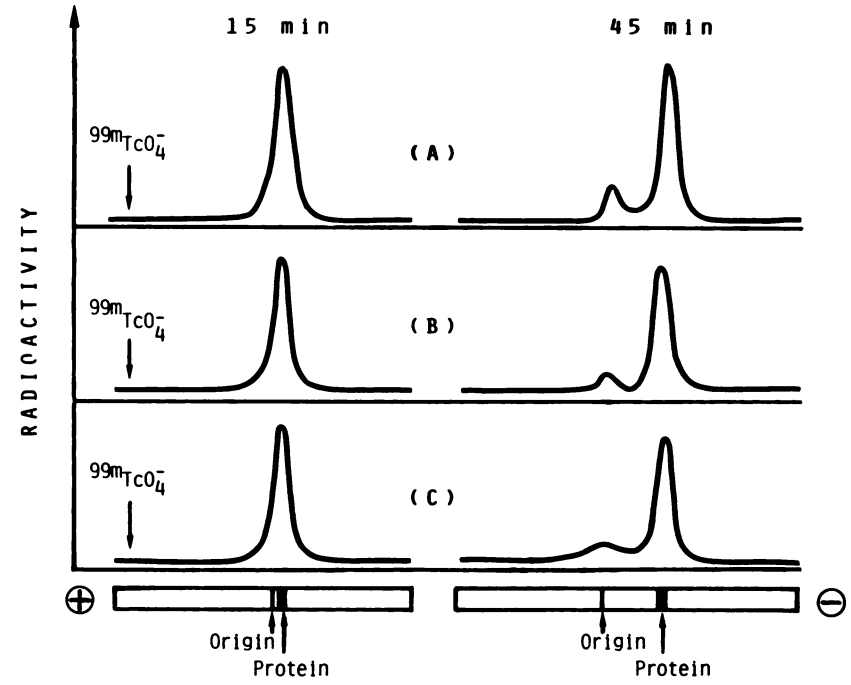

FIGURE 4
Cellulose acetate electrophoresis profile of [^{99m}Tc]CE-DTS-IgG (56C). Technetium-99m labeling was carried out at pH of (A) 4.5, (B) 5.5, and (C) 6.2 for 3 hr in the presence of stannous chloride prepared in tartrate buffer (pH 3.0) (Sn/Tart). Electrophoresis was carried out under the same conditions as described in Figure 3.

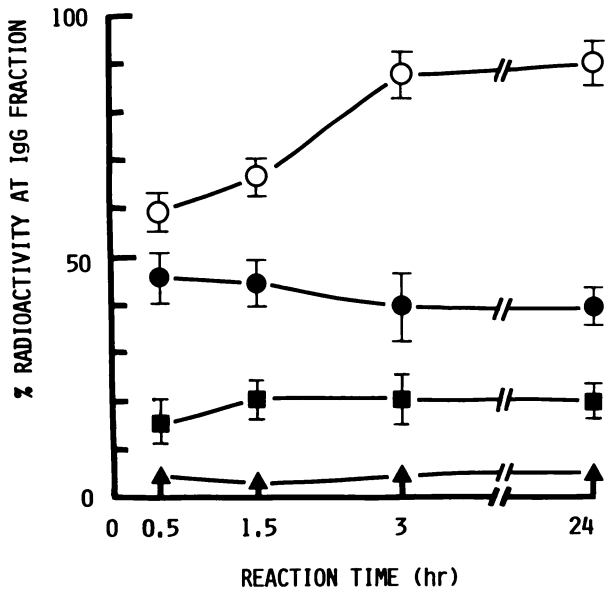

FIGURE 5
Technetium-99m labeling efficiency of CE-DTS-IgG (56C) and uncoupled IgG (56C) as a function of reaction time. Each value was determined by the 45 min electrophoresis. CE-DTS-IgG (56C) (O), uncoupled IgG (56C) in the presence of equimolar amount of free CE-DTS (■), ten fold excess of CE-DTS (▲) and uncoupled IgG (56C) in the absence of free CE-DTS (●). Means and s.d. for triplicate experiments.

number of CE-DTS per molecule of MoAb appeared as a means to increase ^{99m}Tc binding to the CE-DTS part of the molecule. However, heavy BCA conjugation decrease the immunoreactivity of MoAb (*7–11,23*). Therefore, along with the CE-DTS conjugation level, a good balance between in vivo stability and immunoreactivity is most desirable.

In our preliminary work, conjugation of up to one

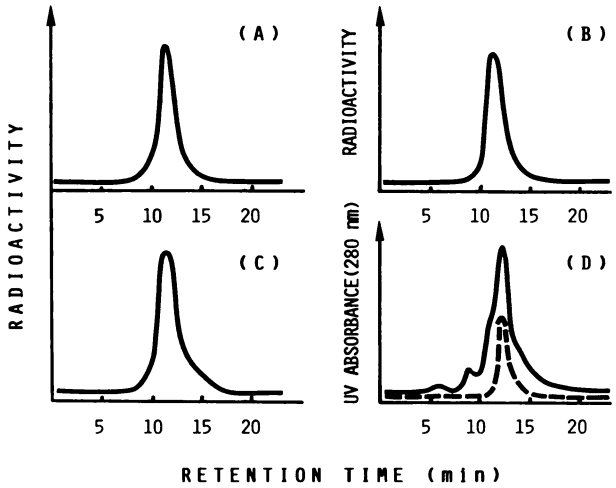

FIGURE 6
HPLC radioactive elution profile of [^{99m}Tc]CE-DTS-IgG (56C) (A) before and after incubation with mice serum for (B) 2 hr, (C) 6 hr, and (D) HPLC uv elution profile of mice serum (—) and [^{99m}Tc]CE-DTS-IgG (56C) (---).

TABLE 2
Biodistribution of [^{99m}Tc]CE-DTS-IgG (56C) and [^{111}In] DTPA-IgG (56C) in Normal Mice at Various Time Postinjection

Organ	Time after injection (hr)* 1	3	6	24
	[^{99m}Tc]CE-DTS-IgG (56C)			
Blood	26.03 (2.81)	18.97 (0.46)	16.77 (1.22)	9.92 (1.43)
Lung	5.30 (0.71)	4.33 (0.49)	4.19 (0.60)	3.09 (0.33)
Liver	13.07 (1.76)	13.02 (2.29)	11.63 (1.64)	5.96 (0.67)
Spleen	3.58 (0.58)	2.93 (0.57)	2.32 (0.34)	2.14 (0.41)
Kidney	8.96 (0.87)	8.17 (0.48)	7.93 (0.83)	5.13 (0.67)
Stomach	2.57 (0.35)	2.55 (0.40)	2.33 (0.34)	2.14 (0.41)
Intestine	1.90 (0.22)	2.50 (0.51)	2.39 (0.45)	0.99 (0.16)
	[^{111}In]DTPA-IgG (56C)			
Blood	26.98 (1.91)	20.72 (2.13)	18.83 (2.03)	10.90 (1.48)
Lung	8.92 (1.19)	7.22 (0.45)	6.54 (1.68)	5.38 (0.69)
Liver	10.58 (1.76)	9.07 (1.34)	8.95 (1.04)	10.65 (1.21)
Spleen	4.05 (0.76)	3.27 (0.54)	3.47 (0.51)	3.62 (0.42)
Kidney	9.12 (0.78)	8.37 (1.01)	9.96 (0.86)	10.31 (1.09)
Stomach	0.87 (0.24)	0.83 (0.30)	1.21 (0.39)	2.22 (0.83)
Intestine	1.45 (0.25)	1.80 (0.14)	1.99 (0.55)	3.17 (0.55)

* Expressed as percent injected dose per gram wet weight as the mean (1 s.d.) for five animals.

CE-DTS molecule per molecule of MoAb against human thyroglobulin has preserved its original immunoreactivity and any CE-DTS conjugation over one has decreased the immunoreactivity (*23*). Thus, in the present study, a molar ratio of 1:1 was selected for the CE-DTS coupling to IgG (56C), using a practical method based on coupling the carboxyl group of CE-DTS with the amino component of the protein by the DPPA. This is a mild and suitable reaction for IgG since the reaction proceeds through the pre-activation of the carboxyl group through the carboxyl azide before mixing with the protein, and this causes no cross-linkage or loss of immunoreactivity of the protein, as experimentaly demonstrated (Fig. 6, Fig. 2, and Table 1).

However, ^{99m}Tc labeling pH appeared as the main factor altering immunoreactivity (Fig. 2, Table 1), and limited the following ^{99m}Tc labeling reaction of CE-DTS-IgG (56C) to a pH range between 4.5 and 6.2. In this pH range, the labeling condition previously selected for the CE-DTS coupled HSA was inapplicable; the chemical state of tin seems to be responsible for undesirable competitive side reactions of ^{99m}Tc (*24–27*). In the previous study with CE-DTS coupled HSA, the use of tin stabilized by ascorbic acid (Sn/Asc system) offered highly stable ^{99m}Tc-labeled HSA (*20*). However, such system proceeds at rather low pH, and in the pH range needed for the IgG of 4.5 to 6.2, some undesirable reaction products appear (Fig. 3). Only the use of stannous chloride prepared in tartrate medium (Sn/Tart) offered high labeling efficiency (Fig. 4). These phenomena may be attributed to the higher stability of Sn-Tart

than the Sn/Asc (*28,29*), avoiding hydrolysis and oxidation of the tin, while a rise of the reaction pH, favored higher reactivity of CE-DTS.

The selected labeling methodology provided a [^{99m}Tc]CE-DTS-IgG (56C) with appropriate stability, with different characteristics from the directly labeled IgG (Fig. 5). The uncoupled IgG (56C) subjected to the Sn/Tart labeling reaction underwent some labeling and labeled IgG (56C) remained unchanged with reaction time. On the other hand, the labeling of CE-DTS-IgG (56C) conjugate displayed an increased yield with an increase of reaction time. Probably, due to some steric interference induced by the bulky IgG (56C) molecule upon coupling, some reduced ^{99m}Tc attached in amino acid residues of the protein undergoes a slow exchange toward the CE-DTS part of the molecule. Another aspect, as also stated by Paik et al. might be attributed to the presence of low affinity and high capacity sites of IgG (*30*). However, above all, the appropriate characteristics of CE-DTS in the selective coordination of ^{99m}Tc was well established by the competitive reaction in the presence of uncoupled IgG (56C) molecule (Fig. 5).

The good stability of [^{99m}Tc]CE-DTS-IgG (56C) can be estimated from the in vitro serum incubation study analyzed by HPLC (Fig. 6) and the comparative mice biodistribution study of [^{99m}Tc]CE-DTS-IgG (56C) with [^{111}In]DTPA-IgG (56C) (Table 2). Unchanged HPLC profile and the comparable blood clearance of ^{99m}Tc and ^{111}In labeled IgG (56C) shows the tight complex of ^{99m}Tc and CE-DTS-IgG (56C). Different liver and kidney clearance of the two labels might be attributed to the different stability of the labels after catabolism at the sites of localization (*31*).

In conclusion, ^{99m}Tc-labeled MoAb with preserved original immunoreactivity and good in-vivo stability was obtained by coupling CE-DTS to MoAb at 1:1 molar ratio by phosphorylazide method, followed by ^{99m}Tc labeling at pH 6.2 in the presence of tin stabilized by tartrate media. High potentiality of CE-DTS as a BCA for labeling MoAb with ^{99m}Tc was observed. Studies to shorten the reaction time and conditions to increase the selectivity of ^{99m}Tc binding is in progress.

NOTES

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