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Radioimmunoscintigraphy of colorectal cancer with technetium-99m-labeled murine anti-carcinoembryonic antigen monoclonal antibody in athymic nude mice

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Technetium-99m (Tc-99m) is an ideal radionuclide for clinical use. A murine monoclonal antibody (Mab) designated F33-104 binds to specific parts of carcinoembryonic antigen (CEA). In the present study, intact Mab F33-104 was labeled with Tc-99m, and the immunoreactivity and biodistribution of Tc-99m-labeled F33-104 were studied in athymic nude mice bearing human colorectal cancer xenografts. Mab F33-104, reduced under optimal conditions, was quickly and stably tagged with Tc-99m without loss of immunoreactivity. Higher tumor uptake of Tc-99m-labeled F33-104 was noted in the biodistribution, resulting in a higher localization index and specific-to-non-specific tumor ratio than those of radioiodinated F33-104. These results suggest the potential of Tc-99m-labeled Mab F33-104 for the radioimmunoimaging of colorectal cancer.

Key words: Tc-99m-labeled Mab F33-104, CEA, radioimmunoscintigraphy

INTRODUCTION

CARCINOEMBRYONIC ANTIGEN (CEA) is one of the most useful human tumor markers.[1,2] Mabs to CEA have been labeled with radionuclides and studied for the diagnosis and therapy of colorectal cancer.[3–7] It is generally accepted that Tc-99m is an ideal radionuclide for clinical use, due to its ready availability, low irradiation of the patients and low cost. Tc-99m-labeled antibodies have therefore been studied for radioimmunoscintigraphic studies of cancer, infection, thrombosis and myocardial infarction.[3–7] However, there have been many problems such as the complexity of the processes, the long time required for preparation and *in vivo* unstableness in clinical use of Tc-99m-labeled Mabs.

Murine Mab designated F33-104 recognizes protein epitopes on domain A3-B3 of CEA, and is able to discriminate CEA in tumor tissues from normal fecal antigen-2, a soluble form CEA-counterpart in normal adult feces. F33-104 is currently used as the radiolabeled tracer in the radioimmunoassay (RIA) of serum CEA levels, resulting in the improvement of cancer specificity, sensitivity and accuracy.[8–10] The present study employed a direct Tc-99m labeling method, where antibody could be quickly labeled with Tc-99m in the presence of a weak competing ligand hydroxyl methylene diphosphonate (HMDP) after the reduction of intrinsic disulphide bonds of Mab by the use of 2-mercaptoethanol.[11]

In the present paper, the *in vitro* and *in vivo* properties of Tc-99m-labeled Mab F33-104 are compared with those of radioiodination, and the potential of Tc-99m-labeled Mab for radioimmunoimaging is described.

MATERIALS AND METHODS

Monoclonal antibodies: The anti-CEA Mab F33-104 IgG_1 was generated by conventional hybridoma methods with highly purified CEA preparations from liver metas-

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Abbreviations: CEA, carcinoembryonic antigen; Tc-99m, technetium-99m; I-125, iodine-125; Mab, monoclonal antibody; 2-ME, 2-mercaptoethanol; HMDP, hydroxyl methylene diphosphonate; In-111, indium-111; PBS, phosphate buffered saline; hCG, human chorionic gonadotropin; RIA, radioimmunoassay.

tases of colon carcinomas as an immunizing antigen.[8,9] The antibody was purified from ascitic fluid by affinity chromatography on protein A (Affi-Gel Protein A®, Bio-Rad Laboratories, CA, USA) and was dialyzed against 0.05 M phosphate buffered saline (PBS), pH 7.5 for 24 hours. The binding affinity of F33-104 to CEA was calculated to be 1.8×10^8 M^{-1} by Scatchard plot analysis. Mab BW431/26, which was used as positive control, recognized CEA. Tc-99m-labeled BW431/26 has been employed mainly in Europe for the diagnosis of patients with colorectal cancer.[12] The Mab to human chorionic gonadotropin (hCG) was used as an irrelevant control Ab. The subtypes of both Mabs were IgG_1.

Cells and tumors: Female athymic nude mice (nu/nu) with a Balb/c background were obtained at approximately 4 weeks of age and were inoculated subcutaneously in the right rear flank with LS-180 human colorectal cancer cells (10^7 cells per animal) or PC-9 human lung cancer cells. LS-180 human colorectal cancer cells expressed CEA, whereas PC-9 cells, which were used as a negative control, did not express CEA. The sizes of LS-180 tumors were from 0.5 to 1.5 cm in diameter by 21 days after the inoculation and by 28 days for PC-9 tumors.

Radiolabeling of monoclonal antibodies

(1) Tc-99m-labeled monoclonal antibodies: Tc-99m labeling was performed by mixing a reduced Ab with Tc-99m-pertechnetate in the presence of a competing ligand HMDP (Clearbone®, Nihon Mediphysics, Nishinomiya, Japan).[11] Antibodies, 5 mg/m*l* of 0.05 M PBS, pH 7.5, were reduced by reaction with a molar excess of 2-mercaptoethanol (Wako Pure Chemical Industries, Ltd, Osaka, Japan) at 100 : 1, 500 : 1, 1000 : 1 and 2000 : 1 (2-ME : Ab) at room temperature for 30 minutes. The reduced antibody was then purified on a PD-10 column (Pharmacia, Uppsala, Sweden) with 0.05 M PBS, pH 7.5 as a mobile phase. The antibody fractions were collected and divided into 0.5 mg aliquots. The antibody was frozen immediately at – 80°C until use. For Tc-99m labeling, 50 μl of HMDP solution reconstituted with a 5 m*l* injection of 0.9% sodium chloride was added to the thawed antibody aliquot and mixed well for 10 seconds. Tc-99m-pertechnetate (740 MBq/m*l*) produced by generator (Ultra-TechneKow®, Daiich Radioisotope Laboratories, Tokyo, Japan) was added to the antibody/HMDP mixture and incubated for 10 minutes. The specific activity of the resultant preparation was about 1,480 MBq/mg.

Labeling efficiency was assessed by cellulose acetate electrophoresis and gel-chromatography. Cellulose diacetate (Separax-S®, Fuji Photo Filum co, Tokyo), cut into 110×10 mm^2 strips was soaked in 0.06 M barbital buffer solution, pH 8.6 and then laid between the electrodes in a flat-bed electrophoresis tank. Tc-99m-labeled Mab, Tc-99m-labeled colloid and Tc-99m-pertechnetate control samples were applied with small pipettes and a current of 0.8 mA/strip was applied for 30 minutes at room temperature. After the strips were dried, the distribution of radioactivity was determined by images in a ZLC7500 gamma camera (Siemens, Ill, USA) equipped with a high resolution collimator for 10 minutes in a 512 by 512 pixel matrix with a digital computer (Scintipac 700, Shimadzu, Kyoto, Japan). Strips were also cut into fractions (10×10 mm^2) and the radioactivity was counted in a well-type gamma counter (Aloka, Japan). Gel-chromatography was performed on a PD-10 columun. Approximately 0.1 μl of the sample was applied to the PD-10 column with 0.05 M PBS, pH 7.5 as a mobile phase. The fractions (0.5 m*l*) were collected into tubes, and counted in a well-type gamma counter.

(2) Radioiodinated antibodies: Mab was labeled with iodine-125 (I-125) by the chloramine-T method.[13] In brief, I-125 sodium iodide (18.5 MBq/5 μl), Mab at 40 μg/180 μl in 0.3 M PBS, pH 7.5 and 10 μl of freshly prepared chloramine-T solution at 0.3 mg/m*l* in 0.3 M PBS, pH 7.5 were mixed, incubated for 5 minutes, and then separated from free I-125 by gel-chromatography on a PD-10 column. The specific activity of I-125-labeled Mab was approximately 440 MBq/mg.

Immunoreactivity of antibodies

(1) Immunoreactivity of reduced antibodies: The effect of reduction on the immunoreactivity of Ab was evaluated by competitive RIA with a commercial available CEA kit (Daiichi Radioisotope Laboratories), where I-125-labeled F33-104 is employed as an I-125-labeled tracer. I-125-labeled F33-104, standard CEA antigen (2.5 ng), and increasing amounts of reduced F33-104, non-reduced F33-104 or control Mab, and a bead covered with anti-CEA Mab were incubated at room temperature for 4 hours, washed 3 times and the radioactivity bound to beads was counted in a well-type gamma counter.

(2) Immunoreactivity of Tc-99m-labeled antibodies: The immunoreactivity of radiolabeled Mab was determined by two methods, antigen binding assays and cell binding assays. In antigen binding assays, each labeled antibody (12 ng) in 100 μl of 0.05 M PBS, pH 7.5, increasing amounts of standard CEA antigen in 50 μl of 0.05 M PBS, pH 7.5 and a bead covered with anti-CEA Mab were incubated at room temperature for 4 hours. After washing 3 times, the radioactivity bound to beads was counted in a well-type gamma counter.[8,9]

Cell binding assays were performed with LS-180 human colorectal cancer cells, which expressed CEA on their surfaces. Labeled antibodies (12 ng) were incubated with increased numbers of LS-180 cells in 200 μl of 0.05 M PBS, pH 7.5 at room temperature for 1 hour in microtubes. These microtubes were centrifuged at 10,000 $\times$ g for 5 minutes. The supernatants were then removed by aspiration and the pellets were cut with a knife and then counted in a well-type gamma counter.

Stability of Tc-99m-labeled monoclonal antibodies

Tc-99m-labeled Mab was incubated with 0.05 M PBS, pH 7.5, human serum with normal CEA levels (1.9 ng/ml; normal range less than 2.5 ng/ml), or human serum with high serum CEA (128 ng/ml) at room temperature for 0, 1, 4 and 24 hours. The stability of Tc-99m-labeled F33-104 was assessed with both gel-chromatography and cellulose acetate electrophoresis as described above.

Biodistribution studies in tumor bearing athymic nude mice and normal mice

Mice were injected intravenously with a mixture of 12.5 μg (18.5 MBq) of Tc-99m-labeled F33-104 and 0.1 μg (44 kBq) of I-125-labeled F33-104 in 200 μl of 0.05 M PBS, pH 7.5. Groups of mice (n = 5) were killed with anesthetic at 3 hours and 18 hours after injection, and the tumors and all internal organs were removed, weighed and counted with a well-type gamma counter. A mixture of 12.5 μg of Tc-99m-labeled anti-hCG Mab and 0.1 μg of I-125-labeled anti-hCG Mab were used as control studies, and groups of mice (n = 5) were killed at 18 hours following the injection. Tc-99m activity was counted in a channel having windows set for 130–150 keV and I-125 activity for 15–85 keV. The samples were initially counted in the Tc-99m activity channel and were then counted in the I-125 activity channel after 7 days. No thyroid blocking agents were administered throughout these biodistribution studies. The results were expressed as % injection dose/gram. The localization index and specific-to-non-specific tumor ratio were described as follows: Localization index = LS-180 tumor-to-blood ratio of radiolabeled F33-104/LS-180 tumor-to-blood ratio of radiolabeled control Mab. Specific-to-non-specific tumor ratio = LS-180 tumor-to-blood ratio of radiolabeled F33-104/PC-9 tumor-to-blood ratio of radiolabeled F33-104.[14–16]

Imaging of athymic nude mice

Athymic nude mice bearing LS-180 human colorectal cancer xenografts were injected through a tail vein with 12.5 μg (18.5 MBq) of Tc-99m-labeled F33-104 for imaging studies. Images were obtained with a ZLC7500 gamma camera equipped with a high resolution collimator positioned at 0.5 cm above the dorsum of the mice, and collected on a 512 by 512 pixel matrix by means of a digital computer (Scintipac 700) for 5 minutes. A 10% energy window was centered over the 140 keV photopeak of Tc-99m.

Statistical comparisons were made by Student's paired t-test.

RESULTS

In order to determine the optimal reduction conditions for F33-104, Mabs were reduced under various conditions (Fig. 1). The reaction with a molar ratio of 2-ME to Ab at above 1000 : 1 was required to obtain a high labeling efficiency of more than 95% (Fig. 1). Immunoreactivity of reduced F33-104 was determined by the competitive radioimmunoassays (Fig. 2). Reduced F33-104 obtained at 1000 : 1 and 2000 : 1 molar ratios and non-reduced F33-104 could equally compete with I-125-labeled F33-104, indicating no difference between the original and reduced F33-104 in immunoreactivity. *In vitro* and *in vivo* properties of Tc-99m-labeled F33-104 were examined in those

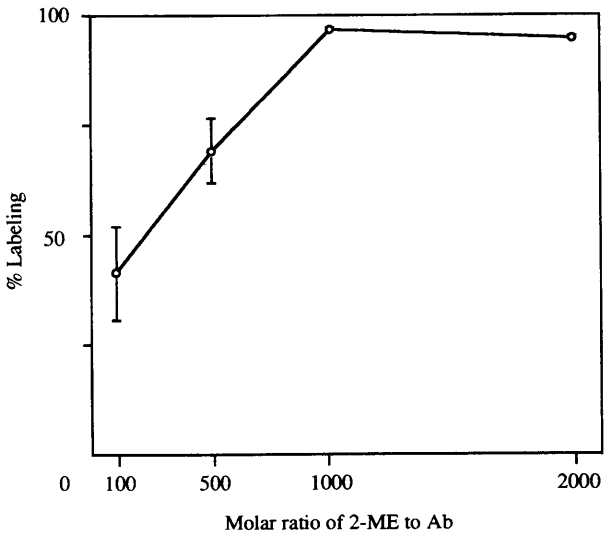

Fig. 1 Labeling efficiency of Tc-99m-labeled F33-104. Antibodies were reduced under conditions of molar excess of 2-mercaptoethanol (ME) to antibodies at 100 : 1, 500 : 1, 1000 : 1 and 2000 : 1. Labeling efficiency was assessed by the cellulose acetate electrophoresis. Vertical bars represent Mean ± S.D. of 3 experiments.

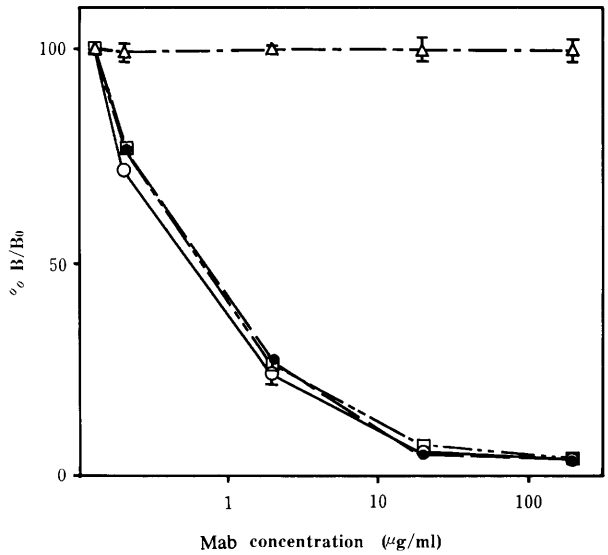

Fig. 2 Effect of reduction on the immunoreactivity of Mab. Competitive inhibition assays were performed by incubating I-125-labeled Mab F33-104 with unreduced F33-104 (○), reduced F33-104 (obtained under 2-ME/Ab molar ratio of 1000) (□), reduced F33-104 (obtained under 2-ME/Ab molar ratio of 2000) (●) and control Mab (△). Vertical bars represent Mean ± S.D. of 3 experiments.

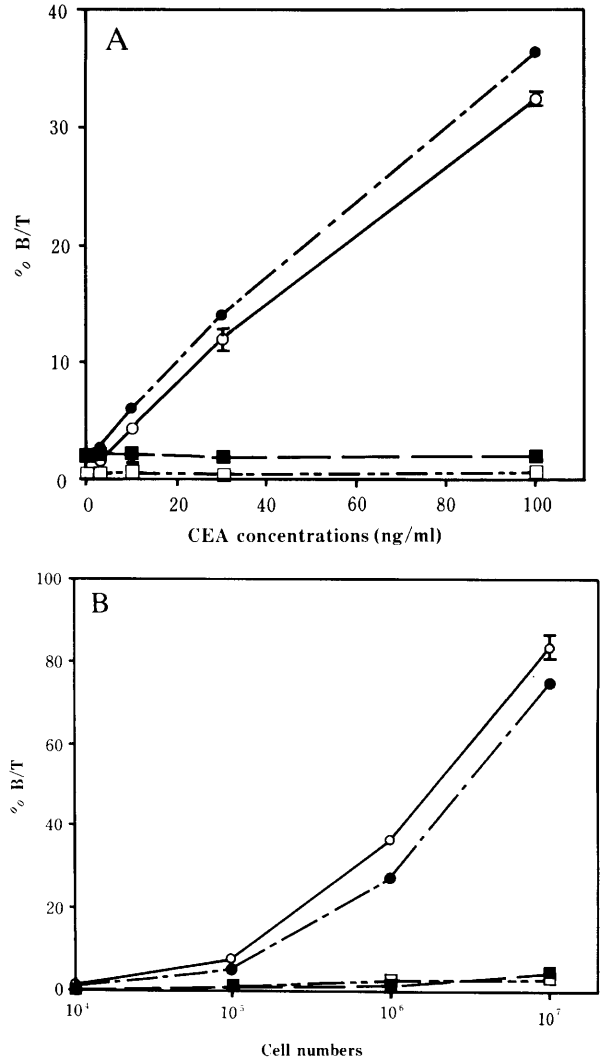

Fig. 3 Immunoreactivity of Tc-99m-labeled F33-104; Antigen binding assays (A) and Cell binding assays (B). Binding of radiolabeled Mab to antigens captured on beads was examined using a commercially available RIA kit. Cell binding assays were performed by incubating increasing numbers of LS-180 human colorectal cancer cells with radiolabeled Mabs. Tc-99m-labeled F33-104 (○), I-125-labeled F33-104 (●), Tc-99m-labeled anti-hCG Mab (□), and I-125 labeled anti-hCG Mab (■). Vertical bars represent Mean ± S.D. of 3 experiments.

at a molar ratio of 1000 : 1. There was little difference between Tc-99m- and I-125-labeled F33-104 as to binding to antigen captured on beads covered with anti-CEA antibodies[9] (Fig. 3A) and in the specific binding to LS-180 human colorectal cancer cells (Fig. 3B).

In cellulose acetate electrophoresis migration of Tc-99m-labeled F33-104 was identical to those of original and reduced F33-104 (Data not shown). There was also no release of Tc-99m-pertechnetate from Tc-99m-labeled Mab nor the formation of Tc-99m-colloid (Fig. 4). The stability of Tc-99m-labeled F33-104 was assessed by gel-filtration chromatography and cellulose acetate electrophoresis after incubation with human serum with or

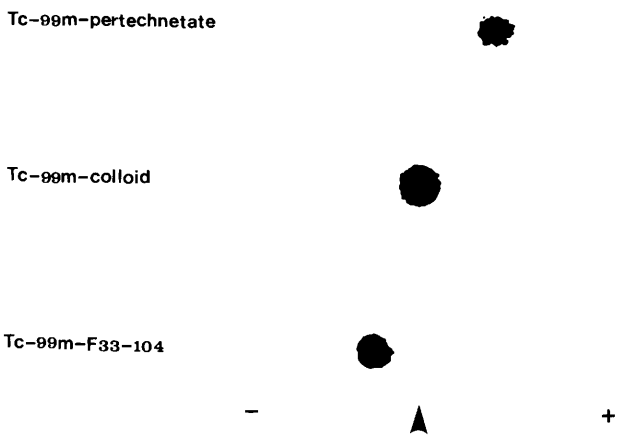

Fig. 4 Cellulose acetate electrophoresis of Tc-99m-pertechnetate, Tc-99m-labeled colloid, and Tc-99m-labeled F33-103. Images were obtained by a gamma camera. Arrow shows the point of application.

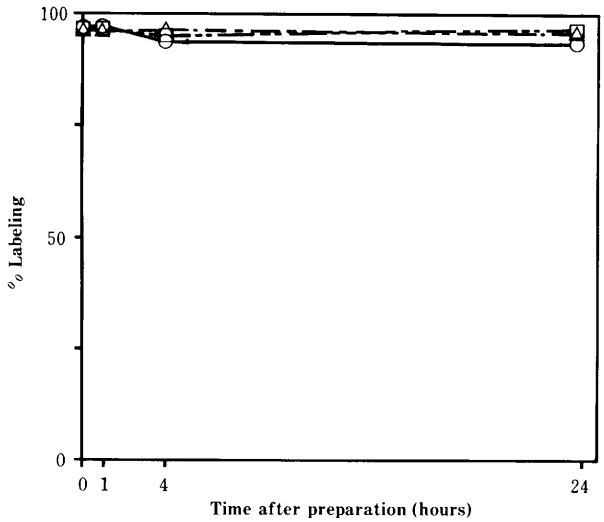

Fig. 5 Radiochemical stability of Tc-99m-labeled F33-104 incubated with 0.05 M PBS, pH 7.5 (○), human serum with normal CEA levels (12.9 ng/ml) (□), or human serum with high serum CEA (128 ng/ml) (△) at room temperature for 0, 1, 4 and 24 hours using the cellulose acetate electrophoresis.

without high CEA values. There was no release of Tc-99m-pertechnetate from Tc-99m-labeled Mab even after 24 hours' incubation (Fig. 5).

Biodistribution studies of athymic nude mice bearing LS-180 human colorectal cancer xenografts showed significant differences between Tc-99m- and I-125-labeled F33-104 in the % injection dose/gram. Higher kidney uptake of Tc-99m-labeled F33-104 was observed at 3 hours after the injection (Fig. 6A). Higher tumor, liver, kidney and gut uptake of Tc-99m-labeled F33-104 was demonstrated at 18 hours, whereas higher stomach uptake of I-125-labeled F33-104 was seen at 18 hours (Fig. 6B). Scintigraphy of a nude mouse bearing a LS-180 human colorectal cancer xenograft demonstrated that a transplanted tumor was clearly visualized by Tc-99m-labeled F33-104, as confirmed by the biodistribution data (Fig. 7).

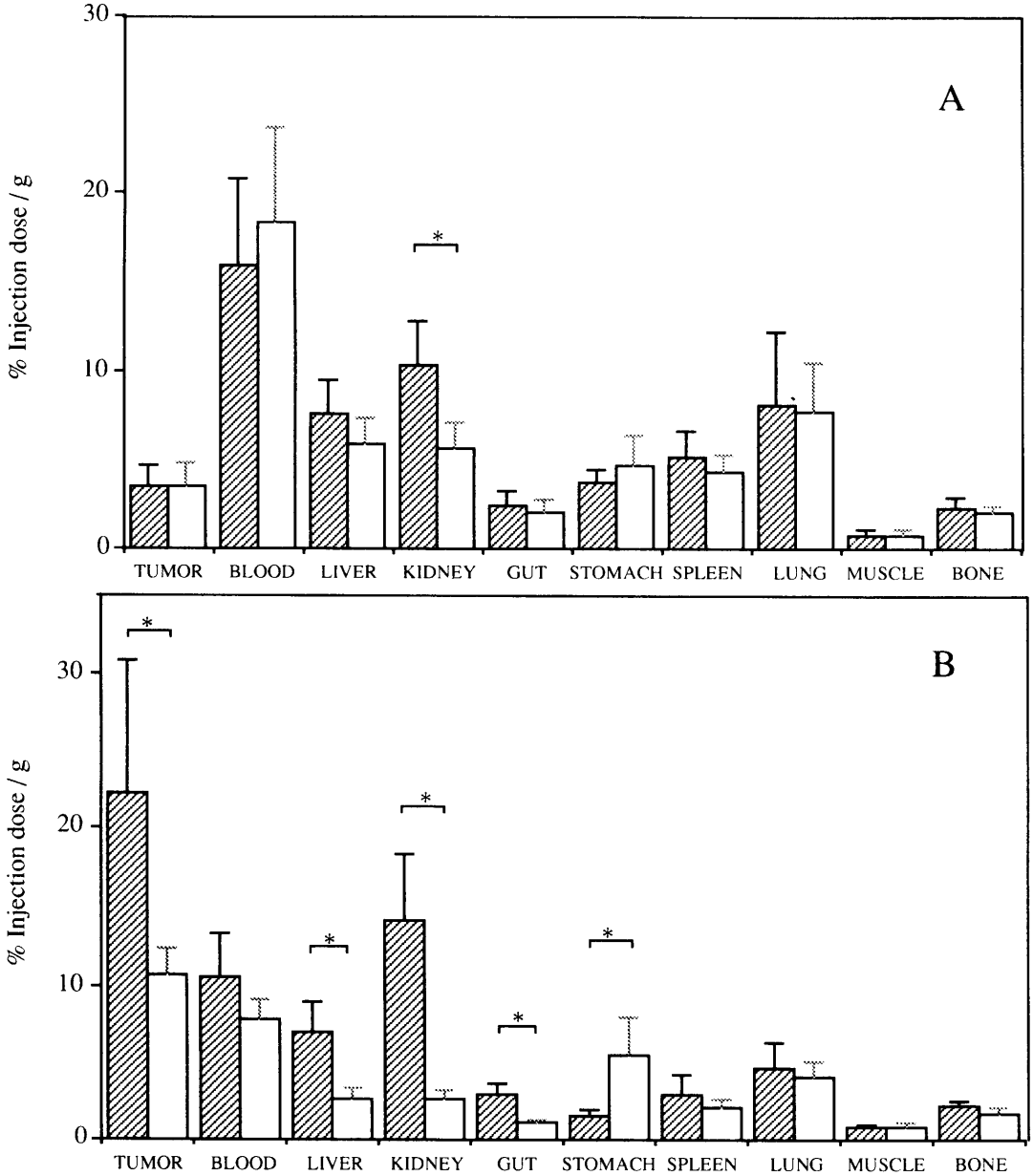

Fig. 6 Biodistributions of radiolabeled Mabs in athymic nude mice bearing LS-180 human colorectal cancer cells at 3 hours (A) and 18 hours (B) following intravenous injection. Tc-99m-labeled F33-104 (▨). I-125-labeled F33-104 (□). Results were expressed as % injection dose/gram. Vertical bars represent Mean ± S.D. *$p < 0.05$.

Table 1 Tumor /blood ratios of radiolabeled Mabs in athymic nude mice bearing specific (LS-180 human colorectal cancer) or non-specific (PC-9 human lung cancer) tumors at 18 hours postintravenous injection

Tumor	Labeled antibody			
	Tc-99m-labeled F33-104	I-125-labeled F33-104	Tc-99m-labeled control Mab	I-125-labeled control Mab
LS-180 human colorectal cancer	2.10 ± 0.45	1.36 ± 0.20	0.51 ± 0.06	0.43 ± 0.06
PC-9 human lung cancer	0.41 ± 0.08	0.33 ± 0.13	—	—

Values are mean ± S.D.
Localization index of Tc-99m-labeled F33-104 was 4.12 and that of I-125-labeled one was 3.16. Specific/non-specific tumor ratio of Tc-99m-labeled F33-104 was 5.12 and that of I-125-labeled one was 4.12.

Table 2 Kidney/blood ratios of radiolabeled Mabs in athymic nude mice bearing tumor xenografts and normal mice obtained 18 hours after the injection

Tumor	F33-104		Anti-hCG Mab		BW431/26
	Tc-99m	I-125	Tc-99m	I-125	Tc-99m
LS-180 human colorectal cancer	1.42 (0.49)	0.32 (0.03)	1.37 (0.37)	0.30 (0.03)	0.90 (0.17)
PC-9 human lung cancer	1.13 (0.12)	0.28 (0.02)	—	—	—
No tumor	0.71 (0.05)	0.33 (0.09)	0.82 (0.09)	0.28 (0.03)	0.44 (0.09)

Values are Mean (S.D.) of 5 animals
*$p < 0.05$.

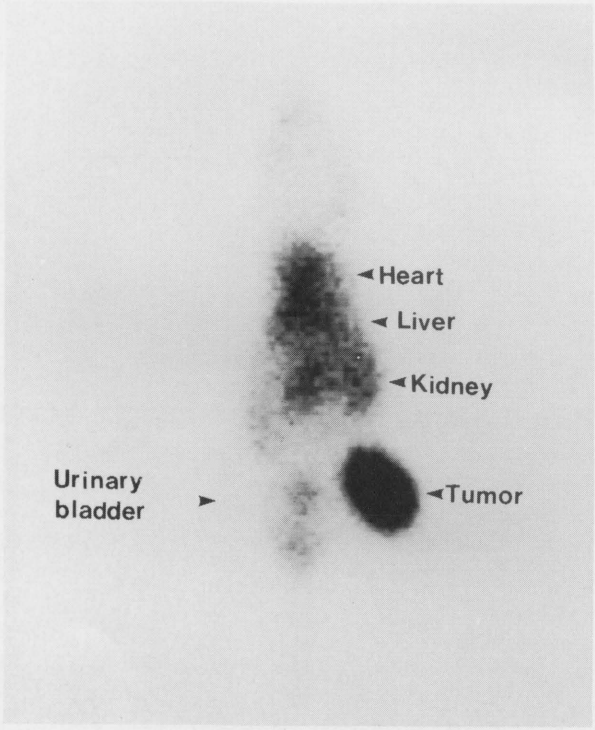

Fig. 7 Scintigraphy of a nude mouse bearing LS-180 human colorectal cancer xenograft. Dorsal gamma scintigraphy of an athymic nude mouse was obtained at 18 hours after intravenous injection of 18.5 MBq of Tc-99m-labeled F33-104.

Uptake of Tc-99m- and I-125-labeled control Mab in human colorectal cancer tissue was significantly lower than those for F33-104 (Table 1). The deposit of F33-104 in PC-9 lung cancer tissue was also significantly lower than in colorectal cancer tissues. The localization index and specific-to-non-specific tumor uptake ratio were calculated as 4.12 and 5.12 for Tc-99m-labeled Mab, respectively. The kidney to blood uptake ratio of Tc-99m-labeled F33-104 and control Mab in athymic nude mice bearing colorectal cancer xenografts was remarkably higher than that of I-125 (Table 2). In addition, the kidney to blood ratios of tumor-bearing athymic nude mice were significantly higher than those of normal nude mice, even when Tc-99m-labeled control Mabs were administered or athymic nude mice were carrying non-specific PC-9 cancer cells.

DISCUSSION

The present study shows that murine anti-CEA Mab, designated F33-104, was quickly and stably labeled with Tc-99m, without damaging antigen-binding activity, by the direct labeling method.[11] Labeling of Mab with Tc-99m was achievable by only mixing reduced Mab and Tc-99m-pertechnetate produced by the generator. Labeling efficiency was so high that Tc-99m-labeled Mab could be injected without further purification, and was very convenient for clinical use. High labeling efficiency of F33-104 with Tc-99m was achieved by increasing the molar ratio of 2-ME to the antibody, probably due to the increased numbers of sulphydryl groups per antibody, cleaved with 2-ME by reducing the number of intrinsic disulphide bonds.[11] The molar ratio of 2-ME to antibody of more than 1000 : 1 seemed to be optimal for Tc-99m-labeling of Mab F33-104. Furthermore, significantly higher tumor uptake was noted at 18 hours after the injection of Tc-99m-labeled F33-104 than for a radioiodinated version in the biodistribution of athymic nude mice bearing specific tumors. This resulted in a significantly higher localization index of 4.12 and specific-to-non-specific tumor uptake ratio of 5.12. Higher tumor activity of Tc-99m-labeled F33-104 than of I-125-labeled F33-104 may be due to the dehalogenation of radioiodine antibody in the circulation.[16] Images of Tc-99m-labeled F33-104 showed predominant specific tumor uptake within 18 hours after injection, confirming the results of biodistribution studies.

Significant kidney uptake of Tc-99m-labeled F33-104 was observed at 3 hours and 18 hours after injection in athymic nude mice, especially in tumor-bearing athymic nude mice. Higher kidney uptake of Tc-99m-labeled Mabs was noted in athymic nude mice bearing both specific and non-specific tumors than that of corresponding iodinated Mab (Table 2). In addition, even Tc-99m-labeled control Mab showed higher kidney uptake in tumor-bearing athymic nude mice than in normal athymic nude mice.

High kidney uptake was already reported when $F(ab')_2$ fragments labeled with indium-111 (In-111) and Tc-99m were administered.[17,18] However, the reasons for high kidney uptake when using intact Mab labeled with Tc-99m are difficult to determine and have not been studied extensively. Immune complex formation of Mab with the circulating CEA seemed unlikely, since significant kidney uptake of Tc-99m-labeled F33-104 in athymic nude mice bearing CEA-negative tumors, and of Tc-99m-labeled control Mab were also observed. Another possible explanation is an *in vivo* breakdown of Tc-99m-labeled Mab by unknown mechanisms at the tumor sites and/or in the circulation which may contribute to significant kidney uptake at a later time after injection.

In-111-labeled Mabs to CEA have been successfully used for the localization of colorectal cancer.[19–22] The antigenic nature of these antibodies has not been described, whereas epitopes recognized by F33-104 have been well characterized.[8,9] In addition Tc-99m has many advantages over In-111 in the imaging of cancer. Tc-99m-labeled anti-CEA Mab F33-104 has the potential for clinical application in diagnosing human colorectal cancer. We are investigating radiolabeled Mab with Re-186, which has favorable nuclear properties for the treatment of cancer.[23,24]

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