In Vivo and In Vitro Characterizations of Three ^{99m}Tc-Labeled Monoclonal Antibody G250 Preparations

Martijn G. Steffens, Egbert Oosterwijk, Marion H.G.C. Kranenborg, Jeroen M.B. Manders, Frans M.J. Debruyne, Frans H.M. Corstens and Otto C. Boerman

Departments of Urology and Nuclear Medicine, University Hospital Nijmegen, Nijmegen, The Netherlands

In previous clinical studies, excellent visualization of tumor lesions has been observed with ^{131}I-labeled monoclonal antibody (mAb) G250 in patients with renal cell carcinoma (RCC). In several cases, ^{131}I-cG250 immunoscintigraphy disclosed tumor lesions that were not visualized by radiography or CT. To improve image quality, we aimed to develop a ^{99m}Tc-labeled mAb G250 preparation for radioimmunodetection of RCC. We studied in vitro stability, biodistribution and imaging potential of three ^{99m}Tc-labeled G250 preparations in nude mice with subcutaneous RCC xenografts. ^{125}I-G250 and the nonspecific mAb ^{131}I-MN14 were used as control antibodies. **Methods:** The mAb G250 was labeled with ^{99m}Tc according to three methods using: (a) S-hydrazinonicotinamide (HYNIC), (b) S-benzoylmercapto-acetyltriglycine (MAG3) and (c) a direct labeling method (Schwarz method). The stability of all preparations was tested in serum at 37°C during 48 h. In addition, diethylenetriamine pentaacetic acid, cysteine and glutathione challenge assays were performed. **Results:** All preparations showed good stability in serum during the 48-h incubation period. ^{99m}Tc-G250 (Schwarz) showed release of the radiolabel at a 100-fold or higher molar excess of cysteine and at a 10,000-fold or higher molar excess of glutathione. ^{99m}Tc-MAG3-G250 showed release of the radiolabel at a 10,000-fold molar excess of cysteine. ^{99m}Tc-HYNIC-G250 was stable under all conditions. Tumors were clearly visualized with all preparations. ^{99m}Tc-G250 (Schwarz) showed significantly lower blood levels (3.8 %ID/g) compared with all other preparations (11.2, 13.4 and 13.4 %ID/g for ^{99m}Tc-HYNIC-G250, ^{99m}Tc-MAG3-G250 and ^{125}I-G250, respectively, 48 h postinjection). At 48-h postinjection, mean tumor uptake was very high with all mAb G250 preparations: 92.4 (^{99m}Tc-HYNIC-G250), 125.9 (^{99m}Tc-MAG3-G250), 29.4 (^{99m}Tc-G250 Schwarz) and 75.4 (^{125}I-G250) %ID/g. Mean tumor uptake of the nonspecific ^{131}I-MN14 mAb was 6.6 %ID/g. **Conclusion:** In this study, ^{99m}Tc-HYNIC-G250 showed excellent in vitro stability and tumor targeting. Moreover, this preparation could be labeled with high efficiency (>95%) at room temperature within 15 min. Therefore, ^{99m}Tc-HYNIC-G250 seems to be an ideal candidate for radioimmunodetection of RCC.

Key Words: monoclonal antibody G250; renal cell carcinoma; radioimmunodetection; mercaptoacetyltriglycine; hydrazinonicotinamide

J Nucl Med 1999; 40:829–836

Renal cell carcinoma (RCC) is the most common malignant disease of the adult kidney. It accounts for 12,000 RCC-related deaths per year in the U.S. (*1*). About one third of all patients present with metastatic disease at the time of diagnosis, and metastases will develop in another 40%, usually within 1 y (*2*). The 5-y survival rate for these patients is <10%, and the median survival time is <2 y (*2*). The treatment of choice for patients with localized disease is radical nephrectomy. Treatment of metastases is more likely to be effective in patients with minimal residual disease. Thus, for both localized and metastatic disease, accurate staging and early detection of metastases are issues of growing importance in the management of patients with RCC.

The routine work-up for patients with primary RCC usually consists of CT scans of the thorax and the abdomen. However, the sensitivity and specificity of CT are limited, and the interpretation of abnormal findings is sometimes difficult. In particular, the distinction between benign, reactively enlarged regional lymph nodes and lymph-node metastases is often cumbersome. In general, radioimmunoscintigraphy has shown excellent sensitivity and specificity in the detection of various tumors (*3*). Such a functional imaging modality may prove to be a useful addition to the standard work-up of patients with cancer (*4*). In previous clinical studies, we observed excellent visualization of tumor lesions with ^{131}I-labeled chimeric monoclonal antibody (mAb) cG250 in patients with RCC (*5*). In a recently completed phase I radioimmunotherapy trial, ^{131}I-cG250 immunoscintigraphy disclosed tumor lesions that were not visualized by CT (*6*). For instance, in one patient, a hot spot in the brain was found on the ^{131}I-cG250 immuno-scintigrams, but was not visualized on a subsequent CT scan of the brain. In two other patients, a total of three suspected bone lesions were found that were not visualized by planar radiography. After 3 mo of follow-up, all four suspected lesions were confirmed radiologically (unpublished data).

^{99m}Tc has ideal characteristics for scintigraphic imaging (140-keV γ-emission, $t_{1/2}$ = 6 h), and there has been much interest in the use of this isotope for antibody-mediated

imaging. Therefore, instead of using ^{131}I, we aimed to develop a ^{99m}Tc-labeled mAb G250 preparation for radioimmunodetection of RCC.

Various techniques have been developed to label antibodies with [99m]Tc. These techniques can be divided into three main categories: direct labeling, the preformed chelate approach and the indirect labeling approach (7). The direct labeling method makes use of reduced disulfide bonds of the protein, which are then reacted with [99m]Tc. Although direct labeling is easy to carry out, the radiolabel linked to the antibody often shows significant instability. The preformed chelate approach involves formation of a ^{99m}Tc complex with a bifunctional chelating agent (BFCA). In a separate step, the ^{99m}Tc-BFCA complex is then conjugated to a protein or a peptide (hot conjugation). In general, this technique leads to more stable products than the direct labeling method, but it is quite complex and time-consuming. In the indirect labeling approach, a BFCA is first conjugated to a protein or a peptide, followed by radiolabeling of this complex at a later time point (cold conjugation). Of all three approaches, indirect labeling seems the most practical. After the conjugation step, the product can be stored (kit formulation), and it combines well-defined chemistry with the ease of a direct labeling method (7).

In this study, we compared the stability, the biodistribution and the imaging characteristics of three ^{99m}Tc-labeled G250 preparations. The mAb G250 was labeled with ^{99m}Tc according to three methods using: (a) S-hydrazinonicotinamide (HYNIC) (indirect labeling), (b) S-benzoylmercaptoacetyltriglycine (MAG3) (preformed chelate approach) and (c) a direct labeling method, as described by Mather and Ellison (8). The latter procedure is generally referred to as the so-called Schwarz method.

MATERIALS AND METHODS

Murine Monoclonal Antibodies G250 and MN14

The generation, characteristics and reactivity of murine mAb G250 (IgG1) have been described previously (9). Briefly, mAb G250 is reactive with the antigen G250, which is expressed in all clear cell RCCs and in the majority of non-clear cell RCCs. Expression in normal organs is restricted to the gastric mucosal cells and the larger bile ducts. Recently, the G250 antigen has been cloned and was found to be homologous with the MN antigen, which is expressed in cervical carcinomas (10). The anticarcinoembryonic antigen mAb MN14 (IgG1) (11), not reactive with the G250 antigen, was used as a control antibody.

Nude Mouse Tumor Model

The NU-12 RCC xenograft was established from a primary tumor by serial subcutaneous transplantations in nude mice (12). Tumor pieces were transplanted subcutaneously into the right flanks of 6-wk-old BALB/c nu/nu mice. Experiments were initiated 8 wk after inoculation.

Conjugation, Radiolabeling and Quality Control

The conjugation of HYNIC with the mAb G250 was carried out as described by Abrams et al. (13). Briefly, 1-µL portions of a HYNIC-dimethylsulfoxide solution (17.2 mg/mL) were added to a mAb G250 solution (10 mg/mL), leading to a final molar ratio of 1.5:1 (HYNIC/mAb G250). The reaction mixture was stirred continuously and incubated for 1 h at room temperature. The HYNIC-G250 conjugate was dialyzed extensively against 0.15-mol/L Na acetate buffer (pH 5.9) and subsequently stored at -20°C. The HYNIC-G250 conjugate (0.2 mg mAb G250) was labeled with ^{99m}Tc using tricine as a coligand (15 min at room temperature, 600 MBq, labeling efficiency >95%). The specific activity of the final product was 2775 MBq/mg mAb G250.

The labeling of the MAG3 chelate with ^{99m}Tc and the conjugation of the radioactive chelate with the mAb G250 were carried out essentially as described by Fritzberg et al. (14), with modifications described by Visser et al. (15). Briefly, 80 nmol MAG3 were labeled with 2775 MBq [99m]Tc (10 min, 100°C). After derivation of MAG3 with 2,3,5,6-tetrafluorphenol (TFP) (100 mg/mL in MeCN/H_2O 9:1), the ^{99m}Tc-MAG3-TFP was purified on a Sep-Pak C18 cartridge (Waters, Milford, MA) and subsequently reacted with 200 µg mAb G250 (2 µg/µL) at a pH of 9.5. The ^{99m}Tc-MAG3-G250 immunoconjugate was then purified in a PD-10 column (Pharmacia, Woerden, The Netherlands). Conjugation efficiency was 50%, resulting in a specific activity of 2220 MBq/mg mAb G250.

Directly labeled ^{99m}Tc-G250 was produced as described by Mather and Ellison (8) (Schwarz method). Briefly, disulfide bonds of the mAb G250 were reduced using 2-mercaptoethanol (2-ME) in a 100:1 molar ratio (2-ME/mAb G250). The reduced mAb G250 was purified in a PD-10 column and stored at -20°C. The reduced mAb G250 (0.2 mg) was labeled with ^{99m}Tc using tricine as a transchelator (15 min at room temperature, 600 MBq, labeling efficiency >95%). The specific activity of the final product was 2775 MBq/mg mAb G250.

The mAbs G250 and MN14 were radioiodinated with [125]I (Amersham International, Buckinghamshire, UK) and with [131]I (Nordion Europe, Fleurus, Belgium), respectively, according to the IodoGen method (16). Specific activity of the radiolabeled preparations were 280 MBq ^{125}I/mg G250 and 185 MBq ^{131}I/mg MN14.

In all preparations, more than 95% of the radioactivity was associated with the antibodies as determined by instant thin-layer chromatography (ITLC) using 0.15 mol/L sodium citrate (pH 5.5) as an eluant.

Immunoreactivity

The immunoreactive fractions of all G250 antibody preparations were determined on SK-RC-52 RCC cells (17) and have been described previously (18).

Stability of Radiolabeled Antibody Preparations

The stability of radiolabeled antibody preparations was monitored in human serum at 37°C for 48 h. At several time points postincubation (0, 1, 2, 4, 8, 20 and 48 h), serum samples were analyzed for the amount of free radiolabel using ITLC. To investigate a possible transchelation to other serum proteins, size-exclusion, high-performance liquid chromatography (HPLC) analysis of the serum samples was performed using a gel filtration column (Biosep Sec 3000; Phenomenex, Torrance, CA). A solution of 0.1 mol/L Tris, 0.15 mol/L NaCl, 0.001 mol/L ethylenediaminetetraacetic acid (pH 7.2) was used as the mobile phase. Fractions of 1 mL were collected, and radioactivity in the fractions was determined.

In addition, the stability of the ^{99m}Tc-labeled mAb G250 preparations was tested in a challenge assay, performed essentially as described by Hnatowich et al. (19). Solutions of all three ^{99m}Tc-labeled mAb G250 preparations (0.1 µmol/L) were incubated

with increasing concentrations of diethylenetriamine pentaacetic acid (DTPA), cysteine and glutathione (0, 100, 1000, 10^4, 10^5 and 10^6 molar excess). After 4 h of incubation at 37°C, the amount of free radiolabel was determined by ITLC.

Biodistribution Studies

Forty mice with NU-12 tumors (0.002–0.15 g) were randomly divided into four groups of 10 mice and were intravenously injected with 3 μg mAb ^{99m}Tc-HYNIC-G250, ^{99m}Tc-MAG3-G250, ^{99m}Tc-G250 (Schwarz) or a combination of ^{125}I-G250/^{131}I-MN14 (3 μg/3 μg per mouse). Of each group, 5 mice were killed by cervical dislocation under ether anesthesia at 24 or 48 h postinjection, and biodistribution of the radiolabel was determined. The amount of radioactivity in the tumor and in the normal tissues (blood, muscle, lung, spleen, liver, kidney and intestines) was determined in a γ-counter (1480 Wizard 3; Wallac Oy, Turku, Finland) along with an injection standard. The percentage of injected dose per gram of tissue (%ID/g) and the tumor-to-blood ratios were calculated.

Immunoscintigraphy

Scintigraphic imaging was performed on 9 mice with NU-12 tumors (0.3–0.9 g). The mice were divided into three groups of 3 mice, and each group received ^{99m}Tc-HYNIC-G250 (3 μg mAb G250, 8.3 MBq ^{99m}Tc), ^{99m}Tc-MAG3-G250 (3 μg mAb G250, 6.7 MBq ^{99m}Tc) or ^{99m}Tc-G250 (Schwarz) (3 μg mAb G250, 8.3 MBq

^{99m}Tc). Mice were anesthetized (halothane/nitrous oxide/oxygen) and placed prone on a single-head gamma camera (Orbiter; Siemens, Inc., Hoffmann Estates, IL) equipped with a parallel-hole, low-energy collimator. Images of the three groups were obtained synchronously at 1, 8 and 24 h postinjection. Images were stored in a 256 × 256 matrix.

Statistical Analysis

All values are expressed as mean ± SD. Statistical analysis was performed using one-way analysis of variance, with Bonferroni post-test correction for multiple comparisons. Differences of $P < 0.05$ were considered to be significant.
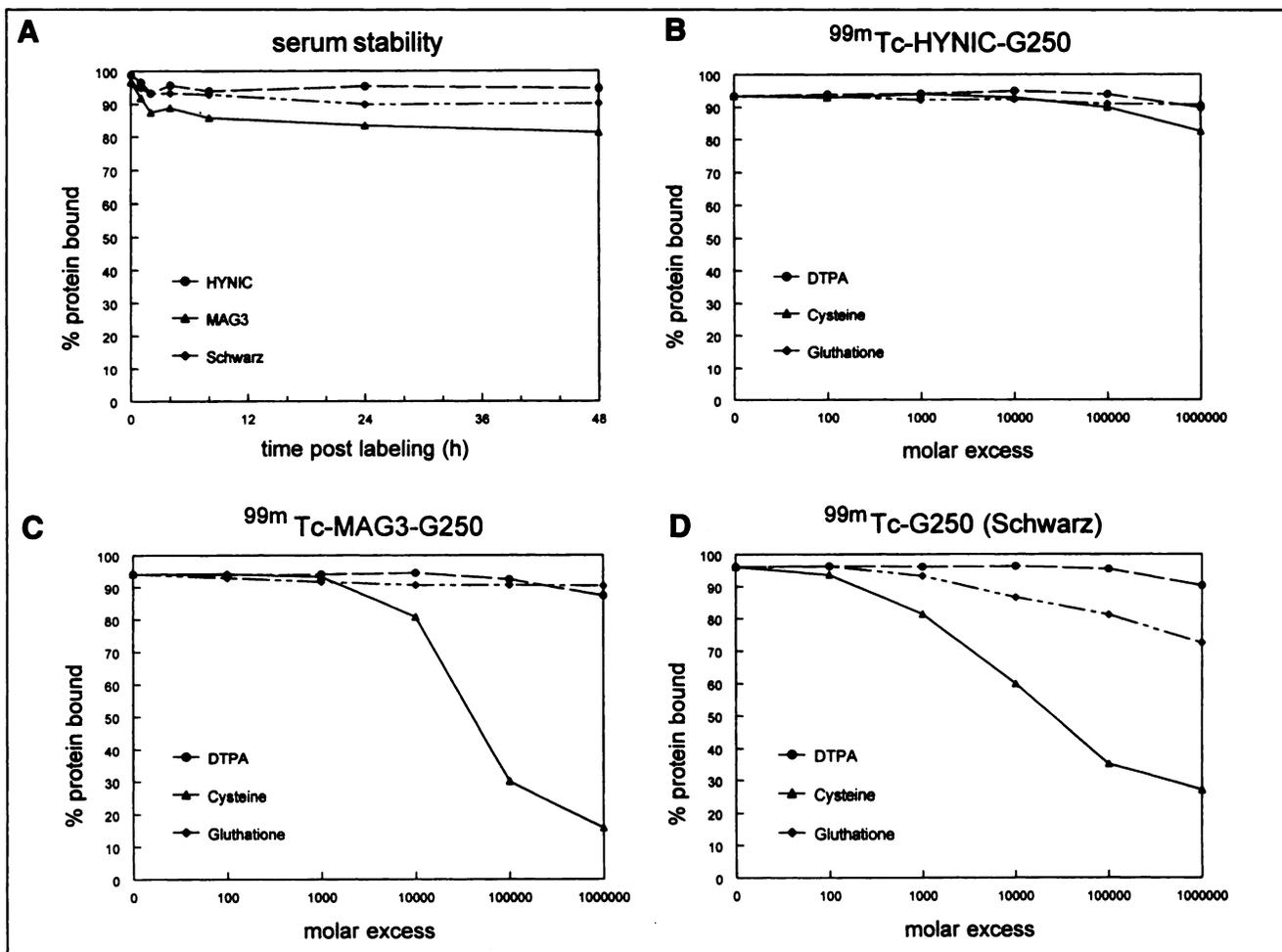
RESULTS

Immunoreactivity

The immunoreactive fraction of the radiolabeled G250 preparations was 86% for ^{99m}Tc-HYNIC-G250, 76% for ^{99m}Tc-MAG3-G250, 81% for ^{99m}Tc-G250 (Schwarz) and 94% for ^{125}I-G250.

In Vitro Stability of Radiolabeled Antibodies

All radiolabeled G250 preparations showed good stability in serum (Fig. 1A). Immediately after labeling, more than


FIGURE 1.   In vitro stability of three ^{99m}Tc-G250 antibody preparations. Stability in serum at 37°C (A). Stability of ^{99m}Tc-HYNIC-G250 (B), ^{99m}Tc-MAG3-G250 (C) and ^{99m}Tc-G250 Schwarz (D) in presence of increasing concentrations of DTPA, glutathione and cysteine.

95% of the ^{99m}Tc was associated with the mAb G250 in all preparations. Figure 2 shows HPLC radiochromatograms of serum samples with ^{99m}Tc-HYNIC-G250, ^{99m}Tc-MAG3-G250 and ^{99m}Tc-G250 (Schwarz) at different time points. The determination of free radiolabel by HPLC matched accurately with the determination of free radiolabel by ITLC (data not shown). All three preparations showed one major peak immediately after labeling, which corresponded with the monomeric IgG peak of the ultraviolet pattern (280 nm) of unlabeled mAb G250 (data not shown). During 48 h of incubation, this peak remained predominantly in all three ^{99m}Tc-G250 preparations. However, at 2 h postlabeling, a small second peak of activity appeared in the radiochromatograms of all three preparations. This second peak (released ^{99m}Tc) increased with time. After 48 h of incubation, the amount of free radiolabel was 5% for ^{99m}Tc-HYNIC-G250, 18% for ^{99m}Tc-MAG3-G250 and 10% for ^{99m}Tc-G250 (Schwarz).

In the challenge assays, ^{99m}Tc-HYNIC-G250 showed virtually no ^{99m}Tc release (Fig. 1B) at any concentration of DTPA, cysteine or glutathione. ^{99m}Tc-MAG3-G250 showed release of ^{99m}Tc at a 10,000-fold or higher molar excess of cysteine (Fig. 1C). ^{99m}Tc-G250 (Schwarz) showed release of ^{99m}Tc at a 100-fold or higher molar excess of cysteine and at a 10,000-fold or higher molar excess of glutathione (Fig. 1D).

Biodistribution

The biodistribution of ^{99m}Tc-HYNIC-G250, ^{99m}Tc-MAG3-G250 and ^{99m}Tc-G250 (Schwarz) was studied and compared with the biodistribution of ^{125}I-G250 and nonspecific ^{131}I-MN14 (Fig. 3, left panels). Table 1 summarizes the uptake of all preparations in the tumor and in seven normal organs. At 24 h postinjection, the blood levels of directly labeled ^{99m}Tc-G250 (Schwarz) were significantly lower ($P < 0.001$) than the blood levels of all other antibody preparations:
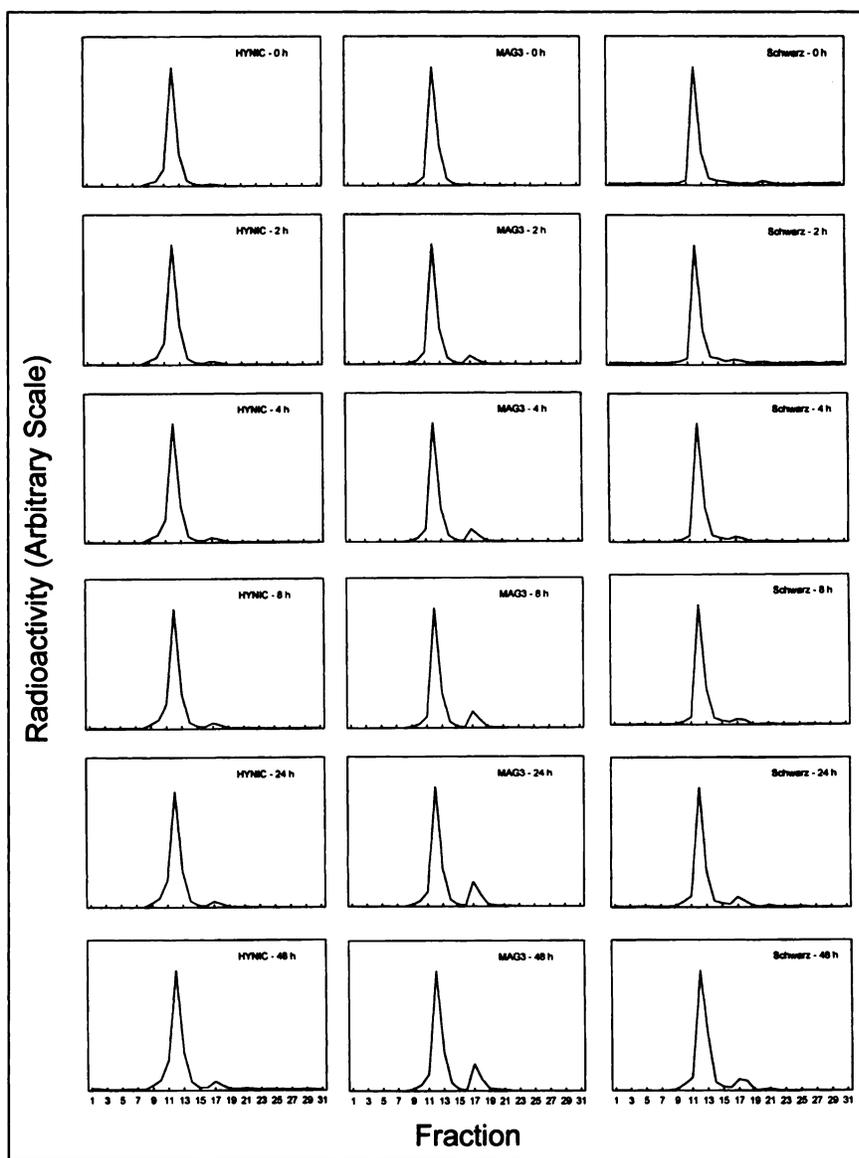
FIGURE 2. HPLC radiochromatograms of three ^{99m}Tc-G250 antibody preparations in serum at various time points. Note small second peak (released ^{99m}Tc) in radiochromatograms of each preparation from 2 h onward.
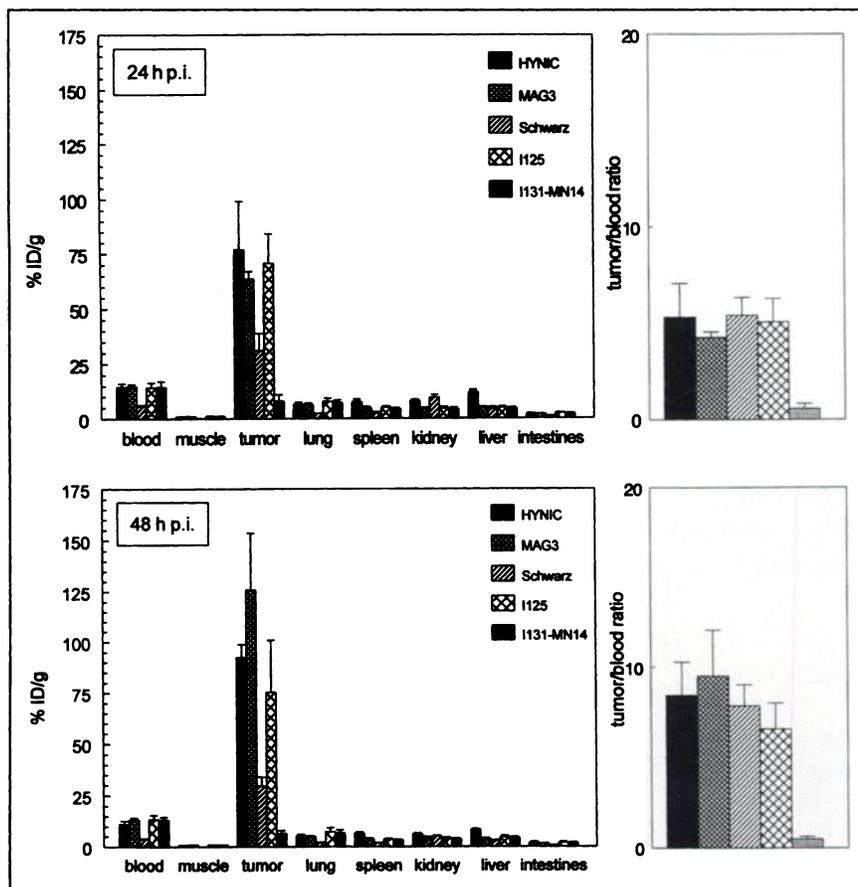
FIGURE 3. Biodistribution (left panels) and tumor-to-blood ratios (right panels) of each antibody preparation at 24 and 48 h postinjection (p.i.). Error bars represent SDs.

5.70 ± 0.56 %ID/g for ^{99m}Tc-G250 (Schwarz) versus 14.66 ± 1.47, 14.88 ± 0.97, 14.25 ± 2.23 and 14.56 ± 2.52 %ID/g for ^{99m}Tc-HYNIC-G250, ^{99m}Tc-MAG3-G250, ^{125}I-G250 and ^{131}I-MN14, respectively. At 48 h postinjection, this difference was even more pronounced: 3.75 ± 0.18 %ID/g for ^{99m}Tc-G250 (Schwarz) versus 11.20 ± 1.58, 13.37 ± 0.93, 13.44 ± 2.08 and 13.23 ± 1.36 %ID/g for ^{99m}Tc-HYNIC-G250, ^{99m}Tc-MAG3-G250, ^{125}I-G250 and ^{131}I-MN14, respectively.

At 24 h postinjection, the mean tumor uptake of all mAb G250 preparations was significantly higher ($P < 0.001$) than that of the control antibody MN14: 76.69 ± 22.37, 63.41 ± 3.59, 31.08 ± 7.81 and 70.72 ± 13.44 %ID/g for ^{99m}Tc-HYNIC-G250, ^{99m}Tc-MAG3-G250, ^{99m}Tc-G250 (Schwarz) and ^{125}I-G250, respectively, versus 8.87 ± 2.92 %ID/g for ^{131}I-MN14. At 48 h postinjection, the tumor uptake of ^{99m}Tc-HYNIC-G250, ^{99m}Tc-MAG3-G250 and ^{125}I-G250 was even higher (92.41 ± 6.41, 125.87 ± 27.75 and 75.41 ± 25.45 %ID/g, respectively), whereas the mean tumor uptake of ^{99m}Tc-G250 (Schwarz) was somewhat lower (29.43 ± 4.49 %ID/g). At both 24 and 48 h postinjection, the mean tumor uptake of ^{99m}Tc-G250 (Schwarz) was significantly

TABLE 1
Biodistribution of ^{99m}Tc-HYNIC-G250, ^{99m}Tc-MAG3-G250, ^{99m}Tc-G250 (Schwarz), ^{125}I-G250 and ^{131}I-MN14 (%ID/g ± SD)

Organ	^{99m}Tc-HYNIC-G250 24 h postinjection	^{99m}Tc-HYNIC-G250 48 h postinjection	^{99m}Tc-MAG3-G250 24 h postinjection	^{99m}Tc-MAG3-G250 48 h postinjection	^{99m}Tc-G250 (Schwarz) 24 h postinjection	^{99m}Tc-G250 (Schwarz) 48 h postinjection	^{125}I-G250 24 h postinjection	^{125}I-G250 48 h postinjection	^{131}I-MN14 24 h postinjection	^{131}I-MN14 48 h postinjection
Blood	14.7 ± 1.5	11.2 ± 1.6	14.9 ± 1.0	13.4 ± 0.9	5.70 ± 0.56	3.75 ± 0.18	14.3 ± 2.2	13.4 ± 2.08	14.6 ± 2.5	13.2 ± 1.4
Muscle	1.01 ± 0.16	0.82 ± 0.09	1.09 ± 0.11	0.98 ± 0.06	0.47 ± 0.08	0.32 ± 0.05	1.13 ± 0.19	0.97 ± 0.08	1.04 ± 0.18	0.90 ± 0.06
Tumor	76.7 ± 22.4	92.4 ± 6.4	63.4 ± 3.6	125.9 ± 27.8	31.1 ± 7.8	29.4 ± 4.5	70.7 ± 13.4	75.4 ± 25.5	8.87 ± 2.92	6.56 ± 1.44
Lung	6.36 ± 0.92	5.31 ± 0.80	6.04 ± 0.93	4.88 ± 0.48	2.57 ± 0.13	1.90 ± 0.24	7.77 ± 1.43	7.34 ± 2.10	7.07 ± 1.29	6.73 ± 1.66
Spleen	7.46 ± 1.26	6.51 ± 0.73	4.46 ± 0.82	3.81 ± 0.42	2.52 ± 0.39	1.83 ± 0.30	4.87 ± 0.67	3.59 ± 0.45	4.21 ± 0.64	3.12 ± 0.39
Kidney	7.84 ± 0.70	6.17 ± 0.60	4.57 ± 0.23	4.21 ± 0.77	9.53 ± 1.19	5.20 ± 0.44	4.47 ± 0.85	4.05 ± 0.62	4.08 ± 0.83	3.75 ± 0.41
Liver	11.9 ± 1.2	8.48 ± 0.44	5.06 ± 0.21	4.00 ± 0.33	4.34 ± 0.90	2.90 ± 0.44	4.79 ± 0.62	4.70 ± 0.83	4.34 ± 0.58	4.36 ± 0.66
Intestines	2.02 ± 0.21	1.93 ± 0.53	1.92 ± 0.07	1.59 ± 0.08	1.08 ± 0.23	0.66 ± 0.06	2.42 ± 0.18	1.92 ± 0.57	2.16 ± 0.18	1.73 ± 0.52

HYNIC = S-hydrazinonicotinamide; MAG3 = S-benzoylmercaptoacetyltriglycine.

lower ($P < 0.001$) compared with all other mAb G250 preparations.

The mean tumor-to-blood ratios were calculated for each antibody preparation and are displayed in Figure 3 (right panels). These ratios were similar for all mAb G250 preparations and were significantly higher ($P < 0.001$) than those of the control mAb MN14 (24 and 48 h postinjection).

Uptake in normal organs was low (<8 %ID/g), with the exception of ^{99m}Tc-HYNIC-G250 uptake in the liver: 11.9 ± 1.22 and 8.48 ± 0.44 %ID/g at 24- and 48-h postinjection, respectively. At 24-h postinjection, uptake of ^{99m}Tc-G250 (Schwarz) in the kidney (9.53 ± 1.19 %ID/g) was significantly higher ($P < 0.03$) than the mean kidney uptake of the other mAb G250 preparations (7.84 ± 0.70, 4.57 ± 0.23 and 4.47 ± 0.85 %ID/g for ^{99m}Tc-HYNIC-G250, ^{99m}Tc-MAG3-G250 and ^{125}I-G250, respectively). At 48-h postinjection, the uptake of ^{99m}Tc-G250 (Schwarz) in the kidney had decreased to 5.20 ± 0.44 %ID/g.
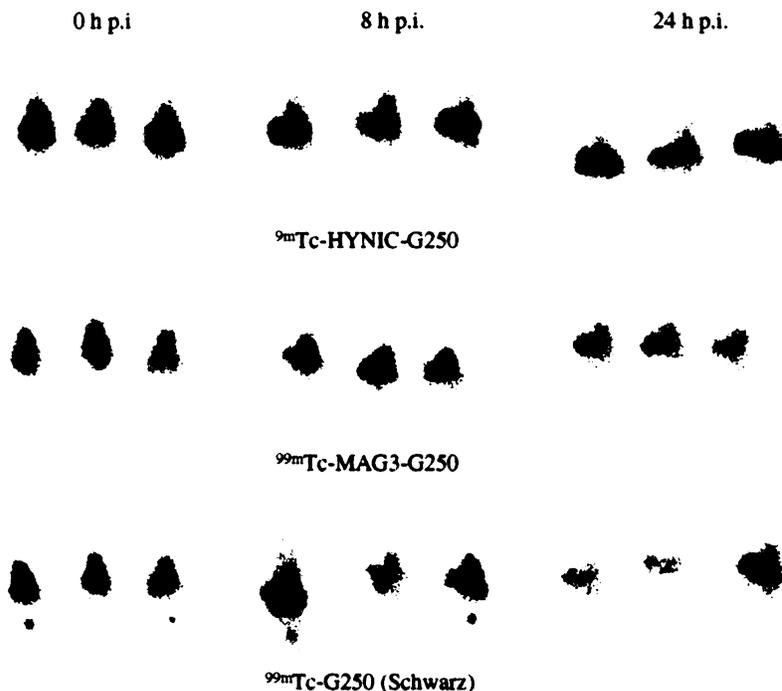
Immunoscintigraphy

Immunoscintigraphic images of the mice injected with the three ^{99m}Tc-G250 preparations are shown in Figure 4. At 24-h postinjection, tumors were clearly visualized with all three preparations. The whole-body retention of ^{99m}Tc-G250 (Schwarz) was lower compared with ^{99m}Tc-HYNIC-G250 and ^{99m}Tc-MAG3-G250. In contrast to the images of the mice injected with ^{99m}Tc-HYNIC-G250 and ^{99m}Tc-MAG3-G250, the images of the mice injected with ^{99m}Tc-G250 (Schwarz) showed enhanced bladder activity as early as 1 h postinjection.

DISCUSSION

At present, no established therapy regimen is available for patients with advanced and progressive RCC. Chemotherapy is ineffective (20); radiotherapy is applied for palliation only; and studies with biological response modifiers, such as interferon-α and interleukin-2, have shown a maximum response rate of only 20% (21,22). Nevertheless, research in this field is flourishing. Several new approaches are currently under investigation, such as combinations of biological response modifiers, the use of tumor-infiltrating lymphocytes, gene therapy, the use of tumor vaccines and radioimmunotherapy (1). Preliminary results of several of these approaches showed higher response rates (up to 47%) as well as significantly increased median survival time. For all of these therapeutic approaches, minimal residual disease offers the best chance for success. Consequently, accurate staging and early detection of metastases are of growing importance.

The physical properties of ^{99m}Tc (140-keV γ-emission, $t_{1/2} = 6$ h), its availability and its low costs make this isotope the ideal radionuclide for radioimmunodetection. As mentioned earlier, various strategies have been developed for linking ^{99m}Tc to antibodies and peptides. Of the direct labeling methods, the pretinning method, using stannous chloride as a reducing agent (23), has been, for the most part, replaced by the Schwarz method (8). Although both direct labeling methods may result in a product that is stable in vitro, several studies have demonstrated instability of these directly radiolabeled antibodies in vivo (19,24). The labeling method using MAG3 as a chelate (preformed chelate

0 h p.i   8 h p.i   24 h p.i

^{9m}Tc-HYNIC-G250

^{99m}Tc-MAG3-G250

^{99m}Tc-G250 (Schwarz)

FIGURE 4. Immunoscintigrams of mice injected with ^{99m}Tc-HYNIC-G250, ^{99m}Tc-MAG3-G250 and ^{99m}Tc-G250 Schwarz at 1, 8 and 24 h postinjection (p.i.). Tumors are clearly visualized with each preparation. Note enhanced bladder activity on images of mice injected with directly labeled ^{99m}Tc-G250 Schwarz.

approach) was originally described by Fritzberg et al. (14) and was later optimized by Visser et al. (15). Labeling of antibodies using the bifunctional chelate HYNIC (indirect labeling approach) has been described by Abrams et al. (13). Both of these approaches have been shown to result in highly stable radioimmunoconjugates.

The stability in serum of all three ^{99m}Tc-G250 preparations was good. With less than 16% loss of radiolabel after 48 h at 37°C, ^{99m}Tc-MAG3-G250 was the preparation with the highest release of ^{99m}Tc. Directly labeled ^{99m}Tc-G250 (Schwarz) showed good stability in serum, with only 6% loss of radiolabel in 48 h. However, this preparation displayed significant instability when challenged with cysteine or glutathione. The observed instability of ^{99m}Tc-G250 (Schwarz) in the cysteine challenge assay showed a much more gradual release of radiolabel (from a 100-fold molar excess and higher) compared with ^{99m}Tc-MAG3-G250. The latter preparation apparently loses the major part of the radiolabel above a certain threshold concentration of cysteine (between 10,000- and 100,000-fold molar excess). ^{99m}Tc-HYNIC-G250 appeared to be the most stable preparation. In serum as well as in all challenge assays, this preparation showed virtually no release of radiolabel. Size-exclusion HPLC analysis of the serum samples containing the three ^{99m}Tc-G250 preparations indicated that transchelation to other serum proteins did not occur with any of the preparations studied.

The scintigraphic images of the mice clearly illustrate the possible use of ^{99m}Tc-labeled mAb G250 as an imaging modality. The most striking difference among the three ^{99m}Tc-labeled mAb G250 preparations was observed in the biodistribution studies. Although directly labeled ^{99m}Tc-G250 (Schwarz) showed good stability in serum, at 24-h postinjection, the blood levels of this preparation were significantly lower than those of all other preparations. This difference was even more pronounced at 48-h postinjection. Furthermore, enhanced kidney uptake was observed at 24-h postinjection, but had normalized by 48-h postinjection. These findings imply that the in vitro stability of directly labeled ^{99m}Tc-G250 (Schwarz) does not reflect the situation in vivo. Mean tumor uptake of all mAb G250 preparations was significantly higher than that of the nonspecific mAb MN14, indicating specific localization of the mAb G250 in the RCC tumor xenografts. At both time points, the mean tumor uptake of directly labeled ^{99m}Tc-G250 (Schwarz) was significantly lower than that of all other mAb G250 preparations. Furthermore, the mean tumor uptake of ^{99m}Tc-G250 (Schwarz) was evenly high at 24 and 48 h postinjection, whereas the mean tumor uptake of the other preparations increased with time. This effect most likely resulted from higher in vivo instability of the directly labeled ^{99m}Tc-G250 (Schwarz) compared with the other mAb G250 preparations. Despite the slightly higher uptake of ^{99m}Tc-HYNIC-G250 in the liver, compared with the other preparations, the tumor-to-liver ratios of ^{99m}Tc-HYNIC-G250 were still very high (>10 at 48-h postinjection). In view of these favorable ratios, liver

uptake of ^{99m}Tc-HYNIC-G250 most likely will not hamper the visualization of liver metastases.

Mather and Ellison (8) reported good stability of antibodies labeled according to the Schwarz method. However, their conclusion was mainly based on DTPA challenge assays (up to 100-fold molar excess DTPA) in which minimal loss of radiolabel was observed. Hnatowich et al. (19) studied the in vitro and in vivo properties of ^{99m}Tc-HYNIC-labeled antibodies in comparison with directly ^{99m}Tc-labeled antibodies using stannous chloride (the pretinning method). These investigators reported a rapid transchelation of ^{99m}Tc in the presence of both cysteine and glutathione for the directly labeled antibodies. In contrast, the ^{99m}Tc-HYNIC-labeled antibodies appeared much more stable. In their studies, these authors also found a higher kidney uptake and a faster blood clearance of the directly labeled antibodies (19). Claessens et al. (24) investigated the imaging potential and biodistribution of ^{99m}Tc-HYNIC-labeled human IgG (hIgG) and directly labeled ^{99m}Tc-hIgG (Schwarz) in rats with a Staphylococcus aureus infection. The results of this study showed an enhanced whole-body clearance of the ^{99m}Tc-hIgG (Schwarz) compared with the ^{99m}Tc-HYNIC-hIgG. Furthermore, enhanced kidney uptake was noted on the scintigrams (12 h postinjection) after administration of directly labeled ^{99m}Tc-hIgG.

In search of a potential blood-pool agent, Verbeke et al. (25) studied ^{99m}Tc-HYNIC-labeled human serum albumin (HSA) and ^{99m}Tc-MAG3-HSA. Cysteine challenge assays revealed a much higher in vitro stability of ^{99m}Tc-HYNIC-HSA. In this assay, approximately 30% loss of radiolabel from the ^{99m}Tc-MAG3-HSA was observed in the presence of a 30,000-fold molar excess of cysteine. The observations of Hnatowich et al. (19), Claessens et al. (24) and Verbeke et al. (25) are strikingly similar to these findings and show that labeling of proteins with ^{99m}Tc by HYNIC results in preparations with excellent in vivo and in vitro stability. A major advantage of the HYNIC method, compared with the MAG3 method, is the easy labeling procedure. The HYNIC-antibody conjugate can be produced as a standard kit and stored at −20°C. The radiolabeling can then be carried out at any time within 15 min at room temperature and thus can be applied in the routine practice of nuclear medicine. So far, our attempts to use the HYNIC chelate for labeling antibodies with ^{186}Re—potentially suitable for radioimmunotherapy—have been unsuccessful. Thus, the use of the cG250-HYNIC conjugate appears to be limited to immunoscintigraphic imaging of RCC.

This study primarily focused on the most suitable radiolabeling technique; determination of the most optimal antibody form was not an objective of this investigation. The biodistribution studies have shown very high tumor uptake of ^{99m}Tc-HYNIC-G250 and ^{99m}Tc-MAG3-G250 at 24 h postinjection. The increased tumor-to-background ratios from 24 to 48 h mainly resulted from clearance of the antibody from the circulation. When images are being obtained from patients with RCC, 24 h would be the latest

possible time point. In view of these considerations, the use of antibody fragments, e.g., F(ab')$_2$ with a 3–4 times more rapid blood clearance (26), could be beneficial. However, the use of antibody fragments will inevitably result in lower tumor uptake. Thus, the most optimal antibody form remains to be determined. A clinical grade F(ab')$_2$ fragment of the chimeric mAb cG250 will be available in the near future, and its tumor targeting capacity, compared with the intact mAb cG250 IgG, will be assessed in clinical study in patients with RCC. Both chelate-based labeling techniques used in this study are suitable for stably labeling antibody fragments (7).

CONCLUSION

^{99m}Tc-HYNIC-G250 appears to be the preparation best suited to image RCC. It showed good stability and excellent tumor targeting. Moreover, this preparation can be labeled with high efficiency (>95%) at room temperature within 15 min. Therefore, ^{99m}Tc-HYNIC-G250 seems to be an ideal candidate for radioimmunodetection of RCC in patients.

ACKNOWLEDGMENTS

REFERENCES

1. Mulders P, Figlin R, deKernion JB, et al. Renal cell carcinoma: recent progress and future directions. *Cancer Res.* 1997;57:5189–5195.
2. DeKernion JB. Renal tumors. In: Walsh PC, Gittes RF, Perlmutter AD, Stamey TA, eds. *Campbell's Urology.* 6th ed. Philadelphia, PA: WB Saunders; 1993:1294–1342.
3. Delaloye AB, Delaloye B. Radiolabelled monoclonal antibodies in tumour imaging and therapy: out of fashion? *Eur J Nucl Med.* 1995;22:571–580.
4. Goldenberg DM. Perspectives on oncologic imaging with radiolabeled antibodies. *Cancer.* 1997;80:2431–2435.
5. Steffens MG, Boerman OC, Oosterwijk-Wakka JC, et al. Targeting of renal cell carcinoma with iodine-131 labeled chimeric monoclonal antibody G250. *J Clin Oncol.* 1997;15:1529–1537.
6. Steffens MG, Boerman OC, Oosterwijk E, et al. Phase I/II radioimmunotherapy with I-131 labeled chimeric monoclonal antibody cG250 in patients with metastasized renal cell carcinoma [abstract]. *J Nucl Med.* 1998;39:247P.
7. Liu S, Edwards DS, Barrett JA. ^{99m}Tc labeling of highly potent small peptides. *Bioconjug Chem.* 1997;8:621–636.
8. Mather SJ, Ellison D. Reduction-mediated technetium-99m labeling of monoclonal antibodies. *J Nucl Med.* 1990;31:692–697.
9. Oosterwijk E, Ruiter DJ, Hoedemaker PJ, et al. Monoclonal antibody G250 recognizes a determinant present in renal cell carcinoma and absent from normal kidney. *Int J Cancer.* 1986;38:489–494.
10. Oosterwijk E, de Weijert M, van Bokhoven A, Brakenhoff RH, Peelen WP, Debruyne FMJ. Molecular characterization of the renal cell carcinoma associated antigen G250 [abstract]. *Urol Res.* 1995;23:253.
11. Hansen HJ, Goldenberg DM, Newman ES, Grebenau R, Sharkey RM. Characterization of second-generation monoclonal antibodies against carcinoembryonic antigen. *Cancer.* 1993;71:3478–3485.
12. Beniers AJ, Peelen WP, Schaafsma HE, et al. Establishment and characterization of five new human renal tumor xenografts. *Am J Pathol.* 1992;140:483–495.
13. Abrams MJ, Juweid M, tenKate CI, et al. Technetium-99m-human polyclonal IgG radiolabeled via the hydrazino nicotinamide derivative for imaging focal sites of infection in rats. *J Nucl Med.* 1990;31:2022–2028.
14. Fritzberg AR, Abrams PG, Beaumier PL, et al. Specific and stable labeling of antibodies with technetium-99m with a diamide dithiolate chelating agent. *Proc Natl Acad Sci USA.* 1988;85:4025–4029.
15. Visser GW, Gerretsen M, Herscheid JD, Snow GB, van Dongen G. Labeling of monoclonal antibodies with rhenium-186 using the MAG3 chelate for radioimmunotherapy of cancer: a technical protocol. *J Nucl Med.* 1993;34:1953–1963.
16. Fraker PJ, Speck JC Jr. Protein and cell membrane iodinations with a sparingly soluble chloroamide, 1,3,4,6-tetrachloro-3a,6a-diphrenylglycoluril. *Biochem Biophys Res Commun.* 1978;80:849–857.
17. Ebert T, Bander NH, Finstad CL, Ramsawak RD, Old LJ. Establishment and characterization of human renal cancer and normal kidney cell lines. *Cancer Res.* 1990;50:5531–5536.
18. Lindmo T, Boven E, Cuttitta F, Fedorko J, Bunn PA. Determination of the immunoreactive fraction of radiolabeled monoclonal antibodies by linear extrapolation to binding at infinite antigen excess. *J Immunol Methods.* 1984;72:77–89.
19. Hnatowich DJ, Mardirossian G, Rusckowski M, Fogarasi M, Virzi F, Winnard P Jr. Directly and indirectly technetium-99m-labeled antibodies: a comparison of in vitro and animal in vivo properties. *J Nucl Med.* 1993;34:109–119.
20. Yagoda A, Abi-Rached B, Petrylak D. Chemotherapy for advanced renal-cell carcinoma: 1983–1993. *Semin Oncol.* 1995;22:42–60.
21. Yang JC, Topalian SL, Parkinson D, et al. Randomized comparison of high-dose and low-dose intravenous interleukin-2 for the therapy of metastatic renal cell carcinoma: an interim report. *J Clin Oncol.* 1994;12:1572–1576.
22. Wirth MP. Immunotherapy for metastatic renal cell carcinoma. *Urol Clin North Am.* 1993;20:283–295.
23. Rhodes BA, Martinez-Duncker C. Direct labeling of antibodies with Tc-99m. *Am Biotechnol Lab.* 1990;8:50–53.
24. Claessens RA, Boerman OC, Koenders EB, Oyen WJ, van der Meer JW, Corstens FH. Technetium-99m labelled hydrazinonicotinamide human non-specific polyclonal immunoglobulin G for detection of infectious foci: a comparison with two other technetium-labelled immunoglobulin preparations. *Eur J Nucl Med.* 1996;23:414–421.
25. Verbeke KA, Vanbilloen HP, De Roo MJ, Verbruggen AM. Technetium-99m mercaptoalbumin as a potential substitute for technetium-99m labelled red blood cells. *Eur J Nucl Med.* 1993;20:473–482.
26. Buist MR, Kenemans P, den Hollander W, et al. Kinetics and tissue distribution of the radiolabeled chimeric monoclonal antibody MOv18 IgG and F(ab')2 fragments in ovarian carcinoma patients. *Cancer Res.* 1993;53:5413–5418.