Successful Imaging of Malignant Melanoma with Technetium-99m-Labeled Monoclonal Antibodies


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F(ab')2 and Fab fragments of murine monoclonal antibody 9.2.27, that recognizes the 250 kD melanoma-associated antigen, were labeled with 99mTc using the bifunctional chelate method of Fritzberg et al. Twenty-seven (27) patients received, intravenously, 10 mg of either F(ab')2 (8), or the Fab (27), labeled with up to 30 mCi of 99mTc. These doses were preceded by an infusion of cold irrelevant antibody. The average serum T1/2 of the F(ab')2 and the Fab were 11 hr and 2 hr, respectively. Twenty-two percent (22%) of the total injected F(ab')2 dose was excreted in the urine in 2 hr, compared to 55% for the Fab group. Imaging was optimal 6–9 hr postinjection for the Fab patients. No nonspecific uptake in liver, spleen, bone marrow, or lung was observed for either antibody form. Overall, (43/53) 81% of known metastases were seen with visualization of tumors as small as 250 mg and tumor localization as high as 0.03% injected dose/g. Immunoperoxidase staining of freshly-frozen tumor nodules removed 24 hr postinjection confirmed antibody deposition in the tumor. Thirty-six previously unknown ("occult") metastatic sites were detected. To date, 12/36 of these sites have been confirmed. We conclude that 99mTc-labeled antibody to melanoma produces high resolution images with a high sensitivity of detecting metastatic melanoma. The detection of previously unknown sites of disease has proven helpful in directing additional diagnostic studies (i.e., CT) as well as planning of therapeutic options.


The advent of monoclonal antibodies stimulated a renewed interest in tumor imaging using radiolabeled antibodies (1–5). This technology may provide a simple, single test to stage all visceral organs and soft tissues for the presence of cancer simultaneously. Suspicious areas may then be confirmed by other imaging procedures directed to those areas. In addition, the overall biodistribution of the antibody, including tumor uptake, can also be used to predict those patients most likely to respond to a therapeutic immunonjugate. Because of its specificity, this diagnostic tool may also distinguish benign from malignant masses.

Prior studies using indium-111 (111In) and iodine-131 (131I) to label antibodies suggested that the 6-hr half-life (T1/2) of technetium-99m (99mTc) was too short for sufficient tumor localization and background clearance (6). As a transition metal, 99mTc has complex chemistry that has presented an obstacle to stable and controlled labeling of proteins. Nonetheless, the physical properties of 99mTc make it the imaging radiolabel of choice if these disadvantages could be overcome. Conventional nuclear medicine studies use 99mTc because its 140 keV principle gamma photon produces superior detection efficiency and image resolution with modern nuclear gamma cameras. Its 6-hr half-life and lack of particulate radiation give low radiation exposure. In addition, it is readily available and relatively inexpensive. Recently, Fritzberg et al. developed a diamide dimercapto
(N₂S₂) tetradedante chelating agent that allows specific, stable binding of the ⁹⁹ᵐTc to proteins (7,8). We undertook this study to determine the feasibility of using this novel stable chelate to label antibodies with ⁹⁹ᵐTc for tumor imaging in humans. We report here the first systematic study that demonstrates successful tumor imaging using this approach.

MATERIALS AND METHODS

Murine monoclonal antibody 9.2.27 (IgG₂a,k) recognizes a 250 kD glycoprotein/proteoglycan surface antigen present on over 90% of human melanoma tumors (9). Clinical grade antibody was produced according to current good manufacturing practices by Damon Biotech (Needham Heights, MA), using its patented Encapcel system and by Karyon Technology, Inc. (Norwood, MA), using its Geltrap method. The immunoglobulin was purified by ion exchange chromatography following preliminary enrichment by ammonium sulfate precipitation.

Technetium-⁹⁹ᵐ Labeling

Technetium-⁹⁹ᵐ 4,5-bisthioacetamide pentaoante was formed by reducing [⁹⁹ᵐTc]pertechnetate with dithionite in the presence of the thioacetate protected form of the ligand. The ⁹⁹ᵐTc complex was esterified with 2,3,5,6-tetrafluorophenol using carbodiimide. The active ester of the complex was reacted with 2.5 mg of antibody to produce the [⁹⁹ᵐTc]N₂S₂ antibody conjugate. After purification by gel filtration (Sephadex PD-10), the labeled antibody preparation was monomeric and had 96–100% (mean of 99%) of the radioactivity bound to protein as determined by thin layer and high performance liquid chromatography (HPLC) gel permeation chromatography.

Immunoreactivity assay of the final product was determined by an in vitro cell binding assay using antigen positive A375 MetMix human melanoma target cells (10). On the basis of the flow cytometry, there was no diminution in binding to target cells compared to unlabeled control. Eighty-five percent of the counts bound to the target cells in vitro.

Immunoperoxidase Evaluation of Tissue Sections

Fresh frozen sections obtained the day after injection were submitted for routine hematoxylin/eosin staining and immunohistochemistry using an indirect, two-stage immunoperoxidase procedure as previously described (11). Cryostat sections were incubated with 9.2.27 whole antibody or an appropriate control antibody and washed prior to addition of horseradish peroxidase conjugated goat antimouse immunoglobulin (KPL, Gaithersburg, MD). Peroxidase activity was visualized with diaminobenzidine hydrochloride (Sigma, St. Louis, MO) and tissues were counterstained in Mayer's hemalum. Endogenous binding of 9.2.27 was assessed by comparing the staining of sections with and without the addition of 9.2.27 in vitro.

Human Antimouse Antibody (Antiglobulin) Assay

Human antiglobulin was measured in dilutions of serum with an enzyme-linked immunosorbent assay (ELISA) employing 9.2.27, the irrelevant antibody, or control antibodies as the solid phase target (12). Antiglobulin was detected with a F(ab')₂ preparation of a mouse antiserum to human IgG (heavy and light chain, Jackson Labs, West Grove, PA). Antiglobulin activity was quantitated by comparing the activity of any given specimen to that of a pooled human sera standard run with each assay and expressed as normal human serum (NHS) equivalents.

Patient Studies

Twenty-seven patients with documented metastatic melanoma participated in the study. All were evaluated at study entry by history and physical examination and with selected radiographic images for evidence of metastatic disease. An ECG, serum electrolytes, SGOT, SCPT, alkaline phosphatase, bilirubin, creatinine, urine analysis, complete blood count with platelets, hematocrit, red blood cell indices, as well as screening tests for hepatitis were performed. Each patient gave written, informed consent. The studies were conducted under the auspices of an IND with the Office of Biologics (FDA) and with the approval of University of Washington Human Subjects and Radiation Safety Committees.

Patients received 2.5 mg radiolabeled 9.2.27 F(ab')₂ fragment (eight patients) or the F(ab')₂ or Fab (19 patients) fragment labeled with 20–30 mCi ⁹⁹ᵐTc by i.v. injection (Table 1). This was followed by 7.5 mg unlabeled F(ab')₂ 9.2.27 for a total dose of 10 mg 9.2.27 over 5–10 min. Lower doses of 2.5 mg labeled antibody alone had been found in prior studies to be insufficient for tumor imaging. One hour prior to the injection of the specific labeled antibody, 23 of these patients also received pre-injections of 41–50 mg of an unlabeled irrelevant murine whole IgG₂a. This antibody was infused intravenously over ~10 min. Vital signs were recorded before and up to 2 hr postinfusion. Patients received a cathartic to clear the bowel 3 hr prior to imaging at the 8-hr timepoint. Immediately postinfusion serial blood and fractional urine samples were obtained at intervals over the next 24 hr. Plasma radioactivity was expressed as the percent of injected dose/g.

![Table 1](image)

<table>
<thead>
<tr>
<th>Patient group</th>
<th>No. patients</th>
<th>No. lesions</th>
<th>No. image positive</th>
<th>Serum T½ (hr)</th>
<th>Urinary excretion % ID in 20 hr</th>
<th>Occult tumors seen</th>
<th>Occult tumors confirmed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. F(ab')₂</td>
<td>3</td>
<td>8</td>
<td>0</td>
<td>6.1 ± 0.60</td>
<td>49.5% ± 39%</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2. F(ab')₂ + Wab</td>
<td>5</td>
<td>11</td>
<td>9</td>
<td>11.2 ± 3.8</td>
<td>22.6% ± 10.8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3. Fab or F(ab') + Wab</td>
<td>19</td>
<td>44</td>
<td>35</td>
<td>2.1 ± 0.7</td>
<td>60% ± 17.5</td>
<td>36</td>
<td>12</td>
</tr>
</tbody>
</table>

*Whole irrelevant antibody.*
of serum or as a percentage of the immediate postinjection serum radioactivity level. After the completion of the study (24 hr), a repeat full set of serum chemistry, urine, and hematologic tests was obtained.

Whole body excretion of the radiolabel was determined by obtaining repetitive total body counts at 0, 3, and 8 hr postinfusion using a 3-in sodium iodide crystal at a distance of 14 ft. A 99mTc comparator standard was counted at each time at a fixed distance to correct for radioactive decay and changes in detector sensitivity.

Planar images of chest, abdomen, and pelvic regions (extremities where indicated) in both anterior and posterior views of the patients were obtained immediately and at 3 and 8 hr postinfusion on either a General Electric 400T or a 500Q gamma camera, interfaced with an ADAC 3300 or a General Electric Star dedicated computer. Digital and/or analog images were acquired and stored on floppy disk or magnetic tape for processing. Changes in normal organ and tumor concentrations of activity were determined by placing a fixed region of interest (ROI) over a standard site on each organ or tumor and recording the number of radioactive counts in that region. At each imaging timepoint, a standard 99mTc source was imaged for 1 min at a fixed distance of 6 in from the camera face to correct the ROI counts for radioactive decay and changes in camera sensitivity. Relative amounts of radioactivity in the tumor or organs were expressed as counts/pixel/second.

RESULTS

No clinical toxicity was observed in any of the patients studied, and no significant changes were noted in the laboratory studies of the blood or urine obtained 24 hr postinfusion.

Eight patients received 10 mg of the antibody in the form of F(ab')2 (Table 1). The first three patients received no irrelevant antibody. The resultant serum T½ of the slower clearing dose fraction was ~6 hr with 50% of the injected dose excreted in the urine in 20 hr. None of the known tumor sites were visualized.

The next five patients received 50 mg (one patient received 200 mg) of unlabeled whole irrelevant antibody 1 hr prior to the infusion of the labeled F(ab')2. Subsequently, the mean serum T½ increased to 11 hr with only 23% of the injected dose excreted in the urine at 20 hr. Concomitant with this increase in serum T½, 9/11 (81% sensitivity) known tumor sites were imaged 7–10 hr postinjection. This is in direct contrast to F(ab')2 without pre-injected antibody; no tumors were visualized. Of the two tumor sites not visualized, one 0.5-cm-diameter superficial lesion was a relatively avascular primary melanoma, and the other was a 1.0-cm suprasellar metastatic lesion. An image obtained 10 hr postinjection of a patient in this group is shown in Figure 1. This patient had a 2 cm × 2 cm subcutaneous metastasis of the angle of the right mandible and a 1.5 cm × 1.0 cm palpable lymph node metastasis in the right inferior mandibular area. This figure shows avid localization in the two melanoma metastases, with relatively low background activity in the surrounding tissues.

Nineteen administrations of 99mTc-labeled 9.2.27 Fab or Fab' fragments (10 mg) were given to 19 patients. All but two received 41–50 mg of whole irrelevant antibody intravenously 1 hr before administration of the labeled antibody. One individual received 82 mg of irrelevant antibody, and one did not receive the irrelevant antibody. The average serum clearance T½ for this patient group was 2 hr, and the average 20-hr urinary clearance was 60% of the injected dose. In this group, 35/50 (70%) of known metastatic sites were imaged. Of the 19 individuals in this patient group, two had small (≥3mm) superficial cutaneous lesions only. A third patient received a dose of radioactivity (9 mCi) which was suboptimal due to decreased radiolabeling yield. The overall tumor detection rate in this patient group (excluding these three patients) is 78% (35 of 44) of all known viable tumors detected. Within this group, 25/28 (89%) subcutaneous or lymph node metastases were detected. Of 12 lung lesions, 58% were detected, as were 2/2 of liver tumors (multiple metastases in one patient; solitary nodule in the other), 0/1 of bone tumors, and 1/1 of spleen tumors. No false-positive lesions were noted.

Of note was that an additional 36 occult tumor sites were detected. At least 12 of these presumed occult tumor sites (33%) were later putatively confirmed as metastatic tumor by physical examination, biopsy, or other imaging modality. In this Phase I study, the patients were referred back to their primary physicians who pursued confirmatory studies only when it seemed useful in prognosis or treatment planning. In one patient (Fig. 2), three of the image positive sites became palpable subcutaneous masses 4 wk after the completion of the study.
FIGURE 2
Image of anterior chest 8 hr after infusion of $^{99m}$Tc-9.2.27 Fab. Numerous non-palpable subcutaneous lesions are visualized along with axillary adenopathy and two pulmonary lesions. The heart is also clearly seen.

During the first 60 min after intravenous administration, 98–100% of the radiolabeled remained bound to antibody as determined by thin layer chromatography in butanol solvent. Immunoreactivity was comparable to the starting material, ranging from 70–100% of the pre-injection value.

Despite no demonstrable hepatic localization of the antibody in some patients, early (2–4 hr) images of the abdomen showed gall bladder visualization. Later images in all patients showed various degrees of bowel activity.

The effect of varying the amount of specific Fab was tested in one patient in this group, who had a palpable lymph node metastasis in the left submandibular region. Images after infusion of only 2.5 mg showed no visualization of the tumor (Fig. 3) and a change in the biodistribution into the reticuloendothelial system, especially bone marrow and spleen. In a subsequent study 3 days later, the same patient received 2.5 mg of labeled antibody fragment with the addition of 7.5 mg of unlabeled specific antibody. There was now clear visualization of the known lymph node metastasis and additional unknown disease present in the submandibular area and marked relative decrease in RES distribution. The previous occult disease was confirmed by neck dissection 2 days later. In both cases, infusion of the specific antibody was preceded by a 41 mg dose of irrelevant whole antibody.

Metastatic tumors from eight patients were biopsied within 24 hr of imaging and further analyzed (Table 2). Patient 4 who imaged positive with F(ab')₂ had an average uptake of 0.029% (0.033 and 0.025) of the injected dose/gm tumor (percent ID/g). Patient 3 who imaged negative with F(ab')₂ despite antigen positive tumors averaged 0.008% ID/g. In comparison, patients in the Fab group and Fab' group had an average of 0.0041% (range 0.0043–0.0039) of the percent ID/g tumor in image-positive tumors, with 0.0011% ID/g in two patients who were image negative. Histologic examination of patient tissues with specific antibody peroxidase staining showed the presence of the 250 kD melanoma surface antigen in five of six cases. Examination of the same tissues for the presence of the infused mouse immunoglobulin by immunoperoxidase staining showed diffuse penetration of the tumor tissues with the 9.2.27 antibody with less than 10% of tumor cells demonstrating in vivo binding of the murine antibody in two patients (Fig. 4) and undetectable in vivo localization by peroxidase in the remaining four patients. This is consistent with previously reported variable results using immunoperoxidase when 10 mg of unlabeled 9.2.27 were administered to patients. One of the two patients who were image negative was also 250 kD antigen negative.

The development of human anti-mouse antilobulin following antibody infusion in 26 patients is shown in Figure 5. Weekly blood samples were obtained for as long as 250 days postinfusion of antibody. Only four of 26 (15%) individuals developed a significant antiligulin response to the 9.2.27 antibody fragments. In contrast, 11 of 21 (52%) individuals who received the irrelevant antibody developed an antiligulin response to that antibody, and the mean peak level of antiligulin was 26.7 normal human serum units as compared to a peak value of 9.0 units against the 9.2.27. A patient with anti-irrelevant antibody of 15 times over baseline, seven times over normal human serum equivalents, was successfully re-imaged 6 mo after the first study, a second and third time by slowly infusing (60 min) the irrelevant antibody prior to the injection of specific antibody.

DISCUSSION

Technetium-99m is the radiolabel of choice for the majority of nuclear medicine procedures because of its relatively short half-life (6.2 hr), low radiation exposure, and its 140 keV gamma emission for which modern nuclear medicine gamma cameras have high detection efficiency and resolution. It has found very little use in antibody imaging due to the complex chemistry involved in achieving a stable chemical linkage to protein and because its short half-life was considered incompatible with the time required for tumor localization and background clearance. The $^3$H.S₄ ligand reported here provides stable binding of $^{99m}$Tc to the antibody. The resulting product has a high degree of radiochemical purity and retains its immunoreactivity. The preformed chelate approach involves first chelating $^{99m}$Tc to the $^3$H.S₄ ligand and then conjugating the chelate to the antibody. This ensured that all the radiolabel was bound to the antibody via the ligand and not by nonspecific interactions. This labeling methodology has been adapted to a kit formulation for routine use and
has been shown in the laboratory to be adaptable to a variety of monoclonal antibodies (7,8).

A sufficient quantity of radiolabeled antibody accumulated in tumor and cleared from nontumor sites rapidly enough to accommodate the use of a radionuclide with a physical half-life of 6 hr. Previous reports of antibody imaging using $^{131}$I or $^{111}$In labels have indicated optimal uptake of labeled antibody in the tumor between 24–72 hr following infusion (3,6). Our studies have demonstrated that imaging with $^{99m}$Tc monoclonal antibody fragments can be achieved in 8 hr or less following infusion. $F(ab')_2$ fragments yielded good tumor uptake at 8 hr but tumor-to-background tissue ratios were suboptimal for imaging due to the 11-hr serum $T_\alpha$ of $F(ab')_2$. Use of the smaller fragments, Fab and Fab', proved effective in delivering sufficient quantity of labeled antibody to tumor sites combined with rapid clearance from nontumor tissues. In most

**FIGURE 3**

A: Upper photo: 8 hr images using 2.5 mg $^{99m}$Tc-Fab showing marked marrow uptake, with no visualization of the left submandibular nodal metastasis, while lower photo: a repeat image of the same patient 3 days later using 2.5 mg $^{99m}$Tc-Fab along with 7.5 mg unlabeled Fab, shows reduced reticuloendothelial uptake and visualization of submandibular palpable nodal metastases (arrow). B: Upper photo: 8 hr images using 2.5 mg $^{99m}$Tc-Fab showing marked marrow, liver, and spleen uptake. Lower photo: a repeat image of the same patient 3 days later using 2.5 mg $^{99m}$Tc-Fab along with 7.5 mg unlabeled Fab shows reduced reticuloendothelial uptake.
cases, tumors that were imaged at 8 hr were initially visible by 3–4 hr following infusion.

Prior studies have reported that increased antibody quantity improved tumor imaging and diminished background accumulation. This has previously been accomplished by adding unlabeled specific antibody. We demonstrated here that an irrelevant antibody that does not compete for antigen sites in tumor could be used to prolong the serum half-life and thereby improve the tumor uptake of F(ab')2 fragments, an unexpected finding. The imaging results reflected this. No tumors were imaged without the addition of irrelevant antibody, while the combination of irrelevant and specific antibody yielded a sensitivity of 81%. The serum clearance of the Fab was not affected by the irrelevant antibody. The percentage of the injected dose per gram achieved with F(ab')2 is approximately an order of magnitude higher than previously reported for labeled antibody uptake in solid tumors in humans. We believe this was due in part to nonspecific uptake of the monoclonal antibody by the reticuloendothelial system that was partially “blocked” by the irrelevant antibody. Nonetheless, it was still necessary to administer a threshold dose of specific antibody in addition to 2.5 mg of labeled antibody to achieve optimal tumor localization analogous to results obtained with unlabeled 9.2.27 (11).

It is well documented that administration of murine

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Fragment</th>
<th>Time (hr)</th>
<th>Conc.</th>
<th>Tissue specimens</th>
<th>Antigen stain</th>
<th>Image results</th>
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<tbody>
<tr>
<td>1</td>
<td>Whole 9.2.27</td>
<td>26</td>
<td>0.0010</td>
<td>Melanoma nodule +5%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>+ Irrelevant</td>
<td>22</td>
<td>0.0025</td>
<td>Two melanoma nodules +++100%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>F(ab')2-irrelevant antibody</td>
<td>24</td>
<td>0.0091</td>
<td>Involved node +++100%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>+ Irrelevant</td>
<td>23</td>
<td>0.0037</td>
<td>Submandibular node</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Fab</td>
<td>22</td>
<td>0.0043</td>
<td>Two melanoma nodules +++100%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Fab</td>
<td>24</td>
<td>ND</td>
<td>Normal skin</td>
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</tr>
<tr>
<td>7</td>
<td>Fab</td>
<td>23</td>
<td>0.0039</td>
<td>Axillary node 1 +50%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Fab</td>
<td>20</td>
<td>0.0011</td>
<td>Axillary node 2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 Percent of the injected dose/g.
2 Intensity grades —, +, ++, +++ heterogeneity 0–100%.
3 Hours after labeled antibody infusion.
monoclonal antibodies in man frequently is accompanied by development of an antiglobulin response and that the incidence of the response appears to be related to the patient disease group under investigation and possibly to the antibody administered (12). The antiglobulin response is undesirable not only for subsequent antibody imaging studies, but also in the repeated use of monoclonal antibody conjugates for therapy. In this study, pre-administration of irrelevant antibody in higher doses appeared to reduce the immunogenicity of the specific antibody. This diminished immune response could be related to several factors, including the greater dose of the irrelevant antibody or that the irrelevant antibody as intact while the specific antibody was a fragment. The use of irrelevant antibody may therefore provide a means of alleviating antiglobulin responses to a specific antibody, since the bulk of the antiglobulin responses in these subjects was directed to the irrelevant antibody rather than the specific antibody (13). This may allow multiple doses by repeated substitution of a new irrelevant antibody at each administration or by using a slow infusion of irrelevant antibody to bind the antiglobulins prior to infusion of the specific antibody. This latter strategy has already proved valid in one patient imaged a second and third time in the presence of an increased level of circulating antiglobulins.

In 30% of the patients, a total of 36 previously unknown sites of tumor were identified. To date, 12 (33%) of these have been confirmed as metastatic sites. It is possible that many of the occult tumors imaged were smaller than 1 cm since a known subcutaneous lesion that weighed 0.25 gm, for example, was easily detected by the imaging procedure. The large number of occult tumors was unexpected based on prior studies imaging melanoma in comparably evaluated patients with 111In- or 131I-labeled antibodies (2,6,14) and suggests that 99mTc antibody imaging may be more sensitive. Since radioimmunoimaging lends itself well to whole body scanning, the procedure has been helpful in directing other diagnostic procedures such as computed tomography and magnetic resonance imaging to specific areas. This has clinical significance as patient management decisions may be affected.

The lung appeared to be the organ with the lowest rate of detection of metastatic disease. In our study 60% of known pulmonary lesions have been imaged; this was lower than any other site, but higher than previously reported using 111In-labeled antibodies (15). Lesions as large as 1 cm diameter failed to sequester radionabeled antibody, but mediastinal disease imaged quite well. The reason for this is not clear.

Liver, spleen, and marrow did not accumulate substantial radioactivity and were not visible above blood pool. Various degrees of intestinal activity were identified in the majority of patients due to biliary excretion; visualization of the gall bladder was seen at earlier timepoints. Since the liver as a whole did not show increased activity, this is most probably related to continuous hepatobiliary clearance of low concentrations of the radiolabeled ligand complex. Cathartics were not invariably successful in purging the bowel of accumulated counts prior to imaging. Techniques to eliminate biliary excretion are under study. As expected, the kidney was the major route of excretion for Fab.

In summary, the N_2S_2 ligand system has overcome the technical problem of labeling proteins with 99mTc. This is the first report demonstrating reproducible tumor imaging with 99mTc-labeled antibodies that is suitable for routine use.

ACKNOWLEDGMENT

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REFERENCES