Technetium-99m-Labeled Monoclonal Antibodies: Influence of Technetium-99m Binding Sites

E. John, M.L. Thakur, S. Wilder, M.M. Alauddin and A.L. Epstein

Department of Radiation Oncology and Nuclear Medicine, Thomas Jefferson University Hospital, Philadelphia, Pennsylvania

A number of ^{99m}Tc-labeled monoclonal antibodies (Mabs) are being evaluated for diagnostic applications. Preparations are carried out using both direct and indirect (bifunctional chelating agent, BFCA) labeling procedures in which nonspecific ^{99m}Tc binding has been postulated. **Methods:** By using the ascorbic acid (AA) reduction mediated direct labeling procedure and diaminotetrathiol (N_2S_4) as a BFCA method, we examined the role of specific binding sites and aliphatic E amino groups of lysine as nonspecific binding sites for ^{99m}Tc. **Results:** Labeling yields for the direct and N_2S_4 "regular" preparations averaged 73% ± 2.8% and 91% ± 2%, for the "specific" preparations, 60% ± 3.5% and 75% ± 2% and for the "nonspecific" preparations 13% ± 1.0% and 16% ± 1% respectively. All preparations were evaluated in tumor-bearing mice. The control and specific preparations permitted excellent scintigraphic visualization of tumors; the percentages of specific preparations in the tumors being 2.1% ± 0.5% and 3.1% ± 0.4%, respectively. With nonspecific preparations, tumors were not visualized and the percentages of administered radioactivity per gram of tumor were only 0.9% ± 0.2% and 0.9% ± 0.3%, respectively. **Conclusions:** In these ^{99m}Tc labeling procedures, the amino group-mediated nonspecific binding of ^{99m}Tc to Mabs can be as high as 16% and contributes to increased liver uptake and decreased tumor uptake of radioactivity.

Key Words: technetium-99m-labeled monoclonal antibodies; technetium-99m binding sites; FITC blocking; iodoacetate blocking

J Nucl Med 1994; 35:876–881

The unique receptor specificity of monoclonal antibodies (Mabs) combined with the excellent physical characteristics and ready availability of the radionuclide have made ^{99m}Tc Mabs highly desirable for scintigraphic imaging. A number of Mabs have been labeled with ^{99m}Tc and evaluated for imaging a variety of tumors (*1–6*) and inflammatory foci (*7–10*). The chemistry by which Mabs are labeled with ^{99m}Tc can be divided into three groups. In group one, a preformed ^{99m}Tc complex is conjugated to the Mab molecule (*11*). In group two, the native disulfide groups are reduced to sulfhydryls to provide strong coordination bonds to reduced ^{99m}Tc (*12–15*) and in group three, a variety of bifunctional chelating agents (BFCAs) are covalently bound to Mab molecules for subsequent chelation with ^{99m}Tc (*16*).

In addition to the functional groups such as those provided in methods two and three, the protein molecules possess other coordinating groups to which ^{99m}Tc ions may bind in their reduced form. These groups have been previously described as low affinity/high capacity and high affinity/low capacity binding sites (*17*). These binding sites probably involve the terminal amino groups of N-terminal amino acids and E-amino groups of lysine residues (*18*). However, neither the quantity of ^{99m}Tc bound to these groups in Mab molecules, nor the influence of such binding on the tissue distribution has been critically examined.

We have attempted to thoroughly examine two types of methods to obtain insight into this phenomenon: one from the direct and the other from the BFCA group. These include ascorbic acid disulfide reduction technique developed in our laboratory as a typical direct labeling method and a diaminotetrathiol (N_2S_4) as a strong BFCA method. Using these two methods we have determined the quantity of ^{99m}Tc bound specifically to the intended functional groups as well as nonspecifically to the native Mab groups and studied their influence on tissue distribution and tumor uptake in experimental animals. Results of these and other related findings form the basis of our study.

MATERIALS AND METHODS

Antibody

TNT-1 (IgG 2a), used as a prototype Mab, is a murine antibody with a specificity for nuclear histones developed by Epstein et al. (*19*). TNT-1, obtained from Dr. Epstein's laboratory (10 mg/ml saline), was divided in 100-μg portions and kept frozen until use.

Received Aug. 18, 1993; revision accepted Dec. 20, 1993.
For correspondence or reprints contact: M.L. Thakur PhD, Dept. of Radiation Oncology and Nuclear Medicine, Thomas Jefferson University Hospital, Philadelphia, PA 19107.

Tumors

Embryonal carcinoma grown in Balb/c mice was used as a prototype tumor model. Approximately 10^7 NF-1 cells obtained from Dr. Damjanov's laboratory (*20*) in the Dept. of Pathology and Cell Biology were implanted in the right thigh of the animals and the tumors were allowed to grow for 8–10 days to approximately 1 cm in diameter.

N,N,N',N', Tetrakis (2-Mercaptoethyl)Ethylene-Diamine (N_2S_4)

This chelating agent was prepared in Dr. Epstein's laboratory using the method of Najafi et al. (*21*). This method has been used extensively to label such antibodies as Lym-1 and B72.3 with ^{99m}Tc and ^{186}Re in high yields (*22*). In this process, the chelating agent is conjugated to Mab via a disulfide exchange with one of the thiol groups and the remaining three thiols and two amino groups were employed to form a coordination complex with reduced ^{99m}Tc or ^{186}Re (*21*). Chemicals and reagents were obtained from Sigma or Aldrich chemicals and used without further purification. Centricon-30, a molecular filtration unit, was obtained from Amicon Inc. (Cambridge, MA).

Labeling of TNT-1 with Technetium-99m

TNT-1 was labeled with ^{99m}Tc by three separate methods for each of the two (direct or BFCA) methods chosen. These are called "regular preparation," "specific preparation," and "nonspecific preparation" and are described as follows.

Radiolabeling of Antibodies by the Direct AA Method

Regular Preparation. The protocol for this preparation was essentially the same as described previously (*15,22–24*). Briefly, 100 μg of TNT-1 were incubated with 3500 molar excess of AA at pH 6.5 for 1 hr at 22°C. A required quantity of pertechnetate was reduced with a freshly prepared solution of $Na_2S_2O_4$ (0.1 *M* $NaHCO_3$, pH 11) for 1 min at 22°C and added to the protein for 30 min incubation at 22°C. The sodium dithionite concentration in the final reaction mixture was 5 μg/μl.

Specific Preparation. Fluoroscein isothiocyanate (FITC) was used as the antibody amine modification reagent as described by Means and Brinkley (*25–27*). In a typical preparation, 400 μl of 0.1 *M* $NaHCO_3$, pH 9.0, were added to 0.5 mg TNT-1 in 125 μl isotonic saline. This was cooled in an ice bath and 26 μg FITC in 13 μl DMF (20 × molar excess) was added. The reaction mixture was stirred for 2 hr as the ice bath gradually reached 22°C. Excess reagents were removed by Centricon-30 filtration and samples were washed twice with 1 ml 0.15 *M* NaCl. FITC dye-to-protein ratio was then determined by a spectrophotometric assay (*25,26*). The modified IgG (100 μg) was then labeled with ^{99m}Tc using the AA procedure as described above. The unused portion of the amino-blocked antibody was stored at −20°C for future use.

Nonspecific Preparation. In this preparation, the antibody was reduced with 3500 molar excess of AA, pH 6.5, for 1 hr at 22°C and then treated with 3500 molar excess of sodium iodoacetate to block the sulfhydryl groups (*27*). The pH of the final reaction mixture was raised to 8 with triethylamine and incubated for 2 hr at 22°C. The excess reagents were then removed by Centricon-30 filtration. The number of thiols before and after blocking the sulfhydryl groups were determined by Ellmans assay (*28*). The sulfhydryl blocked antibodies were labeled with ^{99m}Tc by incubation for 30 min as described previously.

Radiolabeling of Antibodies by the BFCA Method

Regular Preparation. Approximately 0.55 μg (1:1) N_2S_4 was added to 200 μg TNT-1 in 50 μl phosphate buffer, pH 7.0, and the mixture was incubated for 5 min at 22°C. A required quantity of ^{99m}Tc O_4^- was then reduced with $Na_2S_2O_4$ in pH 11 (final concentration 5 μg/μl) and quickly transferred to the conjugated antibody solution. All reagents were purged with oxygen-free nitrogen. The incubation was allowed to proceed for 30 min and any unreacted N_2S_4 or ^{99m}Tc was eliminated by Centricon-30 filtration. The labeled protein was collected, analyzed by ITLC and HPLC and prepared for animal injection. This method is based upon the procedures developed by Najafi et al. (*22*) and Thakur et al. (*15,23,24*).

Specific Preparation. In this preparation, as before, some of the amine groups were blocked with fluoroscein isothiocynate (FITC). Excess reagents were removed and the modified Mab was divided into suitable portions for future use. The number of FITC molecules bound per molecule of protein was determined spectrophotometrically (*25,26*).

A required quantity of this protein was then conjugated with N_2S_4 and labeled with ^{99m}Tc as described previously.

Nonspecific preparation. In this preparation, the thiol groups available for ^{99m}Tc binding were blocked with iodoacetate by the method of Gurd et al. as described previously (*27*). Briefly, the pH of the N_2S_4 conjugated Mab was raised to 8 with triethyl amine and 3500 molar excess iodoacetate added for a 2-hr incubation at 22°C. The excess of iodoacetate was then removed by Centricon-30 filtration and blocking of thiols was confirmed by Ellmans assay (*28*). The sulfhydryl-blocked Mab was then labeled with ^{99m}Tc and analyzed as described above.

FITC Modification of ^{125}I-Labeled Mab

It is possible that FITC modification may alter the Mab charge which may result in a different tissue distribution. In order to assess this possibility, TNT-1 was labeled with ^{125}I and a portion of it was subjected to FITC modification. Following adequate analysis, tissue distribution of ^{125}I-TNT-1 and ^{125}I-FITC-TNT-1 was studied in normal animals. Iodination was accomplished by the iodogen method established in our laboratory (*29*).

Quality Control Tests

These routine tests in our laboratory were designed to determine the proportion of unbound ^{99m}Tc and the colloid formation, if any. HPLC was performed using Waters protein pak SW 300 column and phosphate buffer pH 6.8. HSA-impregnated ITLC strips were developed in EtOH:NH_4OH:H_2O (2:1:5) for determination of colloid formation (*15,23,24*).

Imaging and Tissue Distribution Studies

These were carried out in normal and experimental embryonal carcinoma bearing Balb/c mice weighing between 18 and 25 g. Each group consisted of five animals and received approximately 20 μg protein-labeled either with 40 μCi ^{99m}Tc or 50 μCi ^{125}I through a lateral tail vein. Four hours later, the animals were killed in a halothane gas chamber, imaged in the posterior position using a gamma camera equipped with a pinhole collimator and then dissected. A number of tissues were harvested, weighed and concomitant radioactivity was counted using a Packard (5000 series) automatic gamma counter. Appropriate radioactivity standards counted along with the samples allowed us to determine the radioactivity (%ID/g) associated with each tissue. These were evaluated using the Student's t-test. The ^{125}I-TNT-1 and ^{125}I-FITC-TNT-1 were evaluated only in normal animals.

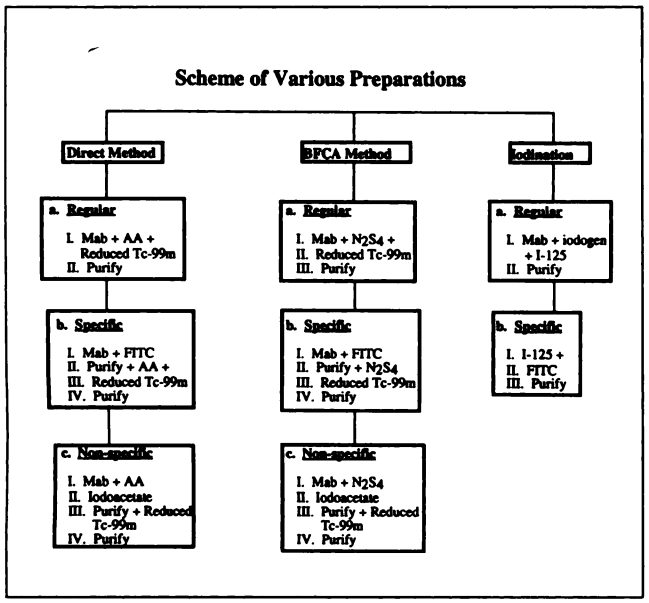

FIGURE 1. Scheme of various preparations.

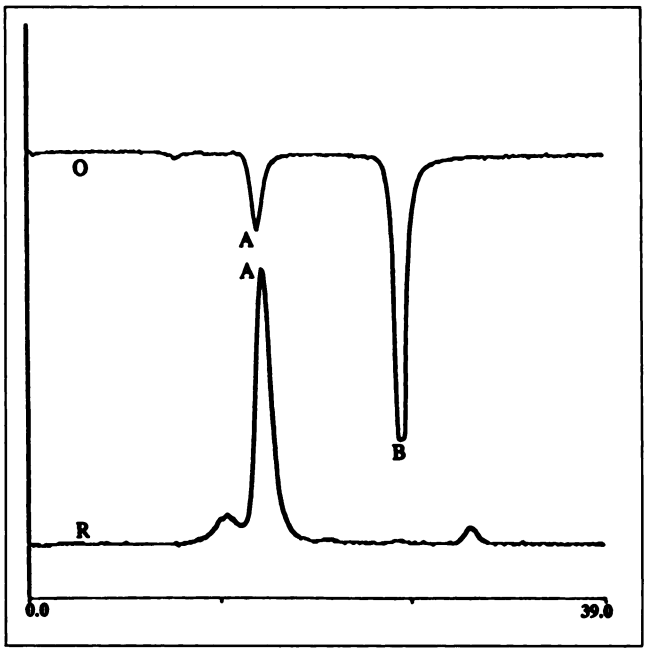

FIGURE 2. HPLC chromatogram of ^{99m}Tc-TNT-1 (O = optical density and R = radioactivity) showing that greater than 95% of the radioactivity is associated with the protein. Radiolabeled TNT-1 is eluted from the column first (A, retention time = 15.8 min) followed by low molecular weight ascorbic acid (B, retention time = 25.3 min). The first small peak (2%) in the radiochromatogram represents high MW impurity and the third peak (2.5%) is free from ^{99m}Tc.

RESULTS

Various preparations are schematically presented in Figure 1. The spectrophotometric assays showed that five FITC molecules were bound to each Mab molecule and upon iodoacetate blocking, there were only less than 0.2 free sulfhydryls per Mab molecule as compared to that of approximately two sulfhydryl groups before blocking. The ^{99m}Tc labeling efficiencies for the direct and N_2S_4 regular preparation averaged 73% ± 2.8% and 91% ± 2%; for the specific preparations: 60% ± 3.5% and 75% ± 2%; for the nonspecific preparations: 13% ± 1% and 16% ± 1%, respectively. In the preparations where reduced ^{99m}Tc was incubated alone with native protein, approximately 20% of the radioactivity was bound to the protein, indicating that $Na_2S_2O_4$ does not reduce the protein disulfide groups to provide sulfhydryls for significant ^{99m}Tc binding. Results of our experiments also indicated that nonspecific binding of similar magnitude also took place in Mabs conjugated with c-DTPA and strengthened our observation that such nonspecific binding was not limited to only N_2S_4 conjugated protein. The colloidal formation in all preparations was less than 5% and radiochemical purity was greater than 95%. These results were also in good agreement with HPLC, a typical elution profile of which is given in Figure 2.

The tissue distribution of these preparations in normal and tumor-bearing mice is given in Tables 1, 2 and 3. Tables 1 and 2 conclusively indicate that in both direct or the N_2S_4 BFCA method, liver uptake with nonspecific preparations was consistently higher than the regular ($p = 0.002$) and specific preparations ($p = 0.003$). Similarly, the blood clearance of nonspecific preparations was significantly faster ($p = 0.002$ and $p = 0.001$) than that of the regular or specific preparations in both methods. Both tables also indicate that the tumor uptake of radioactivity was lowest (0.9% ± 0.27%) with the nonspecific and highest (3.1% ± 0.4%) with the specific preparations. The values were intermediate for the regular preparations and slightly lower for the direct method. Scintigraphic images of animals representing each group for both types of methods are given in Figures 3 and 4. These provide additional evidence for the poor tumor uptake of radioactivity with the nonspecific preparations.

The results with ^{125}I-labeled TNT-1 and ^{125}I-labeled FITC-modified TNT-1 given in Table 3 showed no difference in tissue distribution, thus supporting the hypothesis that it is the nonspecifically bound ^{99m}Tc that contributes to altered tissue distribution in both direct as well as BFCA methods of labeling Mab with ^{99m}Tc.

DISCUSSION

Over the past few years much has been invested in developing the use of radiolabeled Mabs for scintigraphic imaging and therapy. In scintigraphic imaging, Mabs labeled with ^{99m}Tc lead the list. The ^{99m}Tc labeling is achieved either by reduction of Mab disulfide groups to provide strong covalent binding for ^{99m}Tc or by conjugation with bifunctional chelating agents. Although the functional groups provided facilitate a strong chelation with the major quantity of ^{99m}Tc, our data strongly indicate that approximately 15%–20% of the radioactivity is probably bound to the amino groups of the lysine residues.

The pH 9 chosen for FITC conjugation was such that lysine amines will easily react but not the terminal amino groups which are important for antibody-antigen binding.

TABLE 1
Tissues (%ID/g) in Tumor-bearing Mice Four Hours after Intravenous Administration of ^{99m}Tc (AA)-TNT-1 (n = 5)

Organ	^{99m}Tc-TNT-1-FITC (Specific)	^{99m}Tc-TNT-1 (Regular)	^{99m}Tc-TNT-1 (Nonspecific)	P (Specific vs. nonspecific)
Muscle	0.37 ± 0.03	0.33 ± 0.06	0.28 ± 0.07	
Intestine	1.45 ± 0.45	1.43 ± 0.39	0.85 ± 0.22	
Urine	60.4 ± 52.5	46.6 ± 25.4	41.0 ± 5.4	
Heart	1.47 ± 0.33	2.19 ± 1.3	0.49 ± 0.07	
Lungs	2.51 ± 0.49	2.83 ± 0.65	0.75 ± 0.20	
Blood	6.37 ± 0.80	4.26 ± 1.4	1.87 ± 0.89	
Spleen	13.2 ± 3.5	32.5 ± 3.3	16.6 ± 2.4	
Kidneys	13.3 ± 2.6	14.2 ± 1.2	7.96 ± 0.81	
Liver	15.3 ± 2.0	23.5 ± 4.4	25.8 ± 5.4	
Tumor	2.10 ± 0.55	1.9 ± 0.76	0.92 ± 0.19	

These groups are known to be reactive at pH 7, close to their pKa (*25,26*). It was interesting to note that only 5 or 6 NH_2 groups per Mab molecule were blocked by the 20-fold excess FITC. One can only assume that the other groups were unavailable in the unfolded Mab molecule. Although it is possible that other native functional groups, such as the imidazole (*28*) from histidine, may also participate in ^{99m}Tc binding, our results indicate that such binding is relatively small. Our results have also shown that such nonspecific binding takes place when other BFCAs such as the c-DTPA are used, as well as when Mabs are labeled with ^{99m}Tc by the other reduction-based direct methods. Perhaps many of these questions can be better answered by x-ray crystallographic examinations of proteins labeled with ^{99m}Tc. However, our attempts to build large enough crystals of such preparations have not yet been successful.

In experiments in which sulfhydryls were blocked, we chose pH 8 and a short reaction time so that carboxymethylation of the antibody thioether, amino and imidazole groups would be negligible (*27*). At this pH, histidyl side chains and amino groups may be unprotonated. Carboxymethylation of these groups even in protonated form requires an exceedingly long period of time (*27*). We believe therefore, that because of the short reaction time even at the high concentration of the iodoacetate we chose, carboxymethylation of these groups may not occur in any significant quantity.

The hypothesis that Mabs modified with FITC may result in altered tissue distribution was not sustained as evidenced by similar tissue distribution of the ^{125}I Mab and ^{125}I FITC-Mab as shown in Table 3. However, it is evident that uptake of ^{125}I in such tissues as the liver, kidneys and urine is consistently lower than the corresponding values for ^{99m}Tc. The lower ^{125}I values may arise from the dehalogenation and rapid elimination of ^{125}I from these tissues whereas the higher ^{99m}Tc values may result from rapid renal clearance (*6*) of ^{99m}Tc and perhaps from its binding to the glutathione pool in the liver. This hypothesis is currently being investigated in our laboratory.

We did not include tissue distribution of ^{125}I carboxymethylated Mab as control because the Mab was modified with less than two carboxymethylation group per Mab molecule and the added carboxymethyl group is quite similar to structures already present in proteins. Furthermore, it is known that the addition of a carboxymethyl group to the native protein often has little effect on the stability or biological activity of the proteins (*27*).

What draws curious attention to the outcome of these experiments are the important observations that nonspe-

TABLE 2
Tissues (%ID/g) in Tumor-Bearing Mice Four Hours after Intravenous Administration of ^{99m}Tc-(N_2S_4)-TNT-1 (n = 5)

Tissues	Specific	Regular (control)	Nonspecific	P (specific vs. nonspecific)
Muscle	0.6 ± 0.22	0.42 ± 0.02	0.27 ± 0.07	
Intestine	2.5 ± 0.46	2.85 ± 0.74	0.84 ± 0.03	
Urine	70.7 ± 37	70.0 ± 26.7	38.4 ± 5.7	
Heart	2.27 ± 0.24	1.94 ± 0.14	0.54 ± 0.07	
Lungs	5.4 ± 0.75	3.79 ± 0.89	1.5 ± 0.24	
Blood	10.6 ± 1.49	7.9 ± 1.3	1.65 ± 0.21	
Spleen	25.9 ± 1.74	26.6 ± 4.7	27.9 ± 4.8	
Kidneys	10.2 ± 3.3	11.7 ± 1.9	8.83 ± 1.47	
Liver	21.9 ± 1.6	23.3 ± 1.6	46.0 ± 3.7	
Tumor	3.1 ± 0.4	2.8 ± 0.47	0.9 ± 0.27	

TABLE 3
Four-Hour Tissue Distribution in Normal Balb/c Mice (%ID/g)

Organ	^{125}I-TNT-1	^{125}I-TNT-1-FITC
Muscle	0.39 ± 0.07	0.37 ± 0.06
Intestine	2.38 ± 0.41	2.10 ± 0.40
Urine	51.5 ± 24.9	56.7 ± 20.4
Heart	2.32 ± 0.36	2.13 ± 0.24
Lungs	3.08 ± 0.63	3.81 ± 0.71
Blood	10.5 ± 1.1	11.1 ± 2.4
Spleen	19.8 ± 3.3	21.3 ± 5.6
Kidney	4.94 ± 0.64	5.12 ± 1.2
Liver	5.33 ± 0.70	5.53 ± 1.6
n	5	4

cifically bound ^{99m}Tc has the highest liver uptake and the lowest tumor uptake. Contrary to this, when these E-amino groups are blocked with FITC prior to the ^{99m}Tc labeling, the preparations gave the lowest liver uptake and the highest tumor uptake. The rapid clearance of nonspecifically bound ^{99m}Tc may be due to its poor in vivo stability. The in vitro (37°C, 4 hr) serum stability of nonspecific preparation was lower (approximately 50%) than that of the regular preparation (85%). However, its high liver uptake was surprising, even though careful in vitro examinations of each preparation showed less than 5% colloid formation. It is possible, however, that the in vivo instability of the nonspecifically bound ^{99m}Tc may contribute to the high liver uptake by its binding, for example, to the large liver glutathione pool (100–1500 μM).

Soon after the explosive growth in a number of publications in which radiolabeled Mabs were evaluated in animals for various applications, the low tumor and high liver uptake of radiolabeled Mabs emerged as the most significant problem. Our data and that of others suggest that liver uptake of ^{99m}Tc Mabs is significantly higher for the first 4–6 hr postinjection, the time at which the diagnostic imaging is most commonly performed. There are several factors that can affect liver uptake, many of which, including charge modification on Mab molecules (*31*), have been

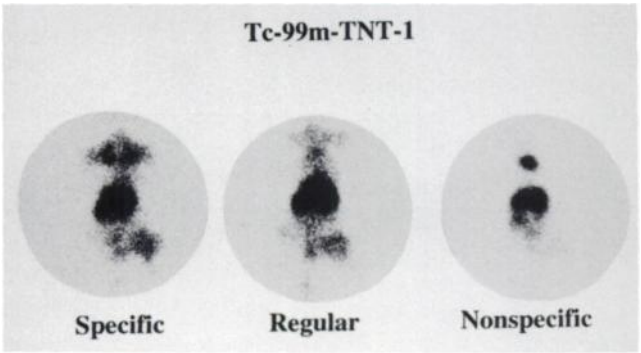

FIGURE 3. Four-hour posterior images of mice bearing embryonal carcinoma in the right thigh. The figure represents three separate animals injected with specific (FITC), regular (control) and nonspecific (IA) preparations. The tumor is not detectable with the nonspecific preparation.

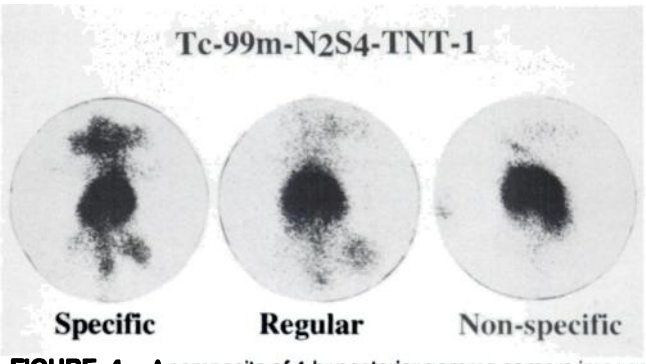

FIGURE 4. A composite of 4-hr posterior gamma camera images of animals which had received. ^{99m}Tc-N_2S_4 TNT-1, specific, regular (control) or nonspecific preparations. Low tumor uptake with nonspecific preparation is clearly evident.

examined by several investigators using ^{111}In Mabs. However, not much has been done with ^{99m}Tc-Mabs.

The observations presented here confirm that nonspecific binding appears not only to increase liver uptake but also to reduce tumor uptake. This is undesirable not only for scintigraphic imaging applications of ^{99m}Tc Mab, but also for therapeutic application of ^{186}Re.

In order to minimize liver uptake and enhance tumor uptake, it is apparent that nonspecific binding should be eliminated. One method is to label Mabs by the preformed ^{99m}Tc chelation technique. However, such agents are not available commercially and involve a procedure that is generally cumbersome. The other method would be to block the E-amino groups before reduced ^{99m}Tc is added to Mabs. Such a formulation for a kit preparation with all direct methods and some BFCA methods is readily feasible and warrants further evaluation.

ACKNOWLEDGMENTS

The authors thank Dr. Ivan Damjanov and Mrs. Andrea Damjanov of the Pathology and Cell Biology Department at Thomas Jefferson University for the continued supply of NF-1 cells and Ms. Kate Musselman for typing the manuscript. This work was supported by NIH grant R01CA51960 and DOE-FG02-92ER61485.

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