which collapsed to one triplet when selective decoupling (^3J = 3.6 Hz to two ortho protons H_2 and H_6) was applied at 3.8 ppm, the chemical shift of $NHCH_2CH_2N$; (b) ^1H NMR shift predictions based on parameters for substituted benzenes gave values of H2 (8.17 ppm), H5 (6.67 ppm) and H6 (7.78 ppm) which agreed well with the measured values. Predictions for the alternative 2-iodo structure were not consistent with the observed values.

MS: *m/z* 376 ([M^+], 2). Elemental analysis as the HCl salt: theoretical (%) C 40.74, H 5.37, N 6.79; Found (%) C 40.54, H 5.34, N 6.76.

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Tumor Imaging with Technetium-99m-Labeled Hydrazinonicotinamide-Fab' Conjugates

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This study compares the in vivo properties of direct versus indirect ^{99m}Tc-labeling for two Fab' fragments from antibodies that recognize tumor-associated antigens. **Methods:** Fab' fragments of two IgG2a monoclonal antibodies were either radiolabeled directly or via the linker bromoacetyl hydrazinonicotinamide hydrobromide (BAHNH) conjugated site specifically at protein thiols. A thiol assay was used to determine the number of thiols in the Fab' and to monitor their consumption during conjugation with BAHNH. Both preparations were labeled to > 95% incorporation of ^{99m}Tc, with the isotope tracking the single 50 kD absorbance peak seen on size-exclusion HPLC. The labeled preparations were tested in tumor-bearing and control mice, with dissections at 4 and 24 hr and gamma scintigraphy of the tumor-bearing mice. **Results:** The major difference between the two labeled preparations for either antibody fragment was the greater accumulation of isotope in the tumor for the indirectly labeled preparations. This increase ranged from 1.5- and 2.7-fold at 4 hr to 2.6- and 3.2-fold at 24 hr for the two antibodies, respectively. Since blood clearance was similar for the two labeling methods, the higher tumor accumulation with the indirectly labeled fragments resulted in higher tumor to blood ratios. Tumors could be imaged with both antibodies with either type of labeling with greater clarity and sensitivity at the 24 hr time point. **Conclusion:** While both labeling methods resulted in tumor detection through imaging, the images obtained with the indirectly labeled antibody fragments were more easily visualized due to the combination of higher radioisotope accumulation in the tumor and similar blood clearances compared to the direct labeled fragment.

Key Words: antibody fragments; technetium-99m; immunoscintigraphy; cancer imaging

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Ever since the introduction of ^{99m}Tc as an isotope for nuclear medicine imaging there has been intense interest in developing methods to label proteins with this radionuclide. These methodologies are the subject of several reviews and can be divided into two categories: direct and indirect methods (*1,2*). In the direct method, the proteins are treated with a reducing agent (e.g., Sn^{2+}, ascorbate, 2-mercaptoethanol, dithiothreitol, bisulfite, etc.) that reduces disulfide bridges within the protein molecule. The resulting sulfhydryl moieties readily bind technetium, consistent with the studies that indicate the high affinity of the oxotechnetium(V) core for thiolate ligands. Proteins labeled with ^{99m}Tc by direct methods have undergone extensive clinical evaluation. Indirect labeling involves either the modification of the protein with a technetium binding ligand (e.g., metallothionein) and subsequent reaction of this conjugate with a labile technetium precursor (e.g., Tc-glucoheptonate) or the reaction of a stable technetium complex containing a protein-

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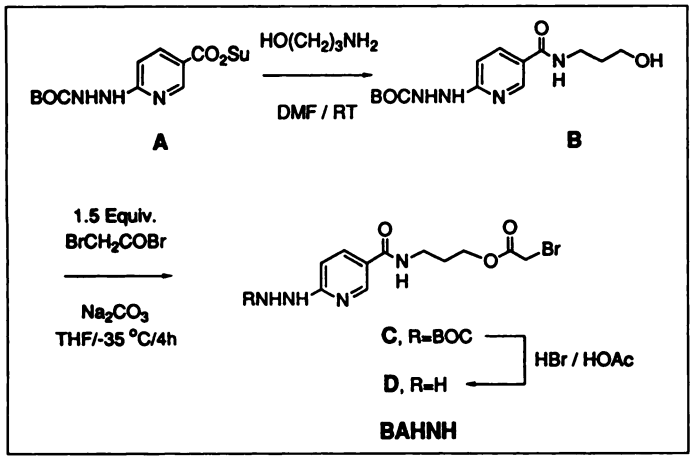

FIGURE 1. Synthesis of BAHNH.

reactive group with the protein to be labeled. Examples of systems using this "preformed chelate" approach include diamide dithiols, BATO ligands and cyclam.

A new indirect labeling approach using hydrazino nicotinamide modification of proteins has been described (*3*). This method has been used to label polyclonal human IgG (*3,4*) fragment E1 (*5*), monoclonal antibodies (*6*) and chemotactic peptides (*7*). In this paper we describe the synthesis of a hydrazino nicotinamide analog (BAHNH) capable of modifying proteins site selectively at sulfhydryl residues. This reagent was used to modify Fab' fragments from two monoclonal antibodies, 15A8-2a and C46.3. The corresponding BAHNH-Fab' conjugates were labeled with ^{99m}Tc and the in vivo (in tumor and nontumor bearing mice) biodistributions were compared with the same Fab's labeled with ^{99m}Tc by a direct method.

MATERIALS AND METHODS

Monoclonal Antibodies

The murine monoclonal antibodies C46.3 (IgG2a) and 15A8-2a (IgG2a) are products of Cytogen. C46.3 was developed by Amersham International (*8*), and 15A8-2a by R. Allen et al. (*9*). F(ab')$_2$ fragments of C46.3 and 15A8-2a were generated by pepsin digestion as previously described (*10*). Fab' fragments were generated by reduction of F(ab')$_2$ fragments with 0.3 m*M* dithiothreitol for 16–20 hr at room temperature.

Preparation of BAHNH

The synthesis of BAHNH (bromoacetic acid 3-[[(6-hydrazino-3-pyridinyl) carbonyl]amino]propyl ester dihydrobromide) is summarized in Figure 1. Succinimidyl 6-(BOC-hydrazino)nicotinate (A) was prepared as previously described (*3*). 3-Amino-1-propanol, bromoacetyl bromide and anhydrous hydrogen bromide were purchased from Aldrich Chemicals (Milwaukee, WI). ^{1}H NMR spectra were recorded on a Bruker AC-300 spectrometer. FAB Mass spectral analyses were carried out by M-Scan, West Chester, PA and elemental analyses were performed at Atlantic Microlabs, Norcross, GA.

6-(BOC-Hydrazino)-3-[(3-Hydroxy) Propyl]Pyridinecarboxamide (B)

To a stirred solution of succinnimidyl 6-(BOC-hydrazino)-nicotinate (350 mg) in DMF (4 ml) cooled to 0–5°C was added a solution of 3-amino-1-propanol (90 mg, 1.2 equiv.) in DMF (2 ml) and the reaction mixture was allowed to stir at 0–5°C for 1 hr and then at room temperature for 16 hr. The mixture was concentrated to dryness and the residue was dissolved in EtOAc (100 ml) and washed with H_2O (2 × 25 ml). The organic phase was separated, dried (Na_2SO_4) and evaporated under reduced pressure to give B (330 mg, 90%) as a white powder: ^{1}H NMR (DMSO-d_6) δ 1.42 (s, 9H), 1.65 (m, 2H), 3.25 (m, 2H), 3.45 (m, 2H), 4.48 (t, 1H, J = 5.2 Hz), 6.51 (d, 1H, J = 8.8 Hz), 7.93 (dd, 1H, J = 8.7, 2.1 Hz), 8.26 (t, 1H, J = 5.4 Hz), 8.52 (d, 1H, J = 2.1 Hz), 8.65 (s, 1H), 8.92 (br. s, 1H). Anal. Calcd for $C_{14}H_{22}N_4O_4$: C, 54.18; H, 7.14; N, 18.05. Found: C, 54.20; H, 7.17; N, 18.12.

Bromoacetic Acid 3-[[[6-(BOC-Hydrazino)-3-Pyridinyl]Carbonyl]Amino]Propyl Ester (C)

To a stirred solution of B (5.0 g, 16.1 mmol) and anhydrous Na_2CO_3 (3.5 g, 33.0 mmol) in dry THF (50 ml) cooled to −30 to −40°C under argon was added dropwise bromoacetyl bromide (5.03 g, 24.9 mmol) and the reaction mixture was allowed to stir at −35°C to −40°C for another 4 hr. The solvent was evaporated under reduced pressure and the residue was dissolved in EtOAc (300 ml) and washed with H_2O (75 ml). The organic phase was separated, dried ($MgSO_4$) and concentrated to approximately 50 ml during which time a white precipitate formed. The solid was collected by filtration, washed with ether and dried in vacuo to give C (4.9 g, 70%) as a white powder: ^{1}H NMR ($CDCl_3$) δ 1.42 (s, 9H), 2.01 (m, 2H), 3.51 (m, 2H), 3.87 (s, 2H), 4.29 (t, 2H, J = 6.1 Hz), 6.59 (br. s, 1H), 6.64 (br. s, 1H) 6.78 (d, 1H, J = 8.9 Hz), 8.05 (dd, 1H, J = 8.8, 2.2 Hz), 8.60 (d, 1H, J = 2.1 Hz); FAB MS *m/z* 433 (M^{81}Br + H, 21), 431 (M^{79}Br + H, 21), 375 (*100*). Anal. Calcd for $C_{16}H_{23}N_4BrO_5$: C, 44.56; H, 5.38; N, 12.99; Br, 18.53. Found: C, 44.62; H, 5.39; N, 12.92; Br, 18.59.

Bromoacetic Acid 3-[[(6-Hydrazino-3-Pyridinyl) Carbonyl]amino]Propyl Ester Dihydrobromide (D) (BAHNH)

A solution of hydrogen bromide in acetic acid was prepared by passing anhydrous hydrogen bromide (gas) through acetic acid (10 ml) at a moderate rate for 5 min. To a cooled (5–10°C) solution of C (90 mg) in acetic acid (1 ml) was added an aliquot of the HBr/acetic acid solution (2 ml) resulting in the immediate formation of a white precipitate. The reaction mixture was allowed to stand at room temperature for 3 min and the precipitate was then collected by filtration, washed with ether and dried in vacuo to give D (65 mg, 71%) as a white solid: ^{1}H NMR (D_2O) δ 1.80 (m, 2H), 3.30 (t, 2H, J = 6.6 Hz), 3.82 (s, 2H), 4.09 (t, 2H, J = 6.1 Hz), 6.83 (d, 1H, J = 9.2 Hz), 7.91 (dd, 1H, J = 9.2, 2.0 Hz), 8.12 (d, 1H, J = 2.0 Hz); FAB MS *m/z* 333 (M^{81}Br + H, 33), 331 (M^{79}Br + H, 33), 136 (*100*). Anal. Calcd for $C_{11}H_{15}N_4BrO_3 \cdot 2HBr \cdot 1/2H_2O \cdot 1/2HOAc$: C, 27.09; H, 3.79; N, 10.53; Br, 45.06. Found: C, 27.04; H, 4.02; N, 10.60; Br, 44.91.

Conjugation of Fab' Fragments

C46.3 F(ab')$_2$ and 15A8-2a F(ab')$_2$ fragments in PBS-EDTA (10 m*M* Phosphate, 150 m*M* NaCl, 1 m*M* EDTA, pH 7) were concentrated to 5–20 mg/mL using either a Centricon-10 concentrator (Amicon, Beverly, MA) or Amicon Stirred Ultrafiltration Cell with a YM10 membrane.

Before conjugation, the F(ab')$_2$ was reduced to Fab' with an 8-fold molar excess of dithiothreitol for 14–18 hr at room temperature in the dark. The reduction was monitored by isocratic size exclusion HPLC using a TSK-3000SW$_{XL}$ column (TosoHaas, Montgomeryville, PA) equilibrated with PBS + 1 m*M* EDTA pH 6. The DTT was not removed after reduction.

The reduced F(ab')$_2$-DTT mixtures were reacted with a 3-fold molar excess of BAHNH to the DTT thiol content. BAHNH was added as a 30 m*M*-100 m*M* solution in water and the pH adjusted to pH 6.0 with 1 N NaOH. The F(ab')$_2$-DTT-BAHNH mixtures were allowed to react for 16–23 hr at 15–30°C after purging the reaction vessel with nitrogen or argon to prevent oxidation.

The conjugates of C46.3 Fab'-BAHNH and 15A8-2a Fab'-BAHNH were purified from their respective reaction mixtures by size-exclusion chromatography on a column of Superose-12 (Phar-

macia, Piscataway, NJ). The maximal reaction mixture load volumes were 3% of total bed volume. The column was eluted at a linear flow rate of 0.5 cm/min with 10–20 m*M* sodium citrate pH 5.0 containing 1–2 m*M* EDTA. The Fab′-BAHNH conjugates were concentrated to 2–5 mg/ml using a Amicon Centricon concentrator. The conjugates were stored at 4°C.

Protein Concentration Determination

The protein concentrations of the antibody samples were determined by either calculation from the UV absorbance using extinction coefficients of $E_{280,\ mg/ml}$ of 1.51 for C46.3 Fab′ and $E_{280,\ mg/ml}$ of 1.36 for 15A8-2a Fab′, or by the Bio-Rad Protein Determination Assay (Bio-Rad, Richmond, CA) as described by the manufacture using C46.3 Fab′ or 15A8-2a Fab′ as protein reference standards.

4,4′-DTDP Residual Thiol Assay

Residual thiols remaining after reaction were determined by reaction with 4,4′-dithiodipyridyl [DTDP] (*11*). Samples to be analyzed were diluted to a total volume of 0.9 ml in thiol assay buffer (100 m*M* phosphate pH 7, 1 m*M* EDTA) and the UV absorbances at 280 nm and 324 nm were determined. One hundred microliters (100 μl) of 2 m*M* DTDP in H_2O was added to the diluted sample and the absorbance at 324 nm was again determined. The thiol concentration was calculated using the published extinction coefficient of 4-pyridinethiol ($E_{324,\ M}$ = 23,000) after subtraction of the pre-DTDP OD324 absorbance from the post-DTDP OD_{324} absorbance, correcting for dilution.

The number of thiols per antibody fragment is expressed as the ratio of the thiol concentration (μ*M*) to the protein concentration (μ*M*).

o-Sulfonic Benzaldehyde Hydrazine Assay

Hydrazine content of the BAHNH modified Fab′ was determined by reaction with o-sulfonic acid benzaldehyde. Samples to be analyzed were diluted into 1 ml of o-sulfonic benzaldehyde (10.4 mg/100 ml 0.1 *M* acetate, pH 4.7). The reaction was incubated at room temperature in the dark overnight. The absorption of the hydrazone adduct was read at 343 nm. The hydrazine concentration was calculated using an extinction coefficient of $E_{343,\ M}$ = 26,500 for the adduct.

The molar substitution ratio (MSR) of hydrazine conjugated to antibody was defined as the ratio of the hydrazine concentration (μ*M*) to the protein concentration (μ*M*) as determined by a Bio-Rad protein determination assay.

Radiolabeling of BAHNH Conjugates

Solutions of C46.3 Fab′-BAHNH or 15A8-2a Fab′-BAHNH conjugates were radiolabeled at 50 mCi/mg by mixing with a ^{99m}Tc-Tricine solution (36 mg/mL tricine, pH 7, 50 μg/mL $SnCl_2 \cdot 2H_2O$, 50 mCi/mL $^{99m}TcO_4$, preincubated for 15 min at room temperature). The conjugate solutions were incubated for 1 hr at room temperature. Radiopurity of the ^{99m}Tc-tricine solution was determined by thin-layer chromatography (TLC) (8 cm) using saline as the mobile phase to determine colloid and methylethylketone as the mobile phase to determine free pertechnetate. Radiopurity of the radiolabeled Fab′ fragments was determined by ITLC using saline as the mobile phase (*12*). All ITLC strips were cut at R_f = 0.5. If the radiopurity was less than 95% as was the case for the C46.3 antibody conjugate (78%), the labeled product was purified by desalting on a Sephadex-G25 NAP column (Pharmacia, Piscataway, NJ) into pH 6 phosphate-buffered saline to give a radiopurity of 99%. In addition, the radiolabeled fragments were tested by size-exclusion HPLC using a TSK-30005W column (TosoHaas, Montgomeryville, PA) fitted with a radioisotope detector.

Direct Radiolabeling of Fab′ Fragments

Solutions of C46.3 Fab′ or 15A8-2a Fab′ fragments were direct-labeled at 50 mCi/mg using ^{99m}Tc-glucoheptonate as a transchelator. The labeling mixtures were incubated for 1 hr at room temperature. Radiopurity of the ^{99m}Tc-glucoheptonate solution was determined by TLC (1 × 8 cm) using saline as the mobile phase to determine colloid and acetone as the mobile phase to determine free pertechnetate. Radiopurity of the radiolabeled Fab′ fragments was determined by ITLC using saline as the mobile phase. All ITLC strips were cut at R_f = 0.5. For consistancy, the radiopurity of the labeled C46.3 Fab′ was boosted from 95% to 98% by the same gel filtration procedure used for the BAHNH conjugate of this antibody, as described above. HPLC analyses were performed as for the BAHNH conjugates.

Immunoreactivity Analysis

Immunoreactivity of C46.3 was determined using the RhoChek (Rhomed, Albuquerque, NM) solid-phase binding assay as described by the manufacturer.

Immunoreactivities of 15A8-2a and its conjugates were determined by radioimmunoassay using live ME180 cells. Trypsinized ME180 cells were washed and diluted to 2 × 10^6 cells/ml in Earle's modified essential medium containing 10 m*M* HEPES and 10% fetal bovine serum (MEM-FBS). The cells were serial diluted in MEM-FBS. To each cell dilution was added 1 × 10^5 dpm of ^{99m}Tc-labeled 15A8-2a. The total radioactivity for each tube was determined by counting in a gamma counter. The cells were incubated for 1 hr at 4°C. The cells were collected by centrifugation and washed 3 times with MEM-FBS. The radioactivity bound to the cells was determined in a gamma counter. The immunoreactive fraction was determined by plotting the inverse of the bound fraction compared with the inverse of the cell concentration. The Y intercept, the fraction of radioactivity at infinite antigen excess, was determined by linear regression analysis (*13*).

Biodistribution Determination

Nude mice bearing either LS174T or MCF-7 xenografts were used to determine the biodistribution, pharmacokinetics and tumor imaging of ^{99m}Tc-labeled C46.3 Fab′ and 15A8-2a Fab′ fragments, respectively (*14,15*). Mice were injected intravenously with 20 μg Fab′ fragments containing 0.5–1 mCi ^{99m}Tc. Mice were dose calibrated and bled for initial blood and whole-body pharmacokinetics immediately after injection of the ^{99m}Tc-Fab′ fragments. Blood pharmacokinetics and whole-body clearances were determined by bleeding or dose calibrating the mice at 2, 4, and 24 hr postinjection. Tissue biodistibution was determined 4 and 24 hr postinjection. Dissected tissues were weighed and the amount of ^{99m}Tc in them determined by gamma counting. Gamma camera imaging was performed at 4 and 24 hr immediately before dissection.

Statistical Analysis

Data are expressed as means and s.d.

RESULTS

The preparation of the new bifunctional reagent BAHNH is described in Figure 1. In contrast to the active ester containing reagent SHNH, BAHNH (D) is a hydrazino nicotinamide derivative linked to a thiol-reactive bromoacetate group designed for the site-selective modification of protein sulfhydryls.

The characteristics of the modified Fab′ fragments are described in Table 1. In both BAHNH modified fragments the residual thiol level was < 0.2 thiol equivalents per protein molecule. The molar substitution ratio for the C46.3 and 15A8-2a Fab′ fragments were 1.5 and 3.5, respectively. Both DTT-reduced Fab′ fragments used for direct labeling contained 3–4 thiol groups per protein molecule. Radiochemical purities

TABLE 1
Characterization of Antibody Fragments

Antibody	Thiols	MSR*	Immunoreactivity (%)	^{99m}Tc Incorporation (%)
Direct Fab'-C46.3	3–4	N/A	42†	99
BAHNH Fab'-C46.3	<0.2	1.5	48†	97
Direct Fab'-15A8-2a	3–4	N/A	93‡	98
BAHNH Fab'-15A8-2a	<0.2	3.5	90‡	96.5

*Molar substitution ratio.
†Average of two replicates (RhoChek).
‡Radioimmunoassay with M180 cells.

of the ^{99m}Tc-labeled fragments were ≥ 96% before injection into the mice (Table 1). As shown in Figure 2, size-exclusion HPLC analyses indicated that all four radiolabeled preparations were relatively homogeneous in molecular size, showing a dominant Fab' fragment peak with little aggregate.

Immunoreactivity values were 42% and 48% for the ^{99m}Tc-labeled C46.3 Fab' and 93% and 90% for the ^{99m}Tc-labeled 15A8-2a Fab', for the direct and indirect labeling methods, respectively. While the value for the labeled C46.3 Fab' was low, it was within the typical range (30%–60%) reported for this assay for a series of radiolabeled antibodies (*16*). In any event, the relative immunoreactivities of the direct and indirect labeled fragments were similar for each antibody.

As shown in Figure 3 for each antibody fragment, the blood clearance characteristics were similar for both the direct and indirect labeling methods. There was, however, a difference (independent of labeling method) between the antibodies in that ~95% of the labeled C46.3 had been cleared from the blood by 4 hr whereas ~80% of the labeled 15A8-2a had cleared by that time. By 24 hr, the blood clearance of both radiolabeled fragments was similar at ~98%. Whole-body clearance demonstrated more of a difference between the labeling methods. For both antibodies, whole-body clearance was greater for direct labeling. In the indirect method, ~40% of the injected dose was retained at 24 hr, while 20%–25% was retained with direct labeling.

Biodistribution results in tumored mice are displayed in Figure 4. Uptake in normal organs was higher with the indirect conjugates at both 4 and 24 hr compared to direct labeling. In all cases except kidney, this uptake was relatively low (<~2% ID/g) and was lower at 24 hr compared to 4 hr. Kidney uptake was high with both fragments although both direct and indirect labeled C46.3 showed some kidney clearance over 24 hr

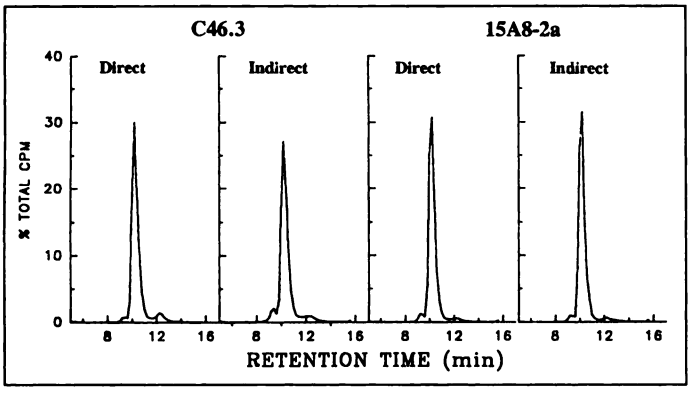

FIGURE 2. Size-exclusion HPLC profiles of radiolabeled Fab' fragments. Marker protein positions were as follows: IgG = 8.1 min, $F(ab')_2$ = 9.4 min, and Fab' = 10.2 min.

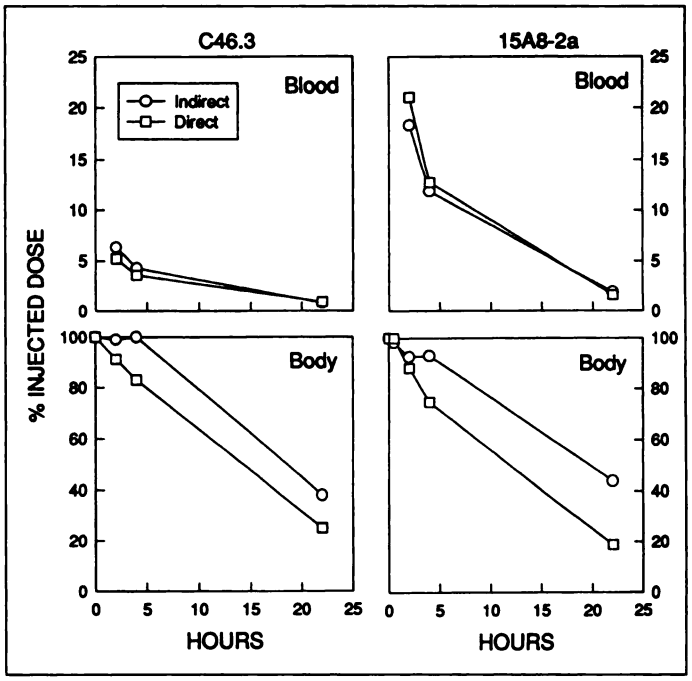

FIGURE 3. Comparison of blood and whole-body clearance of direct (□) and BAHNH (○) ^{99m}Tc-labeled monoclonal Fab' fragments.

compared to labeled 15A8-2a which showed little change in kidney uptake between 4 and 24 hr. In both tumor models, tumor uptake and tumor-to-blood ratios were significantly higher with the indirectly labeled fragments (>95% confidence), particularly at the 24-hr point. The improvement in the tumor-to-blood ratio shown with indirect labeling at 24 hr in the ^{99m}Tc-labeled C46.3 and ^{99m}Tc-labeled 15A8-2a Fab' fragments were ~3.5- and ~1.5-fold, respectively.

Gamma camera images of tumor-bearing mice are shown in Figure 5 for both antibodies. At the 4-hr time point, there was extensive uptake of radioactivity throughout the animal, with concentrations noted in the kidneys, bladder and tumor. By 24 hr (Day 1), only the kidneys and tumor were visualized on the

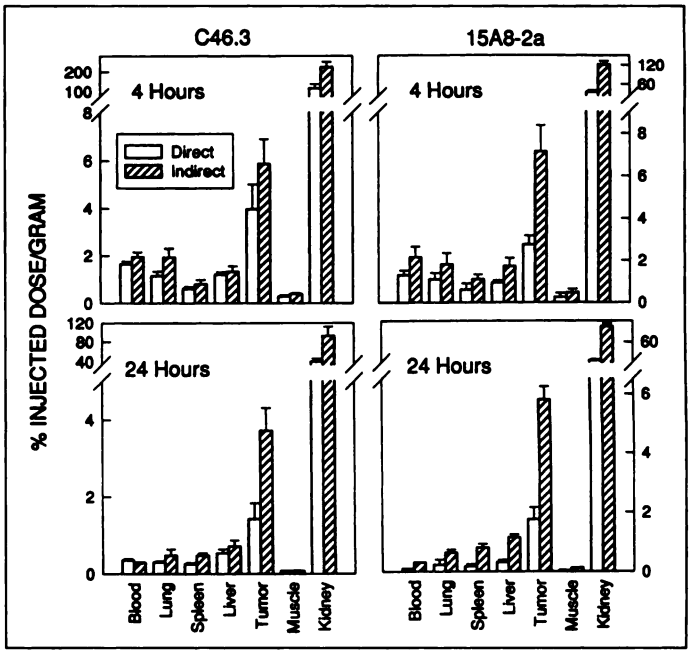

FIGURE 4. Comparison of organ distribution (%ID/g tissue) at 4 and 24 hr following administration of direct and BAHNH ^{99m}Tc-labeled monoclonal Fab' fragments.

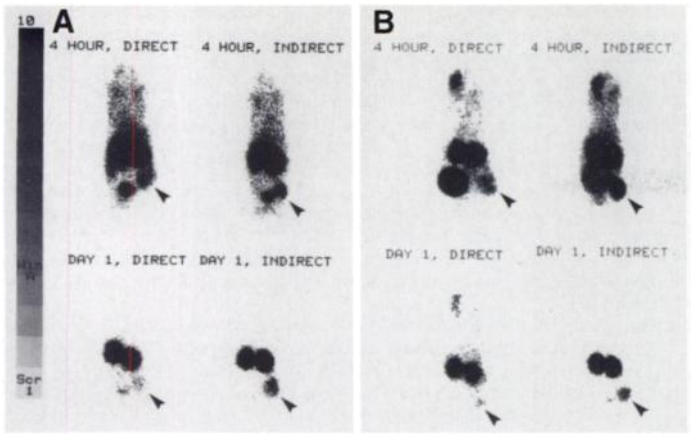

FIGURE 5. A = C46.3 antibody, B = 15A8-2a antibody. Each image is oriented so that the mouse is face up with its head at the top of the picture. The dark area on some images on the right side of the head is an artifact due to spillage during the retro-orbital injection. Each image is from a separate mouse representative of its group of five.

images. While tumor could be seen in all of the images, it was more pronounced in the Day 1 images of the mice given the indirectly labeled conjugates.

DISCUSSION

Recently, we described the synthesis and in vivo tumor localization properties of a series of ^{99m}Tc-labeled hydrazino pyridine-linked monoclonal antibody conjugates in which the functional groups connecting the hydrazinopyridine group to the antibody fragment were systematically varied (*17*). The ester-linked monoclonal antibody conjugate derived from reaction of sulfhydryl residues with BAHNH (compound D, Fig. 1) exhibited lower kidney uptake, faster whole-body clearance and higher tumor localization than the corresponding linkers containing amide groups. These results may be interpreted as evidence for cleavage of the ester in vivo consistent with several other literature reports of antibodies radiolabeled through metabolically cleavable linkers (*18–20*). In order to further optimize a radiolabeled antibody conjugate for radiopharmaceutical applications, ^{99m}Tc-labeled BAHNH-Fab′ conjugates of the monoclonal antibody fragments C46.3 and 15A8-2a were prepared and their respective in vivo biodistributions in tumor- and nontumor-bearing mice were compared with the corresponding fragments ^{99m}Tc-labeled by a direct method.

The use of hydrazino nicotinamide conjugates for indirect labeling of proteins with ^{99m}Tc retains the convenience and high radiolabeling efficiency of direct labeling. Using a combination of residual thiol and hydrazine assays, the site-specific reaction of BAHNH with protein sulfhydryls can be monitored to assess the consumption of thiols in the starting Fab′ and to determine the hydrazine molar substitution ratio of the antibody conjugate. The BAHNH-Fab′ conjugates can be radiolabeled with the precursor complex ^{99m}Tc-tricine at a specific activity of at least 50 mCi/mg protein in high (>96%) radiochemical purity with a 1-hr room temperature incubation. Use of ^{99m}Tc-glucoheptonate as a precursor complex for radiolabeling hydrazino nicotinamide groups is less efficient (*12*). Furthermore, the specificity of ^{99m}Tc-tricine labeling of hydrazino nicotinamide groups compared with residual (nonconjugated) thiols was confirmed by direct incubation of the starting Fab′ fragments with ^{99m}Tc-tricine under a variety of labeling conditions. In all cases, ^{99m}Tc-tricine proved to be a poor precursor complex for direct radiolabeling free sulfhydryl groups on antibody fragments (*17*). For this reason, direct labeling of the C46.3 and 15A8-2a Fab′ fragments for biodistribution experiments was accomplished with ^{99m}Tc-glucoheptonate.

There are at least two other reports in the literature comparing ^{99m}Tc-hydrazino nicotinamide labeled proteins with direct labeling. Solomon et al. (*21*) described a comparison between ^{99m}Tc-hydrazino nicotinamide polyclonal human IgG and a polyclonal human IgG ^{99m}Tc labeled via a direct method in a rat infection model. In this model, the indirectly labeled protein had improved uptake at the site of infection and lower nonspecific uptake compared to the direct labeled protein. Similarly, Hnatowich et al. (*6*) compared hydrazino nicotinamide labeling with direct labeling for two monoclonal antibodies, B72.3 and C110. The indirectly labeled antibodies demonstrated higher tumor uptake, higher in vitro and in vivo stability, but similar tumor-to-blood ratios compared to their direct-labeled analogs.

In the present study, improved tumor-to-blood ratios compared to direct labeling were obtained using a hydrazino nicotinamide derivative attached to the Fab′ fragment via sulfhydryl residues, although no improvement was noted in the characteristically high kidney uptake typical of radiometal-labeled Fab′ fragments. Kidney-to-blood ratios were lower with the directly labeled fragments at all time points. However, the retention of radiometals in the kidney from Fab′ fragments may be greatly reduced by the systemic administration of lysine, as reported recently for an ^{111}In-labeled Fab′ (*22*). A similar technique has been used in patients to block renal tubule uptake of an ^{111}In-labeled somatostatin analog (*23*).

The tumor images were generally sharper and more easily visualized with the indirectly labeled conjugates of either antibody, especially once the background cleared on Day 1. This finding can be attributed to the combination of greater retention in the tumor yet similar blood clearance for the indirect compared to the direct conjugate.

CONCLUSION

Two monoclonal antibody Fab′ fragments were modified at their free sulfhydryl residues using the bifunctional hydrazino nicotinamide reagent, BAHNH. These modified Fab′ fragments were readily labeled with ^{99m}Tc through the ^{99m}Tc-tricine precursor complex. Both indirectly labeled fragments showed improved tumor-to-blood ratios at 24 hr compared to their directly labeled analogs. Kidney uptake was high with all fragments studied. While both labeling methods resulted in tumor detection through imaging, the greater tumor uptake and comparable blood clearance of the indirectly labeled fragments resulted in superior tumor images.

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Interaction of Technetium-99m-N-NOET with Blood Elements: Potential Mechanism of Myocardial Redistribution

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Technetium-99m-N-NOET is a new ^{99m}Tc-labeled, neutral cardiac perfusion imaging agent which has been shown to undergo apparent redistribution in animal models and in humans. The purpose of this study was to investigate the interaction of ^{99m}TcN-NOET with red blood cells (RBCs) and to determine the effects of these interactions on myocardial uptake and clearance of ^{99m}TcN-NOET. **Methods:** After bolus administration, myocardial ^{99m}TcN-NOET clearance was monitored for 1 hr using a sodium iodide detector in 22 isolated, buffer-perfused rat hearts. Hearts were perfused as follows: seven controls with Krebs-Henseleit (KH) buffer (Group 1), five hearts with KH containing RBCs (Group 2), five hearts with KH containing RBCs and albumin (Group 3), five hearts with KH containing RBCs and dextran (Group 4). In a separate protocol, RBCs were incubated in ^{99m}TcN-NOET and then perfused through five hearts (Group 5). **Results:** Technetium-99m-N-NOET myocardial uptake (%ID) was significantly lower in all RBC groups (RBCs = 5.0% ± 1.7%; RBCs+albumin = 8.2% ± 2.1%; RBCs + dextran = 4.0% ± 0.8%; incubated RBCs = 8.8% ± 1.5%) compared with controls (72.2% ± 2.8%; $p < 0.05$). Retention (99.4% ± 0.6%) was near linear in the KH control group with virtually no fractional clearance at 60 min. Retention in groups whose perfusates contained RBCs (RBCs = 62.2 ± 4.2%; RBCs+albumin = 29.9 ± 4.3%; RBCs + dextran = 69.3 ± 3.6%) were all significantly lower than control. Addition of albumin to RBC perfusate resulted in significantly lower retention (29.9% ± 4.3%; $p < 0.01$) than was observed in RBC perfusate alone (62.2% ± 4.2%). Substitution of dextran for albumin produced retention similar to RBCs alone (69.3% ± 3.6%; p = ns). In a separate protocol, RBC binding of ^{99m}TcN-NOET was high (64.4% ± 8.6%) in triple-washed RBCs. Technetium-99m-N-NOET bound to RBCs was subsequently extracted from red cells by the myocardium when those cells were infused into hearts. **Conclusion:** Technetium-99m-N-NOET has high binding affinity to blood elements and transfers bidirectionally between myocardium and blood. The interaction of ^{99m}TcN-NOET with blood elements represents a potential mechanism of redistribution.

Key Words: technetium-99m-N-NOET; myocardium redistribution; erythrocytes

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Technetium-99m-N-NOET (bis(N-ethoxy, N-ethyl dithiocarbamato) nitrido technetium(V)) is a novel myocardial perfusion imaging agent (*1,2*) which is currently under preliminary clinical evaluation (*3*). It is a member of a class of neutral myocardial imaging agents,^{99m}Tc-nitrido dithiocarbamates (*1,2*), characterized by the presence of the Tc-N triple bond group $(Tc\text{-}N)^{2+}$. It is lipophilic and has been shown to be efficiently extracted by the myocardium in preliminary studies using rats, rabbits, dogs, monkeys and humans (*4,5,6*). Technetium-99m-N-NOET has demonstrated favorable biodistribution in animals (*4*) and in humans (*7*). The relationship of microsphere-determined myocardial blood flow and ^{99m}TcN-NOET myocardial activity in dogs was found to be linear over a wide range of flows induced by dipyridamole (*8*). Good diagnostic accuracy in comparison with ^{201}Tl and coronary arteriography has been demonstrated (*3,9*). This is the first neutral ^{99m}Tc myocardial perfusion imaging agent showing

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