Directly and Indirectly Technetium-99m-Labeled Antibodies—A Comparison of In Vitro and Animal In Vivo Properties

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To investigate the in vivo and in vitro properties of ^{99m}Tc when labeled to antibodies via one direct and one indirect method, the B72.3 and C110 IgG antibodies were radiolabeled directly via stannous ion reduction and indirectly via the hydrazino nicotinamide chelator and compared in vitro and in vivo. Antibody avidity (but not immunoreactive fraction) appeared to be independent of labeling methods for both antibodies. Following stannous ion reduction, antibodies were fragmented by denaturing SDS PAGE although only slight evidence of fragmentation was found in vivo. The direct label was instable to transchelation to cysteine and glutathione in vitro and in vivo. Following intravenous administration, urinary excretion of activity was threefold greater for the direct label and was almost exclusively labeled cysteine and glutathione. Significant differences in the biodistribution of ^{99m}Tc were also observed: liver levels were lower, kidney levels were higher and clearance of label from blood and tissues was faster for the direct label. At Day 1, tumor accumulation was threefold lower for the direct label although most normal tissues were also lower. In conclusion, when labeled to two antibodies by one direct method, ^{99m}Tc is unstable towards transchelation relative to one indirect method. These relative instabilities greatly influenced the biodistributions in mice and may influence the quality of images obtained in patients.

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Although suffering from a maturation period which was longer than expected, the use of radiolabeled monoclonal anti-tumor antibodies for radioimmunoscintigraphy has finally achieved widespread acceptance as a clinically useful diagnostic tool. In the view of many, the final hurdle which has now been crossed was the development of satisfactory methods of labeling these antibodies with ^{99m}Tc. Despite its relatively short 6 hr physical half-life, this radionuclide is often considered to be the superior label for radioimmunodiagnosis for reasons of cost, availability and imaging properties. As has been discussed elsewhere (*1,2*), the existing labeling methods may be divided into directly, in which disulfide bridges within the antibody are reduced to generate the endogenous sulfhydryl groups which are thought to serve as the binding sites and indirectly, in which an exogenous chelator is covalently attached to the antibody to serve this function. One direct method was developed by Rhodes et al. (*3*) and relies upon an excess of stannous ion to achieve the reduction of both the antibody disulfide bridges and ^{99m}Tc-pertechnetate. Antibodies so labeled have been used successfully in clinical trials (*4,5*). One example of an indirect method is that of Fritzberg et al. in which a diamino dimercaptide chelator is covalently attached to antibodies (*6*). Antibodies radiolabeled in this way have also been employed successfully in clinical trials (*7*). Another indirect approach was developed recently by Abrams et al. (*8*) in which a hydrazino nicotinamide chelator is covalently attached and the ^{99m}Tc added as a glucoheptonate complex. Antibodies radiolabeled by this method have been administered to a small number of volunteers (*9*).

Because of the importance of ^{99m}Tc-labeled antibodies to radioimmunodiagnosis and because of the large and potentially significant differences between the direct and indirect methods in which antibodies are labeled, this comparison study was performed. We report herein on studies in normal and tumored mice and on in vitro studies designed to assess the properties of ^{99m}Tc when labeled to two intact IgG antibodies, the B72.3 and C110, following published procedures for labeling directly via stannous ion reduction and indirectly via the hydrazino nicotinamide chelator.

MATERIALS AND METHODS

This investigation was performed with B72.3 and C110 in their intact IgG form. The B72.3 antibody, an IgG1, is directed against the antigen tag-72 which is expressed in tumors of colorectal origin (*10*) while the C110, also an IgG1, is an anti-CEA antibody. Both have been used in patients while labeled with ^{111}In (*11,12*). The C110 was a gift from Abbott Laboratories (Abbott Park, IL), while the B72.3 was provided by both Abbott Laboratories and Cytogen Corp. (Princeton, NJ). The C110 conjugated with a benzyl-diethylenetriaminepentaacetic acid (DTPA) chelator for labeling with ^{111}In (*13*) was also supplied by Abbott Laboratories.

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The hydrazino nicotinamide chelator was a gift from Johnson Matthey Pharmaceutical Research (West Chester, PA) and was provided as the t-BOC protected N-hydroxysuccinimide ester of the chelator (SHNH) for conjugation according to published procedures (*8*).

Direct Radiolabeling

Both antibodies were radiolabeled directly via stannous ion reduction as described elsewhere (*3*). Briefly, a stannous tartrate-phthalate solution was first prepared by diluting 1 to 100 a 0.5 *M* $SnCl_2 \cdot 2H_2O$ solution in concentrated HCl with a solution of 10 mM sodium tartrate and 40 m*M* potassium phthalate (Sigma Chemical Co., St. Louis, MO). Solutions were previously degassed by boiling in a nitrogen atmosphere. The solution was adjusted to pH 5.6 with 0.1 and 1 *M* NaOH and stored under nitrogen. The antibody solution to be labeled was first buffer exchanged using a centrifugal filter (Millipore Corp., Bedford, MA) into saline to a final concentration of 3–5 mg/ml. The stannous-tartrate-phthalate solution was added such that the weight ratio of $SnCl_2 \cdot 2H_2O$ to antibody was 0.71 (i.e., 500-fold excess of tin). The preparation was sealed under nitrogen and left to incubate overnight at room temperature. Finally, the preparation was aliquoted into single-use vials. The vials were filled with nitrogen and stored at −20°C.

For radiolabeling, the reduced antibody was allowed to warm to room temperature and a ^{99m}Tc-pertechnetate (NEN Dupont, N. Billerica, MA) solution was added so that the final specific activity of antibody was no greater than 50 mCi/mg. The solution was agitated for about 15 min to dissolve the contents and the preparation was purified on a small column of Sephadex DEAE A25 anion exchange (Aldrich, Milwaukee, WI) with 1–3 ml of saline wash. Approximately 80% of the radioactivity was recovered.

Indirect Labeling

The t-BOC protected N-hydroxysuccinimide ester of the hydrazino nicotinamide chelator was deprotected prior to use by adding a saturated solution of HCl in dioxane to a solution of the chelator in dioxane. The precipitate which formed was recovered by filtration to provide the desired product (*8*). After purification, the ester was stored at −20°C under dessication until needed. Antibody conjugation was achieved at an ester to protein molar ratio between 3 and 8 to 1 by adding the ester in DMF dropwise to a 20 mg/ml solution of antibody in 0.1 *M* phosphate buffer, pH 7.8. The solution was gently stirred for 5 hr at room temperature and then purified by dialysis and exchanged into 10 m*M* citrate buffer at pH 5.2 using a centrifugal filter (Millipore Corp., Bedford, MA). The average number of chelator groups attached per antibody molecule was determined by reacting an aliquot of the coupled antibody at a 2.1 μM concentration with 0.5 m*M* benzaldehyde-2-sulfonic acid as the sodium salt in 0.1 *M* acetate buffer, pH 4.7 for 5 hr at room temperature. The conversion of hydrazino groups to the corresponding hydrazones was measured by optical density at 344 nm using an extinction coefficient of 2.65×10^4 l/mole-cm (Abrams MJ, *personal communication*, 1992).

The conjugated antibody was radiolabeled with ^{99m}Tc by adding freshly prepared ^{99m}Tc-glucoheptonate (NEN Dupont) to a specific activity of antibody of 10 mCi/mg or less and incubating for 1 hr at room temperature. When presented in the glucoheptonate chemical form, nonspecific binding of the label to antibodies does not occur (*14*). The labeled antibody was purified by passage through a Sephadex G50 column. Recovery was approximately 80%.

All labeled antibodies, whether prepared by direct or indirect methods, were evaluated for radiochemical purity by high-performance liquid chromatography (HPLC) analysis using a single size exclusion 1 × 30 cm column of Superose-12 (Pharmacia-LKB, Piscataway, NJ) in which recovery was routinely determined.

Immunoreactivity Measurements

Measurement of immunoreactive fraction and relative avidity of indirect and direct B72.3 and C110 was established by linear extrapolation to binding at infinite antigen excess, using the double inverse plot method as reported by Lindmo et al. (*15*). After labeling with ^{99m}Tc to a specific activity of 25–40 mCi/mg (directly) and 4–5 mCi/mg (indirectly), 1–3 ng of antibody in 10 μl of 1% human serum albumin (HSA) in 0.5 *M* phosphate-buffered saline (PBS) was added in duplicate to test tubes containing serial dilutions of either RhoChek positive test antigen beads or negative control beads (RhoMed, Albuquerque, NM) both suspended in 200 μl of the same solution. After incubating 1 hr at room temperature, the beads were washed twice with cold HSA-PBS solution and counted in a NaI(T1) well counter. Values for specific binding were determined by subtracting binding for the positive beads from binding of the corresponding negative control beads. The results were plotted as the inverse of solid phase volume versus total applied/specific bound antibody and a linear regression analysis was performed. In this presentation, the binding at infinite antigen excess is indicated by the intercept value of the regression line at the ordinate and the fraction of immunoreactive antibody is determined by the inverse of the intercept value. The slope of the line is proportionate to antibody avidity, however, because the concentration of antigen on the beads was not known, only relative values for avidity could be determined. In addition, the assay could not be applied to the native, unlabeled antibody.

SDS PAGE Studies

Because of the possibility that antibody reduction in connection with direct labeling may fragment IgG antibodies (*16*), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) was performed under nonreducing conditions on the native B72.3 and C110 as well as on these antibodies after radiolabeling by either the direct or indirect methods. Samples for assay were prepared at a SDS concentration of 0.5% and immersed in a boiling water bath for 5 min prior to gel loading. Molecular weight standards (BioRad, Richmond, CA) were prepared in the same manner. Samples were loaded at 1.5–6 μg protein per lane and were resolved in a 3.5% acrylamide stacking gel and a 8.0% running gel prepared in a Mighty Small (Hoefer Scientific, San Francisco, CA) apparatus. Gels were run at 7.5 mA/gel (0.75 mA/lane) until the tracking dye reached the bottom of the gel (requiring approximately 2–3 hr). Each gel was then fixed for 1 hr with 12% trichloroacetic acid, stained for 12–16 hr with Coomassie Brilliant Blue G-250 and destained in 25% methanol-distilled water for 1.5 hr with a solvent change after the first 30 min. For autoradiography, the developed gel was dried and placed flat upon a gamma-ray sensitive film (Ektascan MEM-1, Kodak, Rochester, NY) within an evacuated black plastic bag and exposed for 16–20 hr. A recording camera interfaced to a personal computer operating Image 1.41 software (National Institutes of Health, Bethesda, MD) was used to obtain a 512 by

512 pixel digital image of both the autoradiographs and the gels themselves. Each lane within each image was scanned 16 times across its width and the scans summed to produce the density profiles.

Cysteine and Glutathione Challenge

In earlier investigations from this laboratory of the FO23C5 antibody labeled with ^{99m}Tc, it was established that ^{99m}Tc-labeled cysteine is often the most prominent radiolabeled species in the urine of animals (*17*) and patients (*18*) following the administration of this antibody labeled by two direct methods. Labeled cysteine is likely to result from transchelation in vivo of ^{99m}Tc from antibody since cysteine is one of the sulfhydryl-containing molecules present at high concentration in plasma and tissues (*19,20*). It is therefore possible that transchelation may also occur with reduced glutathione, a tripeptide sulfhydryl-containing molecule also present at high concentrations in tissues (*21,22*). Solutions of cysteine and glutathione (Sigma) were added to a solution of the radiolabeled antibodies so that their final concentrations were 0.64 m*M* and 64 m*M* and the protein concentration was 2.4 μM (representing a 300 and 30,000 molar excess relative to antibody). Incubations were conducted at room temperature. At various times over the next 5 hr, aliquots were removed for analysis on Whatman No. 1 paper using as eluant 0.1 *M* phosphate buffer, pH 7.0. In this system, labeled proteins remain at or near the origin while labeled cysteine and glutathione migrate. After developing, each paper was cut in half and each half counted separately in a NaI(T1) well counter.

Animal Studies

The biodistribution of ^{99m}Tc at 2.5 and 24 hr postintravenous administration for both antibodies labeled by both methods was established in normal CD-1 male mice (Charles River Laboratories, Wilmington, MA). Each mouse received 12–13 μg of antibody with about 400 μCi (directly) or about 60 μCi (indirectly) of ^{99m}Tc by tail vein administration. After death by spinal dislocation, samples of tissues were obtained and, after rinsing, were counted along with blood samples in a NaI(T1) well counter against an aliquot of the injectate.

In a separate study, after each of the four labeled antibody preparations was intravenously administered, each mouse was counted within a well-type ionization dose calibrator at intervals over the next 5–6 hr. Loss of whole-body radioactivity not accounted for by physical decay was assumed to be due to urinary excretion.

Swiss male nude mice (Taconic, Germantown, NY) were each implanted with 10^6 LS174T cells (American Tissue Culture Collection, Rockville, MD) subcutaneously in the left flank. Approximately 2–3 wk later when the tumor had grown to an average size of about 1 cm in the longest dimension, each animal received 15 μg of B72.3 antibody radiolabeled with either 600 μCi (directly) or 100 μCi (indirectly) of ^{99m}Tc and were killed 24 hr later by spinal dislocation. Tissues were removed and, after rinsing and weighing, were counted in a NaI(T1) well counter against an aliquot of the injectate.

In an investigation of the effects on biodistribution of stannous ion reduction, C110 previously conjugated with a benzyl-DTPA chelator (*13*) was reduced by stannous ion reduction and, after radiolabeling with ^{111}In (NEN Dupont), 12–13 μg of either the reduced or nonreduced antibody was administered intravenously to each CD-1 male mouse. Animals were killed at 5 hr postinjection and the biodistribution of the label was determined for both preparations. To confirm that ^{111}In does not bind nonspecifically to antibodies reduced by stannous ion, native C110 antibody without the benzyl-DTPA groups was radiolabeled with ^{111}In after reduction. As determined by Sephadex G50 chromatography, only 8% of the label was antibody bound versus 100% for the coupled antibody.

HPLC Studies

In addition to the use of size-exclusion HPLC for assessing the radiochemical purity of the ^{99m}Tc-labeled antibodies, HPLC was also employed to help establish the chemical forms of this label in mouse serum and urine and in homogenates of mouse liver and kidney tissues. Directly and indirectly labeled B72.3 and C110 were intravenously administered to mice so that urine, serum, kidneys and livers could be obtained at 2.5 hr postadministration for HPLC analysis of the radiolabeled species present. The urine and serum samples were injected directly into the HPLC with minimal sample preparation, the kidney and liver tissues were homogenized for analysis as previously described (*17*). Immediately following death, liver (after perfusion of the portal vein with cold saline) and kidneys were minced and ground in a 15 ml Dounce tissue grinder (Wheaton, Milville, NJ). Soluble activity was extracted with 1 ml of 0.1 *M* phosphate buffer, pH 7.0 and, after filtration through a 0.22 μm filter, 100 μl aliquots were analyzed by HPLC using the Superose-12 column. The eluant was 0.1 *M* phosphate buffer pH 7.0 flowing at 0.5 ml/min and 0.35 ml fractions were collected for counting in a well NaI(T1) counter. Recovery was determined in each case by summing the total counts in all fractions and comparing this against an aliquot of the injectate. In this system, IgG elutes in fraction 39, immune and other high molecular weight complexes earlier, ^{99m}Tc-glutathione in fraction 56 and ^{99m}Tc-cysteine in fraction 76 (*17*). Radiolabeled pertechnetate is retained by the column and ^{99m}Tc-glucoheptonate (occasionally present as a radiocontaminant in preparations of indirectly labeled antibodies) also elutes in fraction 56.

To obtain an estimate of molecular weight by HPLC, five molecular weight standards (BioRad) were applied to the column and their retention times recorded. When plotted on a semi-log scale, a linear relationship was obtained.

RESULTS

Radiolabeling

No important differences were observed between B72.3 and C110 in the ease of direct radiolabeling with ^{99m}Tc via stannous ion reduction. For both antibodies, approximately 20% of the added radioactivity was retained on the anion exchange column. After purification in this manner, the radiochemical purity as determined by HPLC was 95% ± 2% (recoveries 100%). Specific activities of 40 mCi/mg of antibody were readily achievable with both antibodies. As discussed below, the HPLC radiochromatographic profiles showed evidence of small amounts of a radiolabeled species of intermediate molecular weight.

Conjugation of B72.3 with the hydrazino nicotinamide chelator in connection with indirect labeling was more facile than C110. As a result B72.3 was conjugated with an average of 2–7 groups per molecule and the C110 with an average of 1–2. Radiolabeling of the conjugated antibodies was not as efficient as direct labeling, therefore

lower specific activities of about 5 mCi/mg for both antibodies were achieved (although no attempt was made to optimize the labeling efficiency of either). Purification by Sephadex G50 was necessary to remove free ^{99m}Tc-glucoheptonate. After purification, radiochemical purity as determined by HPLC was 95% ± 2% (recoveries 100%). As discussed below, the HPLC radiochromatographic profiles of both indirectly labeled antibodies showed evidence of small amounts of radiolabeled aggregates of high molecular weight.

Immunoreactivity Measurements

Figure 1 shows representative results of a double inverse plot analysis using the method reported by Lindmo et al. (*15*) to measure immunoreactive fraction and relative avidity. A linear regression line with regression coefficients of 0.945 or greater has been fitted to each curve. Only minor differences within the errors of the measurement were observed in the slopes for direct versus indirect labeling of both antibodies. Accordingly, no difference in avidity of either antibody was observed in this investigation related to the method of radiolabeling. However, as is evident from the intercept at the ordinate in the figure, binding at infinite antigen excess—and therefore the immunoreactive fraction—is clearly dependent upon the labeling method: the immunoreactive fraction, as determined by repeated measurements (n = 2–3) was reduced for C110 from an average of 91% (range 85%–96%) to 49% (range 43%–59%) for direct versus indirect. The magnitude of this decrease averaged 3% for the B72.3 antibody, from 45% (range 44%–46%) to 42% (range 40%–43%).

SDS PAGE Studies

Figure 2 is a photograph of two SDS gels, that for B72.3 above and for C110 below. The seven lanes in each are identified and a molecular weight scale is shown on the right. Also in the figure on the left of each gel is the corresponding autoradiograph. For both antibodies, analysis following indirect labeling shows substantially the same pattern as that of the native, unmodified antibody except for two bands due to high molecular weight species. For C110, one of these bands appears in the analysis of the native antibody (Fig. 1B), however the remaining band for C110 and both for B72.3 are absent for the native antibodies and therefore are the result of antibody conjugation. Analysis of the direct labeled antibodies shows the absence of these high molecular weight species and the presence of five lower molecular weight species in addition to the IgG antibody. These features are also evident in Figure 3 which presents the summed scans of the gels and autoradiographs. In each, the bold tracings are that of the autoradiograph and thus, the label. For both indirect labeled antibodies (top panels), the label is predominantly on IgG with activity also present on the two higher molecular weight species. In the direct case (bottom panels), the label is clearly not on IgG but is distributed among five lower molecular weight species. Stannous ion reduction followed by denaturing SDS PAGE has produced several antibody fragments with estimated molecular weights of 23, 54, 78 and 100 Kd and another fragment of about 120–140 Kd.

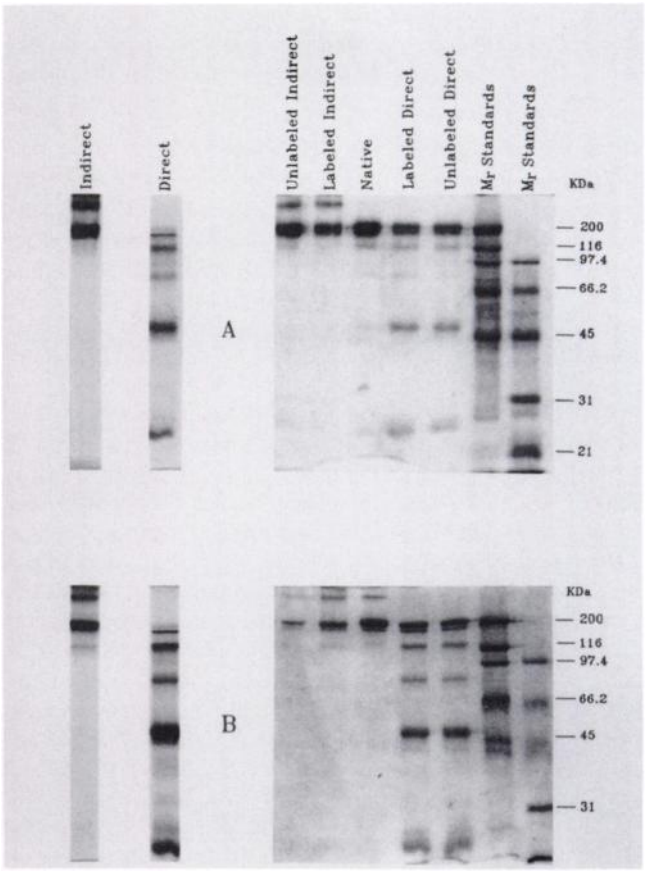

FIGURE 2. On the right, electrophoretograms of B72.3 (A) and C110 (B). In each, the lanes are identified above and the molecular weights of the standards are listed. To the left of each gel is the corresponding autoradiograph.

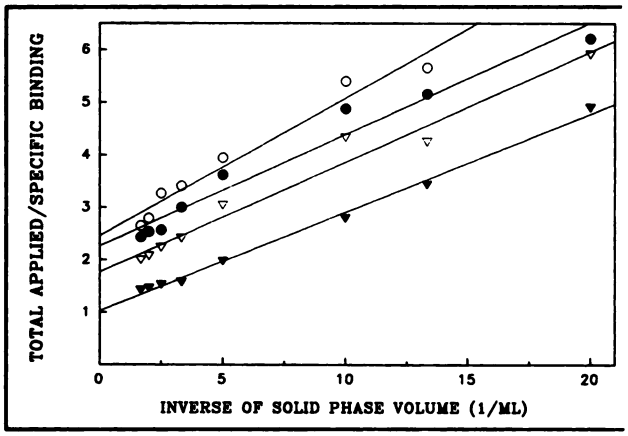

FIGURE 1. Double inverse plots of binding versus increasing volumes of a suspension of beads (solid phase) for ^{99m}Tc-labeled direct B72.3 (○), indirect B72.3 (●), direct C110 (▽) and indirect C110 (▼) (see text). Linear regression lines have been fitted to each curve.

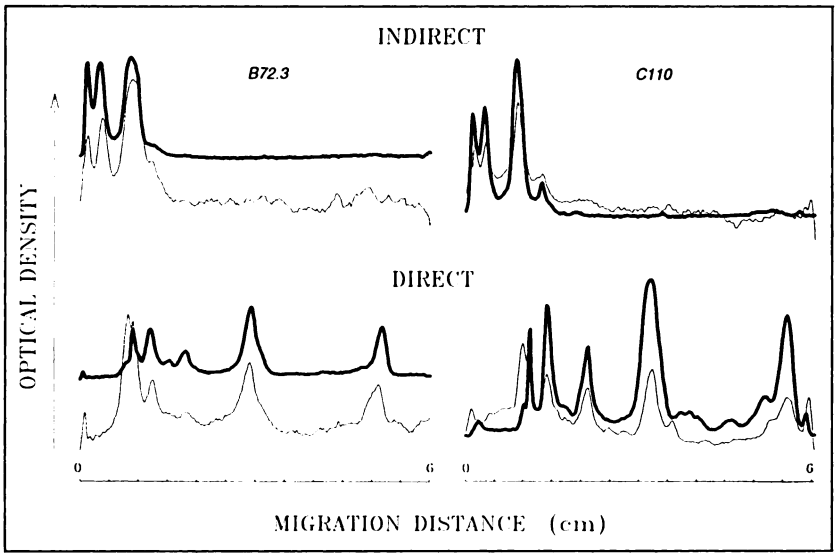

FIGURE 3. Scans of optical density of the autoradiographs (bold line) and the gels from the previous figure. Results are presented for indirectly (top) and directly (bottom) labeled B72.3 (left column) and C110 (right column).

Cysteine and Glutathione Challenge

Figure 4 presents the results of challenging with cysteine and glutathione at two concentrations of B72.3 and C110 after indirect and direct labeling. Clearly, cysteine is more effective than glutathione in promoting transchelation of the ^{99m}Tc from both antibodies regardless of labeling method. Furthermore, for both antibodies the direct label was more susceptible to transchelation than the indirect

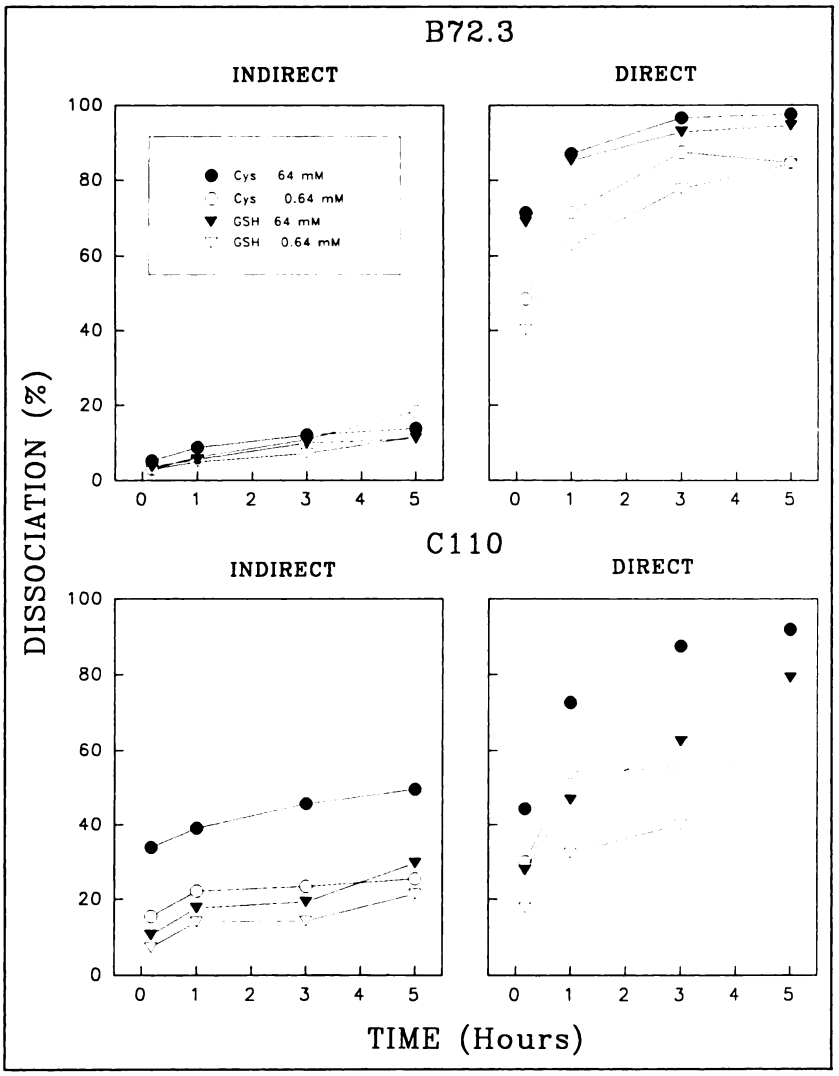

FIGURE 4. The percent dissociation of ^{99m}Tc from antibody versus time of incubation with 0.64 m*M* cysteine (○), 64 m*M* cysteine (●), 0.64 m*M* glutathione (▽) and 64 m*M* glutathione (▼) for B72.3 (top panels) and C110 (bottom panels). Results for both indirect labeling (left column) and direct labeling (right column) are presented.

label. This is most clearly seen for B72.3 but is also readily apparent for C110.

Animal Studies

Figure 5 presents whole body activity corrected for decay over 5–6 hr plotted individually for each animal and for both B72.3 (top panel) and C110 (bottom panel). The open circles and triangles refer to the direct label and the closed circles and triangles refer to the indirect label. It is clear that urinary excretion, the only means of whole body elimination operative at these early times, is much more pronounced for the direct versus the indirect label. For both antibodies, approximately 7%–8% of the administered ^{99m}Tc was excreted over 5 hr after indirect labeling versus 25%–31% after direct labeling.

The biodistributions at 2.5 and 24 hr postintravenous administration are listed in Table 1 for both antibodies. Differences with labeling methods are clearly evident. At each time point, liver levels are lower and kidney levels higher for direct versus indirect labeling. It is also evident that the clearance of label between the two time points is more rapid for direct versus indirect labeling. This difference in clearance was particularly evident from the whole body activities: the percentage of the injected activity remaining in the animals at 24 hr was 30 (1 s.d., n = 5) and 40 (4 s.d., n = 5) for directly labeled B72.3 and C110, respectively and 73 (10 s.d., n = 5) and 83 (9 s.d., n = 5) for indirectly labeled B72.3 and C110.

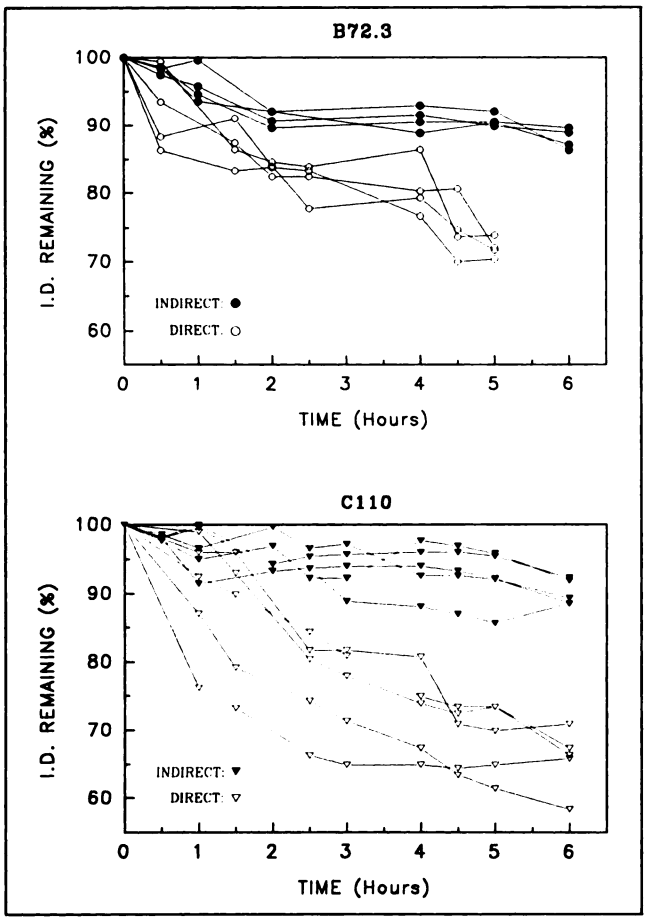

FIGURE 5. Percentage of injected activity (corrected for decay) versus time postadministration in mice receiving ^{99m}Tc-labeled B72.3 antibody (top panel) and C110 (bottom panel). Results are presented for indirect labeling (●, ▼) and direct labeling (○, ▽).

Table 2 lists the biodistribution results obtained in nude mice bearing the LS174T tumor 24 hr after administration of directly and indirectly labeled B72.3. The tumor/normal tissue ratios are also presented. Liver levels are again lower and kidney levels are higher for the direct relative to the indirectly labeled antibody. Furthermore, blood and all tissues are significantly higher ($p < 0.001$) in the indirect case. Tumor values averaged 13% ID/g for indirect and only 4% ID/g for direct. However, despite this difference in tumor levels, the tumor to tissue ratios are largely independent of the method of radiolabeling because of corresponding differences in the levels of most tissue.

Table 3 lists the biodistribution results obtained in normal mice 5 hr postintravenous administration of C110 labeling with ^{111}In through the benzyl-DTPA chelator (*13*). Half the animals received an antibody which had been reduced with stannous ion in the identical manner as performed in connection with direct labeling with ^{99m}Tc. Differences were observed in only three tissues. Thus, although stannous ion reduction generates antibody fragments following SDS PAGE (Figs. 2, 3), the antibody most likely survives excessive fragmentation in vivo. The higher kidney values do indicate that some fragmentation may have occurred with the fragments accumulating in this organ.

HPLC Studies

The HPLC radiochromatographic profiles of mouse sera, urines and homogenates of liver and kidney obtained at 2.5 hr postadministration are presented in Figure 6. In this chromatographic system, IgG antibody elutes in a peak centered at fraction 39, immune complexes earlier and ^{99m}Tc-cysteine in fraction 76 (*17*). When reduced glutathione (Sigma) is radiolabeled with ^{99m}Tc by stannous ion reduction of pertechnetate, one prominent peak centered in fraction 56 appears. When cysteine (Sigma) is labeled in the same manner and analyzed, a number of radiolabeled species are produced in addition to the species eluting in fraction 76, one of which coelutes with labeled glutathione in fraction 56 (*17*). Attempts to find an alternative chromatographic system capable of resolving species within fraction 56 were unsuccessful. A further complication concerning fraction 56 in the case of the indirect label is potential contamination with ^{99m}Tc-glucoheptonate, the weak complex of ^{99m}Tc used to radiolabel the hydrazino nicotinamide-conjugated antibodies (*8*). As a kidney imaging agent, labeled glucoheptonate will appear in urine rapidly after intravenous administration (*23*).

TABLE 1
Biodistribution (%ID/g) in Normal Mice at 2.5 and 24 hr Postadministration of B72.3 and C110 Radiolabeled Directly and Indirectly with ^{99m}Tc

	B72.3					
	2.5 hr			24 hr		
Organ	Direct	P. max*	Indirect	Direct	P. max	Indirect
Liver	5.0 (0.7)	0.001	27.1 (2.1)	2.9 (0.4)	0.001	15.8 (3.7)
Heart	4.2 (0.7)	0.05	5.1 (0.7)	1.2 (0.2)	0.001	3.0 (0.6)
Kidneys	12.2 (0.4)	0.001	7.3 (0.5)	13.3 (1.7)	0.001	5.0 (1.1)
Lungs	4.1 (0.5)	0.001	6.1 (0.5)	1.5 (0.2)	0.001	3.7 (0.9)
Stomach	0.9 (0.2)	0.001	2.2 (0.3)	0.7 (0.1)	0.01	1.4 (0.4)
Spleen	3.6 (0.1)	0.001	6.9 (0.8)	1.5 (0.3)	0.001	4.6 (0.8)
Muscle	0.8 (0.6)	NS†	1.2 (0.3)	0.6 (0.1)	0.001	1.3 (0.2)
Blood	21.7 (3.5)	0.01	31.4 (5.4)	5.5 (0.8)	0.001	16.3 (3.4)
	C110					
	2.5 hr			24 hr		
Organ	Direct	P. max	Indirect	Direct	P. max	Indirect
Liver	7.7 (0.5)	0.001	10.1 (0.7)	3.7 (0.7)	0.001	18.3 (3.2)
Heart	5.8 (0.8)	0.02	4.7 (0.3)	2.1 (0.4)	0.001	3.3 (0.3)
Kidneys	19.2 (3.3)	0.001	6.1 (1.1)	8.2 (0.4)	0.001	6.5 (0.5)
Lungs	7.2 (0.9)	0.001	4.9 (0.4)	2.9 (0.4)	NS	4.0 (1.5)
Stomach	1.8 (0.5)	0.02	1.0 (0.2)	1.4 (0.2)	NS	1.6 (0.3)
Spleen	5.4 (0.7)	0.02	4.4 (0.3)	2.3 (0.5)	0.02	3.8 (0.9)
Muscle	0.7 (0.2)	0.05	0.5 (0.1)	1.0 (0.0)	0.01	1.7 (0.4)
Blood	28.8 (2.0)	0.02	25.9 (1.6)	9.6 (0.8)	0.001	14.1 (1.3)

* Maximum probability (Student's t-test).
† NS = not significant.
One standard deviation in parentheses (n = 5)

However, because directly labeled antibodies were purified by Sephadex G50 chromatography before administration, no more than about 5% of the administered activity was in this chemical form in the injectate and, as such, no more than this percentage may be attributed to labeled glucoheptonate within the peak centered at fraction 56 in the urine analysis, and presumably even less in the analysis of serum or tissues. Recoveries in the HPLC analyses presented in Figure 6 were approximately quantitative except for the urine which consistently showed a recovery of only 60%–80%. Thus, in the case of urine only, one or more additional species (most likely ^{99m}Tc-pertechnetate which is retained by the HPLC column) were present.

It is apparent from the figure that all four injectates were of high radiochemical purity with 90%–95% of the

TABLE 2
Biodistribution in Tumor-Bearing Nude Mice at 24 hr Post-administration of B72.3 Radiolabeled Directly and Indirectly with ^{99m}Tc

	Direct		Indirect	
Organ	% ID/gm	Ratio	% ID/gm	Ratio
Liver	2.3 (0.9)	1.7	9.6 (3.4)	1.3
Heart	0.5 (0.1)	7.3	1.9 (0.5)	6.6
Kidneys	5.4 (1.2)	0.7	3.3 (0.5)	3.9
Lungs	0.8 (0.2)	5.1	2.3 (0.6)	13.0
Stomach	0.5 (0.2)	7.8	1.0 (0.3)	11.9
Spleen	1.0 (0.3)	3.8	4.2 (1.3)	3.1
Muscle	0.2 (0.1)	16.0	1.1 (0.2)	12.9
Bone	0.5 (0.1)	7.8	1.4 (0.4)	9.4
Tumor	3.8 (1.4)	—	12.7 (4.3)	—
Blood	2.0 (0.5)	1.9	9.0 (2.3)	1.4

Tumor-to-tissue and tumor-to-blood ratios are presented. 1 s.d. in parentheses (n = 11–12).

TABLE 3
Biodistribution (%ID/g) in Normal Mice at 5 hr Postadministration of ^{111}In-Labeled C110 Unreduced Antibody and Antibody After Reduction by Stannous Ion

Organ	Reduced	Unreduced	P Max*
Liver	5.7 (0.4)	5.1 (0.3)	0.02
Heart	4.5 (0.8)	4.0 (0.6)	ns
Kidneys	8.5 (1.0)	6.5 (0.9)	0.02
Lungs	4.9 (0.5)	5.5 (0.3)	0.05
Stomach	1.2 (0.2)	1.2 (0.2)	ns
Spleen	4.3 (0.6)	4.0 (0.9)	ns
Muscle	1.1 (0.2)	1.3 (0.4)	ns
Blood	24.1 (1.9)	25.0 (1.8)†	ns

* Maximum probability (Student's t-test).
† n = 4.
One standard deviation in parentheses (n = 5). ns = not significant.

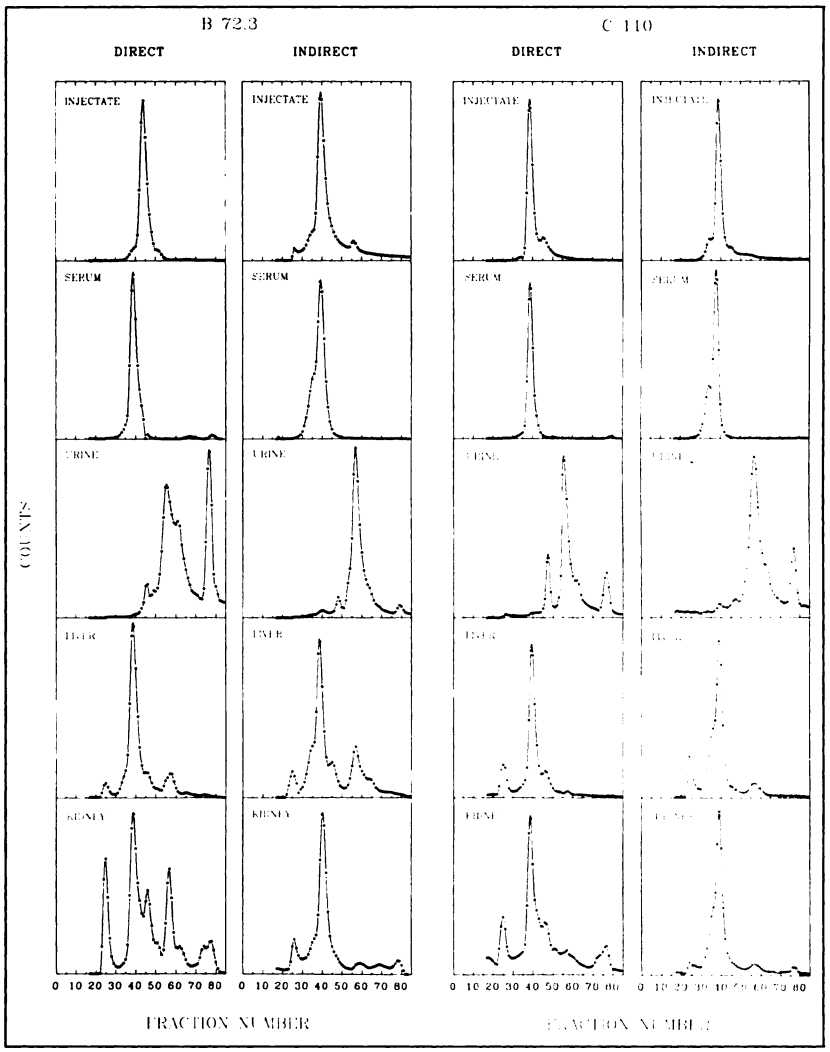

FIGURE 6. Radiochromatograms obtained by size-exclusion HPLC analysis of serums, urine and homogenates of liver and kidneys obtained at 2.5 hr postadministration of labeled B72.3 (left columns) and C110 (right columns). The radiochromatograms obtained by analysis of the injectates are also presented. Results are presented for both direct and indirect labeling.

label coeluting with the antibody in fraction 38–40. Both indirectly labeled antibodies show a low abundance peak centered in fraction 35 with an estimated molecular weight of greater than 300 Kd. This peak is more evident in the serum samples possibly because of more rapid clearance of IgG antibody. As described above, SDS PAGE also showed evidence of two high molecular weight species after conjugation of both antibodies (Figs. 2, 3). Also apparent in Figure 6 for the directly labeled antibodies is a peak centered in fraction 47 with an estimated molecular weight of about 20 Kd. SDS PAGE showed evidence of several low molecular weight fragments, including one estimated at 23 Kd. This species appears to be clearing rapidly from circulation. The presence of a peak centered in fraction 47 in the urine radiochromatograms suggest that the clearance is into urine.

The radiochromatographic profiles differ more with labeling methods than with antibodies although in the case of liver, differences are not observed with labeling methods either. Species with molecular weights high enough to void the column are present in each liver (and kidney) homogenate. These species may be due to transchelation of the ^{99m}Tc label to proteins of this size or may be due to immune complex formation in these organs. Larger differences due to the method of labeling are apparent in urine and kidneys. The peak at fraction 47 is prominent in the urine of animals receiving the directly labeled C110 but not the indirectly labeled antibodies. Likewise, the ^{99m}Tc-cysteine peak (fraction 76) is far more prominent in urine obtained from mice receiving directly labeled B72.3. The fraction of urine activity present as labeled cysteine and as what is likely to be labeled glutathione (fraction 56) was estimated from the radiochromatograms and the result was multiplied by the percentage of the injected dose in urine at this time (Fig. 5). Cumulative release of labeled cysteine during this period in percentage of injected dose was therefore calculated to have been 3–4 for direct versus 0.2–0.4 for indirect. Cumulative release of glutathione was 3 (B72.3) and 9 (C110) for direct versus 2–3 for indirect. Values for glutathione in the indirect case should be considered as upper limits since some contamination of fraction 56 with labeled glucoheptonate must be assumed. Therefore, the increased urine excretion of ^{99m}Tc with directly labeled antibodies (Fig. 5) may be attributed to

the release of labeled cysteine and what is possibly labeled glutathione as well. From these results, the rate of transchelation to cysteine may be estimated to have been between 7 to 20 times greater for the direct label.

The biggest differences with radiolabeling, however, are in the radiochromatograms of kidney: whereas only labeled antibody and a high molecular weight species appear in the indirect case, at least six other peaks are obvious in the direct case.

DISCUSSION

Both direct (*4,5*) and indirect (*7*) labeled anti-tumor antibodies have now been employed in the clinic with encouraging results. One important unanswered question is whether one of these two approaches is superior. The undoubtedly large differences in chemical states by which the ^{99m}Tc is bound to antibodies may be expected to result in significant differences in in vitro stability and therefore in biodistribution of the label. In addition, the requirement that disulfide bridges be reduced in connection with direct labeling may result in some fragmentation of antibody. It is now established that the treatment of F(ab')$_2$ antibody fragments by procedures standard in direct labeling results in almost quantitative conversion to Fab' fragments (*17, 18*). Furthermore, there is the possibility that the relatively harsh reducing conditions inherent in direct labeling may alter the immunoreactivity of antibodies.

This investigation considered only two IgG antibodies and only two methods of radiolabeling with ^{99m}Tc. Nevertheless, certain observations may prove to be generally true of direct and indirect labeling methods when applied to a variety of different antibodies. For both antibodies, avidity may have been independent of the labeling method. However, an important decrease in immunoreactive fraction was observed following direct versus indirect labeling of C110 (Fig. 1). Antibody "immunoreactivity" has been measured by competitive binding assays for antibodies other than those used in this research and found to be unchanged following reduction by stannous ion (*3*) and other reducing agents (*24*) in connection with direct labeling. The results of this investigation suggest that the effects on immunoreactivity of direct labeling may be antibody dependent since the B72.3 and C110 antibodies were altered by the reduction to different extents. The measurement of avidity and immunoreactive fraction by an assay such as that performed herein may be required to assess adequately the immunoreactivity of reduced antibodies. Assuming that indirect labeling does not significantly reduce the immunoreactive fraction from that of the native antibody (which could not be assayed), then direct radiolabeling converted approximately 40% of C110 and a much smaller percentage of B72.3 to unreactive protein. It has been suggested that immunoreactive fraction is an important determinant of immunolocalization (*25*).

Important differences of labeling methods on antibody structures were revealed by SDS PAGE (Figs. 2, 3). Conjugation with SHNH, the N-hydroxysuccinimide ester of the hydrazino nicotinamide chelator, resulted in some aggregation of both antibodies. Further investigations will be necessary to establish the nature of these aggregates. Since the antibodies may be preconjugated, these large proteins could be removed by size exclusion chromatography prior to labeling. Another difference was the extensive fragmentation observed for both antibodies following stannous reduction and denaturing SDS PAGE. The molecular weight estimates of these fragments suggest that they consist of light and heavy chains and combinations thereof (*26*). Fragmentation is well documented following antibody reduction under milder conditions than those employed in this investigation (*26*) and has been observed recently by SDS PAGE of other directly labeled antibodies (*16,27*). That this fragmentation is observed by SDS PAGE of only reduced, and not native or conjugated antibodies, suggests that interchain disulfide bonds have been rendered more susceptible to dissociation by the reduction. However, the only evidence in this research that fragmentation of directly labeled antibodies is occurring in vivo is the small differences in biodistribution of ^{111}In for reduced versus nonreduced C110 antibody (Table 3). Analysis by HPLC of urine and kidney homogenates before and after the addition of carcinoembryonic antigen (in the case of C110) and anti-mouse antibodies failed to show the shift to earlier retention times (i.e., higher molecular weight) which would identify antibody fragments (data not presented). Therefore, under the conditions of this investigation, interchain disulfide bonds were apparently reduced to permit ^{99m}Tc binding but without the introduction of major instabilities of the IgG structure. The effect of antibody reduction on intrachain disulfides was not investigated; alteration of these disulfides would not contribute to fragmentation but could contribute to the decreased immunoreactive fraction which was observed.

A potentially more important difference of labeling methods was the increased instability of ^{99m}Tc towards transchelation to cysteine and glutathione when directly labeled. Several studies have reported that directly and indirectly labeled ^{99m}Tc is stable towards reoxidation to pertechnetate or transchelation to serum proteins (*24,28*). However, ^{99m}Tc labeled antibodies have not generally been tested for stability towards transcomplexation to sulfhydryl-containing molecules such as cysteine and glutathione. Both are present in high concentration in plasma and in tissues; tissue concentrations of free cysteine are in the range of 10–100 μM (*19*) whereas tissue concentrations of free glutathione are in the range of 500 to 10,000 μM with the highest levels in liver (*29*). Plasma levels have been reported to be 10 μM for free cysteine (*30,31*) and about 20 μM for glutathione (*29*). If present in mice at these concentrations, then the molar ratio to antibody was between 100 and 50,000 immediately following antibody administration. As shown in Figure 4, rapid transchelation of ^{99m}Tc occurs in the presence of both cysteine and

glutathione at even a 300 to 1 molar ratio and therefore transchelation may be expected to occur in vivo. It may be estimated from the increased urinary excretion of label and HPLC analysis of urine that the rate of transchelation to cysteine was 7–20 times higher for the direct relative to the indirect label. Although the species responsible for a peak at fraction 56 in the HPLC radiochromatograms was not positively identified in this research as labeled glutathione, the expectation that transchelation to glutathione should occur suggests that this was the case. Transchelation to glutathione of ^{99m}Tc from the HMPAO chelate has been suggested as a possible mechanism, whereby this radiopharmaceutical is retained in brain tissue (*32*).

Major differences in in vivo behavior of ^{99m}Tc were observed between direct and indirect labeling. The label cleared more rapidly from blood and tissues (including tumor) following direct labeling. This more rapid clearance is evident from the biodistribution results (Table 1) and also from the whole body activity levels (Fig. 5). It is likely that the relative clearance is a result of the increased instability of the label when directly labeled to antibody. As a result of greater instability, the label may be rapidly transferred to cysteine and thereafter rapidly cleared into urine. After labeling with ^{99m}Tc, cysteine has been shown to clear rapidly through the kidneys (*33*). In addition to the more rapid clearance for both antibodies and at each time point, direct labeling resulted in lower activity in liver and higher activity in kidneys. An important detoxification mechanism for xenobiotics is conjugation to glutathione in the liver with release into the hepatobiliary tree and into the circulation to be cleared by the kidneys as the cysteine adduct, the ultimate metabolite of glutathione (*29*). Consequently, the increased instability towards transchelation to glutathione of the direct label may be expected to result in increased clearance in the liver and thus lower levels in this organ. The observation that kidney levels are higher for the direct label may be due, in part, to accumulation in this organ of antibody fragments generated by the reduction (Table 3), but is more likely to be related to the clearance of the higher concentrations of labeled cysteine and glutathione in this case.

In summary, important differences in vitro and in vivo in mice have been observed for ^{99m}Tc labeled to two IgG antibodies by one direct and one indirect labeling method. Although the observed instability towards transchelation may be expected to be largely independent of the antibody and its valency, the effects of antibody conjugation and reduction may be more dependent on the nature of the antibody. Furthermore, the results of this investigation certainly depended on the methods employed for direct and indirect labeling. A recent report describes ^{99m}Tc as stable towards cysteine transchelation when ascorbic acid is used for antibody reduction (*34*).

It is interesting to speculate whether image quality in patients would be superior for the direct or the indirect label assuming that the relative instabilities observed in this work apply to all antibodies and to all patients. If so, it is possible that levels of ^{99m}Tc in tissues and especially in the liver may be reduced following administration of the direct label. Because of the short physical half-life of ^{99m}Tc, this clearance may be an advantage, however, if a similar or greater reduction occurs in tumor, then the clearance may be a net disadvantage. Incubation for 2 hr of directly and indirectly labeled B72.3 and C110 with fresh and frozen human tumors and analysis of the homogenates by HPLC consistently showed increased concentrations of labeled cysteine with direct labeled antibodies (data not presented). In as much as the glutathione concentration varies widely among human tumors (*35–37*), it is possible that the effect of labeling methods on tumor uptake may depend on tumor type. Furthermore, rapid blood clearance may also diminish the time available for antibody diffusion into tumor. Moreover, hepatobiliary clearance of ^{99m}Tc has been observed in patients following administration of directly labeled antibodies (*18*) which, if due to biliary excretion of labeled glutathione, may be more pronounced in the direct case. Finally, kidney levels may be higher in the direct case which may obscure lesions in proximity. Further studies are required to establish the properties for tumor imaging in patients of these and other direct and indirect antibody labels.

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