Technetium-99m Labeling of Murine Monoclonal Antibody Fragments

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$F(ab')_2$ fragments of several murine monoclonal antibodies have been labeled with ^{99m}Tc by a direct, pretinning method. The fragments were incubated with stannous ions overnight to split disulfide groups—a process which converts dimeric $F(ab')_2$ to monomeric fragments. The pretinned fragments were then either directly labeled with ^{99m}Tc, frozen for subsequent labeling, or lyophilized to make kits for ^{99m}Tc-labeling at some later date. The ^{99m}Tc-labeled fragments were shown to be stable against transchelation when challenged with ethylenediaminetetraacetic acid, retained immunoreactivity, and were capable of binding to human tumor xenografts in nude mice.

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Because of its favorable physical characteristics, low cost, and ready availability, technetium-99m (^{99m}Tc) is a choice radionuclide for gamma scintigraphy. Technetium-99m-labeled antibodies or their fragments should be superior to other radiolabeled antibodies for use in tumor detection if localization and blood clearance is rapid enough to take advantage of the short half-life (6-hr) of ^{99m}Tc. One way to achieve faster blood clearance is to use antibody fragments (*1*). Previously, we have shown that tumors in patients can be localized within the useful biologic life of ^{99m}Tc (*2*). However, direct methods for the labeling of proteins with ^{99m}Tc have been subject to criticism because of assertions that Tc directly bound to proteins (i.e., bound without the use of bifunctional chelates) is not stable (*3*). The notion that the Tc/protein bond is unstable has persisted even though a previous report comparing the pretinning method with other direct radiolabeling methods clearly demonstrated that it is possible to achieve high-affinity Tc-bonding (*4*). We have now used the pretinning method to successfully label a number of murine monoclonal antibodies and have used a rapid transchelation challenge test which can quickly assess the stability of a Tc-labeled protein.

This paper also describes the use of the pretinning method to prepare labeling "kits" for making ^{99m}Tc-labeled antibody fragments suitable for clinical applications. In addition, we describe a transchelation challenge test to demonstrate the stability of the label of a particular preparation. Several murine monoclonal antibodies labeled by this method have been tested to show that they retain their immunoreactivity and that they can be used to localize human tumor xenografts in nude animals.

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MATERIALS AND METHODS

Preparation of Monoclonal Antibodies and $F(ab')_2$ Fragments

Several monoclonal antibodies were used in this study; a list of their relevant characteristics is supplied in Table 1. The monoclonal antibodies were isolated from murine ascites or spent tissue culture medium using methods tailored to each monoclonal antibody. The monoclonal antibody PE-4* (*5*) was isolated by precipitation in 50% ammonium sulfate. The antibody CTP-2 was isolated by precipitation in 50% ammonium sulfate, resolubilizing in 0.9% NaCl and reprecipitation in 13% polyethylene glycol (PEG, mol wt ~8,000), and then ion exchange chromatography on DEAE-Sephadex (specific elution in 0.01 *M* phosphate buffer, pH 7.2, containing 100 m*M* NaCl). The monoclonal antibody 50H.19 (*6–8*) was isolated using affinity chromatography on protein A-Sepharose with specific elution at pH 4.5 (*9*). The MOPC-21 antibody was isolated from spent tissue culture fluid by precipitation in 50% ammonium sulfate, followed by ion exchange chroma-

TABLE 1
Characteristics of Murine Monoclonal Antibodies Used to Make F(ab')$_2$ Fragments for Radiolabeling with ^{99m}Tc

Antibody designation	Antigen	Antibody class and subclass	Antibody isolation method†
PE-4*	hCG, Beta subunit	IgG_1	AS precip.
CTP-2	hCG, Beta subunit	IgG_1	AS precip., PEG precip., DEAE
50H.19	22K Protein kinase	IgG_{2a}	Protein A-Sephadex
MOPC-21	Unknown	IgG_1	AS precip., DEAE

* PE-4 and CTP-2 recognize unique, and different epitopes.
† See Methods section for details; AS precip.: ammonium sulfate precipitation; PEG precip.: polyethylene glycol precipitation; DEAE: Ion exchange chromatography on DEAE-Sepharose or DEAE-cellulose.

tography on DEAE-cellulose (specific elution in 0.01 *M* phosphate buffer, pH 8.0, containing 25 m*M* NaCl), and subsequently by molecular sieve chromatography on Sephadex G-150. Isolation methods for the antibodies are summarized in Table 1. Analysis by high pressure liquid chromatography (HPLC) indicated that after isolation as described above, the PE-4 preparation contained a large antibody peak along with several other molecular species. The 50H.19 and CTP-2 antibody preparations were found to contain essentially one antibody peak and no other contaminants.

The IgG fractions were concentrated and dialyzed into 0.9% NaCl in an Amicon concentration unit. The IgG was digested with pepsin† by adjusting the pH through the addition of a 1:20 volume of 2.4 *M* acetate buffer pH 4.0 and adding sufficient pepsin to give a final pepsin-to-protein ratio of 1:33 (w/w). The mixture was incubated at 37°C, serially monitored by HPLC, and the reaction terminated when the IgG peak had disappeared. The pH was readjusted to 7.0 and the antibody fragments isolated from the digest by molecular sieve chromatography on Sephadex G-150. The F(ab')$_2$ fragments were collected and concentrated in an Amicon concentration unit (PM-30 membrane) and dialyzed into a pretinning solution composed of 40 m*M* potassium hydrogen phthalate and 10 m*M* sodium potassium tartrate pH 5.6. Analysis of the purified F(ab')$_2$ fragments by HPLC indicated that all preparations, regardless of the route of antibody isolation, contained a single, homogeneous component with a molecular weight of ~100,000 dalton.

Preparation of Pretinned Labeling Kits

The preparation of antibody kits using the pretinning method has been described in detail elsewhere (*4,10*). Briefly, 0.5 *M* $SnCl_2$ in concentrated HCl and 10 N NaOH was added to the solution of phthalate and tartrate described above to give a final concentration of 0.005 *M* Sn^{+2} and pH of 5.6. Two parts of this solution were added to three parts of antibody solution (1 mg/ml of protein in the same phthalate/tartrate solution) to give a final Sn^{+2} concentration of 237 μg/ml and a protein concentration of 600 μg/ml. The reaction vial was purged with nitrogen to remove oxygen, after which the vial was sealed and allowed to stand at room temperature for 21 hr. At the end of the incubation period, 150 μg (0.25 ml) aliquots of the pretinned antibody solution were placed in 10-ml serum vials, lyophilized, sealed under N_2, and stored at −70°C until ready for use, providing a stock of kits for subsequent ^{99m}Tc-radiolabeling. In some cases, the pretinning reaction was allowed to proceed at a reduced temperature (4°C) for 21 hr to produce an "insufficiently tinned" antibody preparation for use in comparative quality control studies.

Technetium-99m Labeling

Approximately 2 mCi of ^{99m}Tc in 0.5 ml normal saline was added to each kit to be radiolabeled. Care was taken in all steps to minimize the introduction of air. The radiolabeling reaction was allowed to proceed for 30 min, then 2 ml of 1% human serum albumin was added; the reaction solution was then passed through a radiopharmaceutical purification column‡ designed to remove pertechnetate ions and other technetium contaminants. The yield of ^{99m}Tc from the filter column was used to measure radiolabeling efficiency.

Quality Control Tests

After radiolabeling and filtration, the radiolabeled product was quality control tested (*4,11*) using thin layer chromatography (TLC), gel column scanning (GCS) (*12*) with Sephadex G-25 columns, and biodistribution studies in mice. TLC was performed on 6 × 63 mm silica gel-impregnated glass filter strips§ (prepared from ITLC product #61886, Gelman Instrument Company) using acetone as a developing agent. The solvent was allowed to migrate to within 2 mm of the end of the strip; the strips were then removed and cut in half, and the radioactivity of each section was measured by gamma scintillation counting. The percent of radioactivity at the origin was calculated as:

$$\frac{\text{cpm at origin}}{\text{cpm at origin + cpm at solvent front}} \times 100.$$

The GCS was performed by adding a 0.2-ml aliquot of sample to a minicolumn (12 cm) of Sephadex G-25

(15 × 0.9 mm diam) pre-equilibrated in 0.9% NaCl, allowing the material to migrate into the gel using 0.9% saline as eluent until the front was ~3/4 the length of the gel. The column was then scanned to determine the distribution of radioactivity as a function of column length, using a multichannel analyzer coupled to a gel column scanning apparatus similar to that described by Jansholt et al. (*13*).

The biodistribution was performed by injecting ~5 μg of radiolabeled protein into the tail vein of female Swiss-Webster mice which were killed by Halothane inhalation 5 hr later. Selected organs were then dissected and weighed, and the radioactivity counted in a gamma scintillation counter. A 100-μl aliquot of blood was taken for counting and the femur was used to estimate the radioactivity associated with the skeleton. The blood pool was assumed to be 7% of the total body weight, the skeleton to be 10%, and the muscle to be 40%. The data from the biodistribution was computed as percent injected dose/g of organ.

Measurement of Transchelation to EDTA

A 0.1-ml aliquot of the ^{99m}Tc-labeled product was mixed with 0.1-ml of 3 m*M* ethylenediaminetetraacetic acid (EDTA) (pH = 7.4) and allowed to incubate for 30 min at room temperature. The entire solution was used for GCS. For a control, another 0.1-ml aliquot was mixed with 0.1-ml of distilled water and also allowed to incubate for 30 min at room temperature prior to GCS. The protein labeling percentage was calculated using the following equation:

$$\frac{P - (B \times C1)}{T - (B \times C2)} \times 100,$$

where B = background cpm per channel;
P = cpm in the void volume peak;
C1 = number of channels in the void volume peak;
T = total in the cpm void volume peak; and
C2 = total number of channels.

High Pressure Liquid Chromatography

High pressure liquid chromatography (HPLC) was performed using a model 8300 uv/visable optic detection control unit supported by a 8700 series solvent delivery system and a model 4270 computing integrator/recorder.[¶] Chromatographic separations were performed using a 300 × 7.5 mm Bio-Sil TSK-250 molecular weight sizing column preceded in series by a 100 × 7.5 mm Bio-Sil TSK 125 guard column.[**] Samples were injected into the unit and eluted using a 50 m*M* Na_2SO_4/10 m*M* NaH_2PO_4 buffer, pH 6.8, at a flow rate of 1.0 ml/min, with protein adsorbance monitored at 280 nm. For combined HPLC and gamma radiation detection, the HPLC efferent tubing was simultaneously monitored by a variable slit-width gamma detector and recorded on a model 30 multichannel analyzer[††] interfaced with an IBM personal computer used for data processing.

Determination of Percent Immunoreactivity of Preparations

Antigen-containing solutions (sonicated homogenates of LoVo cells or human chorionic gonadotropin (hCG)[‡‡] were adsorbed onto polyvinylidene fluoride (Kynar, grade 301F[§§]) to prepare the solid phase immunoadsorbent needed to determine the percentage of immunoreactive, radiolabeled antibody. Kynar immunoadsorbents were prepared by binding antigens to activated beads (*14*). For the immunoassay, aliquots of the Kynar suspension were dispensed into 13 × 100 mm glass test tubes. Two milliliters of phosphate buffered saline pH 7.3 containing 1% bovine serum albumin and ~100,000 cpm of the radiolabeled antibody preparation were added to the suspension. The tubes were mixed and incubated for 1 hr at 37°C with periodic mixing, then counted in a gamma scintillation counter. The Kynar beads were then collected by centrifugation at 1,000 *g* for 15 min, washed twice in 5 ml of PBS, and recounted in a gamma counter. The percent binding is expressed as the ratio of the cpm bound, over the initial cpm, times 100%.

Human Tumor Xenografts in Nude Mice

All cell lines were obtained commercially[¶¶] and grown in RPMI medium containing 10% fetal bovine serum and 50 μg of gentamycin/ml. The cell lines were routinely monitored and found to be negative for mycoplasma contamination by use of the stain Hoescht 33258[***] (*15*). When the cell number was adequate, the cells were collected by trypsinization into centrifuge tubes containing growth medium. After centrifugation the cells were resuspended in serum-free RPMI to a concentration of 2 × 10^7 cells/ml and maintained on wet ice until injection.

Nude mice (*nu/nu*), obtained commercially[†††] were injected with 1 × 10^7 cells subcutaneously in the midline just below the nape of the neck. Palpable tumors were observed within 7 days and the animals were used in experiments when the tumors were at least 1 cm in diam (2–3 wk). Biodistribution studies were carried out as described above, except that the animals were killed at various time intervals after injection and tumor samples were also obtained. For these studies, each 0.1-ml injection contained 5 μg of protein labeled with ~70 μCi of ^{99m}Tc.

RESULTS

Antibody and F(ab')$_2$ Isolation

Though several different methods were used to isolate the monoclonal antibodies, chromatographically pure (by HPLC analysis), F(ab')$_2$ fragments were obtained following pepsin digestion and re-isolation by molecular sieve chromatography. The F(ab')$_2$ fragments were par-

tially converted to monomeric Fab' fragments during the reaction with tin prior to Tc labeling. An example of an in-process HPLC analysis of the isolation of a monoclonal antibody from ascites fluid is given in Fig. 1.

Effects of Pretinning on Composition of $F(ab')_2$ Fragmentation

In all cases, the pretinning of the $F(ab')_2$ fragments was found to cause a reduction of the $F(ab')_2$ to a mixture of $F(ab')_2$, Fab', and peptides, as illustrated in Fig. 1. The ratio of the largest components, the $F(ab')_2$ and Fab', was distinctive for each type of antibody examined. An example of the conversion of $F(ab')_2$ fragments to Fab' fragments as a function of time is shown in Fig. 2. The addition of sodium pertechnetate caused a further increase in the relative amount of Fab', an increase which was pronounced at later time periods.

Radiolabeling of Murine Monoclonal Antibody $F(ab')_2$ Fragments with ^{99m}Tc

After radiolabeling, unbound reaction products were removed by passage of the final reaction mixture through a filter column (Filtech filter) specially designed to remove stannous ions, colloids, and residual pertechnetate, thus allowing only technetium which is irreversibly bonded to proteinaceous material to pass through the filter. The yield of technetium passing through the column was, then, a measure of the yield of radiolabeling. Typical radiolabeling yields for several monoclonal antibodies are given in Table 2. The percentage of reduced technetium in the final product as determined by thin-layer chromatography was found to exceed 98.0% in all cases. To confirm that the transfilter material was protein-associated and not associated with colloid, reduced Tc, or free pertechnetate, gel chromatography column scanning was performed (Table 2 and Fig. 3). Typically, upwards of 80% of the total radioactivity was found to be associated with the protein peak near the bottom of the gel. Preparations of pretinned antibody fragments of 50H.19 have been consistently labeled with ^{99m}Tc for periods up to a year (the longest time examined for any of the antibody preparations) with no significant loss of labeling efficiency or immunoreactivity.

Transchelatable Technetium

Figures 3A–F show that EDTA is not able to remove ^{99m}Tc from the labeled protein when the labeling pro-

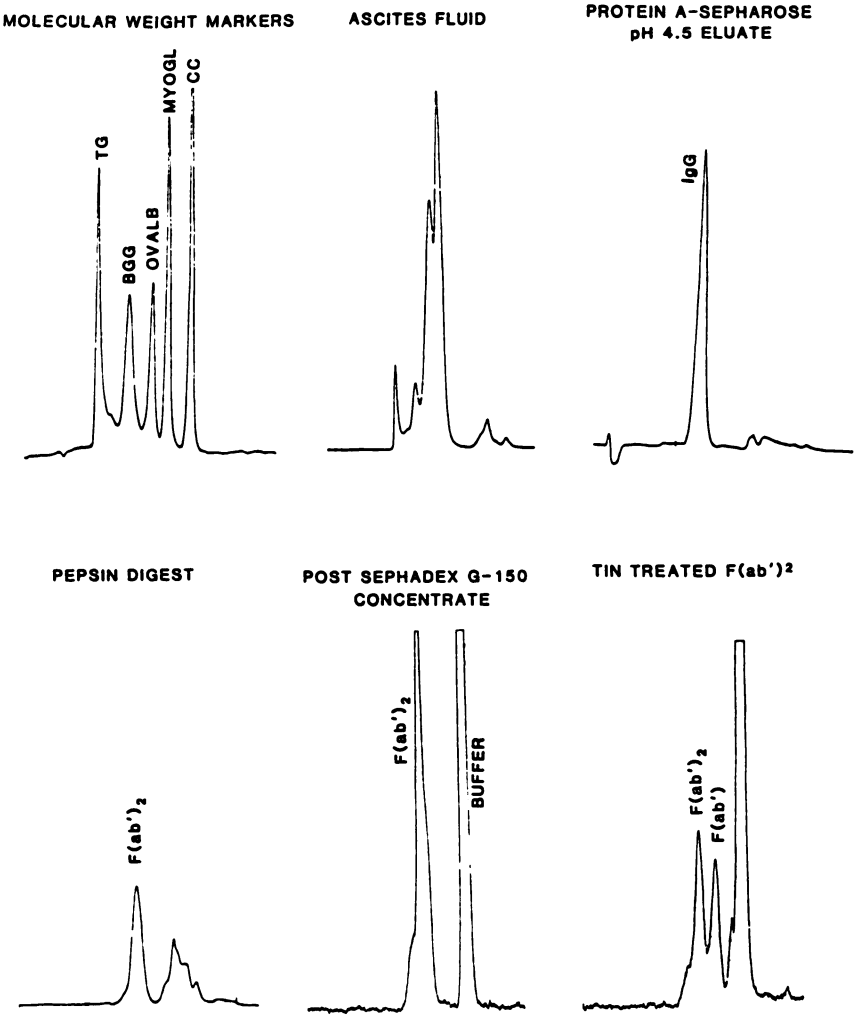

FIGURE 1
Analysis by HPLC of processing of 50H.19 from ascites fluid to pretinned monoclonal antibody fragment preparation. Thyroglobulin (TG), bovine gamma globulin (BGG), ovalbumin (OVALB), myoglobin (MYOGL) and cytochrome C (CC) were used as molecular weight markers

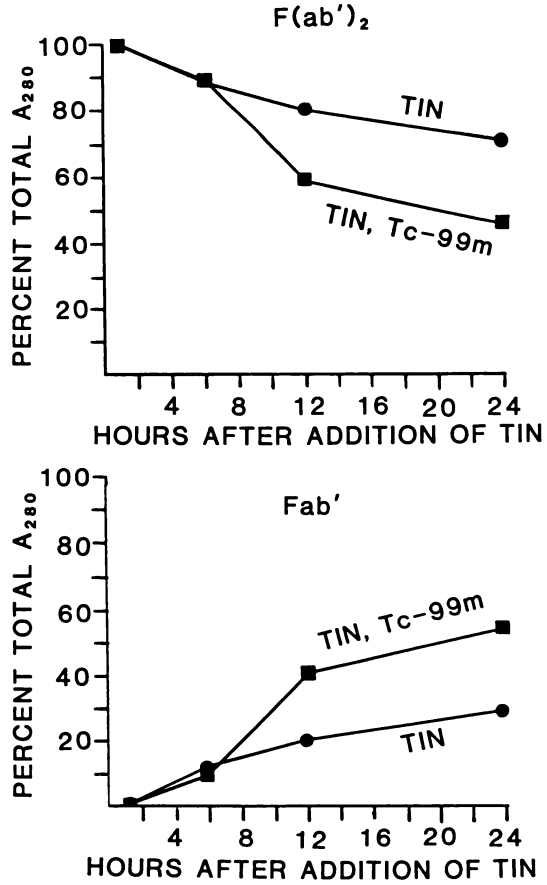

FIGURE 2
Relative amounts of $F(ab')_2$ (top) and Fab' (bottom) in solution of $F(ab')_2$ fragments of 50H.19 antibodies as function of time of exposure to Sn^{+2} in phthalate/tartrate solution

cedure is carefully followed. To illustrate preparations which are vulnerable to transchelation, two antibody preparations (MOPC-21 and 50H.19) were tinned at a reduced temperature (4°C). In such cases, a substantial amount of the ^{99m}Tc was found to be reversibly associated with the protein as determined by incubation of the radiolabeled preparations in the presence of EDTA followed by gel column scanning (Fig. 3F).

The MOPC-21 preparations and the 50H.19 preparations had similar migration profiles. To identify which molecular species were radiolabeled with ^{99m}Tc, pre-

TABLE 2
Quality Control Properties of ^{99m}Tc-Labeled Murine Monoclonal Antibody Fragments (All Values Reported ± s.d.)

Antibody	Radiolabeling reaction yield (Filtech yield)	% Reduced Tc in final product (TLC)	% Radiolabeled protein (GCS)
BGG	90.1 ± 5.3	99.9 ± 0.1	80.6 ± 5.4 (n = 5)
PE-4	93.2 ± 1.2	96.9 ± 1.7	74.6 ± 1.7 (n = 3)
50H.19	93.5 ± 5.0	98.5 ± 1.0	88.5 ± 4.0 (n = 5)
CTP-2	92.0 ± 7.4	98.8 ± 0.8	83.5 ± 1.2 (n = 3)
MOPC-21	95.1 ± 1.0	98.5 ± 0.7	79.2 ± 5.2 (n = 5)

tinned 50H.19 and PE-4 antibody preparations were radiometrically analyzed by HPLC. The elution profiles indicated that primarily one molecular species was being labeled and that the molecular weight of that species was consistent with Fab' fragments (Fig. 4).

Immunoreactivity of ^{99m}Tc-Labeled Monoclonal Antibody Fragments

After fragmentation and radiolabeling, antibody 50H.19 was used for immunoreactivity measurement. A ^{99m}Tc-binding assay using LoVo cells fixed to Kynar showed ~55% immunoreactivity (Fig. 5).

Biodistribution of ^{99m}Tc-Labeled Antibody Fragments

The biodistribution of one ^{99m}Tc-labeled antibody fragment preparation (50H.19) was compared at 5 hr after injection using four different injection sites: (a) intravenous, (b) intraperitoneal, (c) intramuscular, and (d) subcutaneous (Table 3). The biodistribution of the antibody preparation was similar regardless of the route of administration. The largest amount of radioactivity was associated with the kidneys (nearly 40% of the injected dose/g) and the large intestine (~3%). The i.v. injection resulted in the lowest amount of radioactivity in the circulation after 5 hr.

Tumor Localization of ^{99m}Tc-Labeled Antibody Fragments in Athymic Mice Bearing Xenografted Human Tumors

Athymic mice bearing human colon tumors from the cell line LoVo were used in tumor localization studies with the ^{99m}Tc-labeled 50H.19 preparations (Table 4). Tumor localization was observed at 10 hr after injection and was maximal at 15 hr. The preparation localized at the tumors more than at any organs examined at both 10 and 15 hr after injection, except the kidneys. The tumor-to-blood ratio at 15 hr was 2.3 and the tumor-to-muscle ratio was 13.8. By 20 hr there was a trend toward reduction of the total amount of radioactivity associated with the tumor.

A second monoclonal antibody preparation (PE-4; anti-hCG, beta subunit-specific) was used in tumor localization studies using the cell lines BeWo or LoVo grown as xenografts in athymic mice. Cell line BeWo was used because in culture it exhibits a high amount of cell surface hCG. The cell line LoVo was used because in culture it exhibits a very low amount of cell surface hCG (data not presented). Tumor localization was detected in mice bearing either BeWo or LoVo (Fig. 6), although the localization tended to be higher in nude mice bearing BeWo tumors.

DISCUSSION AND CONCLUSIONS

During our original studies of technetium labeling of gamma globulin (*4*), we observed that at least two reaction products were produced. One product or set of products was characterized by the reversibility of the

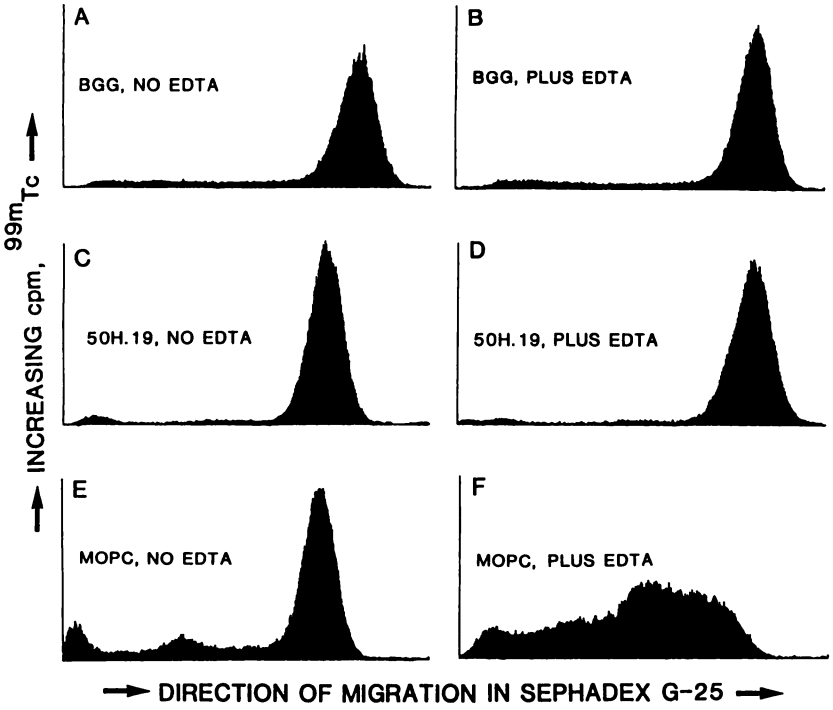

FIGURE 3
GCS showing migration profiles of ^{99m}Tc-labeled antibody preparations preincubated in presence or absence of 3 m*M* EDTA—comparison of sufficiently pretinned and nonreversibly bound ^{99m}Tc to insufficiently pretinned and reversibly bound ^{99m}Tc preparations. A: Bovine gamma globulin (labeling control) (BGG) in absence of EDTA. B: BGG in presence of EDTA. C: Sufficiently tinned 50H.19 F(ab')$_2$ in absence of EDTA. D: Sufficiently tinned 50H.19 F(ab')$_2$ in presence of EDTA. E: Insufficiently pretinned MOPC-21 F(ab')$_2$ in absence of EDTA [compare with A and C]. F: Insufficiently pretinned MOPC-21 F(ab')$_2$ in presence of EDTA [compare with E, B, and D]. Note poorly well-defined peaks indicative of poor radiolabeling

Tc-bond; the reversibly labeled proteins would exchange-label Sephadex G-25 and disassociate when subjected to thin layer chromatography using 1–6 N HCl to develop the chromatograms. A second type of product was also observed: nonreversibly Tc-bonded proteins, i.e., a fraction of the Tc-labeled protein would not release Tc to Sephadex nor disassociate in HCl. A similar observation has been reported by Paik et al. (*16*), although these investigators examined reaction products at one set time after initiating the reaction while we observed that the relative amount of nonreversibly bonded Tc was highly dependent on reaction

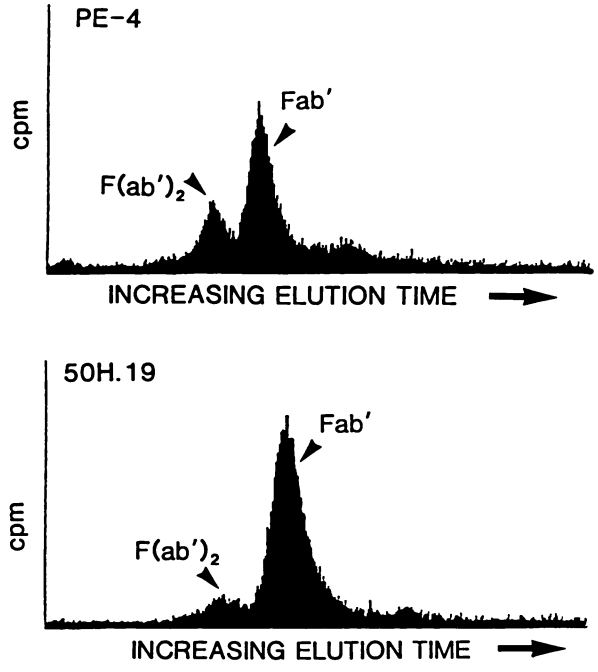

FIGURE 4
Analysis by HPLC of radioactivity associated with ^{99m}Tc-labeled monoclonal antibody preparations. A: Profile using 50H.19 preparation. B: Profile using PE-4 preparation

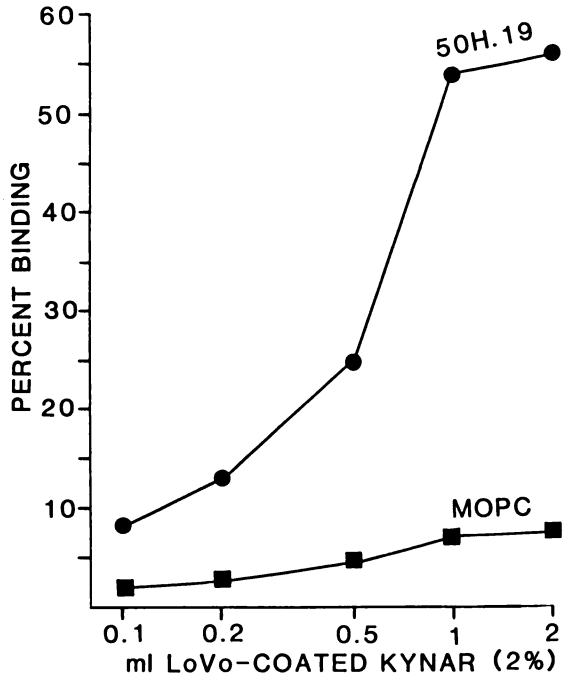

FIGURE 5
Percent binding of ^{99m}Tc to solid phase antigen as function of total amount of antigen in sample (ml of LoVo-coated Kynar bead suspension). 50H.19 is radiolabeled antibody fragment which recognizes antigenic determinant on LoVo cell membranes and MOPC-21 is radiolabeled control antibody fragment

TABLE 3
Biodistribution of ^{99m}Tc-Labeled 50H.19 at 5 hr After i.v., s.c., i.m., or i.p. Injection in Female Swiss-Webster Mice (Five Animals per Experimental Group, All Values Reported ± s.d.)

Organ	% Injected dose/g			
	i.v.	s.c.	i.m.	i.p.
Blood	1.32 ± 0.10	2.03 ± 0.42	1.89 ± 0.25	2.43 ± 0.39
Kidneys	42.84 ± 2.20	40.04 ± 15.90	37.99 ± 3.95	41.84 ± 6.99
Liver	1.54 ± 0.31	1.09 ± 0.14	1.04 ± 0.13	1.45 ± 0.19
Lungs	0.93 ± 0.16	1.43 ± 0.36	1.32 ± 0.19	1.71 ± 0.27
Stomach	0.27 ± 0.16	1.44 ± 1.51	0.45 ± 0.15	1.36 ± 0.70
Spleen	0.67 ± 0.33	0.54 ± 0.06	0.65 ± 0.20	0.63 ± 0.18
Large intestine	3.33 ± 1.20	4.59 ± 1.22	2.62 ± 0.66	4.93 ± 2.26
Small intestine	0.54 ± 0.18	0.95 ± 0.21	0.69 ± 0.18	1.45 ± 0.38
Heart	0.71 ± 0.20	0.73 ± 0.12	0.83 ± 0.14	1.14 ± 0.95
Ovaries	0.79 ± 0.19	0.94 ± 0.25	1.03 ± 0.39	1.16 ± 0.54
Muscle	0.21 ± 0.03	0.19 ± 0.05	0.22 ± 0.04	1.22 ± 0.33
Skeleton	0.37 ± 0.06	0.49 ± 0.15	0.50 ± 0.13	0.80 ± 0.23
Thyroid	0.74 ± 0.35	2.06 ± 1.68	1.20 ± 0.32	0.82 ± 0.12

time. The time-dependency of the reaction suggested that conditions might be optimized in such a way that most of the product would be of the second or nonreversibly bonded type—or what Paik et al. (*16*) called high affinity. This led to the development of the pretinning method and a Sephadex column device—Filtech radiopharmaceutical filter—which can be used to remove any reversibly bound Tc from a Tc-radiopharmaceutical. The column, which contains Sephadex G-25 with adsorbed stannous phthalate, is also capable of reducing and quantitatively binding pertechnetate. Thus, after labeling with Tc, the reaction product is passed through the Filtech to provide a radiochemically pure product. In addition, by measuring the percentage of the ^{99m}Tc radioactivity which passes through the column, the radiolabeling reaction yield is determined.

The original strategy was (a) to optimize the reaction yield of nonreversibly bonded Tc, (b) to determine if the immunoreactivity of the protein had been altered by the labeling (should the latter take place, the strategy entailed determining whether a method could be devised which would produce the nonreversibly bonded Tc without compromising immunoreactivity), and then, (c) to determine if a further modification of the method could be devised which would increase the clearance time of the Tc-labeled globulins from the blood so that the short half-life of ^{99m}Tc would not limit the clinical utility of the product. This type of protein modification had previously been demonstrated by Harwig et al. (*17*). Fortunately, the original method accomplished both of the latter objectives; i.e., the method did not change the immunoreactivity of the immunoglobulin (*4*), but it did modify the protein in some way which caused it to clear more rapidly from the blood stream (unpublished data).

The chemical modification of immunoglobulins

TABLE 4
Time Course for Localization of ^{99m}Tc-Labeled F(ab')$_2$ Fragments of Monoclonal Antibody 50H.19 in Nude Mice Bearing LoVo Tumors (Data Presented is Decay Corrected and Shows % Injected Dose per g of Wet Organ Weight ± s.d.)

Organ	% Injected Dose/g (n = 5)			
	5 hr	10 hr	15 hr	20 hr
Blood	1.88 ± 0.22	1.02 ± 0.15	0.78 ± 0.15	0.57 ± 0.04
Kidneys	66.28 ± 10.51	72.00 ± 15.31	44.57 ± 3.71	43.21 ± 7.39
Liver	1.71 ± 0.30	1.17 ± 0.34	1.02 ± 0.19	1.05 ± 0.18
Lungs	1.71 ± 0.30	0.98 ± 0.17	0.85 ± 0.16	0.78 ± 0.42
Stomach	0.29 ± 0.07	0.13 ± 0.04	0.36 ± 0.17	0.27 ± 0.14
Spleen	0.93 ± 0.32	0.81 ± 0.29	0.65 ± 0.13	0.45 ± 0.15
Large intestine	2.78 ± 1.25	1.17 ± 0.38	0.80 ± 0.55	0.85 ± 0.46
Small intestine	0.55 ± 0.14	0.30 ± 0.11	0.33 ± 0.13	0.30 ± 0.06
Heart	0.93 ± 0.19	0.52 ± 0.17	0.34 ± 0.06	0.28 ± 0.02
Ovaries	0.95 ± 0.49	0.87 ± 0.22	0.57 ± 0.23	0.43 ± 0.21
Muscle	0.30 ± 0.06	0.20 ± 0.04	0.13 ± 0.04	0.10 ± 0.03
Skeleton	0.58 ± 0.15	0.42 ± 0.06	0.38 ± 0.02	0.23 ± 0.05
Thyroid	0.83 ± 0.14	0.64 ± 0.36	0.58 ± 0.38	0.49 ± 0.23
Tumor	1.90 ± 0.45	1.61 ± 0.26	1.80 ± 0.40	1.17 ± 0.36

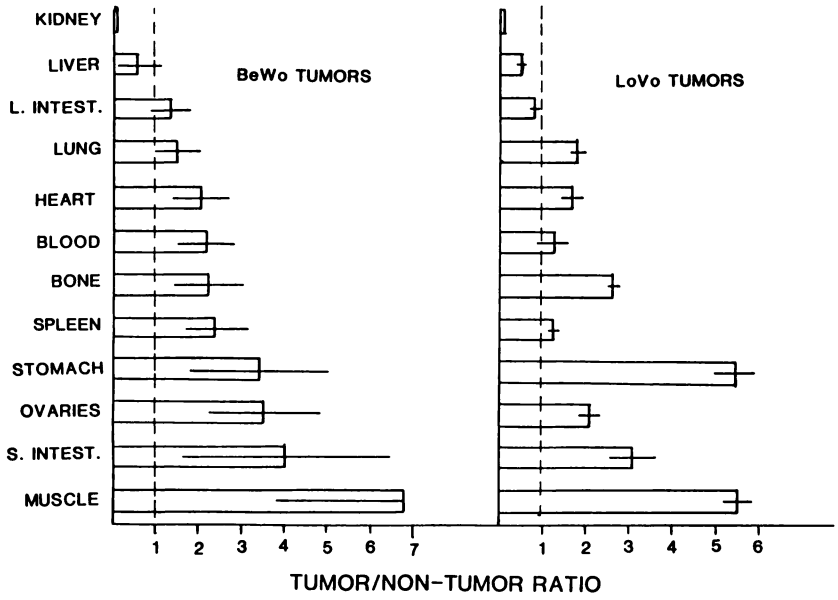

FIGURE 6
Tumor-to-nontumor ratios obtained from biodistribution studies showing localization of ^{99m}Tc-labeled antibody fragments reactive with beta-hCG in athymic mice (*nu/nu*) bearing xenograft tumors of choriocarcinoma cell line BeWo or colon carcinoma cell line LoVo. Monoclonal antibody was derived from hybridoma line PE-4. Animals were killed 20 hr after injection (BeWo, n = 3; LoVo, n = 5)

which results from prolonged exposure to stannous ions has not been fully explored. Very early in the development of the method we switched to the use of F(ab')$_2$ to avoid the problems which we assumed to be associated with inclusion of the Fc portion of the molecule in the product, i.e., the immunodominance of the Fc region, and nonspecific binding through the Fc region. HPLC analysis of the final reaction product, produced by the pretinning of F(ab')$_2$ followed by labeling with ^{99m}Tc, is shown in Fig. 3. What is observed is that the Fab' fragments are produced during the pretinning reaction and that the monomeric fragments preferentially label with Tc. The second observation, shown in Fig. 2, is that the Tc-labeling reaction itself further increases the relative amount of monomeric fragment in the final product.

The experiments of Paik et al. (*16*) have shown that Tc-labeling through the sulfhydryl groups produces bonding that is more stable than the Tc-diethylenetriaminepentaacetic acid (DTPA) bond. From these observations we can assume that during the reaction of the stannous ions with the IgG, disulfide bonds are reduced. We have not actually measured this, as Paik et al. did, but we have observed that the stannous ions are oxidized and that the reaction terminates once the supply of stannous ions is exhausted. The generation of monomeric fragments from F(ab')$_2$ in the reaction mixture as a function of time also supports this interpretation. The preferential labeling of the monomers by the Tc further suggests that the bonding is occurring through the sulfhydryl groups [as originally suggested by Steigman et al. (*18*)] which are being formed by the reduction of the disulfide bonds and which bridge the two monomers to form the dimeric, F(ab')$_2$ fragment. Perhaps the reason there is an increase in monomers after Tc-labeling is that the Tc prevents the reformation of the disulfide bonds.

Studies in which proteins are labeled with ^{99m}Tc in the presence of chelating agents, such as DTPA (*3,16, 19*) have revealed that some of the technetium is bonded directly to the protein and that this bonding is stronger than that of the Tc-DTPA bond. The relative amount of strongly bonded, i.e., Paik's high affinity bonded, technetium is dependent on reactions conditions—the predominate variable being exposure time of the proteins to stannous ions (*4,16*). When this is optimized, the protein can be labeled exclusively at the high affinity sites. The high affinity sites are most likely free sulfhydryl groups produced by the reduction of disulfide bonds by stannous ions. Our current observations support the original hypothesis of Steigman et al. (*18*) that technetium can be bound to proteins through free sulfhydryl groups.

The principle findings of studies on the Tc-labeling of IgG and fragments over the last 5 yr are presented in this report. The conclusions from these studies are the following.

1. IgG fragments can be reacted with stannous ions to produce a chemical species of the protein capable of forming a very strong bond with technetium.

2. The pretinned fragments can be stored either frozen or lyophilized and used for subsequent radiolabeling with ^{99m}Tc; i.e., an "instant labeling kit" can be prepared.

3. The ^{99m}Tc-labeled fragments retain their immunoreactivity and can be used as a radiopharmaceutical.

4. The clearance rate of the ^{99m}Tc-fragments from the circulation is rapid.

FOOTNOTES

*Bio-Yeda, Israel.
†Cooper Biomedical Inc., Malvern, PA.
‡Filtech, Summa Medical Corp., Albuquerque, NM.

§Gelman Sciences Inc., Ann Arbor, MI.
¶Spectra-Physics, San Jose, CA.
**Bio-Rad, Richmond, CA.
††Canberra Industries, Inc., Meriden, CT.
‡‡Sigma Chemical Co., St. Louis, MO.
§§Penwalt Corp., King of Prussia, PA.
¶¶American Type Culture Collection, Rockville, MD.
***Calbiochem-Behring, San Diego, CA.
†††Harlan Sprague Dawley, Indianapolis, IN.

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REFERENCES

1. Burchiel SW, Burke D, Breslow K, et al: Biodistribution of radiolabeled antibodies and their fragments: An immunopharmacologic approach to in vivo tumor imaging. *Proc SW Pharm Assoc* 25:405–408, 1982
2. Morrison RT, Lyster DM, Alcorn L, et al: Radioimmunoimaging with ^{99m}Tc monoclonal antibodies: Clinical studies. *Int J Nucl Med Biol* 11:184–188, 1984
3. Childs RL, Hnatowich DJ: Optimum conditions for labeling of DTPA-coupled antibodies with technetium-99m. *J Nucl Med* 26:293–299, 1985
4. Rhodes BA, Torvestad, DA, Breslow, K, et al: ^{99m}Tc-labeling and acceptance testing of radiolabeled antibodies and antibody fragments. In *Tumor Imaging: The Radioimmunochemical Detection of Cancer*, Burchiel SW, Rhodes BA, Friedman B, eds. New York, Masson Publishing USA, Inc., 1982, pp 111–123
5. Rahamin N, Inbar M, Hexter CS: Monoclonal antibody specific to the beta subunit of human chorionic gonadotropin (hCG) and capable of agglutinating hCG-coated red blood cells. *Hybridoma* 3:49–55, 1984
6. MacLean GD, Seehafer J, Shaw ARE, et al: Antigenic heterogeneity of human colorectal cancer cell lines analyzed by a panel of monoclonal antibodies. I. Heterogeneous expression of Ia-like and HLA-like antigenic determinants. *J Nat Cancer Inst* 69:357–364, 1982
7. Seehafer J, Longenecker BM, Shaw ARE: Human tumor cell membrane glycoprotein associated with protein kinase activity. *Int J Cancer* 34:815–819, 1984
8. Seehafer J, Longenecker BM, Shaw ARE: Biochemical characterization of human carcinoma surface antigen associated with protein kinase activity. *Int J Cancer* 34:821–829, 1984
9. Ey PL, Prowse SJ, Jenkin CR: Isolation of pure IgG_1, IgG_{2a}, and IgG_{2b} immunoglobulins from mouse serum using protein A-Sepharose. *Immunochem* 15:429–436, 1978
10. Rhodes BA, Burchiel SW: Radiolabeling of antibodies with technetium-99m. In *Radioimmunoimaging and Radioimmunotherapy*, Burchiel SW, Rhodes BA, eds. New York, Elsevier, 1983, pp 207–222
11. Breslow K, Rhodes BA, Burchiel SW, et al: Quality control of radiolabeled antibodies. In *Tumor Imaging: The Radioimmunochemical Detection of Cancer*, Burchiel SW, Rhodes BA, Friedman B, eds. New York, Masson Publishing USA Inc., 1982, pp 157–166
12. Persson BRR, Darte L: Gel chromatography column scanning for the analysis of ^{99m}Tc-labeled compounds. *J Chromatogr* 101:315–326, 1974
13. Jansholt AL, Krohn KA, Vera DR, et al: Design and validation of a radiochromatogram scanner with analog and digital output. *J Nucl Med Tech* 8:222–227, 1980
14. Primus FJ, Newell KD, Blue A, et al: Immunological heterogeneity of carcinoembryonic antigen distinguished by monoclonal antibodies. *Cancer Res* 43:686–692, 1983
15. Chen TR: In situ detection of mycoplasma contamination in cell cultures by fluorescent Hoechst 33258 stains. *Exp Cell Res* 104:255–262, 1977
16. Paik CH, Phan LNB, Hong JJ, et al: The labeling of high affinity sites of antibodies with ^{99m}Tc. *Int J Nucl Med Biol* 12:3–8, 1985
17. Harwig JF, Coleman RE, Harwig SS, et al: Highly iodinated fibrinogen: A new thrombus-localizing agent. *J Nucl Med* 16:756–763, 1975
18. Steigman J, Williams HP, Soloman NA: The importance of the protein sulfhydryl group in HSA labeling with technetium-99m. *J Nucl Med* 16:573, 1975 (abstr)
19. Lanteigne D, Hnatowich DJ: The labeling of DTPA-coupled proteins with ^{99m}Tc. *Int J Appl Radiat Isot* 35:617–621, 1984