RADIOCHEMISTRY AND RADIOPHARMACEUTICALS

Technetium-99m Labeling of Antibodies to Cardiac Myosin Fab and to Human Fibrinogen

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We have developed a method of labeling biologically active labile macromolecules, such as human fibrinogen (HF) and anticardiac-myosin Fab (AM-Fab), with Tc-99m at neutral pH. This method uses dithionite reduction of pertechnetate and subsequent labeling, to test the method with acid-labile macromolecules. Complexes of diethylene triamine pentaacetic acid with macromolecules such as human fibrinogen (D-HF) and anticardiac-myosin Fab (D-AM-Fab) were labeled and utilized in in vitro and in vivo studies. In biodistribution studies, the Tc-99m D-HF had a two-component blood clearance (half-times 1 hr and 15 hr) and was 80–88% coagulable. The Tc-99m AM-Fab retained its immunoreactivity as tested by affinity chromatography; also during in vivo localization in experimental myocardial infarction. This labeling technique provides an easy and efficient approach to the Tc-99m labeling of other biologically active and acid-labile macromolecules.

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Biologically active macromolecules—such as antibodies specific for cardiac myosin (*1–4*), carcinoembryonic antigen (*5,6*), α-fetoproteins (*7*), fibrinogen (*8*), and mitochondria (*9*)—have been labeled with iodine-125 and iodine-131 and used clinically or experimentally to locate and/or visualize specific target organs. These nuclides, however, are not ideal tracers for most nuclear imaging (*10*). Another candidate, iodine-123, is not widely available in high radionuclidic purity. A radionuclide that is well matched to present imaging instruments is technetium-99m, with its most abundant photon emission at 140 keV, $T_{1/2}$ of 6 hr, and no β emission (*10,11*). Unfortunately, the methods used to bind Tc-99m to macromolecules usually require relatively harsh conditions such as electrophoresis using zirconium electrodes (*12*) or reduction in an acidic environment (*13,14*), and these frequently result in excessive denaturation of the labeled biomacromolecules.

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To attach Tc-99m to labile macromolecules such as rabbit antibodies to cardiac myosin Fab (AM-Fab) and human fibrinogen (HF), we investigated a three-step process: (a) derivation (D) of the macromolecules with a bifunctional chelate as described by Krejcarek and Tucker (*15*) and Khaw et al. (*16*); (b) reduction of the technetium and sodium dithionite; and (c) labeling by addition of the reduced technetium to the buffered derived protein. In this report we will discuss our initial in vitro and in vivo experience with this labeling technique.

METHODS

Preparation of proteins. *Preparation of antibody Fab to cardiac myosin (AM-Fab).* This was performed as previously described (*1–4*). An aliquot of cardiac myosin was purified by the method of Katz et al. (*17*), with purity determined by 10% polyacrylamide SDS gel electrophoresis (*18*). It was then used in complete Freund's adjuvant to immunize rabbits. Another aliquot of cardiac myosin was used to prepare a myosin-Seph-

arose affinity column by the cyanogen bromide activation procedure of Cuatrecasas (*19*). Rabbit antibody to canine or human cardiac myosin (AM) was purified by appropriate myosin-Sepharose affinity (*1*), with elution by 5*M* guanidine-HCl (*1*). The protein was subjected to renaturation over several days through serial dialysis in 3.0*M* KCl, and then against 0.3*M* phosphate buffer saline solution (PBS). The product was tested for antibody activity by its binding capacity for the homologous cardiac myosin (*1*), labeled with I-125 by the lactoperoxidase method of Marchalonis (*20*).

Fab from antimyosin were prepared by papain digestion at an enzyme-to-substrate ratio of 1:100 at 37°C for 1.5 hr (*21*). Fab were separated from Fc and undigested whole AM by a protein-A Sepharose column, whereas AM-Fab were recovered in the fall-through fractions (*22*). Purity of AM-Fab was demonstrated by double immunodiffusion in 1% agarose gel (*23*) where precipitin lines of partial identity to whole AM or normal IgG, and nonidentity to Fc, where obtained against goat serum antibody to rabbit IgG. Antibody activity of Fab was determined as previously described (*1*). AM-Fab were concentrated by vacuum dialysis to 4–5 mg/ml, then dialyzed against 0.1*M* $NaHCO_3$ and frozen until used.

Purification of human fibrinogen (HF). Initially HF was isolated from plasma collected in acid-citrate-dextrose buffer according to the method of Blomback et al (*24,25*). Fibrinogen thus prepared was 97% coagulable by thrombin.* The protein concentration in the clots was assayed by Kjeldahl (*26*). Purified fibrinogen was lyophilized and stored at 4°C. Recently, HF was purchased commercially.† Immunological purity was also determined by immunoelectrophoresis, providing one precipitin arc with rabbit antiserum against human fibrinogen (*27*).‡

Labeling procedure. *Step 1: Covalent coupling with diethylene triamine penta-acetic acid (DTPA).* This ligand was covalently coupled to either AM-Fab or HF by the carboxycarbonic anhydride reaction of DTPA as described by Krejcarek and Tucker (*15*), with minimal modification for coupling small quantities of protein. An average of 5 mg antibody protein was used for coupling with DTPA (minimum 3 mg/ml).

An aliquot of 100 μl (12.5 μ*M*) of carboxycarbonic anhydride of DTPA (the presence of a crystalline precipitate, which is used in the following reactions, is an indication of acid anhydride formation) was added to 1 ml of AM-Fab (0.06–0.1 μ*M*) with thorough stirring; 15 min later another 100-μl aliquot of the anhydride was added with constant stirring. After 1 hr at room temperature, it was dialyzed at 4°C overnight against a large excess (3–6 l) of 0.3*M* PBS, pH 8.0. An aliquot of this D-AM-Fab was then tested for its antibody activity as described previously (*1*). The AM activity of D-AM-Fab for I-125 myosin was unchanged from that of unmodified AM-Fab and normal IgG and indicates DTPA coupling has no denaturing effect on the AM-Fab (*28*).

Derivation of fibrinogen was achieved by addition of 100 μl (12.5 μ*M*) of carboxycarbonic anhydride to 5 ml of 50 mg fibrinogen (≃0.15 μ*M*) in 0.1 *M* $NaHCO_3$. Fifteen minutes later another aliquot of 100 μl was added. (Excessive amounts of anhydride D can cause varying degrees of denaturation of the derived fibrinogen (D-HF) prepared.) D-HF was then dialyzed against 0.3 *M* PBS, pH 8.0, and treated as described above for the preparation of D-AM-Fab.

Step 2: Determination of optimal dithionite concentration for reduction of pertechnetate. A. Each $^{99m}TcO_4^-$ sample obtained from the Mo-99—Tc-99m-generator was assayed for total technetium molarity/ml by the method of Lamson et al. (*29*). To aliquots of $^{99m}TcO_4^-$ (usually 4–5 mCi) the following molar excesses of solid sodium dithionite ($Na_2S_2O_4$) were added: 10^2, 10^3, 10^4, 4×10^4, 5×10^4, 10^5, and 5×10^5 in enclosed test tubes and vortexed vigorously.

B. After incubation at room temperature for 10 min, the reduced technetium was added dropwise quickly to 20 μg of D-AM-Fab in 500 μl of 0.3*M* PBS, pH 8.0, with vigorous stirring. Bound and free technetium were separated by Sephadex G-25 column chromatography (0.5 × 10 cm). Tc-99m D-AM-Fab was recovered in the void volume. The efficiency of reduction of pertechnetate with $Na_2S_2O_4$ was determined as the efficiency of formation of Tc-99m D-AM-Fab recovered in the void

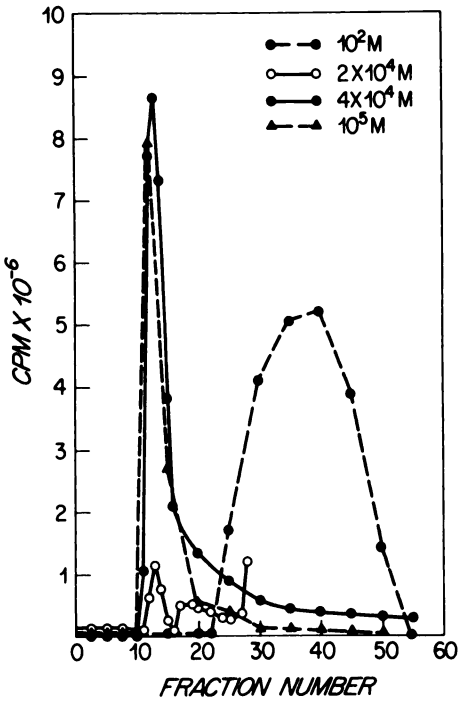

FIG. 1. Reduction of pertechnetate with 10^2, 2×10^4, 4×10^4, and 1×10^5 molar excesses of $Na_2S_2O_4$, with subsequent chelation of reduced Tc with D-AM-Fab and chromatography on 10-ml Sephadex G25 column.

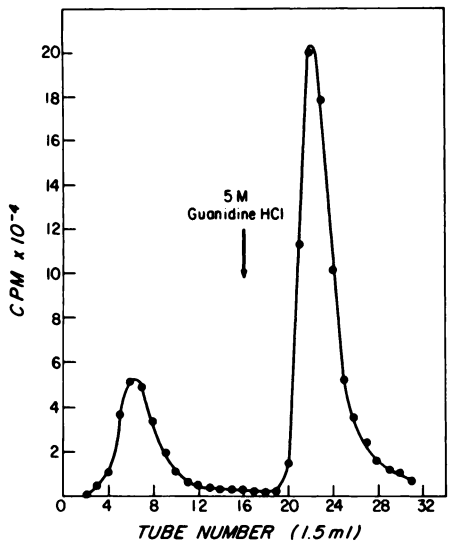

FIG. 2. Affinity chromatography of Tc-99m-D-AM-Fab on a 5-ml myosin-Sepharose column. Specifically bound radioactivity was dissociated with 5*M* guanidine-HCl.

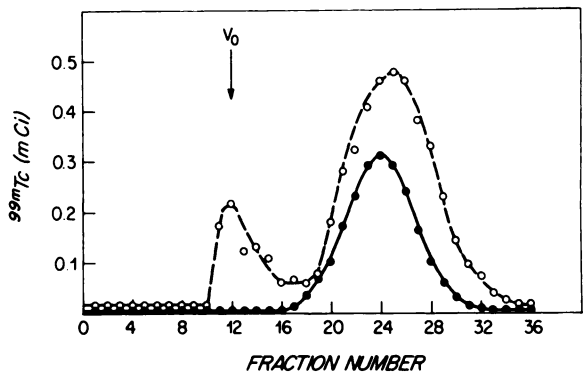

FIG. 3. Sepharose-4B (10 ml) chromatograms of Tc-99m-D-AM-Fab prepared at two different times. One preparation showed presence of colloids; the other showed no colloidal contamination.

volume of Sephadex G-25 column chromatography. Each reduction and coupling reaction was performed at least three times, and Fig. 1 shows chromatograms from one such series of reductions, where optimal reduction was obtained at a $Na_2S_2O_4$ molar excess of 4×10^4 or greater. In the following studies, therefore, this molar excess or greater was used as standard concentration for reduction of pertechnetate.

Step 3: Chelation of reduced Tc-99m by D-AM-Fab or D-HF. Generator-eluted pertechnetate (20–40 mCi) was added to sufficient $Na_2S_2O_4$ to provide a 5×10^4 to 5×10^5 molar excess and incubated at room temperature for 10 min. A. To label D-AM-Fab, the reduced Tc-99m solution was then continuously added dropwise to 100–200 μg of D-AM-Fab in 300–500 μl of 0.3 *M* phosphate, pH 8.0, 0.15 *M* NaCl (0.3 *M* PBS, pH 8.0), while vortexing the antibody solution vigorously. The final solution was approximately neutral. Bound and free Tc-99m were separated by Sephadex G-25 column chromatography (0.5 × 10 cm). To determine the antibody activity, an aliquot of Tc-99m D-AM-Fab was applied to a 5-ml myosin-Sepharose column (*1*). Figure 2 shows one such affinity chromatogram where the column was eluted with lactated Ringer's to obtain the fall-through fraction, then 5*M* guanidine-HCl was used to elute specifically bound Tc-99m D-AM-Fab. B. To label D-HF with Tc-99m pertechnetate was reduced with 2.5×10^5 molar excess of $Na_2S_2O_4$. The reduced technetium was added to 2–6 mg of D-HF in 0.4 ml of 0.3*M* PBS, pH 8.0, with vortexing. Bound and free Tc-99m D-AM-Fab and Tc-99m D-HF were eluted in the void volume with lactated Ringer's solution.

Further purification of Tc-99m-labeled compounds. The technetium-tagged antibodies to myosin Fab or fibrinogen were tested for contamination with Tc-99m colloids that are co-eluted in the void volume of Sephadex G-25 column chromatography. An aliquot of the labeled compound was applied on a Sepharose-4B column (10 ml). Lower concentrations of $Na_2S_2O_4$, or higher total Tc concentrations, encourage colloid formation (Fig. 3). The Tc-99m labeled proteins are eluted in the included volume according to their molecular weights. To reduce contaminating colloids, the following procedure was added to the labeling process. A. Tc-99m D-AM-Fab was chromatographed on a 2.5 × 20 cm Sepharose 4B column. The peak in the void volume was discarded and the included peak used for in vivo detection and visualization of myocardial infarction. Figure 3 shows Sepharose 4B chromatograms (10-ml col) of two separate labelings: one showing presence of colloids and the other without contaminating colloids. B. Tc-99m D-HF can be purified by addition of saturated $(NH_4)_2SO_4$ to a final 25% $(NH_4)_2SO_4$ saturation. Labeled fibrinogen is precipitated out, recovered by centrifugation, and resolubilized in 1 ml of 0.3*M* PBS, pH 8.0. The sample is desalted on a second Sephadex G-25 column. The Tc-99m D-HF recovered in the void volume was used for subsequent studies. Figure 4 shows the protocol used to prepare Tc-99m-labeled fibrinogen, and Table 1 shows the expected yields in the various labeling steps.

Confirmation of labeled compounds. *Determination by column chromatography.* The Tc-labeled compounds were applied to a precalibrated Sephadex 4B column (50 × 1.5 cm) to ensure that Tc-99m activity associated with peaks in the void volume (50 ml) of the Sephadex G-25 column chromatography were associated with either D-HF or D-AM-Fab. Fractions (2.5 ml) were collected. Figure 5 shows the semilog plot against molecular weight of the elution volumes of Tc-99m D-HF (mole wt 340,000), normal rabbit IgG (NRIgG) (mole wt 150,000), I-125 AM(Fab')$_2$ (mole wt 100,000), Tc-99m D-AM-Fab (mole wt 50,000), and bovine serum albumin (BSA) (mole wt 64,000). It indicates that the Tc-99m-labeled molecular species are in their monomer

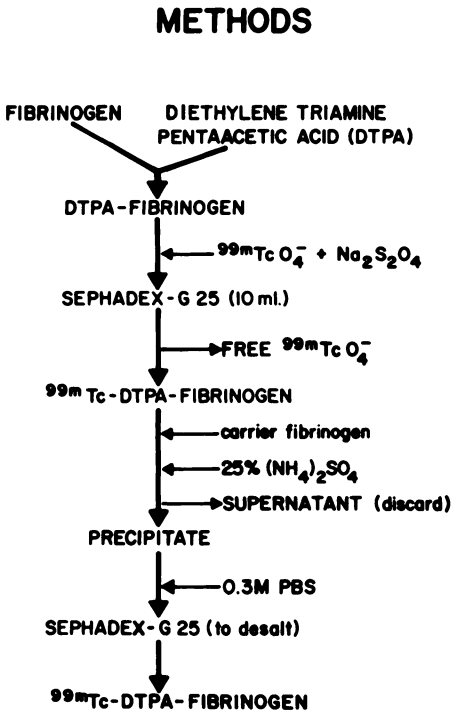

FIG. 4. Schema of protocol used to prepare and label D-HF with Tc-99m.

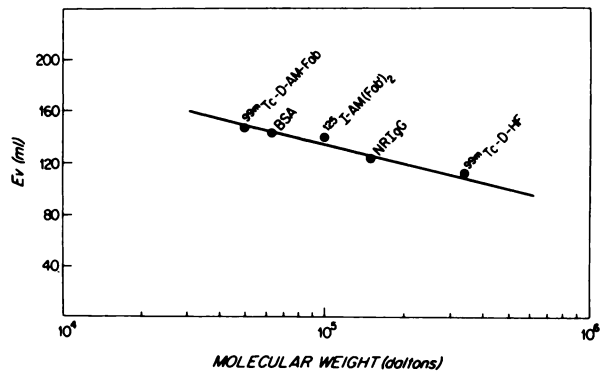

FIG. 5. Determination of molecular species of Tc-99m-labeled compounds by Sepharose 4B-column chromatography. Standards used were NR IgG: I-125 AM(Fab')$_2$, and BSA.

conformation.

Coagulability and precipitability of Tc-99m D-HF. A. Coagulability in human and canine whole blood (Table 2): To 1 cc of fresh human or canine blood, an aliquot of Tc-99m D-HF was added; then, the mixture was incubated either overnight at 4°C, or at 37°C for 1 hr, then at 4°C for 1 hr to shrink the blood clot. After centrifugation, serum was removed with a Pasteur pipette. The precipitate was washed twice with 1 ml of saline, recentrifuging and the supernatant added to the serum. Coagulability was determined as the ratio of radioactivity in clot to total radioactivity used. Four studies (two performed in duplicate and two in triplicate) were performed for coagulability in fresh human blood. In two of these studies, I-125 fibrinogen[§] (100,000 cpm) was also added to the blood for comparison. Coagulability in fresh canine blood was performed in quadruplicate in four sets.

B. Coagulability in plasma was examined in two studies performed with human plasma and three with canine plasma in triplicate (Table 2). To 0.5–1 ml of fresh human or canine plasma, an aliquot of Tc-99m D-HF or I-125 fibrinogen was added. The plasma was allowed to clot at room temperature for 1 hr, or overnight at 4°C. Serum was separated from clot by centrifugation, washed twice in 1 ml saline, then clot and supernatant were counted in a gamma counter to determine percent radioactivity in the clot.

C. Coagulability of Tc-99m D-HF in citrated pooled human plasma with thrombin was tested in 25 studies performed in triplicate (Table 2). To 200-μg aliquots of D-HF in 100 μl of 0.3*M* PBS, pH 8.0, were added 10 μl

TABLE 1. % YIELD OF Tc-99m-D-HF

Number of Trial	1	2	3	4	5	6	7	
Step	% of Total Tc-99m O_4^- Used							Yield Mean ± SD
1. TcO_4^-	100	100	100	100	100	100	100	100
2. Seph G-25* (column 1)	64.1	67.8	71.0	65.9	63.3	58.7	73.3	66.3 ± 4.9
3. 25% $(NH_4)_2SO_4$ SAT-ppt†	38.8	45.5	52.7	45.3	44.7	43.1	45.2	45.0 ± 4.1
4. Seph G-25 (column 2)	20.8	34.1	43.6	31.3	24.8	37.0	31.9	31.9‡ ± 7.6
5. Coagulability	84.6	89.3	86.6	96.5	82.0	85.1	85.4	87.2 ± 4.6

* Sephadex G-25.
† Saturation precipitate.
‡ Mean specific activity 4m Ci/mg.

TABLE 2. COAGULABILITY OF Tc-99m-D-HF

Samples	Radiolabel	% Activity in Clot (±SD)
1. Human Blood, n = 4	Tc-99m	80.4 (75.7–85.1)†
2. Human Blood, n = 2	I-125	92.8 (91.3–93.3)†
3. Human Plasma (fresh), n = 2	Tc-99m	82.0 (81.5–82.5)†
4. Human Plasma (fresh), n = 2	I-125	88.8 (84.5–92.8)†
5. Human Plasma (citrated), n = 20	Tc-99m	84.3 ± 12.4*
6. Human Plasma (citrated), n = 5	I-125	82.8 ± 3.8*
7. Canine Blood, n = 4	Tc-99m	82.6 (78.6–85.9)†
8. Canine Plasma, n = 3	Tc-99m	88.4 (84.4–92.6)†
9. Human Plasma (citrated), n = 8	$^{99m}TcO_4^-$	10.5 ± 1.1

* Plasma clotted with 50 units of thrombin.
† Range of values.

TABLE 3. Tc-99m ASSOCIATED WITH $(NH_4)_2SO_4$ PRECIPITABLE FIBRINOGEN

Number	Sample	Carrier D-HF	% $(NH_4)_2SO_4$ Saturation	% Activity In Precipitate (± SD)
1. n = 14	Tc-99m-D-HF*	—	25	88.6 ± 6.2
2. n = 4	Tc-99m-D-HF	1.25 mg	25	81.7 (77.0–84.5)†
3. n = 4	I-125-HF	1.25 mg	25	92.9 (91.9–93.8)†

* No carrier used due to 1–2 mg D-HF used in labeling.
† Range of values.

(≃1 μCi) of Tc-99m D-HF (n = 20) or 10 μl (0.1 μCi) of I-125 fibrinogen (n = 5). Then 0.5–1 ml of blood-bank human plasma in citrate was added and 10–50 units of thrombin (1000 μ/ml) added to the above mixture. It was then allowed to clot at room temperature for 2 hr, after which the clot was washed twice in 1 ml saline. Supernatant and washes were pooled, and radioactivities in the clot and the pooled mixture were determined by scintillation counting.

A control study using $^{99m}TcO_4^-$ in place of Tc-99m D-HF was performed in triplicate (n = 8) (Table 2). Coagulability was calculated as the ratio of percent of radioactivity in the clot to total activity in the reaction mixture. For each time D-HF was labeled with Tc-99m, coagulability in human plasma was performed in triplicate sets.

D. Precipitability of radiolabeled fibrinogen (Table 3): To 100 μl of Tc-99m DTPA-fibrinogen (≃4.4 μCi) or I-125 fibrinogen (1 μCi) was added 1.25 mg of stable D-HF. Then a saturated solution of $(NH_4)_2SO_4$ was added to give a final $(NH_4)_2SO_4$ saturation of 25% (*30*). The precipitate was washed twice with 25% saturated $(NH_4)_2SO_4$ solution and supernatant and precipitate counts were determined by gamma scintillation counting (n = 4). In more recent studies with 2 mg D-HF as starting material, precipitability was performed by direct addition of saturated $(NH_4)_2SO_4$ to give a final saturation of 25%, and precipitate and supernatant activities counted in a dose calibrator. The results from 14 trials are shown in Table 3.

Biodistribution in mice. CD mice were divided into five groups of six mice each. Twenty μCi of Tc-99m D-HF per mouse was administered by tail vein, with an aliquot reserved for a standard. Separate groups of mice were killed at 15 min, 30 min, 1 hr, 3 hr, and 24 hr, and the following organs were excised, weighed, and counted: blood, brain, thyroid, heart, lungs, liver, spleen, gastrointestinal tract, kidneys, bone, tail, and carcass. Total blood activity was estimated from an aliquot, assuming blood volume to be 8% of body weight. Blood activity in the carcass was not corrected in the calculation. Percent dose per organ was calculated as

$$100 \times (\text{cps/organ}) \div \text{standard dose}.$$

Coagulability of the Tc-99m D-HF used in this biodistribution study was 86%.

Experimental myocardial infarction. Four dogs were anesthetized with intravenous pentobarbital, intubated, and put on a Harvard respirator. Following a left thoracotomy, the left anterior descending coronary artery (LAD) was dissected free approximately 2/3 the distance from apex to base and was occluded with a silk ligature (*1–4*). The thoracotomy was then closed and the animal allowed to recover. After 4 hr of coronary occlusion, 8–10 mCi of Tc-99m D-AM-Fab were injected intravenously. Tc-99m D-AM-Fab prepared for in vivo studies used 1% BSA in lactated Ringer's solution to elute the columns in order to minimize nonspecific adsorption of the small concentration of AM-Fab used (200 μg AM-Fab, specific activity 50 mCi/mg). Ten-minute images containing at least 500K counts (usually 5 million) were obtained, using an Anger camera with a low-energy, parallel-hole, all-purpose colimator. The earliest localized area of uptake was obtained at 12 hr after injection of Tc-99m D-AM-Fab. Experimental myocardial infarction was confirmed by histochemical triphenyltetrazolium chloride (*31*), which stains for

dehydrogenase activity in normal myocardium. Following sacrifice, the heart was excised, cut into ventricular slices parallel to the atrioventricular groove, and reimaged for 100,000 counts. The images were compared with the histochemical delineation of infarction.

RESULTS

Covalent coupling of antimyosin Fab with DTPA does not reduce the binding capacity for the homologous antigen, in that D-AM-Fab and unmodified AM-Fab both showed identical binding for I-125 canine cardiac myosin (*28*).

The optimal concentration of $Na_2S_2O_4$ for reduction of $^{99m}TcO_4^-$ was observed to be 4×10^4 molar excess of total Tc molar concentration to be reduced. Figure 1 shows one series of Sephadex G-25 column chromatograms of D-AM-Fab coupled to Tc-99m reduced with varying concentrations of dithionite. Maximum labeling recovered in the void-volume activity occurred with 4×10^4 molar excess of $Na_2S_2O_4$; however, not all technetium activity is in labeled antibody, some activity may be due to contaminating Tc-99m colloids. These were removed by Sepharose 4B column chromatography which showed another peak of radioactivity eluted in the void volume (Fig. 3) (see Methods) or with Tc-99m D-HF by ammonium sulfate precipitation.

To demonstrate that Tc-99m D-AM-Fab thus prepared retained its antibody activity, an aliquot of Tc-99m D-AM-Fab was applied to a 5-ml cardiac-myosin-Sepharose affinity column. Figure 2 shows one such affinity chromatogram where colloids and/or denatured antibody were eluted in the fall-through peak. Specific antibody with its associated radioactivity was eluted in the guanidine.

The conservation of the molecular size of the DTPA-derived proteins when labeled with dithionite-reduced Tc-99m was demonstrated by chromatography on a precalibrated Sepharose 4B column. Figure 5 shows that Tc-99m D-AM-Fab eluted in the 50,000-dalton molecular-weight range, and Tc-99m D-HF in the 340,000-dalton range. This indicates that the labeled proteins are still in the monomer forms and that any excessive $Na_2S_2O_4$ did not reduce intrachain disulfide bonds of either the Fab or fibrinogen.

The biological activity of Tc-99m D-HF was determined by its coagulability in whole blood, in fresh plasma, or in citrated plasma with thrombin. When Tc-99m D-HF was used as tracer (Table 2), coagulability was about 80% in whole human blood and ~82½% in canine; it was ~85% in fresh human plasma and ~84% in citrated plasma clotted with thrombin. Precipitability of the radiolabeled fibrinogen in 25% $(NH_4)_2SO_4$ was 81.7% and 88.6% in whole human and canine blood, respectively, compared with 92.9% for I-125 fibrinogen§ (Table 3). The replication in each experiment was consistently within 5%. The control study (n = 8) in triplicate revealed 10.5% binding of free pertechnetate in the clot. The mean specific activity of Tc-99m D-HF prepared was 4 mCi/mg fibrinogen.

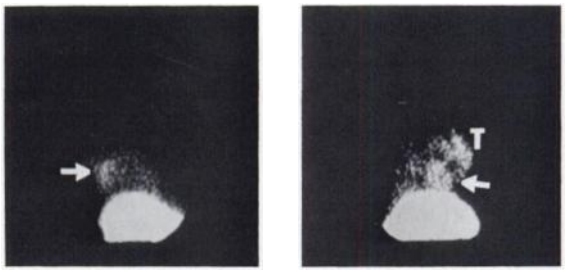

FIG. 6. Gamma scintigraphic images of Tc-99m-D-AM-Fab uptake in experimental canine myocardial infarction. Left lateral view (left) shows anteroapical localization of Tc-99m-D-AM-Fab in infarct (arrow), and anteroposterior view (right) shows localization in infarct (arrow). T denotes area of uptake in thoracotomy due to cross-reactivity of AM-Fab with skeletal myosin.

Biodistribution of Tc-99m D-HF in mice is shown in Table 4. Blood clearance was biexponential, with half-times (decay-corrected) of 1 hr for the fast component and 15 hr for the slow. At 24 hr, 11.5% of the dose was still circulating. Liver activity ranged from 18.6 ± 1.5% to 22 ± 3.2%; thyroid from ~0.2% to ~0.01%. The dogs with ligated LAD coronaries received 8–10 mCi of Tc-99m D-AM-Fab (~50 mCi/mg) after 4 hr of occlusion, and were scintigraphed 12 hr later. (L. lat. and AP). The images showed localization of Tc-99m D-AM-Fab in the anteroapical region of the heart, the site of the experimental infarction (Fig. 6). Liver activity was also visible, probably due to presence of some denatured proteins and/or Tc-99m colloids. The results indicate that in vivo biological activity of Tc-99m D-AM-Fab is preserved after labeling with reduced Tc-99m.

DISCUSSION

Generator-produced pertechnetate (*33*) contains Tc-(VII) in a relatively unreactive ionic state, $^{99m}TcO_4^-$. To couple Tc to various compounds it must be reduced to a Tc^{3+} or Tc^{4+} state. Both electrophoretic and chemical methods such as stannous-HCl reduction have been suggested for the reduction (*34*). Although all these methods are very useful in labeling inorganic and simple organic compounds, they are difficult to apply to acid-labile macromolecules. To minimize the acid denaturation of macromolecules such as AM-Fab or HF, dithionite reduction was used in this study.

Other weak reducing agents such as dithiothreitol have also been tried in our laboratory, but with these the reduction was incomplete, yielding low labeling efficiency (unpublished data). It appears that the more gentle but weaker reducing agents cannot reduce TcO_4^-

TABLE 4. BIODISTRIBUTION OF Tc-99m-D-HF IN MICE

Organ	% Dose/organ* at hours after injection				
	0.25	0.5	1.0	3.0	24.0
Blood	67.0 ± 3.1	59.4 ± 15.0	58.0 ± 6.5	48.8 ± 6.4	11.5 ± 2.6
Brain	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.2	0.2 ± 0.1	0.04 ± 0.008
Thyroid	0.2 ± 0.01	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.006
Heart	0.8 ± 0.3	0.5 ± 0.1	0.7 ± 0.5	0.6 ± 0.2	0.2 ± 0.0
Lungs	1.7 ± 0.3	1.3 ± 0.3	1.6 ± 0.4	1.2 ± 0.2	0.5 ± 0.1
Liver	19.2 ± 3.1	21.5 ± 4.5	22.0 ± 3.2	21.4 ± 3.3	18.6 ± 1.5
Spleen	0.9 ± 0.2	1.0 ± 0.2	1.0 ± 0.2	1.0 ± 0.2	1.1 ± 0.1
GIT†	4.9 ± 0.7	5.8 ± 0.3	6.4 ± 0.6	8.2 ± 0.7	3.3 ± 0.7
Kidneys	6.1 ± 0.3	6.7 ± 0.7	5.9 ± 3.0	7.2 ± 0.6	6.5 ± 0.5
Bone	7.0 ± 1.2	5.9 ± 1.4	5.7 ± 4.5	7.5 ± 1.9	5.7 ± 2.3
Tail	4.0 ± 1.0	2.4 ± 0.5	3.1 ± 2.0	1.8 ± 0.5	1.1 ± 0.2
Carcass	33.4 ± 8.8	36.6 ± 13.6	31.9 ± 7.4	34.5 ± 13.3	18.7 ± 2.5

* Mean of 6 samples, ±s.d.
† Gastrointestinal tract.

completely. $Na_2S_2O_4$ is a stronger reducing agent than dithiothreitol and, at the 5×10^4 to 5×10^5 molar excess concentrations used, was able to produce maximal reduction of Tc-99m to label DTPA-complexed macromolecules. This observation has also been reported by Jones et al. (*35*).

Our study demonstrates, both in vivo and in vitro, that acid-labile macromolecules can be labeled with Tc-99m by this method without appreciable loss of biological activity. Their monomer molecular forms were also maintained (Fig. 5). However, some Tc-99m colloids are produced during the dithionite reduction and subsequent chelating reactions, and they contaminate the final product. The amount of colloid formed in each reaction depends on the molar ratio of $Na_2S_2O_4$ to total Tc used: the greater this ratio, the less colloid formation (unpublished data). The timing between successive elutions of the Mo-99 generator observed also plays a part; we find that a 24-hr rest period minimizes the tendency to colloid formation. The contamination can be minimized by the $(NH_4)_2SO_4$ salting-out process described, or by appropriate column chromatography using molecular sieves that discriminate colloidal forms from smaller radiolabeled proteins (e.g., Fab). Both the in vitro coagulability and content in the $(NH_4)_2SO_4$ which cannot be precipitated with $(NH_4)_2SO_4$ with any consistency precipitable-fractions of the Tc-99m D-HF, as well as the in vivo behavior, indicate that the product labeled with $Na_2S_2SO_4$-reduced Tc-99m is not significantly altered, and behaves like the iodine-labeled compound. Coagulability of Tc-99m-labeled fibrinogen was consistently as high as 92% when labeling used the improved protocol.

The minimal Tc-99m thyroid activity as seen in the biodistribution study (Table 4) also indicates that Tc-99m is not released from the Tc-99m D-HF in vivo as free Tc-99m. The liver activity (approximately 20%) indicates the presence of some denatured fibrinogen. The Tc-99m D-HF used was 86% coagulable in vitro, indicating that the difference between liver activity and coagulability may also be due to use of human fibrinogen in a murine experiment. Whole-blood clearance of Tc-99m D-HF is slower than that of the Tc-99m fibrinogen of Harwig et al. (*36*). In their study only 25% of injected dose remained in circulation at 10 min after injection, whereas we found 67 ± 3.1% of the dose in the blood at 15 min after injection (Table 4). This difference in blood clearance may be due to our minimizing the denaturation of the protein. The purity of the radiolabeled compound is also reflected in the high coagulability ($\simeq$80%) of Tc-99m D-HF (Table 1) compared with the 52% of Tc-99m fibrinogen in the Harwig study (*36*).

Another radionuclide that has been used to label fibrinogen for imaging is iodine-123 (*37*), but this nuclide is not readily available in concentrations suitable for iodination.

The antimyosin antibody activity, on the other hand, appears to be preserved to about 80–90% of the total antibody activity in the in vitro affinity-chromatography method, and in-vivo studies showed specific and quick localization in experimental myocardial infarction. Although the antibody activity was preserved, there is about 20–30% liver activity in the scintigram at 12 hr. This appears to be slightly greater than the 10–20% denatured antibody activity indicated by affinity chromatography, which is probably due to incomplete clearance of colloids. Despite the presence of partial denaturation or minor colloidal contaminants, the usefulness of this technique is apparent in its ability to radiolabel fibrinogen as well as AM-Fab, and its use in

vitro and in vivo to demonstrate myocardial infarction.

FOOTNOTES

* Bovine Thrombin, Parke Davis, Detroit, MI.
† Kabe, Switzerland.
‡ Hyland Laboratories.
§ Amersham.

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A fund has been established in the ERF by friends of Marc Tetalman, M.D., who was a tragic homicide victim while attending the SNM meeting in Atlanta in June 1979. This fund will permit an award of $3,000 to be made in June, 1983 to a young investigator (35 years of age or younger) who is pursuing a career in Nuclear Medicine. This award is to be repeated annually. It is possible that additional contributions to our fund will permit the stipend to be increased in future years. Applicants should submit prior to March 1, 1983 a curriculum vitae together with data supporting current research efforts.

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