

In vitro properties and *in vivo* behavior of technetium-99m labeled fibrinogens

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Fibrinogen was labeled with Tc-99m by two methods and the *in vitro* stability and *in vivo* behavior in mice were studied. The Tc-99m labeling was performed by mixing an unreduced fibrinogen (UnFib) or a reduced fibrinogen (ReFib) with Tc-99m pertechnetate in the presence of stannous chloride. In both of them, chelation with Tc-99m resulted in a single radiochemical product. For the *in vitro* stability studies, Tc-99m labeled fibrinogen (Tc-99m UnFib) was prepared with UnFib, and transchelation with cysteine solution was easy to produce compared to Tc-99m labeled fibrinogen (Tc-99m ReFib) prepared with ReFib. The radioactivity bound to clottable protein for Tc-99m UnFib and Tc-99m ReFib was about 70% and about 69%, respectively. The *in vivo* behavior of these labeled fibrinogens was studied, and their efficiencies for imaging an abscess and Ehrlich tumor in mice were determined with a gamma camera. Technetium-99m UnFib underwent a rapid partial exchange of the Tc-99m with compounds of the blood buffer system *in vivo*, resulting in early urinary excretion. On the other hand, the fraction of Tc-99m ReFib that remained intact *in vivo* was biologically active and would be incorporated into the abscess and tumor. The uptake in the abscess increased slightly over time with Tc-99m ReFib, but the abscess to blood and abscess to muscle ratios were 0.09 and 2.6 at 5 hr, respectively. Clearly delineated images of the abscess were obtained beginning at about 5 hr after injection. The tumor to blood and tumor to muscle ratios were 0.05 and 1.4 at 5 hr, respectively. The Ehrlich tumor image in mice was slightly visible at 10 hr. The short half-life of Tc-99m was inappropriate for fibrinogen with a low pharmacokinetic value, because it was necessary for imaging of the abscess and tumor to take a long time.

Key words: technetium-99m, fibrinogen, abscess, fibrin

INTRODUCTION

FIBRINOGEN, as found after its transformation into fibrin in various specific lesions such as vein thrombosis, heart infarction, rheumatoid arthritis, and malignant tumors, is potentially a very interesting protein.¹ Fibrinogen has, therefore, been labeled with radionuclides and studied for the diagnosis of cancer, infection, thrombosis,^{2,3} rheumatoid arthritis, and myocardial infarction. Fibrinogen labeled with I-125 or I-131 is generally used for thrombosis detection,^{4,6} but imaging cannot be achieved due to the physical properties of these tracers. Although I-123 labeled fibrinogen⁷ has good physical properties, it is too expensive for clinical use. The presence of varying

amounts of free radio iodine in iodolabeled fibrinogen preparations often poses problems for accurate quantifiable assessment of a function.

It is generally accepted that Tc-99m is an ideal radionuclide for clinical use, due to its ready availability, low irradiation of the patient, and low cost. Fibrinogen has therefore been labeled with Tc-99m⁸⁻¹⁰ and studied for the diagnosis of thrombosis.¹¹⁻¹⁴ There have been, however, many problems such as *in vivo* instability and the loss of clottability due to the labeling technique for Tc-99m labeled fibrinogen in clinical use.

We employed two Tc-99m labeling methods with fibrinogen in order to improve the *in vivo* stability and thus to optimize its incorporation into various specific lesions.

This paper reports our investigation of the *in vivo* behavior of Tc-99m labeled fibrinogens (Tc-99m UnFib and Tc-99m ReFib) prepared with an unreduced fibrinogen (UnFib) and a reduced fibrinogen (ReFib), including stability, clearance mechanisms, incorporation into an

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abscess and tumor, and potential as an imaging agent for the diagnosis of cancer and infection.

MATERIALS AND METHODS

Materials

Bovine plasma fibrinogen containing lyophilized fibrinogen, 15% sodium chloride and 10% sodium citrate was purchased from Sigma Chemical Co. (St. Louis, MO) and used with further purification. Ten mg of commercial fibrinogen was dissolved in 1 ml saline and the protein fraction was then separated on a mini-column prepacked with Sephadex G-25M (PD-10) column (Pharmacia, Uppsala, Sweden) by eluting with saline. The protein content of the purified fibrinogen was measured by the bicinchoninic acid method.¹⁵ The separated fibrinogen solution (1.5 ml) was kept at -20°C until use. Its coagulability amounted to about $74.02 \pm 2.56\%$.

Stannous chloride solution: Twenty mg of stannous chloride (Nakalai Tesque, Kyoto, Japan) was dissolved in 0.1 N hydrochloride solution, and the solution brought to 10 ml.

Reducing solution: A 0.08 mg/ml stannous chloride aliquot, 1.2 mg/ml 1-hydroxyethane-1,1-diphosphonate (Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan), and 0.4 mg/ml ascorbic acid (Wako pure chemical Ind. Ltd., Osaka, Japan) were dissolved in saline solution. Stannous chloride was used as a reducing agent, 1-hydroxyethane-1,1-diphosphonate as an exchangeable ligand in order to label the Tc-99m to the sulfhydryl groups, and ascorbic acid as an antioxidant.

Technetium-99m pertechnetate was eluted from a sterile Mo-99-Tc-99m shielded generator (Ultra-TechneKow, Dai-ichi Radioisotope Laboratories, Chiba, Japan) with isotonic saline.

Technetium-99m labeled fibrinogen prepared by using an unreduced fibrinogen (Tc-99m UnFib)

Fifty μl of stannous chloride solution was added to 1.5 ml of saline solution containing purified fibrinogen (8 mg). Two ml of the Tc-99m pertechnetate solution was immediately added. The resultant solution was ready for injection after 10 min incubation at room temperature.

Technetium-99m labeled fibrinogen prepared by using a reduced fibrinogen (Tc-99m ReFib)

One ml of the commercial fibrinogen (10 mg) was reduced with 2-mercaptoethanol at a molar ratio of 5000 : 1 (2-mercaptoethanol : fibrinogen) at room temperature for 30 min. The reduced fibrinogen was then separated by gel filtration on a PD-10 column by eluting with saline. For Tc-99m labeling, 50 μl of freshly prepared reducing solution was immediately added to the reduced fibrinogen aliquot and mixed well for 10 sec. Technetium-99m pertechnetate (55.5 MBq) was immediately added to the fibrinogen mixture and incubated for 10 min at room

temperature. The pH of the final mixture was adjusted to 7.0. The labeling procedure employed in this study is similar to the labeling method for the antibody described by Schwarz.¹⁶

Technetium-99m complexes

Technetium-99m ascorbate, Tc-99m 1-hydroxyethane-1,1-diphosphonate, Tc-99m cysteinate, and Tc-99m cystinate were used as the standards for thin layer chromatography or gel chromatography. These Tc-99m complex solutions were prepared by the mixing of 1 ml of each ligand solution (20 mg/ml), 0.05 ml of stannous chloride solution (2 mg/ml in 0.1 N HCl) and 2 ml of Tc-99m pertechnetate. The pH of the ligand solution was adjusted to 6.0 except for the cystinate solution which was pH 12.0.

Quality Control

The following tests were done to evaluate the yield of the labeling.

1) *Thin layer chromatography:* The labeling efficiencies with Tc-99m were chromatographically evaluated by using a 0.25 mm Silica-gel 60 plate (Merck, Darmstadt, Germany) developed with an acetonitrile : water (7 : 3) solvent system. The plates were counted by images in a gamma camera (Ohio-Nuclear Co., OH) equipped with a high resolution collimator and a digital computer (VP-450). The Rf values were compared to Tc-99m pertechnetate and hydrolyzed Tc-99m colloid.

2) *Gel chromatography:* Gel chromatography was performed on a PD-10 column. Approximately 0.1 ml of the sample was applied to the PD-10 column and eluted with saline. The fractions (0.4 ml) were collected into tubes, and counted in a well-type gamma counter (Packard COBRA II, Meriden, CT). The column was standardized with Tc-99m pertechnetate, Tc-99m 1-hydroxyethane-1,1-diphosphonate, and Tc-99m ascorbate.

3) *Cellulose acetate electrophoresis:* The purity of Tc-99m UnFib or Tc-99m ReFib was determined by cellulose acetate electrophoresis. The cellulose acetate strips (SELECA-V, Toyo Roshi Kaisha, Ltd., Tokyo, Japan) were run at a constant voltage of 600 V for 60 min with 0.1 M sodium barbital buffer, pH 8.6. The cellulose acetate strips were counted as images in a gamma camera equipped with a high resolution collimator and a digital computer. Movement was compared to the unlabeled fibrinogen and hydrolyzed Tc-99m colloid. The position of the protein was detected by Ponceau 3R staining.

4) *Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE):* Samples for assay were prepared under nonreducing conditions at a SDS concentration of 0.4% and allowed to stand at room temperature for 30 min prior to gel loading. Molecular weight standards (Biorad, Richmond, CA) were prepared in the same manner. Samples were loaded at 15 μg protein per lane and were resolved in a 4.0% acrylamide stacking gel and an 8.0% running gel prepared in a Mini-PROTEAN II

(Biorad, Richmond, CA) apparatus. Gels were run at 25 mA/gel until the tracking dye reached the bottom of the gel (approximately 45 min). Each gel was divided into strips 0.5 cm wide, and its radioactivity was counted in a well-type gamma counter.

The standard gel was stained with Coomassie Brilliant Blue (Wako Pure Chemical Ind. Ltd., Osaka, Japan) after electrophoresis.

5) *Reduction efficiency of 2-mercaptoethanol for fibrinogen:* The fibrinogen solutions were reduced by reaction with a molar ratio of 2-mercaptoethanol ranging from 100 : 1 to 10,000 : 1 at room temperature for 30 minutes. The reduction conditions were determined by densitometric analysis of the SDS gels stained with Coomassie Brilliant Blue.

6) *Clottability:* An aliquot of Tc-99m labeled fibrinogen was clotted by the addition of fibrinogen (2 mg) and thrombin (Sigma Chemical Co., St. Louis, MO) solution at a concentration of 1 NIH unit per milligram of clottable protein. The clot was separated after 1 hr incubation and both the clot and supernatant fluid were counted in a well-type gamma counter.

7) *In vitro stability:* Technetium-99m labeled fibrinogen solutions were allowed to stand at room temperature for 24 hr. Their stability was assessed with gel chromatography as previously described.

The solution of Tc-99m UnFib or Tc-99m ReFib was also mixed for transchelation with cysteine solution. A mixing agent to protein molar ratio of 500 : 1 was chosen. Following a reaction time of 1 hr at room temperature, the preparations were analyzed by thin layer chromatography as previously described.

In vivo studies

Abscess bearing mice: Male ICR mice (Clea Japan, Tokyo, Japan) were subcutaneously injected with 0.1 ml of a turpentine oil-liquid paraffin (1 : 1) mixture into the right foreleg and left for 24 hr.

Ehrlich tumor bearing mice: Male ICR mice were subcutaneously inoculated with Ehrlich tumor cells (4×10^7 cells) into the right hindleg and left for a period of 2–3 weeks to allow tumor growth.

Each mouse received 0.20 ml (3 MBq, 0.45 mg) of fibrinogen labeled with Tc-99m through the tail vein. Sequential scintigrams of three mice were obtained at predetermined intervals with a gamma camera equipped with a high resolution collimator positioned at the dorsum of the mice, and collected by means of a digital computer. The animals (4/group) were killed by collecting blood from the heart at 1 hr and 5 hr postinjection. The organs, blood, some muscle and the abscess or tumor were removed, weighed, and the radioactivity was counted in a well-type gamma counter. The radioactivity of all organs, blood and urine was also counted by means of images in a gamma camera to measure the radioactivity of the excreted urine and the total radioactivity. The correction

of each count in two measurements was performed by coincidence of the blood radioactivity. The percentages of the injected dose per organ or gram were determined by the ratio of tissue radioactivity to total radioactivity.

Mice urine aliquots containing the excreted radioactivity were also similarly analyzed by thin layer chromatography.

RESULTS

In vitro studies

During the thin layer chromatography, both Tc-99m UnFib and Tc-99m ReFib remained at origin in the acetonitrile : water (7 : 3) solvent system on silica gel. No radioactivity was observed at the front position. In this solvent system, pertechnetate moved to the front, and the reduced hydrolyzed Tc-99m colloid also remained at origin.

When chromatographed on a PD-10 column, fibrinogen was found in fractions 6 to 10 and Tc-99m pertechnetate in fractions 30 to 40. The total recovered radioactivity of Tc-99m UnFib and Tc-99m ReFib amounted to $46.0 \pm 8.6\%$ and $93.4 \pm 5.1\%$, respectively. The low recovery of Tc-99m UnFib suggested that the Tc-99m atom was not strongly bound to the fibrinogen and could be adsorbed by the gel. But other radiochromatographic peaks were not detected in Tc-99m UnFib (Fig. 1, A). The labeling yield of Tc-99m ReFib was greater than 90% when estimated by gel chromatography (Fig. 1, B).

During electrophoresis, Tc-99m UnFib and Tc-99m ReFib on cellulose acetate strips migrated about 1.8 cm toward the anode. Under the same conditions, the control of hydrolyzed Tc-99m colloid did not migrate.

The SDS-PAGE of Tc-99m ReFib produced three peaks with estimated molecular weights of 340 Kd, 170 Kd and much below 48 Kd. The first peak corresponded to the native fibrinogen and the second peak to the fibrinogen fragment. Another peak showed the dissociation of Tc-99m ReFib. No radioactivity appeared at the position on the α , β , and γ chains of reduced fibrinogen.

Figure 2 shows the influence of the concentration of 2-mercaptoethanol on the reduction in fibrinogen. A molar ratio of 2-mercaptoethanol to fibrinogen of more than 3,000 : 1 seemed to be optimal for the Tc-99m labeling of fibrinogen.

The radioactivity bound to clottable protein, determined by the addition of thrombin solution was $70.4 \pm 4.1\%$ for Tc-99m UnFib and $68.7 \pm 1.9\%$ for Tc-99m ReFib. These are satisfactory clottability results for these agents.

These Tc-99m labeled fibrinogens were stable in neutral saline solution after 24 hr. Following 1-hr incubation with cysteine solution, the TLC results showed that about 70% of Tc-99m UnFib and about 10% of Tc-99m ReFib were removed by cysteine. In the case of Tc-99m UnFib, the peaks of Tc-99m cysteinate and Tc-99m cystinate were present (Fig. 3). The peak of Tc-99m cystinate was

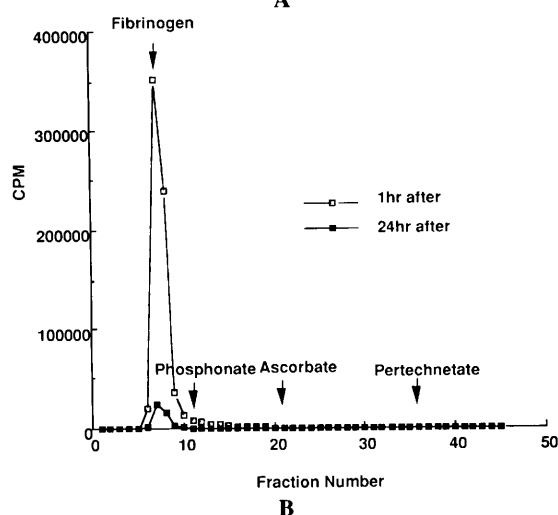
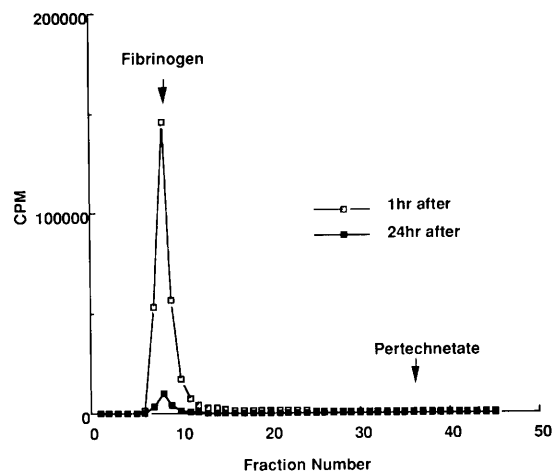


Fig. 1 Gel chromatograms of Tc-99m labeled fibrinogen prepared using an unreduced fibrinogen (A) and a reduced fibrinogen (B). Each fraction was 0.4 ml.

mainly present in the case of Tc-99m ReFib.

In vivo studies

Scintigraphic studies were performed in four mice bearing an abscess or Ehrlich tumor after an intravenous injection of either Tc-99m UnFib or Tc-99m ReFib solution. Sequential scintigrams were obtained at predetermined time intervals for 24 hr with a gamma camera. Typical scintigrams of the mice bearing abscess revealed that the Tc-99m UnFib was rapidly excreted in the urine (Fig. 4, A). The thin layer chromatography of urine activity showed another peak compared with the peak of Tc-99m labeled fibrinogen, indicating that ligand exchange had occurred. Figure 3 shows the results of TLC radiochromatograms of Tc-99m UnFib, Tc-99m cystinate, Tc-99m cystinate and the urine sample. The chromatographic peak in the urine analysis was the peak due to Tc-99m cystinate.

In Tc-99m ReFib, the heart and the liver were immediately visualized after intravenous injection, and the blad-

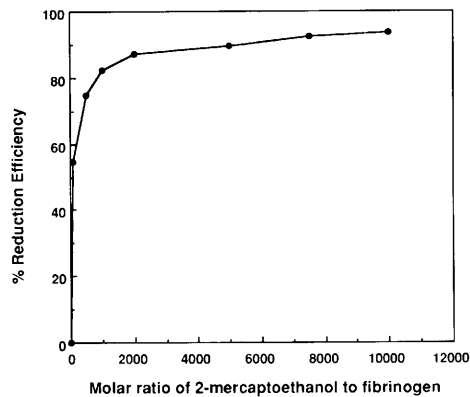


Fig. 2 The influence of the concentration of 2-mercaptoethanol on reduction. The fibrinogen solution was reduced by reaction with a molar ratio of 2-mercaptoethanol ranging from 100 : 1 to 10,000 : 1 at room temperature for 30 minutes. These reduction conditions were determined by densitometric analysis of the gels stained with Coomassie Brilliant Blue.

Table 1 Biodistribution data of Tc-99m ReFib in abscess-bearing mice*

Organ	1 hr	5 hr
Salivary gland	0.19 ± 0.03	0.11 ± 0.06
Spleen	0.63 ± 0.05	0.42 ± 0.15
Stomach	0.54 ± 0.01	0.54 ± 0.17
Intestine	4.61 ± 0.73	10.57 ± 0.47
Liver	22.82 ± 0.68	15.59 ± 2.75
Kidney	5.73 ± 0.39	4.22 ± 0.62
Bladder and Urine	14.77 ± 2.86	26.02 ± 1.65
Muscle 1 g	0.76 ± 0.10	0.48 ± 0.07
Abscess 1 g	1.12 ± 0.44	1.27 ± 0.30
Blood 1 g	23.49 ± 0.79	14.31 ± 2.78

*: Expressed as percentage of injected dose per organ. Each value is mean ± s.d. for four mice.

der after 30 min (Fig. 4, B). The blood clearance of Tc-99m ReFib was biexponential. The half-life of the second components determined by serial images was about 5.2 hr. The Tc-99m ReFib was slowly cleared from the blood into the liver and kidneys after intravenous administration. Technetium-99m activity then moved from the liver into the gallbladder or gut. The image of the abscess became visible in 5 hr and was very clearly visualized at 10–20 hr (Fig. 5, A). The thyroid gland and stomach were not visualized at any time.

The Ehrlich tumor was visualized 10 hr after the administration of Tc-99m ReFib (Fig. 5, B). This image was obscure in comparison with the abscess.

The biodistribution of Tc-99m ReFib was evaluated in the abscess or tumor bearing mice at 1 hr and 5 hr post-injection, respectively. The percentage of the injected dose per organ (% ID/organ) or gram (% ID/g) determined in the abscess or tumor and in all major organs is shown in Table 1 and Table 2. The activity in the liver cleared from 22.82 ± 0.68% at 1 hr to 15.59 ± 2.75% at 5 hr for

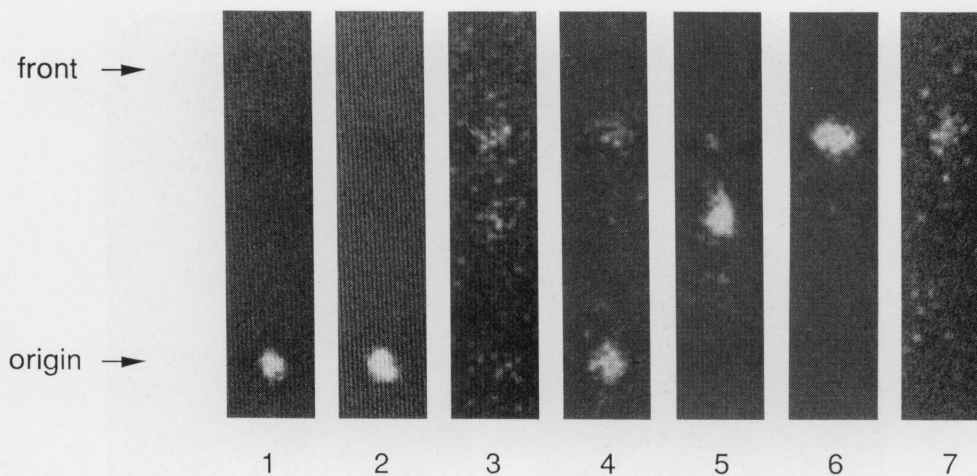


Fig. 3 Thin layer chromatograms of Tc-99m compounds. 1: Tc-99m UnFib solution; 2: Tc-99m ReFib solution; 3: Cysteine solution was added to Tc-99m UnFib solution; 4: Cysteine solution was added to Tc-99m ReFib solution; 5: Tc-99m Cysteinate solution; 6: Tc-99m Cystinate solution; 7: Urine sample collected after administration of Tc-99m UnFib solution.

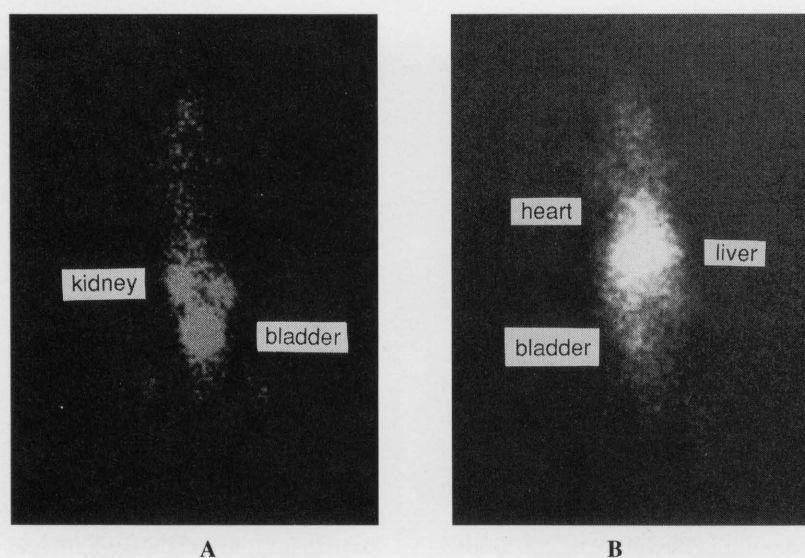


Fig. 4 Scintigrams of abscess-bearing mice 30 min after injection of Tc-99m UnFib (A) or Tc-99m ReFib (B).

Table 2 Biodistribution data of Tc-99m ReFib in mice bearing Ehrlich tumor*

Organ	1 hr	5 hr
Salivary gland	0.21 ± 0.04	0.15 ± 0.07
Spleen	1.56 ± 0.41	0.84 ± 0.15
Stomach	0.57 ± 0.06	0.44 ± 0.07
Intestine	4.27 ± 0.66	9.82 ± 0.63
Liver	21.17 ± 1.85	14.65 ± 1.69
Kidney	10.51 ± 1.23	6.37 ± 1.12
Bladder and Urine	8.29 ± 1.86	23.45 ± 2.49
Muscle 1 g	0.69 ± 0.13	0.49 ± 0.05
Ehrlich tumor 1 g	0.70 ± 0.14	0.68 ± 0.13
Blood 1 g	23.00 ± 1.68	13.01 ± 1.26

*: Expressed as percentage of injected dose per organ. Each value is mean ± s.d. for four mice.

Tc-99m ReFib. The % ID/organ in the bowel (small and large intestine) increased from $4.61 \pm 0.73\%$ at 1 hr to $10.57 \pm 0.47\%$ at 5 hr for Tc-99m ReFib. The uptakes in the abscess slightly increased over time from $1.12 \pm 0.44\%$ at 1 hr to $1.27 \pm 0.30\%$ at 5 hr. These values for abscess accumulation were unsatisfactory for scintigraphic visualization at an early time. The blood clearance of Tc-99m ReFib was slower than that of Tc-99m UnFib; this resulted in a blood retention of $14.31 \pm 2.78\%$ ID/g for Tc-99m ReFib at 5 hr postinjection.

The distribution results for mice bearing an Ehrlich tumor were similar to those for mice bearing an abscess. The tumor uptake of Tc-99m ReFib showed from $0.70 \pm 0.14\%$ at 1 hr to $0.68 \pm 0.13\%$ at 5 hr and these values were relatively low.

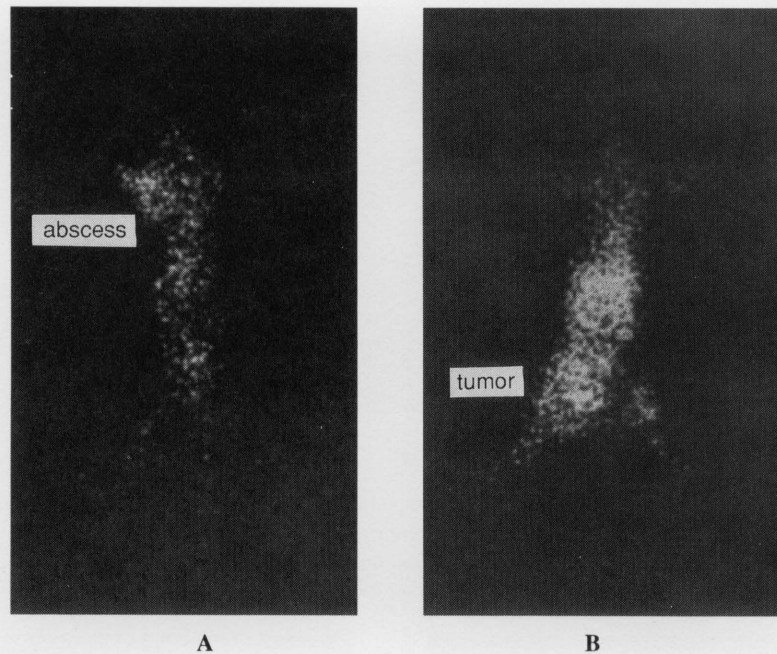


Fig. 5 Scintigrams of mice bearing abscess (A) or Ehrlich tumor (B) 20 hr after injection of Tc-99m ReFib.

DISCUSSION

The present study employed two labeling methods to bind Tc-99m with the substituent amino acid side chains of an unreduced fibrinogen (UnFib) and with the sulfhydryl groups of a reduced fibrinogen (ReFib).

Stannous chloride was the only reducer of Tc-99m pertechnetate in this study, but it did not serve as a ligand between Tc-99m and fibrinogen. In preparations with UnFib, the Sn colloid was not formed. In preparations with ReFib, the stannous ion underwent slight hydrolysis to form an Sn colloid in the absence of 1-hydroxyethane-1,1-diphosphonate as a soluble chelating agent. In this case, the sulfhydryl group in reduced fibrinogen also acted as a reducing agent and so the concentration of stannous chloride was low. Both Tc-99m labeled fibrinogens were stable in neutral saline solution, because other radiochromatographic peaks were undetectable upon standing for 24 hours at room temperature. The low recovery of Tc-99m UnFib during gel chromatography showed that the Tc-99m atom was weakly bonded to the fibrinogen and underwent partial exchange to the gel. During *In vitro* stability studies, Tc-99m UnFib readily produced transchelation by means of the cysteine solution compared to Tc-99m ReFib. High labeling efficiencies in Tc-99m ReFib were achieved by increasing the molar ratio of 2-mercaptoethanol to the fibrinogen. It is probable that the increased numbers of sulfhydryl groups reduced the number of intrinsic disulfide bonds which allowed the fibrinogen to bond with the 2-mercaptoethanol. The molar ratio of 2-mercaptoethanol to fibrinogen of more than 3,000 : 1 seemed to be optimal for the Tc-99m labeling of

fibrinogen. The Tc-99m atom labeled with the sulfhydryl groups was more inert to exchange reactions than that with substituent amino acid side chains.

The radioactivity bound to clottable protein was 70% for Tc-99m UnFib and 69% for Tc-99m ReFib. The clottability of unlabeled fibrinogen was about 74%. The transformation into fibrin did not release Tc-99m from either Tc-99m labeled fibrinogen in the *in vitro* experiment.

To be clinically useful, these preparations must also possess satisfactory *in vivo* characteristics. Technetium-99m should remain firmly bound to the fibrinogen after administration, and the Tc-99m labeled fibrinogen should remain in circulation long enough to be incorporated into the various specific lesions, but be cleared fast enough to minimize the blood background.

Although Tc-99m UnFib was stable *in vitro*, it underwent rapid partial exchange of the Tc-99m with compounds of the blood buffer system *in vivo*, resulting in early excretion. Urinalysis suggested that the radioactivity of Tc-99m may be in a different form from Tc-99m labeled fibrinogen. The chromatographic peak of the excreted radioactivity was the peak due to Tc-99m cystinate. On the other hand, the Tc-99m ReFib reported here was efficient to some extent. Though the transchelation by cysteine solution occurred slowly, *in vivo* experiments on Tc-99m ReFib confirmed its adequate stability as a tracer. The yield and the stability of the labeling, and the reproducibility of this prerelation technique suggested that fibrinogen, after being labeled with Tc-99m according to the proposed method, did not appreciably lose its biologic properties such as clottability *in vivo*, as was

found in the *in vitro* experiments.

Although the specificity of fibrinogen was not as high as the specificity of the antibodies, Tc-99m ReFib may be superior to Tc-99m UnFib for detecting abscesses. Fibrinogen was stuck in an abscess after its transformation into fibrin. Furthermore, the physical properties of Tc-99m are ideal for gamma camera imaging, and clearly delineated images of an abscess can be obtained beginning at about 5 hr after injection. The uptake of Tc-99m ReFib into the Ehrlich tumor was not very high.

These characteristics of Tc-99m labeled fibrinogen prepared with a reduced fibrinogen provide a potential radiopharmaceutical for various specific lesions such as an abscess and inflammation in the surroundings of a tumor. But the abscess to blood and tumor to blood ratios were low at 5 hr, so that the imaging of the abscess and tumor took a long time. Consequently, the short half-life of Tc-99m was inappropriate for fibrinogen with a low pharmacokinetic value.

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