Preparation and Preliminary Evaluation of Technetium-99m-Labeled Fragment E_1 for Thrombus Imaging

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Fragment E_1 labeled with ^{123}I has been previously shown to permit imaging of thrombi in patients within as little as 20 min after injection. Because of the relatively rapid localization and blood disappearance of this protein, ^{99m}Tc would be the most clinically acceptable radionuclide for labeling Fragment E_1. In this study, human fragment E_1 was derivatized with a hydrazino nicotinate function to permit radiolabeling with reduced technetium. The modification reaction was carried out while the fragment E_1 was protected in a complex, so that the modification occurred in nonfunctional regions of the fragment E_1 molecule. After radiolabeling with ^{99m}Tc, the modified fragment E_1 retained its functional activity, as judged by its binding to fragment DD in vitro. The ability of ^{99m}Tc-fragment E_1 to produce images of venous thrombi was demonstrated in animal models. Images were focally positive within 20 min to 1 hr after injection. Thrombus-to-blood ratios exceeded those from ^{125}I-fibrinogen in the same animals. This method of labeling appears to provide an alternative radiolabel to ^{123}I without compromising the function of fragment E_1.

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Fragment E_1 is a plasmic degradation product of human cross-linked fibrin which binds specifically to polymers of fibrin (*1*). Iodine-123 labeled fragment E_1 has previously been shown to permit imaging of thrombi in patients within as little as 20 min after injection (*2*), and its uptake by thrombi was not affected by heparin. A ^{99m}Tc label would be desirable for fragment E_1, but conventional ^{99m}Tc labeling methods which involve reduction of disulfides have resulted in loss of biologic activity, presumably because the functional activity of fragment E_1 requires the unique structure provided by multiple polypeptide chains which are held together by disulfide linkages. Modification reactions on free-fragment E_1 based on reaction with primary amino groups have also been shown to deactivate the protein to thrombus binding (*3*). Therefore, (DD)E (in which fragment E_1's active site is protected) was reacted with a modification reagent for linking reduced Tc, and the complex was then dissociated to yield modified Fragment E_1.

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METHODS

Preparation of Fibrin Fragments

Human cross-linked fibrin was prepared as previously described (*4*). Human (DD)E complex was prepared by suspending one gram of freeze-dried cross-linked fibrin in 20 ml of Tris-HCl buffer, pH 7.4, and digesting it with one unit of plasmin (Kabi Diagnostika, Stockholm). The (DD)E complex was purified from other products by Sepharose CL-6B column chromatography (*1*). Fragment DD for binding assays was prepared by digesting cross-linked fibrin with ten units of plasmin per gram of fibrin, so that in 24 hr the products consisted of fragment DD and fragment E_3 (*4*). Fragment DD was further purified by column chromatography on Sepharose CL-6B. Human fragment E_1 was purified from (DD)E complex by adding glacial acetic acid dropwise to a solution of (DD)E complex with gentle stirring until the concentration of acetic acid had reached 0.55 *M*. Fifteen minutes later, the pH was adjusted to 5.5 by the addition of NaOH. The precipitate was removed by centrifugation, and the supernatant (containing fragment E_1) was concentrated by ultrafiltration.

Modification of Fragments with SHNH

The (DD)E complex was dialyzed into 12.5 m*M* sodium tetraborate buffer, pH 8.5, and then was reacted with a 60-fold excess of SHNH (succinimidyl 4-hydrazino nicotinate hydrochloride [2,5-pyrrolidinedione,1-[(6-hydrazine-3-pyridinyl)carbonyloxy] monohydrochloride], prepared as previously described (*5*) in additional tetraborate buffer, pH 8.5. The reaction mixture was stirred for 3 hr at 4°C. The modified (DD)E was then treated with acetic acid (final concentration 0.55 *M*) followed by partial neutralization to pH 5.5 with NaOH (as described above) to yield modified fragment E_1. The number of hydrazino nicotinate (HN) groups bound to each fragment was determined by a colorimetric assay using p-nitrobenzaldehyde as described by King et al (*6*).

Radiolabeling

Technetium-99m-glucoheptonate was prepared using a commercial kit (DuPont, N. Billerica, MA). Equal volumes of ^{99m}Tc-glucoheptonate (typically prepared at a concentration of 80 mCi/ml) and HN-fragment E_1 (approximately 4-5 mg/ml in pH 5.5 acetate) were combined and incubated for 1 hr at 37°C. The radiochemical purity was assessed by spotting a small aliquot (2 μl) on a strip of instant thin-layer chromatography ITLC-SG media (Gelman, Ann Arbor, MI), and developing it in 0.9% NaCl. The remainder of the labeling mixture was used without further purification. Iodine-125-fibrinogen was prepared by radiolabeling human fibrinogen (Kabi Diagnostika, Stockholm) with Na^{125}I (ICN, Irvine, CA) using the iodine monochloride method (*7*). Iodine-125-fragment E_1 was prepared by labeling purified fragment E_1 with Na^{125}I (ICN, Irvine, CA) using the iodogen method as previously described (*8*).

Stability Testing of the Radiolabel

This study was done to determine whether the technetium bound to the NH-fragment E_1 was labile. Technetium-99m-fragment E_1 (2 mg/ml; 33 μmol) was mixed with an equal volume of buffer (0.05 *M* Tris, 0.1 *M* NaCl, pH 7.4) containing a chelating agent (DTPA, L-cysteine, or sodium diethyldithiocarbamate) at a concentration of 20 m*M*. This represented a 600-fold molar excess of challenge chelator. The mixtures were incubated at 37°C. At 2 hr and 24 hr after mixing, aliquots of the mixtures were analyzed to determine radiochemical purity as described above.

Test for Retention of Fibrin Binding Activity In Vitro

The binding sites for fragment E_1 are found in the paired D domains of fibrin, and the soluble fragment DD contains these binding sites. This assay for binding activity is based on the theory that Fragment E_1 should have a different mobility on gel electrophoresis than would fragment E_1 which has bound to fragment DD, because the (DD)E complex which results would have a higher molecular weight. Tris-glycine polyacrylamide disc gels, 9% crosslinked, were prepared according to the method of Davis (*9*). Technetium-99m-labeled fragment E_1 was loaded onto the gels, either alone or pre-mixed with a tenfold excess of nonlabeled fragment DD. Iodine-125-fragment E_1 was used as a standard for comparison, and was also loaded onto gels, either alone or premixed with nonlabeled fragment DD. After electrophoresis, the gels were sliced into ten equal segments which were counted in a well counter. Functionally active radiolabeled fragment E_1 was expected to appear in slices 3–8 on gels where fragment E_1 was loaded alone, and would be expected to shift to slices 1–2 (top of the gel) upon binding to fragment DD and forming the larger (DD)E complex. The patterns of counts in the gels were analyzed as previously described (*3*) to yield an estimate of the percent of radiolabeled fragment E_1 which was able to bind to fragment DD.

Thrombus Imaging Studies In Vivo

The ability of the ^{99m}Tc-fragment E_1 to image thrombi was assessed in animal models. These studies were reviewed and approved by the Institutional Animal Care and Use Committee before the work was begun.

A model of fresh thrombi was created in nine rabbits by a method similar to that of Collen (*10*). New Zealand white rabbits weighing 1.8–3.2 kg were preanesthetized by intramuscular injection of 40 mg/kg ketamine hydrochloride and 0.4 mg/kg acepromazine maleate. For surgery, sodium pentobarbital (10 mg/kg) was administered intravenously. Additional doses of these drugs were administered as needed to maintain anesthesia. The surgical procedure described by Collen was used, except that a silk thread was used to anchor the clot, and the vein was not cannulated nor was the blood removed from the vein. Ten units of bovine thrombin (Parke-Davis) in 0.1 ml of saline were injected into the blood in the isolated segment of vein using a 27-g needle. After 30 min, the clamps were removed from the vein and the incision was sewn closed. Within 30 min after closing the wound, 1–3 mCi ^{99m}Tc-fragment E_1 was administered through a butterfly infusion set placed in a marginal ear vein on the contralateral side. As a positive control in each animal, 25–50 μCi ^{125}I-fibrinogen were also injected in the same site. Isotonic saline was used to flush the butterfly after injecting each tracer.

Thrombi were induced in five mongrel dogs (11–17 kg) by transcatheter placement of embolization coils in their femoral veins, as previously described (*11*). Multiple coils were placed in some animals. A radiograph without contrast was obtained to document the location of each coil after placement. The thrombi were allowed to age on the coils for 24 hr before administration of the radiotracers. Then 3–6 mCi ^{99m}Tc-fragment E_1 and 40–70 μCi ^{125}I-fibrinogen were injected into a foreleg vein and were flushed in with isotonic saline.

Before injection, each animal was positioned for an anterior view of the chest, using a large field of view gamma camera (General Electric, Milwaukee, WI). The camera was fitted with a low-energy all-purpose collimator and was set to acquire the 140 keV photopeak of ^{99m}Tc with a 20% window. A Macintosh IIx computer was interfaced to the camera using a NucLear Mac A/D board and software (Scientific Imaging, Denver, CO). Initially, the computer was set to acquire a dynamic series of 10-sec frames for a total of 10 min in a 128 × 128 byte mode matrix. The acquisition was begun just before injection of the radiotracers. At hourly intervals, additional 10-sec static anterior views of the chest were acquired. These static images, in addition to the dynamic series, were used to estimate the rate of blood disappearance. In each frame, a region of interest was drawn around the heart. The counts in the region of the heart were decay-corrected and expressed as a percentage of the maximum counts in the heart region. A curve of the form $y = a^*e^{-bt} + c^*e^{-dh} + f^*e^{-gt}$ was fit to the data using a nonlinear curve-fitting program (LabView 2, National Instruments, Austin, TX).

Immediately after completion of the initial dynamic acquisition, and at approximately hourly intervals thereafter, the rabbits were repositioned to obtain anterior views of the head and neck, and the dogs were repositioned to obtain anterior view of both hind legs. Static images were acquired in a 256 × 256 byte mode matrix and 500,000 counts were accumulated in each image.

As a negative control, ^{99m}Tc-glucoheptonate was administered in place of the ^{99m}Tc-fragment E_1 in three additional rabbits and two additional dogs with induced thrombi. The rest of the experiment was carried out in the same way.

At 4 hr postinjection of the radiotracers, a blood sample was drawn and animals were euthanized with a bolus intravenous injection of T-61 euthanasia solution (American Hoechst, Animal Health Division, Somerville, NJ). The vein segment containing the thrombus was removed and the thrombus was separated from vessel wall and coils (dogs) or threads (rabbits). Samples of control vessel (uninjured vein from a comparable site on the contralateral side) and skeletal muscle were also taken. All samples were

TABLE 1
Stability in vitro

	Radiochemical purity (%) of ^{99m}Tc-fragment E_1 after incubation at 37°C in buffer† containing:			
	Control	Cysteine	DTPA	DDC*
2 hr	96.6	94.6	96.5	97.3
24 hr	94.0	92.9	96.4	98.3

Control = no chelating agent added.
* DDC = diethyldithiocarbamate.
† 0.05 *M* Tris-HCl, 0.1 *M* NaCl, pH 7.4.

weighed and counted for ^{99m}Tc content in a NaI(Tl) well counter (Searle, Des Plaines, IL). A saved aliquot of the injected dose was diluted and a portion was counted with the tissue specimens in order to relate the counts in each specimen to the injected dose. Samples were saved and re-counted 1 wk later (after ^{99m}Tc had decayed) to determine ^{125}I content of the samples.

Thrombus uptake indices, such as the percent injected dose per gram of thrombus, thrombus-to-blood ratio and thrombus-to-muscle ratio, were calculated from the count data. These quantities were compared for the different tracers using one-way analysis of variance (ANOVA).

RESULTS

Modification and Radiolabeling

After modification and dissociation of the (DD)E complex, approximately 0.7 HN groups were found to be attached to each fragment E_1 molecule. This material migrated as a single band on polyacrylamide gel electrophoresis. Radiolabeling by adding an equal volume of ^{99m}Tc-GHA consistently resulted in greater than 90% incorporation of ^{99m}Tc by fragment E_1 within 1 hr.

Stability of Radiolabel

Table 1 shows the radiochemical purity of samples of ^{99m}Tc fragment E_1 after incubation with an excess of chelating agents. There was no appreciable loss of technetium to any of the chelators, nor was there appreciable breakdown to free pertechnetate, as the radiochemical purity remained in excess of 92% in all samples after 24 hr incubation.

Retention of Functional Activity

Figure 1 shows the distribution of radioactivity on a polyacrylamide gel following electrophoresis of ^{99m}Tc fragment E_1 which was prepared by reacting SHNH with the (DD)E complex. Eighty-three percent of the ^{99m}Tc fragment E_1 was able to bind to fragment DD (its fibrin binding site) compared with 81% of conventionally prepared ^{125}I-fragment E_1. If SHNH was reacted with purified fragment E_1 instead of with the (DD)E complex, only 23% of the resulting ^{99m}Tc labeled fragment E_1 was able to bind to fragment DD (data not shown).

Imaging of Thrombi in Animal Models

Images of fresh thrombi in rabbit jugular veins were focally positive within 20 min and improved further over 4 hr (Fig. 2). Images of 1-day-old thrombi in the femoral veins of dogs were also focally positive in early images (Fig. 3). Blood clearance was fairly rapid, with approximately 20% of the injected dose remaining in the blood at 30 min (Fig. 4). The blood disappearance kinetics visually approximated three components, and the nonlinear fitting program found the following parameters for the components: 50.7% cleared with a half-time of 0.25 min, 40% cleared with a half-time of 13.1 min and a 9.3% cleared with a half-time of 11.6 hr.

The results of ex vivo counting of the thrombus and other specimens are shown in Tables 2 and 3. Fibrinogen had a higher percent injected dose per gram of thrombus than fragment E_1; however, fragment E_1 thrombus-to-blood ratios were higher than fibrinogen in both rabbits and dogs. Fragment E_1 thrombus-to-muscle ratios were also higher than fibrinogen in dogs. Uptake of fragment E_1 per gram of thrombus was about ten times the uptake of glucoheptonate in rabbits, and about 36 times the uptake in dogs.

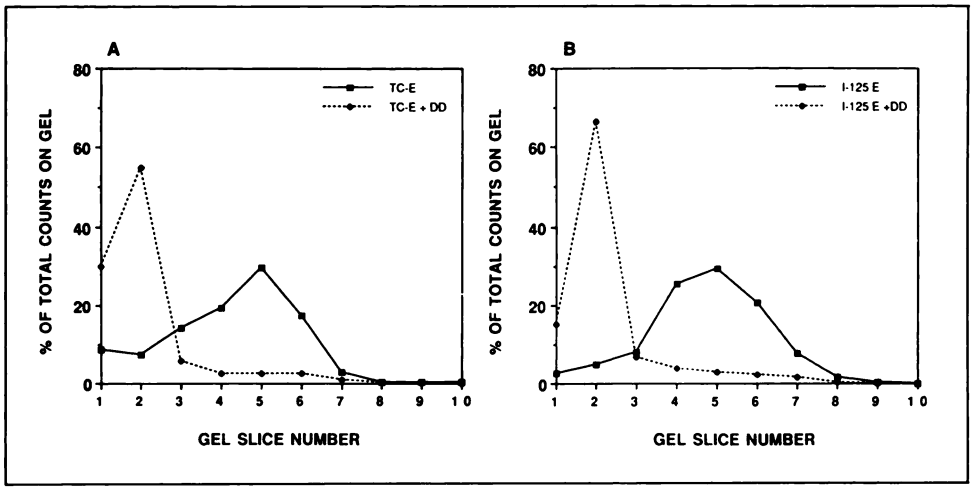

FIGURE 1. Polyacrylamide gel electrophoresis of radiolabeled fragment E_1 alone (solid lines) or premixed with a ten-fold excess of fragment DD (dashed lines). Each gel was sliced into ten segments. The counts in each slice were expressed as a percentage of the total counts on the gel. (A) Technetium-99m-fragment E_1 and (B) ^{125}I-fragment E_1. In both cases, radiolabeled fragment E_1 was able to bind to fragment DD almost quantitatively, as evidenced by the peak shift to lower slice numbers.

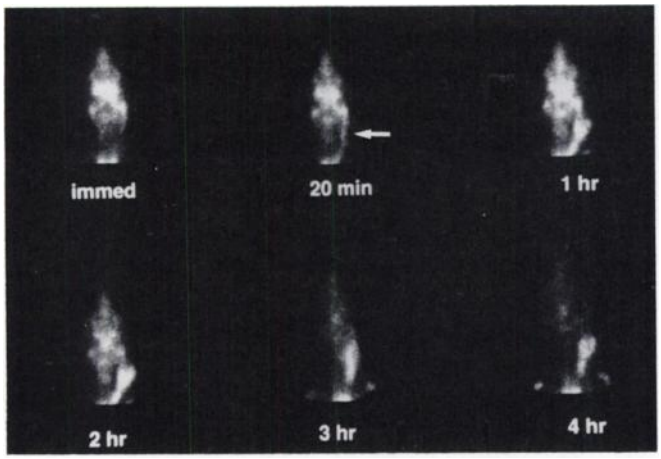

FIGURE 2. Gamma camera images of a rabbit with a thrombus induced in a jugular vein (arrow) at various times following injection of ^{99m}Tc-fragment E_1.

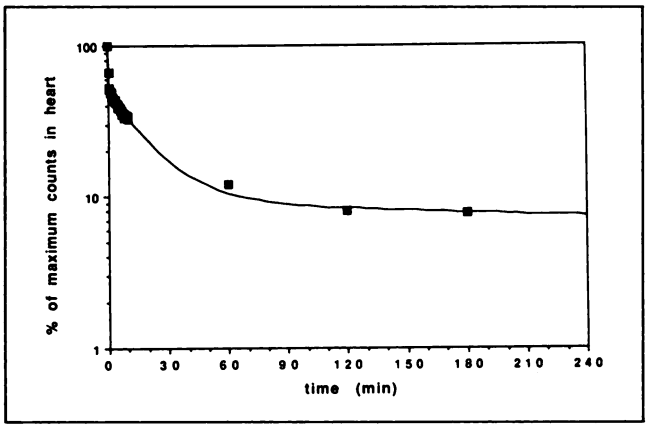

FIGURE 4. Blood disappearance of ^{99m}Tc activity following injection of ^{99m}Tc-fragment E_1 in a dog.

DISCUSSION

A radionuclide test for imaging of pre-existing venous thrombosis should be capable of producing images of diagnostic quality within a few hours after injection. This necessitates adequate binding to the thrombus, coupled with rapid clearance of unbound radiotracer from the blood and soft-tissue background. It has previously been shown that proteins of medium size such as fragments of monoclonal antibodies to fibrin and fragment E_1 have the required affinity for thrombi as well as moderately rapid clearance from the blood (*8,11–13*). For these proteins, ^{99m}Tc is the radiolabel of choice for clinical acceptability.

These studies demonstrated that fragment E_1 can be labeled with ^{99m}Tc without significant loss of functional activity, provided the labeling is site-directed to a region of the molecule which is not critical for binding. These results are in agreement with previous work which showed that protection of the active region of fragment E_1 during reaction with the cyclic anhydride of DTPA was essential for preservation of activity (*3*). Both SHNH and the cyclic anhydride of DTPA react with primary amino groups, which are found not only in mid-chain lysines, but also on the amino termini of all six polypeptide chains of fragment E_1 (*14*). The amino termini of at least two of the chains are believed to be critical for binding activity (*14*). Fortunately, this region of the molecule is protected in the (DD)E complex and Fragment E_1 can be easily separated from the (DD)E complex after attaching the linker groups.

In addition to offering site-specific radiolabeling with ^{99m}Tc, this radiolabeling method avoids reduction of technetium in the presence of the protein. In a protein such as fragment E_1, whose structure depends on multiple disulfide bridges, it is important to avoid reduction steps that would cleave disulfides and thus alter the structure and possibly the binding affinity of the protein. For the above reasons, other techniques that have been developed for radiolabeling proteins with ^{99m}Tc, such as reduction and direct labeling of intrinsic disulfides (*15*) or attachment of pre-formed ^{99m}Tc complexes to protein amine groups (*16*), would not be suitable for labeling fragment E_1.

The hydrazino nicotinate linker provides a stable method for attaching technetium to the protein. In this report, we demonstrated that challenge chelators did not remove ^{99m}Tc from labeled fragment E_1. This linker has previously been shown to provide a stable technetium label for polyclonal IgG (*5*).

Most studies of thrombus-imaging radiopharmaceuticals, including our previous work with fragment E_1 (*3,8*) employed in vitro clot-binding assays to evaluate the potential for binding to thrombi in vivo. In vitro clot-binding assays, however, were not done as a part of this report. Instead, retention of binding affinity was assessed by the ability of radiolabeled fragment E_1 to form a complex with fragment DD, its complementary binding site in fibrin. This technique was felt to be more specific than a test of binding to clots in vitro, as it avoids problems of nonspecific entrapment and variability among blood samples. In our past work, in vitro assays using pre-formed clots have

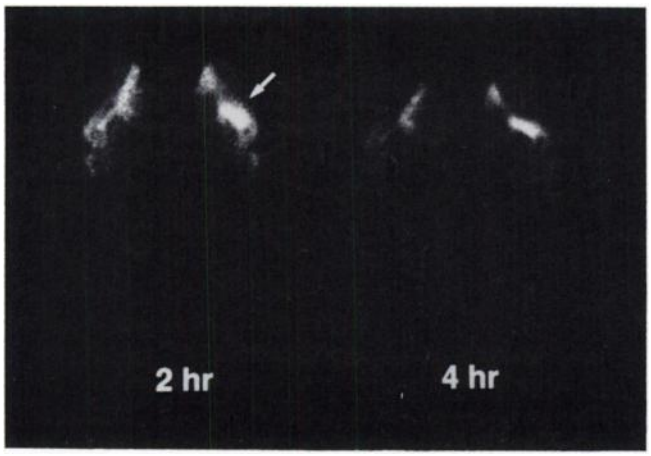

FIGURE 3. Gamma camera images of a dog with a thrombus induced in a femoral vein (arrow) at various times following injection of ^{99m}Tc-fragment E_1.

TABLE 2
Uptake of Radiotracers by Thrombi in Rabbits at 4 Hours Postinjection*

Tracer	%ID/g	Thrombus:Blood	Thrombus:Muscle
^{99m}Tc-fragment E_1	0.301 ± 0.087	7.9 ± 2.0†	218 ± 69
^{125}I-fibrinogen	2.42 ± 0.802	3.1 ± 0.6	234 ± 55
^{99m}Tc-glucoheptonate	0.019 ± 0.008	2.1 ± 0.6	32 ± 16

* Mean ± s.e.
† Significant at 95% level vs. fibrinogen and glucoheptonate.

served only as preliminary indicators of the potential for thrombus uptake in vivo, because many additional factors can affect the ability of a radiotracer to image thrombi in vivo. Thus, the assessment of the ability of ^{99m}Tc-HN-fragment E_1 to bind to a fibrin surface was done in vivo rather than with pre-formed clots in vitro.

The blood disappearance curves obtained with ^{99m}Tc-HN-fragment E_1 were almost superimposable on blood disappearance curves obtained previously with radioiodinated fragment E_1 (*2*). This indicates that modification with the hydrazino nicotinate linker and subsequent radiolabeling did not significantly alter the fragment E_1. Blood clearance curves have been shown to be sensitive indicators of subtle alteration of protein structure (*17*).

Technetium-99m-HN-fragment E_1 retained its ability to bind to thrombi in vivo and to permit rapid imaging of the thrombi over the background. Fragment E_1 thrombus-to-muscle ratios in dogs and thrombus-to-blood ratios in both species were more than adequate for a clearly positive image at 4 hr and were better than fibrinogen, on average. This is in general agreement with previous animal studies with radioiodinated fragment E_1 (*8*). Thrombus-to-blood ratios for fragment E_1 in this study at 4 hr postinjection were not as high as those found in the pig model, perhaps owing to the different thrombogenic characteristics of that model and the later sampling time in the pig study (24 hr postinjection).

In these studies, we used labeled fibrinogen as a positive control compound, because its thrombus imaging characteristics are well documented. It has been shown that ^{123}I-fibrinogen is taken up avidly in actively forming thrombi and can permit imaging of such thrombi within 6 hr (*18*). For a thrombus-imaging radiotracer to be useful, it must produce thrombus-to-background ratios at least as high as iodinated fibrinogen produces. The high thrombus-to-blood ratios for fibrinogen in the models employed in this study indicate that the thrombi in this study should be considered fresh. The uptake of fibrinogen has been shown to decrease with thrombus age (*8,19*). We have previously shown that uptake of fragment E_1 was high in thrombi up to 5-days-old, in which fibrinogen uptake was very low (*8*). In addition, our clinical studies with ^{123}I-fragment E_1 have indicated that thrombi several days old can be readily imaged in patients, even during heparin therapy (*2,20*).

A number of other radiotracers have been evaluated as agents for imaging vascular thrombi. The properties of these compounds have been recently reviewed (*13,21,22*). Until new compounds actually reach clinical trials, it is often difficult to assess the potential of each tracer because of the inadequacy of animal models of thrombosis. Most new thrombus tracers are evaluated in fresh thrombi which are actively depositing blood elements, although thrombi in most patients will be several days old at the time of injection of a thrombus imaging radiopharmaceutical. In the case of ^{99m}Tc-HN-fragment E_1, success in the clinical situation is predicted because radioiodinated fragment E_1 has been previously shown to produce images of clinical thrombi, ^{99m}Tc-HN-fragment E_1 appeared to retain the properties of the iodinated protein, and it achieved thrombus-to-blood and thrombus-to-muscle ratios better than fibrinogen in the dog model.

The percent of injected fragment E_1 that binds per gram of thrombus was two to three times as high as the binding of ^{99m}Tc-labeled antifibrin T2G1s monoclonal antibody Fab′ fragments in the same dog model (*11*). Initial clinical trials with ^{99m}Tc-antifibrin have been moderately encouraging (*23,24*), so one might expect ^{99m}Tc-fragment E_1 to be even more successful. Fragment E_1 also has the potential advantage of more rapid blood disappearance; in this study, approximately 8% of the injected dose remained in

TABLE 3
Uptake of Radiotracers by Thrombi in Dogs at 4 Hours Postinjection*

Tracer	%ID/g	Thrombus:Blood	Thrombus:Muscle
^{99m}Tc-fragment E_1	0.256 ± 0.115	15.7 ± 8.0	904 ± 576
^{125}I-fibrinogen	0.379 ± 0.120	4.8 ± 1.4	88 ± 19
^{99m}Tc-glucoheptonate	0.007 ± 0.004	2.6 ± 1.1	8 ± 6

* Mean ± s.e.

the blood at 4 hr, compared with 24% for T2G1s Fab' (*11*).

This method of labeling with ^{99m}Tc appears to provide an alternative radiolabel to ^{123}I without compromising the function of fragment E_1. We believe it holds promise for rapid imaging of venous thrombi in patients.

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