Radioimmunoimaging of Experimental Thrombi in Dogs Using Technetium-99m-Labeled Monoclonal Antibody Fragments Reactive with Human Platelets

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Monoclonal antibody 50H.19, which reacts with human platelets, was converted to fragments, pretinned, and made into kits for subsequent radiolabeling with ^{99m}Tc. The antibody, which cross-reacts with dog platelets, was used to evaluate in vitro binding to blood clots and in vivo in experimental thrombi in dogs. After radiolabeling, 97.4 ± 6.4% of the ^{99m}Tc was antibody-associated. The preparations retained immunoreactivity, as determined by: (a) binding studies using whole blood and determining the ratio of cell-to-plasma radioactivity (ratios of 57.6–61.2) and (b) binding of the antibody to clots (clot/serum ratios were 57.2–74.6%). Approximately 50% of the radioactivity was cleared from the blood in 3–6 min and 18–24% was excreted in urine within 3 hr. Experimental thrombi in dogs could be visualized consistently within 2–3 hr postinjection in peripheral veins and arteries, pulmonary arteries, and the right ventricle. In addition, damage to blood vessel intima without visible thrombi could also be detected. This method has the following advantages: short and simple pre-imaging preparation, and rapid visualization of thrombi with no need for blood-pool subtraction or delayed imaging.

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Current methods of thrombus detection and localization are neither sensitive nor specific (*1,2*). This problem has been approached using a variety of radiopharmaceuticals some of which remained limited to animal experiments while others were put to use in humans. Among these methods were: Indium-111-(^{111}In) labeled platelets (*3–8*), technetium-99m (^{99m}Tc) plasmin (*9*), iodine-131 (^{131}I) streptokinase (*10*), ^{99m}Tc-labeled red cells (*11*), [^{99m}Tc]macroaggregated albumin (*12*), [^{99m}Tc]heparin (*13*), iodine-125 (^{125}I) fibrinogen (*14*), radioiodinated fibrin fragments (*15*), and ^{111}In-labeled tissue plasminogen activator (*16*). However, only some of these methods have become established in routine diagnostic practice, in large part because of lack of sensitivity and/or specificity and the complexity and lengthy pre-imaging labeling requirements, as in the case of direct radiolabeling of platelets. Moreover, the slow blood clearance of the radiolabeled agents from the circulation causes low thrombus/background ratios which necessitate blood-pool subtraction, delayed imaging, or the use of an additional injection of an antibody (*17*).

An alternative method of thrombus detection by using a radiolabeled monoclonal antiplatelet antibody has been investigated by Oster et al. (*18*) and also by Som et al., in a preliminary report (*19*). In the studies performed by Oster et al., a murine monoclonal antibody which reacts with the platelet membrane glycoprotein IIB/IIIA was used (*20*). This antibody labeled by either ^{111}In or ^{123}I and used in a dog model enabled imaging of thrombi within 3 hr after injection without the need of blood-pool subtraction or delayed imaging. Since this antibody inhibits platelet adhesion (*20*), the amount of antibody injected and needed to obtain an optimal image without compromising the localizing ability of the tagged platelets is critical. The preliminary

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results reported by Som (*19*) and the extension of these studies reported here, involved the use of a different monoclonal antibody preparation, a murine monoclonal antibody fragment mixture reactive with platelets and provided in kit form for labeling with ^{99m}Tc (*19*).

The antibody and its F(ab')$_2$ fragments, but not other components in the kit used in the present study (primarily Fab' fragments), were found to induce platelet activation and aggregation. The biochemistry and physiology of the antibody characteristics and its interaction with platelets will be described separately.

MATERIALS AND METHODS

The murine hybridoma secreting the antibody 50H.19 was prepared using the human melanoma cell line MEL-T as the immunogen (*21*). A detailed description of the antibody reactivity with platelets will be described elsewhere (Zamora et al; work in progress), but can be summarized as follows: The antibody recognizes one low molecular weight tumor cell antigen (*22,23*) and also cross-reacts with human platelets recognizing three proteinase-sensitive, low molecular weight platelet antigens. The antibody does not react with red blood cells (types A, B, O), peripheral lymphocytes, fibroblasts (fetal or neonatal), serum or plasma, but does cross-react slightly with endothelial cells as determined by immunohistochemistry, radioimmunoassay and/or enzyme-linked immunosorbant assay. Specific binding to platelet preparations of the intact antibody F(ab')$_2$ fragments and the antibody kits used in this study have been demonstrated by immunohistochemistry and/or radiolabeling with ^{99m}Tc (intact antibody and antibody kits) in contradistinction to control preparations obtained from the cell line MOPC-21 which produces a nonspecific antibody. The immunoreactivity of the antibody kits was assessed using platelet homogenates or human serum albumin (negative control) adsorbed onto Kynar* (*24*). The immunoreactivity was found to range from 40–60% for the intact antibody and the antibody fragment kits. Each of the single-use kits contained 150 μg of lyophylized antibody protein as a mixture of F(ab') (85%) and F(ab')$_2$ (15%). The ratio between the fragments is a direct result of a "pretinning" (*25*) step which chemically reduces the F(ab')$_2$ used in the kit preparation (*24*) and the ratio of two components did not change extensively after ^{99m}Tc-labeling (the Fab' was the primary species radiolabeled). The labeling with ^{99m}Tc was performed by the method described by Rhodes et al. (*25*). In short, 3–30 mCi of ^{99m}Tc was added to pretinned, lyophilized MAb fragment mixture and incubated for 1 hr at room temperature. The mixture was passed through a radiopharmaceutical filter column† to remove the unbound pertechnetate following which the preparation could be used in experiments. The recovery of radioactivity after filtration was measured and thin layer chromatography (TLC) of the eluates was performed using acetone as a solvent.

In Vitro Studies

Cell binding in vitro was determined by adding labeled antibody to blood/ACD-A (acid citrate dextrose) (7:1) and incubating for 1 hr at room temperature. The mixture was centrifuged at 1,900 *g* and the plasma was removed taking care not to remove the buffy coat. Technetium-99m activity in the cellular fraction and in plasma was assessed in a well-type counter. Cell-binding in vivo was determined in blood samples of dogs 1 hr after the injection of [^{99m}Tc]50H.19. Measurements were made in blood samples to which ACD-A (7:1) was added, as well as in blood samples to which 50H.19 was added prior to allowing it to clot. Activity was measured in the washed clot (×2) and in serum to which the washings were added. Blood samples from human volunteers were also assessed in a similar way. Cell binding in blood/ACD-A and binding to in vitro formed clots were assayed for ^{99m}Tc after 1 hr incubation with the [^{99m}Tc]50H.19 using the same technique as for the dog blood samples.

Immunohistochemical staining was performed by placing platelet-rich-plasma aliquots in individual wells of tissue culture cluster plates. After incubating with 50H.19 for 15 min at room temperature, the unbound cells were removed by washing with phosphate buffered saline to which 0.2% bovine serum albumin (BSA) was added. The specimens were fixed in methanol and air dried. For staining, the specimens were rehydrated and incubated in spent tissue culture media from murine hybridomas producing antibodies directed against either platelets, human chorionic gonadotropin (HCG) or colon ovarian tumor antigen (COTA). The anti-HCG and anti-COTA antibodies served as negative controls. The specimens were rinsed and stained with an immunoperoxidase staining kit.‡ Diaminobenzidine was used as the chromogen and osmium tetroxide as a mordant.

Experimental thrombi were induced in 15 dogs by transcatheter placement (under fluoroscopy monitoring) of either copper coils (seven dogs) or aluminum balls (eight dogs). The dimensions of the copper coils were 1.5 × 0.5 mm and diameter of the balls was 3/32 in. The coils or balls were placed in peripheral veins (femoral or jugular) and arteries (femoral or carotid) as well as in segmental pulmonary arteries and the right ventricle. The antibody mixture was injected 1–3 hr after the coil or balls were inserted. Spontaneous thrombi formed on catheters placed for blood sampling and intimal damage occurred at catheter insertion sites. After the injection of ^{99m}Tc-labeled 50H.19 to six normal dogs, blood samples were obtained at fixed intervals

(1, 2, 3, 4, 5, 10, 15, 20, 30, 45, 60, 90, 120, 180, and 240 min) and urine was collected from time 0 to 1 hr, 1 to 2 hr, and 2 to 3 hr after injection. Technetium-99m activity was measured in whole blood, plasma, and in urine and expressed as percent of the injected radioactivity. Imaging was performed immediately after injection and continued for 3.5–4 hr, using a large field-of-view gamma camera.[§] The images were recorded on Polaroid film. No image enhancement or subtraction techniques were used. At the end of the experiment the animals were killed and the presence of thrombi was ascertained by radiography, dissection, and ex-vivo scintigraphy.

Control imaging experiments were carried out in six dogs using ^{99m}Tc-labeled MOPC-21 (a nonspecific IgG_1 monoclonal antibody), in the same conditions as for the 50H.19 experiments.

RESULTS

Following incubation of the pretinned antibody fragments with ^{99m}Tc and passage through the Filtech, 92.8 ± 2.5% of the radioactivity was recovered in the filtrate. Thin layer chromatography of the eluate showed that 97.4 ± 6.4% was bound to protein.

As seen in Table 1, binding to dog blood cells in vitro was 57.6 ± 2.9% and to human blood cells 61.2 ± 2.1%. In vivo blood cell binding, performed in dogs only, was very similar, 56.2 ± 5.4% of the total blood activity. In vivo labeled blood (Table 2) clotted in vitro showed higher activity (69.9 ± 2.7%) than clots labeled in vitro with [^{99m}Tc]50H.19 (57.2 ± 1.9%) as expected, and binding to human in vitro clotted blood was even higher (75.6 ± 5.8%). Phase contrast microscopy of the tissue culture cluster plates on which platelet-rich-plasma was placed revealed that the only cellular material attached to the plates were platelets. Immunohistochemical staining of these preparations (Fig. 1) showed intense accumulation of 50H.19 but no staining in either of the controls.

The clearance studies in dogs show that 50% of the injected radioactivity was cleared from the blood in 3–6 min (Fig. 2) and 18–24% was recovered in urine collected in the first 3 hr after injection (Fig. 3).

Thrombi in peripheral veins (Fig. 4) and arteries (Fig. 5) could be imaged 1 hr after the injection of the [^{99m}Tc] 50H.19. Although visualization of thrombi in the trunk was possible 1 hr after injection, the optimal imaging time seemed to be 2 hr after injection (Figs. 6–8). At this time lung and blood pool activity had decreased significantly. Although liver activity was seen initially it gradually decreased while activity in the spleen persisted. In all 15 dogs studied with 50H.19 there was consistent visualization of thrombi and areas of endothelial damage. Thrombi became evident at 1.5 hr postinjection but the visualization was optimal at 3.5–4.0 hr after injection.

Experimental thrombi could not be visualized with the nonspecific antibody preparation [^{99m}Tc]MOPC-21 under the same experimental conditions. The thrombus to blood ratio at autopsy 3 hr after 50H.19 injection was on the average of 15.

TABLE 1
Technetium-99m 50H.19 Binding to Cellular Blood Fraction

	In vitro		In vivo	
	Cells (%)	Plasma (%)	Cells (%)	Plasma (%)
Dogs*	57.6 ± 2.9	42.5 ± 2.9	56.2 ± 5.4	43.8 ± 5.4
Humans†	61.2 ± 2.1	38.8 ± 2.1	—	—

*n = 4.
†n = 8.
Each point represents mean ± s.d.

TABLE 2
Technetium-99m 50H.19—Clot Binding Studies

	In vitro	In vivo
	Percent binding to clot	Percent binding to clot
Dogs*	57.2 ± 1.9	69.9 ± 2.7
Humans†	74.6 ± 5.8	—

*n = 4.
†n = 8.
Each point represents mean ± s.d.

DISCUSSION

These data clearly demonstrate that labeling of the monoclonal antibody fragments of 50H.19 with ^{99m}Tc

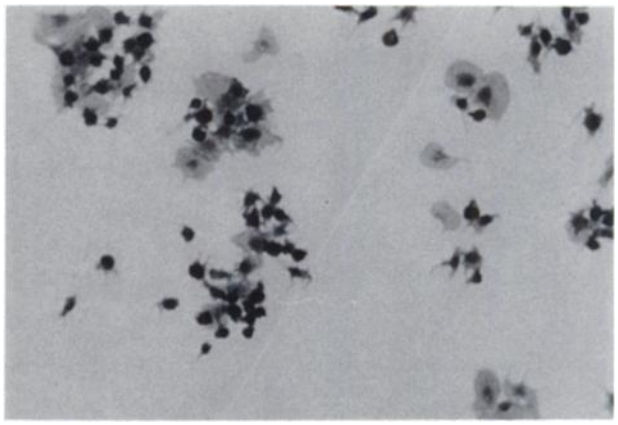

FIGURE 1
Immunohistochemical staining of platelets showing intense binding of ^{99m}Tc-labeled 50H.19

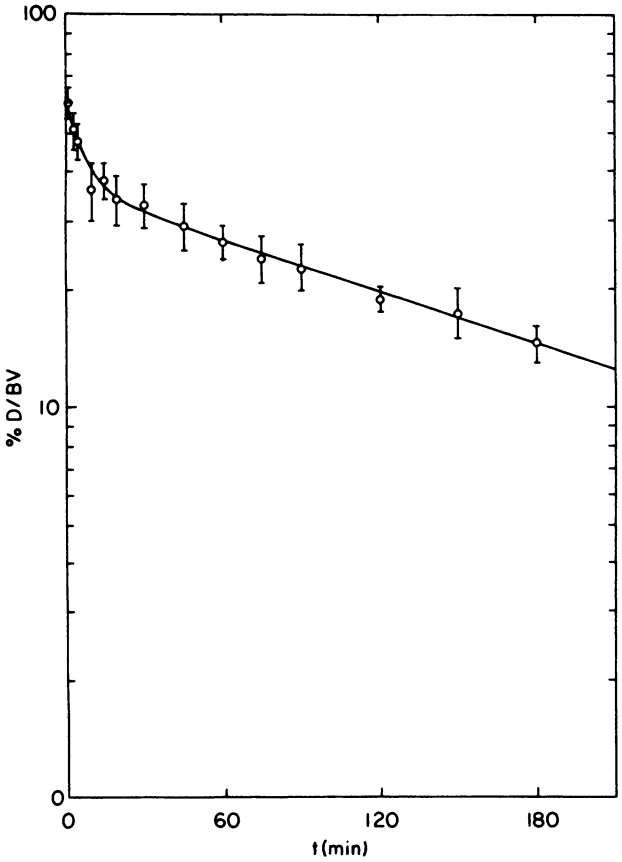

FIGURE 2
Blood disappearance curve (dogs) of ^{99m}Tc-labeled 50H.19. Approximately 50% of injected activity is cleared from blood in 3–6 min

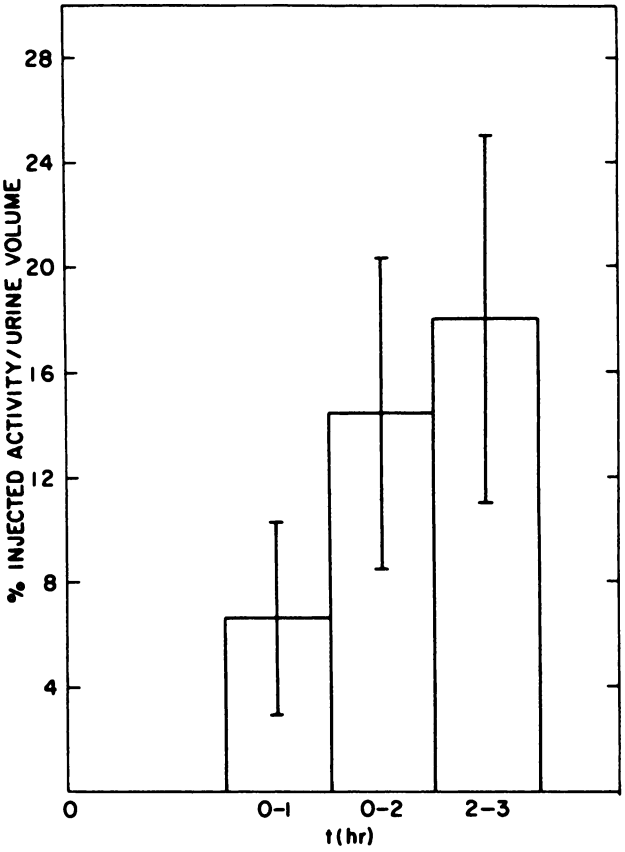

FIGURE 3
Urinary excretion (dogs) after i.v. injection of ^{99m}Tc-labeled 50H.19. Approximately 24% of activity was excreted in urine in 3 hr

is efficient (yield = 97.4%) and that immunoreactivity is preserved during the labeling. The latter was proven by in vitro and in vivo binding of the antibody to platelets and clots as well as by immunohistochemical staining and by antigen binding assays to be reported elsewhere (Zamora et al: work in progress). Imaging of experimental thrombi in peripheral veins and arteries as well as in the trunk could be performed 1–4 hr after injection. Even in the chest, thrombi could be imaged without the need of blood-pool subtraction.

Sites of intimal injury with no visible thrombi at autopsy could also be located. This technique seems to have advantages over the previously published methods (*18*) because of the use of ^{99m}Tc instead of ^{111}In. It is well known that ^{99m}Tc is more suitable for gamma scintigraphy, while ^{111}In has a long half-life for this application, thus increasing the unnecessary radiation dose. The faster blood clearance of the fragments as compared to whole antibody (*18*) is also a favorable characteristic for imaging. Both the antibody and fragments seem to have advantages over ^{111}In-labeled platelets because of the faster blood clearance. In the platelet-antibody complex formed after injecting the antibody, the platelets maintain their function of adherence to damaged endothelium and incorporation into thrombi. In addition, the availability of a two-step (incubation and separation of free TcO_4^-) kit shortens considerably the pre-imaging preparation time and the need of draw-

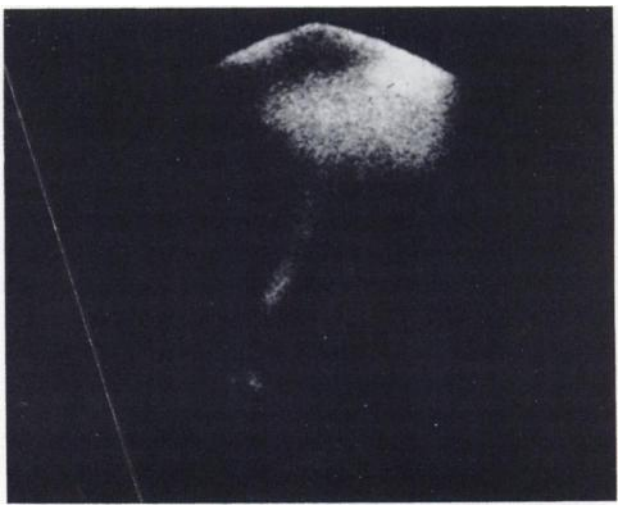

FIGURE 4
Rt. femoral vein thrombus 3 hr postinjection. Thrombus formed spontaneously along indwelling catheter

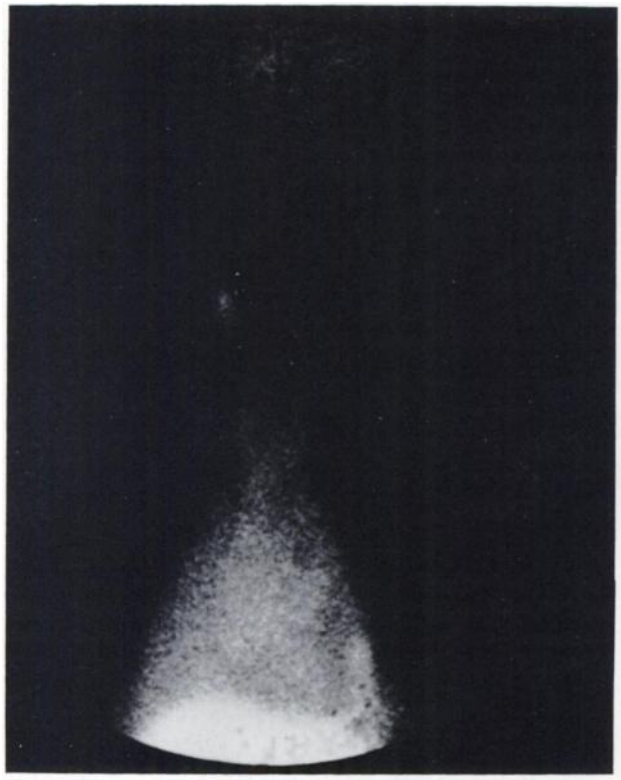

FIGURE 5
Rt. common carotid artery thrombus. Scintigraphy 1 hr postinjection. There still is activity in cardiac blood pool and in lungs

ing 50–80 ml of homologous blood. With the simpler method the risk of platelet damage and contamination is also avoided as is the need of skilled personnel for carrying out the labeling.

The low radioactivity in the cardiac blood pool in the early period is of considerable importance. This suggests that intracoronary thrombi might be visualized by this method while therapy can still be effective.

The radioactivity in the plasma represents that fraction of the preparation which is not immunoreactive and does not bind to the platelets. This fraction, however, is cleared rapidly (within 10 min) from the circulation and does not seem to interfere with imaging.

We are, therefore, of the opinion that this is a very

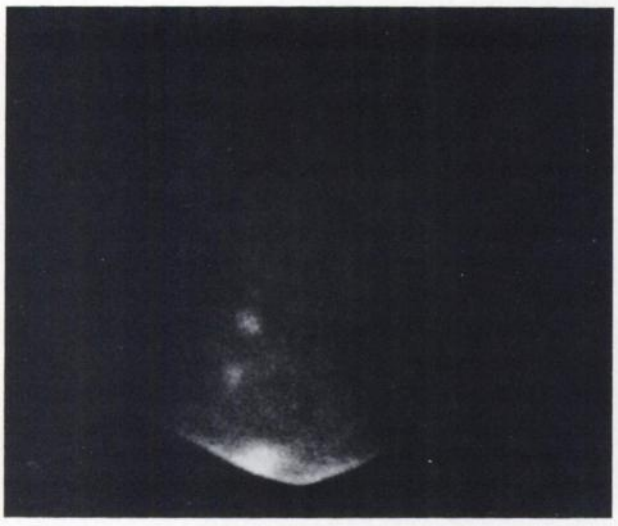

FIGURE 6
Two thrombi in rt. segmental pulmonary arteries 4 hr after injection (anterior view). Focal tissue activity in lower right corner is gallbladder

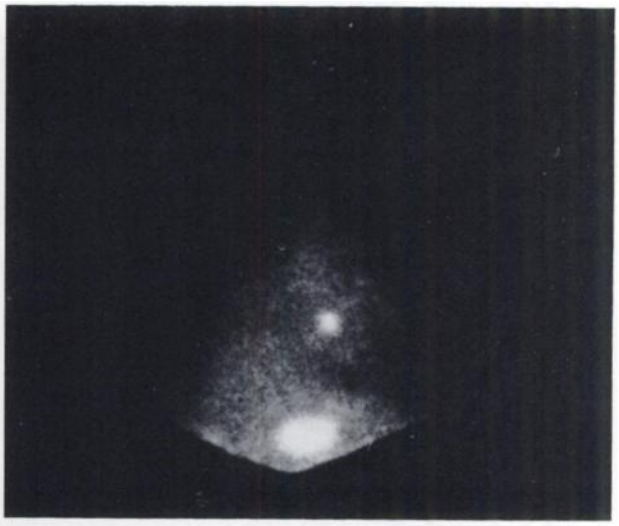

FIGURE 7
Thrombus in rt. segmental pulmonary artery 2 hr after injection (rt. lateral view)

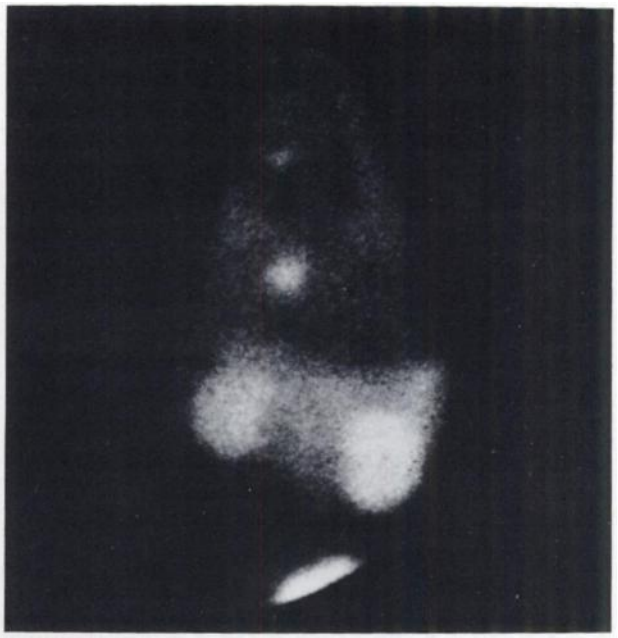

FIGURE 8
Thrombus in rt. segmental pulmonary artery 2 hr after injection (unmagnified view). Notice intense activity in thrombus, cardiac area has less activity than lungs. Liver is not visualized, but spleen, kidneys, and top of bladder have intensive activity

promising technique for thrombus detection and clinical trials in humans are justified.

FOOTNOTES

* Penwalt Corp., (Polyvinylidene fluoride, grade 301F), King of Prussia, PA (fluoride, grade 301F).

† Filtech, Summa Medical Corporation, Albuquerque, NM.

‡ Vector, Burlingame, CA.

§ Ohio Nuclear, Solon, OH.

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