Technetium-99m-White Blood Cell-Specific Imaging Agent Developed from Platelet Factor 4 to Detect Infection

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We have developed a leukocyte-avid, ^{99m}Tc-labeled peptide (P483H) as a potential imaging agent for infection. P483H contains the heparin-binding region of platelet factor-4 (PF-4) and a lysine-rich sequence for rapid renal clearance. Technetium-99m-P483H was evaluated for its ability to selectively label white blood cells (WBCs) in vitro and to detect focal *E. coli* infections in rabbits. **Methods:** Technetium-99m-P483H was incubated with citrated whole human blood, layered onto WBC isolation media and subjected to density gradient centrifugation to measure WBC-associated radioactivity. Indium-111-WBCs and ^{99m}Tc-gluceptate were used as controls. In the in vivo model, *E. coli* infected rabbits were imaged and necropsied 4 hr after administration of ^{99m}Tc-P483H. Infected and contralateral control muscles were evaluated for %ID, %ID/g, I_{max} (muscle sample showing the highest uptake, i.e., %ID/g) and I_{max}-to-blood and I_{max}-to-control muscle ratios. Indium-111-WBCs, ^{111}In-DTPA, ^{131}I-albumin (HSA), ^{99m}Tc-nanocolloid, ^{67}Ga and ^{99m}Tc-gluceptate were evaluated as in vivo controls. **Results:** Technetium-99m-P483H associated predominantly with WBCs in vitro, and ^{99m}Tc-P483H provided high contrast images of infection in vivo. In vitro, 73% of ^{99m}Tc-P483H radioactivity was associated with WBCs. Technetium-99m-P483H outperformed ^{111}In-WBCs, ^{111}In-DTPA, ^{131}I-albumin, ^{99m}Tc-nanocolloid, ^{67}Ga-citrate and ^{99m}Tc-gluceptate with an infection I_{max} average of 0.062 %ID/g (±0.029; n = 48). Technetium-99m-P483H also outperformed all controls, including ^{111}In-WBCs, ^{111}In-DTPA, ^{131}I-albumin, ^{99m}Tc-nanocolloid, ^{67}Ga-citrate and ^{99m}Tc-gluceptate. The I_{max}-to-blood and I_{max}-to-control muscle ratios for ^{99m}Tc-P483H averaged 3.1 (±2.4) and 26.8 (±16.8), respectively, and again outperformed all controls. **Conclusion:** Technetium-99m-P483H associates predominantly with WBCs in vitro and identified focal infections in vivo within 4 hr versus conventional imaging agents. Additionally, the agent showed rapid blood clearance and exclusive renal excretion, which provides a clear abdominal field for imaging abdominal infections.

Key Words: technetium-99m-P483H; leukocytes; infection imaging platelet factor 4

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White blood cells (WBCs), particularly polymorphonuclear leukocytes (PMNLs) and monocytes, accumulate in high concentrations at sites of infection (*1*). A small ^{99m}Tc-labeled peptide capable of binding to leukocytes in vivo would thus represent a significant clinical breakthrough (*2,3*).

Radiodiagnostics currently used in the clinic to detect infection by imaging include ^{111}In-WBCs, ^{67}Ga, ^{131}I-albumin, ^{111}In-DTPA, ^{99m}Tc-gluceptate, ^{99m}Tc-nanocolloid and new monoclonal and polyclonal IgG technologies have been added. The standard clinical procedure is one of tagging WBCs extracorporeally with isotopes, such as ^{111}In using tropolone or oxine (*2–4*). The methodology enabling WBC labeling with ^{99m}Tc has been developed more recently (*4–6*). Both the ^{99m}Tc and ^{111}In procedures involve harvesting, isolating and radiolabeling of WBCs. This process is time-consuming, expensive and carries substantial contamination risks. Moreover, due to WBC retention in the blood, optimal imaging target-to-background ratios often require diagnostic periods of up to 24 hr after injection. These drawbacks thus limit the usefulness of ^{111}In-WBCs in patients with acute conditions.

Each of the other infection imaging agents likewise has significant drawbacks, such as nonspecific uptake, poor target-to-background ratios at early time points and high intestinal uptake. Thus, there still is a need for an in vivo method of labeling WBCs with ^{99m}Tc to produce rapid diagnostic images of occult infection with minimal abdominal interference (*3,7,8*).

Platelet factor-4 (PF-4) is a 29-kDa homotetrameric protein released from the alpha granules of platelets during activation (*9–11*). PF-4 has been called the "body's heparin neutralizing agent." During the course of our preclinical evaluation of PF-4 analogs, we observed that the infection-imaging properties of specific analogs are significantly enhanced once heparin is added. We therefore postulate that PF-4-heparin complexes (PHCs) may have a role in the infection process in that they attach PHCs to circulating and infection-modulating WBCs and possibly modulate WBC function.

We have identified several ^{99m}Tc-labeled PF-4 analogs, particularly those complexed with heparin, which are leukocyte-avid and appear to be capable of tagging WBCs in vivo. One PF-4 analog complexed with heparin, P483H, is a 23 amino acid peptide containing the heparin-binding sequence of PF-4 and can be labeled with ^{99m}Tc. The diagnostic potential of P483H is optimal when P483 is combined with unfractionated heparin in a specific molar ratio.

Although the exact mechanism of action of ^{99m}Tc-P483H in identifying areas of infection is unknown, we postulate that PF-4:heparin complexes, such as ^{99m}Tc-P483H, are bound by circulating leukocytes and carried to the site of infection. Another mechanism, which is not mutually exclusive with the one above, involves attachment of ^{99m}Tc-P483H to WBCs already resident at the infection site.

The purpose of this study was to evaluate ^{99m}Tc-P483H against other clinically useful infection imaging agents for its ability to label WBCs in vitro and to compare the in vivo biodistributions and infection imaging parameters (%ID/g in blood, normal muscle and infected muscle) at a relatively early time point (4 hr) after injection using a rabbit infection model.

MATERIALS AND METHODS

Chemical Structures

Peptide P483 (Acetyl-Lys-Lys-Lys-Lys-Lys-Cys-Gly-Cys-Gly-Gly-Pro-Leu-Tyr-Lys-Lys-Ile-Ile-Lys-Lys-Leu-Leu-Glu-Ser) is an

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analog of the C-terminus of PF-4. In addition, P483 has five lysines (Lys_5) placed on the N-terminus. P483 trifluoroacetate (TFA) salt was provided as a lyophilized powder for these studies.

Solid-Phase Peptide Synthesis

P483 was synthesized by solid-phase techniques (*12*) with a synthesizer using N-α-Fmoc protection, HBTU coupling and hydroxymethyl polystyrene resin. Commercially available protected amino acids were used.

P483 was purified by preparative HPLC on a reversed-phase column (15 μm, 300Å, 40 or 47 × 300 mm). Purified P483 was lyophilized and the purity and composition confirmed by reversed-phase HPLC and fast atom bombardment or electrospray mass spectrometry.

Peptide-Heparin Complexation (P483H)

When P483 (avg. mol. wt. = 10,000) is combined with unfractionated porcine heparin in a specific molar ratio of 9.4 μg peptide to 1 USP unit of heparin, a stable peptide-glycosaminoglycan complex is formed (peptide-heparin complex, PHC). The stoichiometry was determined by both peptide and heparin analysis. The optimal peptide-to-heparin molar ratio for WBC binding and infection localization in vivo was found to be 6.9. Minimal infection uptake (<0.01 %ID/g) has been observed at ratios less than 3 or greater than 60.

Technetium-99m Labeling of P483H

Ad-lib (Unformulated) Protocol. Approximately 0.5–1.0 mg lyophilized P483 (TFA) salt was diluted to 1 mg/ml in saline. Technetium-99m-labeled glucoheptonate was prepared at >100 mCi/ml with 1.0 ml ^{99m}Tc generator eluate incubated for 15 min at room temperature and added to the peptide solutions at 0.25 ml/ml. Specific activities exceeded 20 mCi/mg of peptide. Quality control of ^{99m}Tc-P483 labeling consisted of thin-layer chromatography on two ITLC-SG strips using two solvent systems: (a) saturated saline (SAS; ^{99m}Tc-P483, R_f = 0.0–0.7; [^{99m}Tc]pertechnetate and ^{99m}Tc-glucoheptonate, R_f = 0.7–1.0) and (b) pyridine:acetic acid water (PAH; 5:3:1.5; ^{99m}Tc-microcolloid R_f = 0.0–0.2, all other species R_f = 0.2–1.0). The strips were cut and the specific fractions counted. Once the radiolabeled peptide passed the quality control measures, heparin was added at 30 USP units per ml (~38 units/mg peptide). The PHCs were assayed by thin-layer chromatography on solvent saturation pads using an acetonitrile:acetic acid:water (AAW, 1:1:3) solvent system and cutting at an R_f of 0.5. PHCs remain at the origin (R_f: 0.0–0.5). Technetium-99m-P483H as PHCs could not be assayed by HPLC due to physical properties of the peptide-glycosaminoglycan complex.

Formulated Vial Protocol. Formulated vials containing 150 and 250 μg P483 (TFA) salt were prepared with and without added heparin. The formulation was composed of sufficient glucoheptonate, stannous ion and support buffers to provide radiochemical purity (RCP) > 95% after the addition of 0.5 ml ^{99m}Tc generator eluate. P483 kits without heparin were converted to ^{99m}Tc-P483H by either the addition of 0.5 ml heparinized saline at 30 USP units/ml before or after addition of 0.5 ml ^{99m}Tc generator eluate or, if reconstituted with 1 ml ^{99m}Tc generator eluate, heparin was added in microliter quantities (i.e., 30 μl 500 USP units/ml heparin stock). Formulated vials were also made containing 150 μg lyophilized P483 and 15 units heparin. The kits required only addition of 1 ml ^{99m}Tc generator eluate. Technetium-99m-labeled PHCs in these kits were assayed by thin-layer chromatography using SAS on ITLC-SG strips and solvent saturation pads with AAW as described above.

Criteria for labeling ^{99m}Tc-P483H were defined as >90% RCP, as determined by the ratio of the activity at the origin of the saturated pad with AAW, divided by the total activity applied to the pad. Formulated kits generally exceeded 95% RCP, while ad-lib preparations were required to exceed 90% RCP. Specific activities used for imaging studies were in the range of 60–100 mCi/mg and doses averaged 10–20 μg/kg for nonformulated preparations and 2–4 μg/kg for formulated 150 μg kits.

In Vitro Association of Technetium-99m-P483H WBC

Human whole blood samples (n = 4, 27 ml each) were collected from four volunteers. Each sample was collected into 3 ml 3.8% sodium citrate at pH 7.4. Ad-lib labeled ^{99m}Tc-P483H (@ 20 mCi/mg) was diluted 1000-fold and 300 μl were added to each sample (7–10 μCi; 15–18 μCi/μg) to provide approximately 200–300 nCi/ml. After mixing by inversion, duplicate assays were performed by layering 4 ml of each blood sample over 2.5 ml ficoll-hypaque. The ficoll-hypaque blood mixture was centrifuged at 450× g for 25 min at room temperature using previously described methods (*13*). As controls, ^{111}In-WBCs and ^{99m}Tc-gluceptate were assayed for WBC association using ficoll-hypaque as a micro-ficoll assay system (^{111}In-WBCs only) and the above described neutrophil isolation medium method (^{99m}Tc-gluceptate).

Labeling of WBCs with Indium-111 and Technetium-99m-Gluceptate

Isolated and enriched human WBCs were used for most of these experiments instead of rabbit WBCs because:

1. PMNLs comprise a smaller fraction of all rabbit WBCs (*14*).
2. Although we could isolate rabbit WBCs using the combined ficoll method, human WBCs are more easily isolated with hetastarch and, thus, we would avoid the possibility of functional loss of the WBCs by exposure to ficoll.
3. No immunogenic response to heterologous WBCs would be expected within the 4-hr study period in nonsensitized rabbits.

Indium-111-Oxine-WBCs. Human WBCs obtained from four volunteers were collected into citrate as described. Each of the specimens had 7 ml hetastarch added, which was allowed to sediment in the blood over 2 hr. Suspended WBCs were harvested, pelleted, washed and labeled with ^{111}In according to the oxine method used by Thakur (*15*) and washed again to remove unincorporated ^{111}In. WBCs were assayed using a micro-ficoll assay method. Briefly, isolated and pelleted ^{111}In-WBCs (~100–150 μCi per 5 × 10^6 cells) were diluted into 250 μl PBS. Seventy-five microliters of ^{111}In-WBCs were then placed into three separate 150-μl glass tubes followed by 50 μl neutrophil isolation medium. The tubes were sealed at the ficoll end with hematocrit sealing cement. The tubes were then centrifuged at 500× g for 15 min at room temperature, taped onto a cardboard backing and imaged by contact film radiography for 2–4 hr. Duplicate tubes were cut above and below the WBC bands and the three fractions were counted in a gamma well counter.

Technetium-99m-Gluceptate. Technetium-99m-gluceptate was radiolabeled according to manufacturer's instructions. The labeled material was assayed using the standard ficoll gradient technique described above for ^{99m}Tc-P483H. Applied radioactivity was approximately 200–300 nCi/ml. As with ^{99m}Tc-P483H, 4 ml of the radiolabeled blood were layered onto neutrophil isolation medium and centrifuged at 450× g for 25 min.

To quantify the distribution of ^{99m}Tc-P483H and ^{99m}Tc-gluceptate among blood components, discrete layers (200 μl) were gently siphoned off each neutrophil isolation medium tube and each layer was counted in sequence in a gamma well counter. As a control for ^{99m}Tc-P483H, the peptide was mixed with 30 ml citrated normal saline as a "cell-free" system, and 4 ml of the solution were layered on neutrophil isolation medium and processed as described.

Monocytes and lymphocytes co-migrate upon centrifugation in neutrophil isolation medium. Therefore, to determine the mono-

cyte-associated radioactivity, 4 ml labeled blood were subjected to gradient fractionation with NycoPrep 1.068® (Accurate Chemical and Scientific Corp., Westbury, NY), a monocyte isolation media in the same manner as neutrophil isolation medium.

Rabbit Model of Infection

The animal model used to evaluate our PF-4 analogs is similar to that described previously by Babich et al. (*16*). Leg infections were induced in adult New Zealand White (NZW) rabbits (2–2.5 kg) by injecting 1.0 ml *E. coli* (10^{8-9} organisms/ml) into the left calf muscle. An infection develops within 12–24 hr, typically defined as a lesion with focal purulence, edema and modest hemorrhage (grade III). The infections typically occupied more than 10 cm^3 of muscle tissue 18–24 hr after infection.

At 18–24 hr after induction of infection, ^{99m}Tc-P483H and six reference control agents were tested for their abilities to provide high contrast infection images 4 hr after administration. Technetium-99m-P483H was evaluated in 48 rabbits using various ad-lib and formulated kits. Eight of the 48 animals were examined with a kit formulation (Lot no. 9515M01), which contains 150 μg peptide in a lyophilized PHC kit for preparation of ^{99m}Tc-P483H. The dose of ^{99m}Tc-P483H for ad-lib preparations averaged 8–12 μg/kg (1.5 mCi/kg) per rabbit and the dose for formulated 150 μg kits averaged 2–4 μg/kg (1.5 mCi/kg). The reference agents included: ^{111}In-WBCs (n = 4), ^{67}Ga-citrate (n = 2), ^{111}In-DTPA (n = 1), ^{99m}Tc-nanocolloid (n = 3), ^{131}I-albumin (n = 1) and ^{99m}Tc-gluceptate (n = 5). Infected rabbits were imaged 4 hr postinjection.

The animals were killed 4 hr postadministration by an overdose of anesthetic followed by withdrawal of a terminal blood sample and a 5-min acquisition image of the infection was obtained. Necropsy was performed on each animal to collect six infected muscle samples of 1–2 g each and two muscle samples from the contralateral leg. Tissues were counted in a gamma well counter against diluted dosing solution standards. The %ID, %ID/g, I_{max} (e.g., the infection sample with the highest %ID/g), I_{max}-to-blood and I_{max}-to-control ratios were calculated.

Technetium-99m-P483H In Vivo WBC Association

Pharmacokinetic analysis of blood clearance was performed in three rabbits. Analysis included microhematocrits at each time point to assess radioactivity in the plasma and WBC/RBC fractions.

To determine if an administered dose of ^{99m}Tc-P483H was associated with WBCs on injection, the following experiment was performed. In a deeply anesthetized rabbit, an imaging dose of ^{99m}Tc-P483H was injected into the ear vein immediately after performing rib separation thoracotomy to expose the right heart. Ten milliliters of blood were quickly collected into citrate from the right ventricle during injection. Duplicate 3-ml samples of the recovered blood were layered onto 2 ml of a 1:1 mixture of the two WBC isolation medias. A mixture of neutrophil isolation medium and NycoPrep 1.068® was used since rabbit WBCs typically do not separate adequately from RBCs on either substance alone. The tubes were centrifuged as described above at 450× g for 25 min and samples were serially removed and sequence counted as described.

Three additional experiments were designed to examine whether systemically administered ^{99m}Tc-P483H labels circulating WBCs in vivo are then "carried" to the locus of infection.

In the first experiment, approximately 3×10^6 human WBCs were labeled with ^{99m}Tc-P483H ex vivo and injected intravenously into two rabbits with 18–24-hr-old induced *E. coli* leg infections. Images were obtained over 4 hr postinjection and were followed by necropsy and tissue distributions.

We also examined the possibility that ^{99m}Tc-P483H leaves the vascular space and binds to WBCs that may be in residence at a specific site. Approximately 3×10^6 human WBCs were isolated as described above and injected into the calf muscles of four noninfected rabbits 30 min prior to administration of ^{99m}Tc-P483H. Serial images were obtained over 4 hr and were followed by necropsy. As a control, the opposite leg was injected with cell-free human plasma. Tissues were assayed as described above.

Finally, we examined the uptake of ^{99m}Tc-P483H in WBC-depleted infections versus normal animals. The time course for infection uptake in the normal animals was also determined. Four rabbits were injected intravenously with a single injection of mechlorethamine (1.75 mg/kg) to deplete circulating WBCs to approximately one-fourth normal circulating levels. Six normal rabbits served as controls. The nadir of the mechlorethamine response was between 3–4 days postinjection. The four mechlorethamine-treated and six control rabbits were infected with *E. coli* on the third day after mechlorethamine administration. The uptake of ^{99m}Tc-P483H in the infections on Day 4 was assessed. The time course for infection uptake in the controls was assessed by necropsy of one animal at each hour postinjection and the remaining three at 4 hr. Tissues were assayed as described above.

RESULTS

In Vitro Association of Technetium-99m-P483H with Human WBCs

When ^{99m}Tc-P483H was incubated with citrated whole human blood and subjected to density gradient centrifugation with neutrophil isolation medium, the majority of the labeled peptide was associated with the WBCs. The percent of applied radioactivity associated with WBCs averaged 76.5% (n = 2). Of the cell-associated activity, PMNLs accounted for only 36%, while the monocyte/lymphocyte layer accounted for the remaining 64%. Application of blood samples onto monocyte isolation media showed that of the 54% cell-associated activity found within the monocyte/lymphocyte layer, more than half was associated with monocytes, despite their low abundance in the circulating blood pool (8%–10%). Incubation of ^{99m}Tc-P483H with whole blood at room temperature for several hours (>8 hr) followed by neutrophil isolation medium gradient centrifugation yielded the same distribution of radioactivity as above, demonstrating the stability of the radiolabeled peptide in whole blood.

Table 1 describes the in vitro distributions of ^{99m}Tc-P483H, ^{111}In-WBCs and ^{99m}Tc-gluceptate using gradient centrifugation techniques. Indium-111-WBC ficoll distributions were examined using a neutrophil isolation medium micro-ficoll assay system and ^{111}In-WBCs suspended in PBS. In this constrained system, approximately 73% of the radioactivity was associated with WBC bands. Technetium-99m-gluceptate was assayed in whole blood using neutrophil isolation medium (n = 1) and showed very low WBC association (<15%). Over 80% of the applied radioactivity was found to be associated with the plasma fractions. A "cell-free" assay of ^{99m}Tc-P483H in saline using neutrophil isolation medium (n = 1) showed that a significant fraction of the radioactivity (73%) was associated with ficoll densities greater than the WBC densities (that is, below the PMNL layer and in the ficoll-RBC layers). The plasma layer in this case was found to contain only 5% and the previous WBC layers accounted for only 22%.

Biodistribution in the Rabbit Infection Model

Four hours after ^{99m}Tc-P483H administration, infection sites were visible with significant image contrast. Infection uptake with ^{99m}Tc-P483H in the rabbit model at 4 hr, as well as 4-hr values for ^{111}In-oxine-WBCs, ^{111}In-DTPA, ^{131}I-albumin (HSA),

TABLE 1
Distribution of Technetium-99m-P483H in Whole Blood: In Vitro Separations (Mean Percentage of Total Radioactivity)

Agent	Percentage in gradient layers					Percentage with WBCs
	Plasma	Monos/Lymphs	PMNs	Ficoll	RBCs	
^{99m}Tc-P483H (n = 4)	11.4	45.9* (26% monos/19% lymphs)	27.7	6.9	8.1	73.6
^{99m}Tc-P483H (n = 1) in saline ("cell free")	5.3	10	11.7	47.3	25.7	21.7
Washed ^{111}In-WBCs (n = 2)	15.4	Cell types not isolated		12	n.a.†	72.6
^{99m}Tc-gluceptate (n = 1)	87.4	9.8	1.4	0.8	0.6	11.2

*Monocytes showed 26.8% of applied radioactivity versus 19.1% for lymphocytes using monocyte isolation media section.
†WBCs were isolated cells, labeled per a standard clinical protocol, and applied to neutrophil isolation medium without RBCs present.

^{99m}Tc-nanocolloid, ^{67}Ga-citrate and ^{99m}Tc-gluceptate are presented in Table 2. In all cases except for ^{131}I-albumin, the I_{max} uptake for ^{99m}Tc-P483H was significantly higher. Iodine-131-albumin has a prolonged blood clearance upon injection ($t_{1/2\alpha}$ > 2 hr) relative to small peptides. The higher blood concentration retained at 4 hr by ^{131}I-albumin reduces image contrast. Thus, despite high I_{max}, the resulting I_{max}-to-blood and I_{max}-to-control ratios were only 0.13, the lowest among all agents tested.

Technetium-99m-P483H provided a significantly higher range of I_{max} values, with some I_{max} as high as 0.1 to 0.16 %ID/g. The I_{max} average for ^{99m}Tc-P483H was 0.062 %ID/g (n = 48) with total infection uptake (the sum of six infection samples per animal) approaching 1.0 %ID by 4 hr. The I_{max}-to-blood and I_{max}-to-control ratios averaged 3.1 and 26.8, respectively, at 4 hr. The kit containing 150 μg lyophilized PHCs provided the same I_{max} at 0.062 (±0.22) and I_{max}-to-blood and I_{max}-to-control ratios of 4.7 (±2.5) and 32.4 (±14), respectively. An image of an *E. coli*-infected rabbit at 4 hr postadministration formulated ^{99m}Tc-P483H is presented in Figure 1.

Indium-111-oxine (human) WBCs in the rabbit model provided an average I_{max} of 0.051 %ID/g at 4 hr, with I_{max}-to-blood and I_{max}-to-control ratios of 0.5 (±0.2) and 6.9 (±2.0), respectively. Circulating labeled cells prevent early (<4 hr) identification of infections due to the poor I_{max}-to-blood ratio, which defines image contrast. At 4 hr, ^{99m}Tc-P483H showed slightly higher infection uptake relative to ^{111}In-WBCs but, due to more rapid blood clearance, ^{99m}Tc-P483H showed a sixfold higher I_{max}-to-blood ratio and a more than fourfold increase in the I_{max}-to-control ratio.

Isolated human WBCs, which were labeled with ^{99m}Tc-P483H, washed of unlabeled radioactivity and administered to rabbits with *E. coli* infections, could also identify the locus of infection. The labeled cells showed significant transit delay in the lungs at injection similar to ^{111}In-WBCs and also not unlike a direct injection of ^{99m}Tc-P483H itself. Sufficient image contrast was evident at 4 hr to identify the infection site (Fig. 2). The 4-hr I_{max} average was only 0.0047 (±0.0008; n = 2), or more than 10 times less than ^{99m}Tc-P483H itself; the I_{max}-to-control ratio, however, was 17.4.

Isolated and unlabeled human WBCs injected into the calf muscle of normal rabbits were also identified by 4 hr after direct injection of ^{99m}Tc-P483H (Fig. 3). The percent injected dose per gram of tissue at the WBC injection site averaged 0.0381 %ID/g (±0.022, n = 2). The contralateral leg injected with an equal volume of plasma did not show significantly different

TABLE 2
Performance of Infection Imaging Agents in the Rabbit *E. coli* Model: Biodistribution Parameters at 4 Hours (18–24-Hour Infections)

Test agent	%ID/g			I_{max} ratios		No.
	Blood	I_{max}	Control	I_{max}-to-blood	I_{max}-to-control	
^{99m}Tc-P483H (all formulations)						
Mean	0.020	0.062	0.0023	3.1	26.8	48
(s.d.)	(0.008)	(0.029)	(0.0015)	(2.4)	(16.8)	
^{99m}Tc-P483H (kit)	0.0132	0.062	0.0019	4.7	32.4	8
	(0.005)	(0.022)	(0.0012)	(2.5)	(14)	
^{111}In-oxine-WBCs	0.1102	0.051	0.0074	0.5	6.9	4
	(0.026)	(0.008)	(0.0002)	(0.2)	(2.0)	
^{67}Ga-citrate	0.192	0.0431	0.0172	0.22	2.5	2
^{111}In-DTPA	0.0374	0.0244	0.0035	0.65	7	1
^{99m}Tc-nanocolloid	0.0056	0.0072	0.0007	1.3	11.1	3
	(0.0017)	(0.0025)	(0.0002)	(0.23)	(0.9)	
^{131}I-albumin (HSA)	0.522	0.0665	0.0171	0.13	3.9	1
^{99m}Tc-gluceptate	0.0085	0.0123	0.0011	1.56	12.8	5
	(0.0034)	(0.0036)	(0.0007)	(0.55)	(6.1)	

I_{max} = Infection sample per animal with the highest %ID/g. Numbers in parentheses are mean ± s.d.

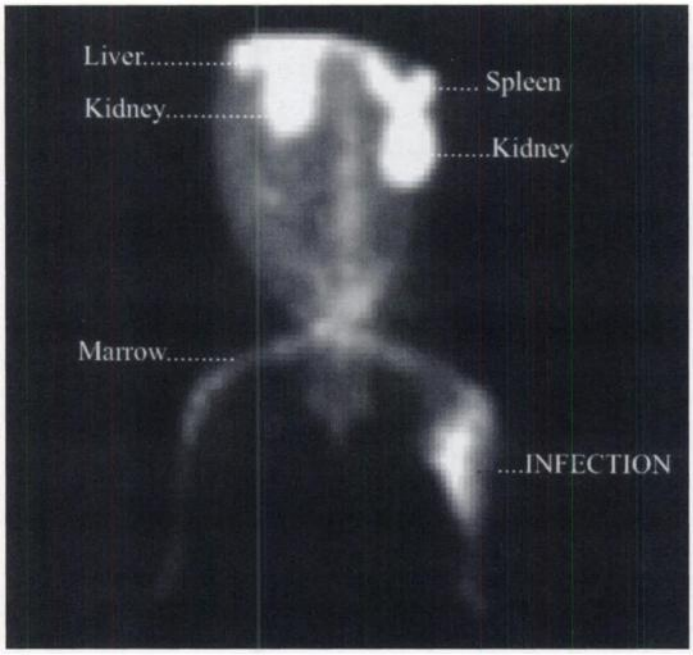

FIGURE 1. Four-hour anterior image produced by ^{99m}Tc-P483H in a rabbit with 24-hr *E. coli* infection of the left calf muscle. There is much greater contrast in the left leg infection (right). The liver, kidneys and spleen are evident, as well as the bone marrow of the spine, pelvis and legs.

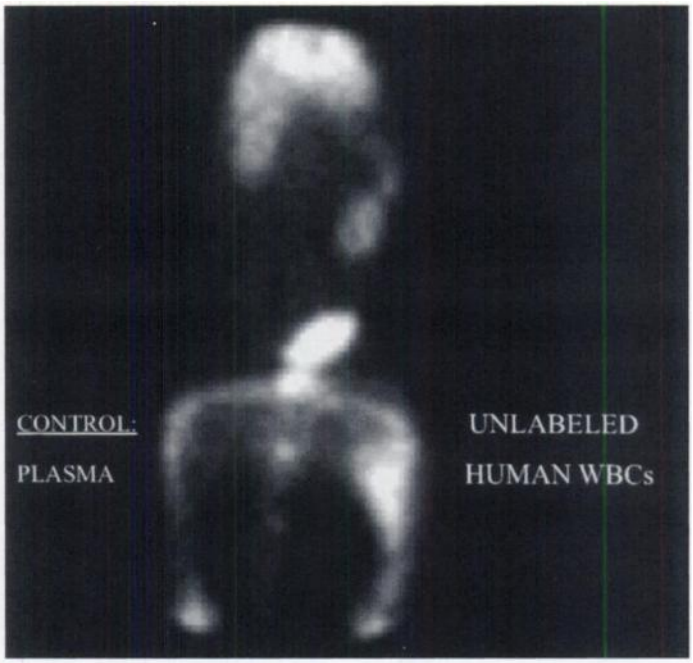

FIGURE 3. Four-hour anterior image of a rabbit injected with washed human WBCs (~10^5 cells suspended in 1 ml PBS) into the left leg 30 min prior to injection of ^{99m}Tc-P483H. Plasma (human, 1.0) was also injected into the contralateral leg 30 min prior to P483H to serve as a control for inflammatory mediators (i.e., complement). There is intense uptake in the left leg and the virtual absence of any activity in the plasma-injected control leg.

uptake relative to normal muscle (0.0017 %ID/g). The I_{max}-to-blood and I_{max}-to-control ratios averaged 2.5 and 24.5, respectively.

WBC depletion studies showed that ^{99m}Tc-P483H requires the presence of WBCs in order to accumulate at the infection site. Four mechlorethamine-treated *E. coli*-infected rabbits with WBC counts depleted to one-fourth that of normal rabbits (infected controls) showed only limited uptake at the site of infection with 0.0136 %ID/g (±0.007, n = 4) versus normal (albeit infected) rabbits with 0.065 (±0.026, n = 3) %ID/g (Table 3).

A second part of this experiment assessed the time-activity uptake curve for ^{99m}Tc-P483H at *E. coli* infection sites. The images of infected animals killed at 1, 2, 3 and 4 hr postinjection of ^{99m}Tc-P483H are shown in Figure 4. Figure 5 shows the infection time-activity curve generated from these animals. The function was defined as a single exponential with the origin forced to zero. The function showed a time-activity C_{max} of approximately 1.8%–2%ID/g by 10–12 hr.

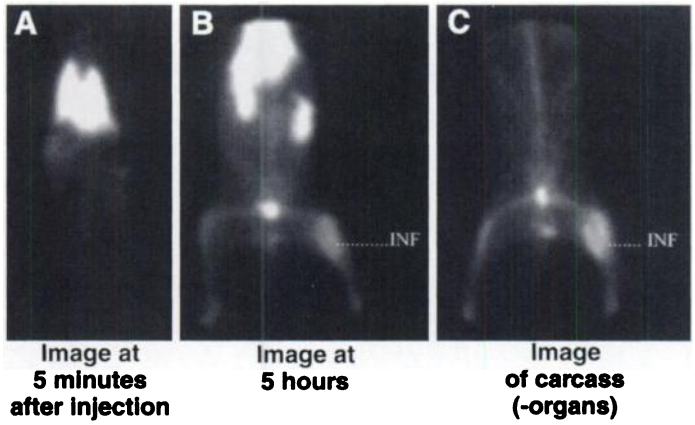

FIGURE 2. Five-minute and 4-hr scintigraphic images produced by human leukocytes labeled ex vivo with ^{99m}Tc-P483H in a rabbit with a 24-hr infection of the left calf muscle (right). (A) Image of WBC lung distribution at 5 min. (B) Infection (INF) uptake at 4 hr. (C) Infection uptake at 4 hr with organs removed to expose bone structures such as spine, pelvis and femurs.

DISCUSSION

The PF-4 analog P483H contains the heparin-binding domain of PF-4 for which receptors have been identified on PMNLs, monocytes, endothelium, fibroblasts and hepatocytes (*17–21*). No evidence exists, however, for receptors on lymphocytes (*14,16,17*). The in vitro distribution in human blood revealed by density gradient centrifugation suggests that ^{99m}Tc-P483H associates with specific WBCs, particularly monocytes.

The percentage of applied radioactivity of ^{99m}Tc-P483H that associated with WBCs in vitro was equal to that of ^{111}In-oxine WBCs (73.6% versus 72.6%, respectively). Since the ^{111}In-WBCs were washed of unbound ^{111}In, the recovery was 27% lower than expected. Isolated and washed ^{111}In-WBCs should have uniquely located to the specific cell bands. Indeed, images of the tubes showed only two bands—PMNLs and the combined monocytes/lymphocytes. The sampling technique for counting may not have provided sufficient separation of cell layers. Technetium-99m-P483H showed band resolution similar to ^{111}In-WBCs. Since both ^{111}In-WBC and ^{99m}Tc-P483H tubes showed virtually identical imaging patterns, and cell recoveries in the assay were virtually identical, it can be argued that ^{99m}Tc-P483H may actually associate quantitatively with WBCs in vitro.

Technetium-99m-P483H distributions among monocytes was accomplished by use of a second assay with monocyte isolation media, which isolates monocytes from lymphocytes. Integrating the WBC results from both isolation media suggests that approximately 38% of ^{99m}Tc-P483H activity is associated with PMNLs, which comprise 60%–70% of human WBCs. Monocytes, which comprise only 8%–10% of human WBCs, however, had 43% of the cell-associated radioactivity. Lymphocytes, which comprise 15%–25% of WBCs, contained the remaining 19%. The data thus reflect a higher affinity for

TABLE 3
Effect of Mechlorethamine on Infection Uptake*

Mechlorethamine†	Blood	Infection I_{max} (%ID/g)	Control	I_{max} ratios		WBC count
				I_{max}-to-blood	I_{max}-to-control	
1.75 mg/kg n = 4	0.0158	0.0136	0.0025	0.8	6.0	avg: 700/mm**3
Controls						
0.0 mg/Kg n = 3	0.0151	0.0651	0.018	4.2	35.4	avg: 3000/mm**3

*Technetium-99m-P483H uptake in Infections of WBC-depleted and control rabbits.
†Mechlorethamine, a methylated-bis (chloroethyl)amine chemotherapeutic agent with dose-dependent myelopoietic suppression, was administered intravenously at 1.75 mg/kg 3 days prior to infection.

monocytes by ^{99m}Tc-P483H based on a normal differential. Indium-111-WBCs were not differentially assayed.

In Vivo Association with WBCs

The ability of ^{99m}Tc-P483H to associate with WBCs in vivo was examined in a normal rabbit. The rabbit was injected with ^{99m}Tc-P483H in an ear vein and blood was collected during injection from the right heart. The NIM-to-MIM distribution showed similar monocyte preference, with >40% of total radioactivity present associated with these cells, even though the rabbit differential for monocytes averages only 10% (range: 2%–16%). The percentage of radioactivity associated with WBCs was, as was the case in the human studies, 73% using the serial sampling technique. The rabbit WBC differential, however, is significantly different from the results obtained in humans (*18*), with >60% of the circulating WBCs as mononuclear (NZW rabbit differential: 20%–30% PMNLs, 30%–50% lymphocytes, 2%–16% monocytes).

Whether PHCs have the capacity to exert chemotactic effects on neutrophils, like f-Met-Leu-Phe based infection imaging agents (*16*), is not presently known, but it does appear unlikely on three grounds. First, even if P483H possessed chemotactic properties equivalent to PF-4, the plasma concentrations of peptide would not be sufficient to exert these effects, since the threshold concentrations for measurable chemotactic effects on neutrophils is at least 50 times greater than the highest plasma concentration attainable (*19*). Second, since the chemotactic effects of PF-4 are, like the heparin-binding domain, in the COOH terminus (*20*), it is unlikely that it can exert these effects when complexed to heparin, since heparin may exert strong conformational changes upon the peptide. Third, PF-4 is one of the weakest known chemotaxins (*19*).

PHCs are known to accumulate in the lungs on injection in a similar manner as ^{111}In-WBCs (i.e., kit Lot no. 9515M01, concentration in the lung at t_0 = 45% ID, $C_{lung = 0}$). The uptake of the agent in the lung may provide sufficient in situ focal concentrations after injection to label lung-resident and lung-transiting leukocyte WBCs. Transient neutropenia has been observed in rabbits administered f-Met-Leu-Phe peptides; however, this has not been observed after administration of PF-4 analogs such as ^{99m}Tc-P483H. These findings were supported in P483H toxicology tests designed to examine changes in pulmonary function and WBC differentials following repeated intravenous doses. Briefly, three normal rabbits were administered mock-labeled P483H (plus three saline volume-matched controls) over the course of 1 hr. Each animal received seven doses 10 min apart, each starting at 10 times the MHD, then 30 times the MHD, and then 5 sequential doses at 100 times the MHD (MHD = maximum human dose, 1 unit vial, 1 μg/kg) to a total dose of 540 times the MHD. Blood counts, blood gases and blood pH were scored before (1 min) and after (3 and 9 min) each dose and no physiologically significant changes were observed in any parameter relative to controls.

Experiments in which human WBCs were labeled ex vivo with ^{99m}Tc-P483H and injected intravenously into infected rabbits showed tenfold lower uptake at the infections relative to directly injected ^{99m}Tc-P483H. This provides indirect evidence that ^{99m}Tc-P483H may localize at sites of infection by two mechanisms: (a) a fast component which likely labels WBCs at the infection in situ and (b) a slower mechanism, possibly mediated by circulating WBCs tagged with ^{99m}Tc-P483H which are attracted to the infection by chemotaxic mechanisms.

Technetium-99m-P483H-labeled WBCs, like ^{111}In-WBCs, shows prolonged residence in the lungs after injection with eventual clearance by slow release back into circulation. Phar-

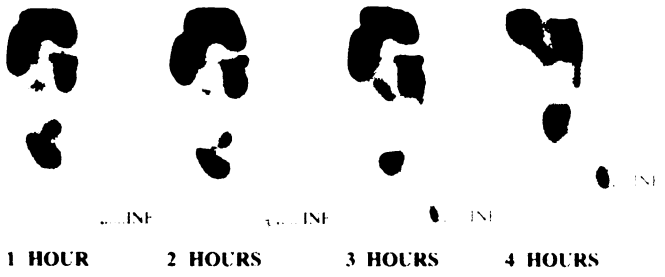

FIGURE 4. Time-activity images of four rabbits injected with ^{99m}Tc-P483H and killed for necropsy at 1, 2, 3 and 4 hr postinjection. Note the accumulation of the agent with time in the infected calf muscle of the left leg.

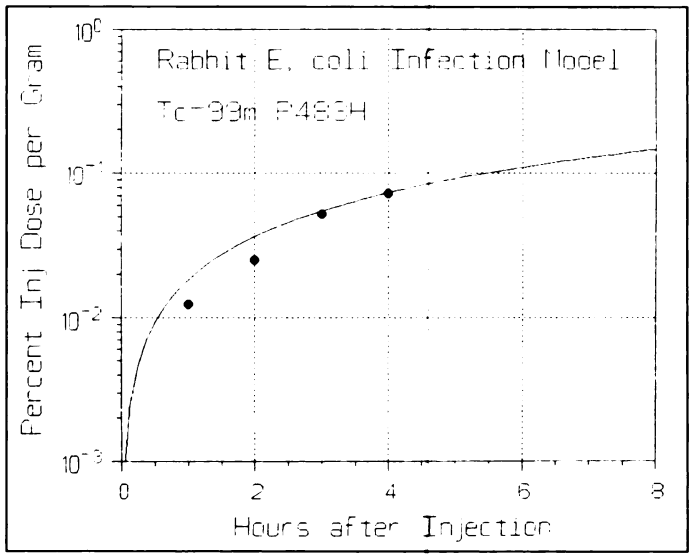

FIGURE 5. The I_{max} time-activity curve from the animals depicted in Figure 4. The curve was fit to a single exponential and predicts, assuming steady-state kinetics, an I_{max} of approximately 1.8%–2%ID/g at 10–12 hr postinjection.

macokinetic studies in normal rabbits, in which timed blood samples were taken and examined for radioactivity in hematocrit fractions, showed a continuous ratio of approximately 70%–80% of the circulating activity in the plasma and 20%–30% in the WBC/RBC fraction over 4 hr. Based on in vivo blood sampling of ^{99m}Tc-P483H followed by 1:1 neutrophil isolation medium analysis, it can be determined that RBC radioactivity is very small and that virtually all WBC/RBC activity is WBC-associated.

Further support for ^{99m}Tc-P483H in vivo WBC association comes from the two experiments in which ^{99m}Tc-P483H was injected in rabbits having unlabeled human WBCs injected into the calf muscle and plasma into the contralateral muscle of normal rabbits and mechlorethamine-treated (e.g., WBC-depleted) infected animals. In the first case, ^{99m}Tc-P483H identified the leg with WBCs and did not accumulate in the plasma-injected leg, which contained no WBCs but retained all other chemotactic and immunomodulating elements (i.e., complement, etc.) which could elicit an inflammatory response. In the second experiment, leukopenic and infected animals showed <25% uptake of ^{99m}Tc-P483 compared to normals. In both cases, the presence of circulating WBCs was important to provide image contrast.

CONCLUSION

We have developed a ^{99m}Tc-labeled peptide that is comprised of the C-terminal sequence of PF-4 and five lysine residues. The peptide, P483, when combined with heparin and labeled with ^{99m}Tc (^{99m}Tc-P483H), is an effective infection-seeking agent. When exposed to human blood in vitro, ^{99m}Tc-P483H rapidly associates with WBCs, predominately monocytes, and is metabolically stable. Technetium-99m-P483H provides high I_{max}-to-blood and I_{max}-to-control ratios early after injection (4 hr or less) in our infection model. These 4-hr ratios are far superior to ^{111}In-labeled WBCs and other available agents. Technetium-99m-P483H represents a promising new clinical candidate for detection of occult infection and inflammation.

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