Assessment of Myocardial Inflammation Produced by Experimental Coronary Occlusion and Reperfusion With 99mTc-RP517, a New Leukotriene B4 Receptor Antagonist That Preferentially Labels Neutrophils In Vivo

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Background—99mTc-RP517 is a new leukotriene B4 (LTB4) receptor antagonist developed for imaging acute inflammation or infection. A unique property of 99mTc-RP517 is its ability to label white blood cells in vivo after intravenous injection. The goals of this study were to determine relative 99mTc-RP517 binding to human leukocyte subtypes and the 99mTc-RP517 uptake pattern in canine myocardium where inflammation was induced by either coronary occlusion and reperfusion or tumor necrosis factor α (TNFα) injection.

Methods and Results—Fluorescence-activated cell sorter analysis was performed on whole human blood (n=2) and isolated neutrophils (n=4) with a fluorescent analog of 99mTc-RP517, [F]-RP517. In whole blood, [F]-RP517 (500 nmol/L) preferentially labeled neutrophils. On isolated neutrophils, [F]-RP517 (10 nmol/L) binding was inhibited by 44% when LTB4 (400 nmol/L) was added. 99mTc-RP517 was injected intravenously in anesthetized, open-chest dogs before coronary occlusion (90 minutes) and reperfusion (120 minutes) (n=9) or before intramyocardial TNFα injection (n=3). Ex vivo images of heart slices were acquired. The left ventricle was divided into 72 segments for flow and 99mTc-RP517 uptake analysis. There was an inverse exponential relationship between 99mTc-RP517 uptake and occlusion flow (r=0.73). In the same 15 segments, 99mTc-RP517 uptake was highly correlated with the neutrophil enzyme myeloperoxidase (r=0.91). Ex vivo images revealed tracer uptake in the reperfused area (ischemic to normal count ratio 2.7±0.2).

Conclusions—RP517 binds to the neutrophil LTB4 receptor after intravenous injection. After reperfusion, 99mTc-RP517 uptake correlated with myeloperoxidase and was observed on ex vivo images, indicating that this tracer may have potential as an inflammation-imaging agent. (Circulation. 2002;106:592-598.)

Key Words: inflammation, myocardium, imaging

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have demonstrated in detail the prominent role of inflammation, particularly neutrophil infiltration, in myocardial ischemia-reperfusion–induced injury,9–13 and the acute inflammatory response to myocardial infarction has been documented by11In-labeled autologous neutrophil imaging in patients with acute myocardial infarction.14 The hypothesis tested in the present experimental study was that 99mTc-RP517 should show preferential uptake in areas of coronary reperfusion showing evidence of inflammation characterized by neutrophil accumulation. Accordingly, our aims were to determine relative 99mTc-RP517 binding to human leukocyte subtypes and to determine whether 99mTc-RP517 could assess neutrophil infiltration in the setting of ischemia reperfusion or direct tumor necrosis factor α (TNFα)–induced myocardial inflammation.

Methods

In Vitro Experiments

Human Blood Preparation

Heparinized venous blood from 6 human donors was used. Whole blood from 2 donors was incubated 30 minutes at 4°C with a fluorescent analog of RP517 ([F]-RP517, 500 nmol/L), and fluorescence-activated cell sorter (FACS) analysis was performed. Neutrophils (10^6/mL) were isolated from the whole blood of 4 donors following a one-step Ficoll-Hypaque procedure15 and stained with fluorescently tagged (CY3) RP517 (10 nmol/L) for 30 minutes at 4°C in the presence or absence of LTB4 (400 nmol/L) or nonfluorescent RP517 (1 nmol/L to 10 μmol/L) before addition of 3 mL of Hanks’ Balanced Salt Solution (HBSS) and centrifugation (200g, 8 minutes). The pellets were resuspended in 0.5 mL PBS containing 0.5% paraformaldehyde for FACS analysis.

FACS Analysis

The FL2 channel (565-nm emission) of a FACS Calibur (Becton Dickinson Biosciences) was used. In whole blood samples, the leukocyte subtypes were identified by antibody staining for CD24-PE and CD14-FITC followed by back-gating to the forward and side scatter plots. The cells were identified on the forward and side scatter plot as follows: neutrophils (CD24+/H11001), monocytes (CD14+/H9262), and lymphocytes (CD14−/H11002). In purified neutrophil preparations, only CD24+ cells were assayed for fluorescent RP517 association. Mean fluorescence intensity (MFI) results were expressed as mean±SEM.

In Vivo Experiments

Surgical Preparation

Twelve fasted adult mongrel dogs (24.9±1.5 kg; range, 17.3 to 31.8 kg; Haycock Kennels, Quakertown, Pa) were anesthetized with sodium pentobarbital (30 mg/kg IV), tracheally intubated, and mechanically ventilated. Open-chest surgery and instrumentation were performed as previously described.16

Experimental Protocol

In 12 dogs, 99mTc-RP517 was injected intravenously during baseline. In 9 of these dogs, the left anterior descending (LAD) artery was totally occluded for 90 minutes and reperfused for 120 minutes. Regional myocardial blood flows were assessed using radiolabeled microspheres during baseline and at the end of the occlusion and reperfusion periods. Myocardial biopsies were obtained in vivo from the regions supplied by the LAD and left circumflex (LCx) arteries for myeloperoxidase assay in 4 dogs. In one dog, an in vivo 99mTc-RP517 image was acquired in the left, lateral projection at 60 minutes after reperfusion using a Siemens Orbiter γ camera. The image was acquired for 5 minutes with a high-resolution collimator and a 20% window centered around the photopeak of 99mTc. Before the animals were euthanized with an overdose of sodium pentobarbital, the LAD was briefly reoccluded and 40 mL of monastral blue dye was rapidly injected into the left atrial catheter to delineate the anatomic risk area.

In 3 additional normal dogs, an intramyocardial injection of TNFα (1.5 to 2.0 μg) was given after 99mTc-RP517 administration. Three hours later, myocardial biopsies were obtained from peri-injection and control regions for myeloperoxidase assay, and the dogs were then euthanized.

Preparation of 99mTc-RP517

Ethanol 0.2 mL was added to a 99mTc-RP517 vial containing 20 μg SG380 (nonlabeled precursor of 99mTc-RP517), 5 mg TPPTS, 6.5 mg tricine, 40 mg mannitol, 0.25 mol/L succinate buffer (pH 4.8), and 0.1% lysolecithin in 1.0 mL of 12% ethanol. After introduction of sodium pertechnetate Tc-99 m (1.75 GBq [50 mCi]), the vial was heated in boiling water for 30 minutes and allowed to cool at room temperature.

Postmortem Analysis

Ex Vivo Image Acquisition and Quantification of Count Ratio

At the end of the experiment, the heart was excised and sliced evenly from apex to base into 4 segments. The left ventricle and septum were separated from the remainder of the heart. In 10 dogs, the heart slices were imaged directly on the collimator of the γ camera. For quantification of 99mTc activity, regions of interest were drawn on the anteroseptal left ventricular wall and on the normally perfused posterior wall. The ex vivo count ratio was computed by dividing the average counts per pixel in the stenotic region by the average counts per pixel in the nonstenotic region.

Determination of Regional Myocardial Blood Flow and 99mTc-RP517 Myocardial Activity

The technique used in our laboratory to quantify regional myocardial blood flow with radioactive microspheres has been described previously.17 In 8 of 9 occlusion/reperfusion dogs, each of 4 myocardial slices was divided into 6 transmural sections, which were then subdivided into epicardial, midwall, and endocardial segments. In one dog dedicated to the assessment of the relationship between 99mTc-RP517 myocardial activity and myeloperoxidase content in the same segments, 15 postmortem biopsies were taken from the normal, border, and central infarct zones. The myocardial segments and arterial blood samples were counted in a γ-well scintillation counter (MINAXI 5550, Packard Instruments). The window settings were as follows: 99mTc, 120 to 160; 113Sn, 340 to 440; 42Sr, 450 to 580; 79Nb, 640 to 840; and 52Sc, 842 to 1300 keV. Tissue counts were corrected for background, decay, and isotope spillover, and regional blood flow was calculated with specialized computer software (PCG-ERDA, Scientific Computing Solutions, LLC).

Determination of Risk Area and Infarct Size

The endocardial and epicardial surfaces of each heart slice and the borders of the blue dye–determined risk area were traced on acetate sheets. The heart slices were then incubated for 10 minutes at 37°C in a 2% solution of triphenyl tetrazolium chloride (TTC) to delineate infarct area, and the infarct area was then traced on the previous acetate sheets. Risk and infarcted area were determined with a digital planimeter program (DigiPlan, Scientific Computing Solutions, LLC).

Myeloperoxidase Assay

Myeloperoxidase (MPO), indicative of neutrophil infiltration into tissue, was measured from myocardial biopsies (25.0 to 58.3 mg) taken in vivo in 7 dogs from the LAD and LCx regions and from 15 postmortem biopsies performed in one dog (59.2 to 194.0 mg). Myocardial specimens were made into suspensions and processed as previously described.18 MPO concentration was determined as described previously19 using a Molecular Devices SPECTRAMax PLUS spectrophotometer. Standards with a known concentration of MPO (Sigma) were also performed, and results were expressed as units of MPO per milligram of wet tissue.
Data Analysis

Myocardial Blood Flows and $^{99m}$Tc-RP517 Activity

Segments with flow ≤0.3, >0.3 and ≤0.7, and >0.7 mL/min per gram were classified as ischemic, border, and normal zones, respectively. When $^{99m}$Tc-RP517 uptake was plotted as a function of occlusion flow, the flow in each segment was expressed as a percentage of the average of the 5 segments exhibiting the highest flows during occlusion observed in each dog. Likewise, $^{99m}$Tc-RP517 activity in each segment was expressed as a percentage of the average $^{99m}$Tc-RP517 activity in the 5 segments exhibiting the highest flow during occlusion.

Statistical Analysis

Mean and SEM computations were performed with SYSTAT software (SPSS, Inc). Comparisons within each group were made with a paired Student’s t test. The in vitro binding data were analyzed using GraphPad Prism software (GraphPad Software, Inc).

Results

The chemical structure of $^{99m}$Tc-RP517 is shown in Figure 1.

In Vitro Experiments

Results of FACS analysis are shown in Figures 2 and 3. The left panel of Figure 2 depicts the 3 distinct subpopulations of leukocytes separated from whole human blood by FACS in the absence of [F]-RP517. Shown in red in the right panel of Figure 2, after incubation of whole human blood with [F]-RP517, there was preferential staining of the neutrophil pool by [F]-RP517. MFI values were 61.7±0.1, 14.6±0.1, and 5.5±0.1 for neutrophils, monocytes, and lymphocytes, respectively. In isolated neutrophils, [F]-RP517 binding was reduced by 44% from 19.3±1.4 to 10.9±0.9 (P<0.001) when LTB4 (400 nmol/L) was present. Likewise, as shown in Figure 3, nonfluorescent RP517 inhibited the binding of [F]-RP517 on isolated neutrophils in a dose-dependent manner, with a 50% effective concentration (EC50) of 26±1 nmol/L.

Figure 2.
Identification of white blood cell populations by FACS analysis in whole human blood (left) and preferential $^{99m}$Tc-RP517 neutrophil binding after incubation with whole blood (right, red dots).

In Vivo Experiments

Hemodynamics

Hemodynamic data for the 9 occlusion/reperfusion dogs are presented in Table 1. Heart rate, left atrial and mean arterial pressures, LCx ultrasonic flow, and maximum positive first derivative of left ventricle (LV) pressure (dP/dt) did not change throughout the experiment. Ultrasonic LAD artery flow fell from 35±7 mL/min at baseline to 0±0 mL/min after the occlusion (P<0.001) and returned to baseline after reperfusion.

Risk Area and Infarct Size

The myocardial risk area was 31.6±2.3% of the LV. By TTC staining, infarct size was 15.1±2.1% and 43.4±6.1% of the LV and risk areas, respectively.

Comparison Between Microsphere Blood Flow and $^{99m}$Tc-RP517 Activity

Table 2 summarizes the absolute regional myocardial blood flows in the ischemic, border, and normal zone samples from 8 of 9 occlusion/reperfusion dogs. Baseline, occlusion, and reperfusion flows were not significantly different in the normal zone. After total LAD occlusion, regional myocardial

Figure 3.
MFI after incubation of fluorescent RP517 ([F]-RP517, 10 nmol/L) with isolated human neutrophils in the presence of increasing concentration of nonfluorescent RP517 (RP517).
blood flow in the ischemic and border zones significantly decreased when compared with baseline. Reperfusion flows were significantly lower than baseline flows in both zones. Table 2 also compares the ischemic to normal zone and border to normal zone occlusion flow and $^{99}$mTc-RP517 activity ratios. The significantly higher $^{99}$mTc-RP517 ischemic to normal zone than border to normal zone ratio demonstrates the graded accumulation of the tracer in previously ischemic tissue.

Figure 4 provides a detailed analysis of the $^{99}$mTc-RP517 uptake pattern after grouping of the endocardial, midwall, and epicardial segments according to the severity of flow reduction during occlusion. As shown in Figure 4, myocardial $^{99}$mTc-RP517 activity increased as occlusion flow decreased in the endocardial and midwall segments ($P<0.05$ versus adjacent flow range for all comparisons). In contrast, tracer uptake in the epicardial layer became significant only when occlusion flow was $<40\%$ of normal ($P<0.05$ versus normal flow range). For flow ranges between $0\%$ and $60\%$ of normal, there was a graded increase in $^{99}$mTc-RP517 uptake from the epicardial to the endocardial segments.

**Relationships Between Occlusion Flow, $^{99}$mTc-RP517 Activity, and Tissue MPO**

The relationship between $^{99}$mTc-RP517 activity and occlusion flow in 573 myocardial segments from 8 dogs is shown in Figure 5A. The dotted curves represent the individual relationships for all animals. The best mathematical curve fit was an exponential with individual $r$ values ranging from 0.77 to 0.92. The overall $r$ value for the family of curves was 0.73 (solid line). In one additional dog, $^{99}$mTc-RP517 activity increased as occlusion flow decreased to $0.92$. The overall relationship between occlusion flow (mL/min per gram) and $^{99}$mTc-RP517 activity was highly correlated ($r=0.91$) with the neutrophil-specific enzyme MPO.

**$^{99}$mTc-RP517 Activity by Imaging**

Figure 6A shows a photograph of a blue dye–stained and TTC-stained heart slice from a representative occlusion/reperfusion dog. Figure 6B shows an ex vivo image of $^{99}$mTc-RP517 in the same slice. $^{99}$mTc-RP517 was localized to the risk area with a higher degree of uptake in the central ischemic zone. Uptake of the tracer in the normal posterior wall was negligible. The ischemic to normal zone count ratio of $^{99}$mTc-RP517 obtained from quantification of ex vivo images was not significantly different from the MPO tissue concentration ratio from the same regions ($2.7 \pm 0.2$ and $2.6 \pm 0.7$, respectively, $P=NS$). Figure 6C depicts a raw and background subtracted in vivo $^{99}$mTc-RP517 image that was acquired 60 minutes after reperfusion in one dog. Note that there was focal $^{99}$mTc-RP517 uptake that delineated the area of inflammation in the occluded-reperfused zone of the

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**TABLE 1. Hemodynamic Variables**

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Occlusion</th>
<th>Reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate, bpm</td>
<td>134±7</td>
<td>144±10</td>
<td>142±11</td>
</tr>
<tr>
<td>AP, mm Hg</td>
<td>114±6</td>
<td>104±5</td>
<td>100±3</td>
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<td>LAP, mm Hg</td>
<td>6±1</td>
<td>7±1</td>
<td>7±1</td>
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<tr>
<td>LAD flow, mL/min</td>
<td>35±7</td>
<td>0±0*</td>
<td>25±4</td>
</tr>
<tr>
<td>LCx flow, mL/min</td>
<td>47±9</td>
<td>42±7</td>
<td>37±7</td>
</tr>
<tr>
<td>dP/dt, mm Hg/s</td>
<td>2739±226</td>
<td>2772±254</td>
<td>2590±260</td>
</tr>
</tbody>
</table>

$AP$ indicates mean arterial pressure; $LAP$, mean left atrial pressure. Values are mean±SEM.

* $P<0.001$ vs baseline.

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**TABLE 2. Absolute Regional Myocardial Blood Flow and $^{99}$mTc-RP517 and Flow Ratios**

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Occlusion</th>
<th>Reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ischemic zone (I)</td>
<td>1.29±0.13</td>
<td>0.11±0.01*</td>
<td>0.89±0.10*</td>
</tr>
<tr>
<td>Border zone (B)</td>
<td>1.30±0.14</td>
<td>0.52±0.02*</td>
<td>0.93±0.06*</td>
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<tr>
<td>Normal zone (N)</td>
<td>1.56±0.18</td>
<td>1.14±0.09</td>
<td>1.21±0.11</td>
</tr>
<tr>
<td>I/N ratio</td>
<td>0.84±0.04</td>
<td>0.10±0.01</td>
<td>0.79±0.11</td>
</tr>
<tr>
<td>B/N ratio</td>
<td>0.84±0.05</td>
<td>0.48±0.04‡</td>
<td>0.79±0.06</td>
</tr>
</tbody>
</table>

Values are expressed in mL/min per gram and are mean±SEM.

* $P<0.05$ vs baseline; † $P<0.001$ vs occlusion flow ratio; ‡ $P=0.001$ vs I/N ratio.

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Figure 4. Myocardial localization of $^{99}$mTc-RP517. The endocardial (Endo), midwall (Mid), and epicardial (Epi) segments from 8 dogs ($n=573$) were grouped according to the flow reduction during occlusion. Note the strong, inverse relationship between occlusion flow and $^{99}$mTc-RP517 myocardial uptake in the endocardial and midwall layers. Also note the graded increase in $^{99}$mTc-RP517 from the epicardial to the endocardial layer in flow ranges $<60\%$ of normal. * $P<0.05$ versus adjacent flow range; † $P<0.05$ versus adjacent layer.
anteroseptal wall. Figure 6D depicts a 99mTc-RP517 image of a heart slice from a dog that received an intramyocardial injection of TNFα. Note the prominent uptake of the tracer observed at the injection site (white arrow). In this slice, there was a 75% increase in tracer uptake at the site of TNFα injection corresponding to a 51% increase in MPO in the same region. For all 3 dogs, the mean 99mTc-RP517 uptake and MPO ratios (injection site/contralateral wall) were 1.55 ± 0.13 and 1.45 ± 0.06, respectively (P=NS).

Discussion

The present study was undertaken to evaluate the potential of 99mTc-RP517 to assess myocardial inflammation in vivo after intravenous injection of the tracer. The major findings of the study are the following: (1) When incubated with whole blood, 99mTc-RP517 binds preferentially to LTB4 receptors on neutrophils. (2) After in vivo intravenous injection, the myocardial uptake of 99mTc-RP517 correlated with the specific neutrophil enzyme MPO tissue content when inflammation was induced by either ischemia and reperfusion or TNFα stimulation. (3) 99mTc-RP517 was localized in the area of myocardial inflammation and could be seen readily on images of the heart.

In Vitro Experiments

LTB4 is a potent inflammatory chemoattractant released by polymorphonuclear neutrophils, monocytes, and macrophages.20 Neutrophils express the LTB4 receptor,21 and binding of LTB4 to its receptor enhances adhesion of neutrophils to the vascular endothelium at the site of injury by activation of the leukocyte adhesion glycoprotein complex CD11/CD18.22 In this study, when the fluorescent LTB4 receptor antagonist [F]-RP517 was incubated with whole human blood, it bound preferentially to neutrophils. The binding of [F]-RP517 to isolated neutrophils occurred specifically on the LTB4 receptor as shown by the 44% decrease in binding when LTB4 was present. There are several possible explanations for the subtotal displacement of [F]-RP517 by...
LTB4. First, there are multiple receptor sites with different affinities for the agonist LTB4 and the tethered antagonist ([F]-RP517). Second, as is typical of all G-protein–coupled receptors, the LTB4 receptor may exist in two different conformational states, one with a relatively low affinity for the LTB4 agonist. In this case, although we used 40-times higher concentration of LTB4 compared with the agonist [F]-RP517, the concentration of LTB4 may have been inadequate to completely displace [F]-RP517 from a receptor in the low-affinity agonist-binding conformation. It is important to note that regardless of the mechanism, the binding of [F]-RP517 is specific to neutrophils as indicated by the FACS data and by the fact that [F]-RP517 was completely displaced by RP517 in isolated neutrophils.

In Vivo Experiments

99mTc-RP517 Myocardial Localization

Myocardial ischemia prompts an acute inflammatory response that is enhanced by reperfusion. A significant part of the myocardial injury resulting from ischemia and reperfusion is attributable to this acute inflammatory response. Chemotactic factors such as TNFα, IL-8, IL-6, PAF, complement, and leukotrienes are released by the myocardium during ischemia.23 These factors initiate a series of interactions between neutrophils and the vascular endothelium on reperfusion via specific adhesion molecules on both cell types,24 leading ultimately to neutrophil recruitment to the reperfused myocardium and neutrophil-induced reperfusion injury through generation of oxygen free radicals, degranulation and release of proteases, and release of arachidonic metabolites and other inflammatory mediators.23

The experimental canine model of myocardial infarction caused by coronary artery occlusion and reperfusion has been shown to induce neutrophil accumulation after reperfusion within the myocardial risk area.10 Neutrophil accumulation, assessed by technetium-99m-labeled autologous neutrophil localization, was found to be inversely correlated with regional myocardial blood flow during occlusion.10 Our data show a similar 99mTc-RP517 uptake pattern.

When the myocardial segments were grouped according to the severity of flow reduction during the occlusion, a graded distribution of 99mTc-RP517 was observed, with segments in the lowest flow ranges showing the highest 99mTc-RP517 uptake. Dreyer et al10 also observed a nonlinear relationship between neutrophil tissue content and occlusion flow. Similarly, we found that 99mTc-RP517 uptake was not linearly related to myocardial occlusion blood flow. Dreyer et al10 also reported that neutrophil recruitment was most prominent in the subendocardium.10 Our data are also consistent with these findings, because 99mTc-RP517 activity was higher in the endocardial than in the midwall and epicardial layers.

Myeloperoxidase and 99mTc-RP517 Activity

To provide additional evidence that 99mTc-RP517 uptake reflects postreperfusion neutrophil recruitment in previously ischemic tissue, we evaluated tracer uptake and MPO, a specific neutrophil enzyme,25 in the same 15 myocardial segments. We found a strong correlation ($r=0.91$) between neutrophil infiltration, assessed by MPO, and 99mTc-RP517 uptake, indicating that 99mTc-RP517 is a tracer of inflammation in our canine model of ischemia-reperfusion.

99mTc-RP517 Imaging

By ex vivo imaging of heart slices, we found that 99mTc-RP517 localized within the area of inflammation induced either by ischemia and reperfusion or by intramyocardial TNFα injection. In the occluded-reperfused dogs, there was negligible uptake occurring in the normal area. In addition, 99mTc-RP517 uptake in the inflammatory region was readily observed by in vivo imaging, indicating that this tracer may have potential as an inflammation-imaging agent. In the TNFα-injected dogs, background activity was higher as a result of systemic inflammation caused by recirculation of TNFα. The mean ex vivo count ratio of 99mTc-RP517 uptake was similar to the MPO activity ratio in both occluded-reperfused and TNFα-injected dogs, suggesting that 99mTc-RP517 could assess the severity of an acute inflammation injury.

Injection Timing for 99mTc-RP517

In the present study, we administered 99mTc-RP517 during the baseline period to create a circulating pool of labeled neutrophils and to avoid any confounding factors that might affect the uptake pattern of 99mTc-RP517, such as the hyperemic phase of reflow. In retrospect, this may have actually reduced our sensitivity somewhat, because a portion of the circulating pool of labeled neutrophils may have marginalized in other regions of the body, such as the site of recent surgery, and thus been unavailable at the time of the inflammatory stimulus at reperfusion. Thus, the optimal time for injection may be at the more clinically relevant time point of reperfusion. Additional studies are needed to address this important question.

Conclusion

99mTc-RP517 is a tracer of neutrophil infiltration in vivo. Additional experimental studies seem warranted to assess the potential of 99mTc-RP517 as an inflammation-imaging agent in vivo by determining the optimal dose, timing for injection and imaging, and usefulness for assessing myocardial inflammation that may be caused by a variety of factors.

Acknowledgments

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References


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