Neutrophil Accumulation in Ischemic Canine Myocardium
Insights Into Time Course, Distribution, and Mechanism of Localization During Early Reperfusion

William J. Dreyer, MD; Lloyd H. Michael, PhD; M. Stewart West, PhD;
C. Wayne Smith, MD; Robert Rothlein, PhD; Roger D. Rossen, MD;
Donald C. Anderson, MD; and Mark L. Entman, MD

Background. We have previously demonstrated that chemotactic factors released from the ischemic canine myocardium peak early during reperfusion and that they elicit neutrophil adherence reactions in vitro that are dependent on the CD18 glycoprotein family. In this study we investigated the hypothesis that neutrophil localization in ischemic canine myocardium in vivo occurs over a similar time course during early reperfusion and involves a CD18-dependent mechanism.

Methods and Results. We occluded the circumflex coronary artery for 1 hour in acute, open-chest dogs, followed by reperfusion for 1, 2, 3, or 4 hours. Regional myocardial blood flow was determined using radiolabeled microspheres, and localization was traced using technetium-99m-labeled autologous neutrophils. In the first hour of reperfusion, neutrophil localization occurred preferentially within the subendocardial region and was inversely related to flow. Neutrophil localization diminished across the ischemic myocardium from epicardium to endocardium but remained negatively related to flow in the midmyocardial region. Regardless of flow, little neutrophil localization occurred in the subepicardial region. Neutrophil localization was greatest in the first hour of reperfusion and diminished thereafter. By 4 hours of reperfusion, the rate of localization was markedly attenuated relative to 1 hour. Dogs given anti-CD18 monoclonal antibody R15.7 (1 mg/kg i.v.) before occlusion underwent 1 hour of occlusion followed by 1 hour of reperfusion. When compared with 1-hour reperfusion controls, the R15.7-treated dogs demonstrated significant attenuation of neutrophil localization in the subendocardial region.

Conclusions. These data support the concepts that rapid neutrophil localization during reperfusion occurs within regions of previous myocardial ischemia and that neutrophils preferentially localize within the subendocardial region. The rate of neutrophil localization is greatest within the first hour after the initiation of reperfusion, and localization is, at least in part, CD18 dependent. Therapies directed against neutrophil-mediated reperfusion injury should be initiated with these considerations in mind. (Circulation 1991;84:400–411)

Neutrophil localization within ischemic myocardium has been implicated as a mechanism of tissue injury capable of extending myocardial infarction. Previous histopathologic findings have shown that after coronary artery occlusion, neutrophils accumulate in an area of infarction after a period of 12–24 hours. With reperfusion, however, neutrophil accumulation is accelerated within the ischemic

From the Section of Cardiovascular Sciences, The Methodist Hospital, and DeBakey Heart Center, Department of Medicine (W.J.D., L.H.M., M.S.W., M.L.E.), the Spero P. Martel Section of Leukocyte Biology and Inflammation Research, Department of Pediatrics (W.J.D., C.W.S., D.C.A.), the Lillie Frank Abercrombie Section of Cardiology, Department of Pediatrics (W.J.D.), the Department of Microbiology and Immunology, Veterans Affairs Medical Center (R.D.R.), Baylor College of Medicine, Houston, Tex., and Boehringer Ingelheim Pharmaceuticals, Inc. (R.R.), Ridgefield, Conn.

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Address for correspondence: William J. Dreyer, MD, Texas Children’s Hospital, Clinical Care Center, Suite 2100, 8080 N. Stadium Drive, Houston, TX 77054.

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region.^{2–4} Previous findings in our laboratory^ have demonstrated that neutrophil chemotactic factors were present in cardiac lymph during reperfusion after a 1-hour coronary artery occlusion. Chemotactic factor generation peaked at 1–2 hours of reperfusion and returned to baseline by 4 hours of reperfusion. Chemotactic activity generated in lymph was capable of inducing increased CD11b/CD18 adherence glycoprotein expression on the neutrophil surface as well as increased neutrophil adherence to canine endothelial cell monolayers in vitro. From this information we hypothesized that in vivo neutrophil localization within the ischemic myocardium would occur with a similar time course during early reperfusion and that it would involve a CD18-dependent mechanism. To investigate this, we used a canine model of myocardial ischemia/reperfusion; regional myocardial blood flow was measured using radiolabeled microspheres, and neutrophil localization was traced using technetium-99m-labeled neutrophils. The dogs were studied during the first 4 hours of reperfusion after a 1-hour coronary artery occlusion, and the effects of systemically administered anti-CD18 monoclonal antibody R15.7 were assessed in dogs undergoing a 1-hour occlusion and 1 hour of reperfusion.

**Methods**

**Surgical Preparation of Animal Model**

Healthy adult mongrel dogs (15–22 kg) of either sex were anesthetized with sodium pentobarbital (30 mg/kg), intubated, and ventilated with an animal respirator with supplemental oxygen to maintain a normal arterial pH and oxygen content. A left thoracotomy was performed to provide access to the heart. The proximal circumflex coronary artery was isolated, and depending on the size of the artery and the extent of visible collateral vessels, a site just proximal or just distal to the first distal branch was prepared for ligation with a suture tourniquet. A Doppler flow probe was placed on the artery distal to the occlusion site to allow for the continuous monitoring of coronary flow. Cannulas were placed in the right and left atria for blood sampling or the infusion of substances as necessary. A femoral arterial line was also placed percutaneously. Arterial blood pressure, heart rate, electrocardiogram (standard limb lead II), and circumflex blood flow were recorded continuously. Regional myocardial ischemia was produced by 60 minutes of circumflex coronary artery occlusion and was followed by 1–4 hours of reperfusion. The suture ligature was released gradually over several minutes at the time of reperfusion to reduce the development of hemorrhagic infarction. After the reperfusion period, hearts were stopped by the infusion of potassium chloride and removed from the chest for sectioning.

Predetermined exclusion criteria were established to assure uniformity between the control and treatment groups. Dogs were excluded from data analysis if they failed to demonstrate an area of substantially reduced myocardial blood flow during ischemia as determined by microsphere analysis (see below) or if they developed ventricular fibrillation at any time during coronary artery occlusion or reperfusion. This protocol was reviewed and approved by the Baylor College of Medicine Animal Care and Use Committee.

**Treatment Groups**

Dogs were assigned to one of five groups.

*Group 1.* This initial “control” group consisted of eight dogs that underwent 60 minutes of coronary artery occlusion followed by 60 minutes of reperfusion. Regional myocardial blood flow determinations were made before occlusion, 50 minutes after occlusion, and during reperfusion at that point in time when hyperemic flow in the occluded vessel diminished to a plateau (generally to within 10% of baseline flow as determined by the Doppler flow probe and generally 10–15 minutes into reperfusion). Immediately after the reperfusion blood flow determination, 4×10^7 to 6×10^7 99mTc-labeled neutrophils (see below) were given over 2–3 minutes by left atrial injection and allowed to circulate 45 minutes before termination of the experiment. 99mTc activity was measured in the plasma, white blood cell (WBC) fraction, and red blood cell (RBC) fraction at 5, 15, 30, and 45 minutes after labeled neutrophil injection.

*Groups 2–4.* Dogs in each of these groups also underwent 60 minutes of coronary artery occlusion; regional myocardial blood flow determinations were made at rest and at 50 minutes into occlusion. In each successive group, the reperfusion period was increased by 1 hour to 2, 3, and 4 hours, respectively. 99mTc-labeled neutrophils were given in each group just 45 minutes before the end of the reperfusion period to reflect neutrophil accumulation occurring during the second, third, and fourth hours of reperfusion only. A reperfusion myocardial blood flow determination was made in each group just before the administration of the 99mTc-labeled neutrophils. Group 2 consisted of five dogs and groups 3 and 4 consisted of four dogs each.

*Group 5.* This group consisted of seven dogs that received a single 1 mg/kg dose of the anti-CD18 monoclonal antibody R15.7 (see below) by peripheral intravenous injection 3 hours before coronary artery occlusion. Dogs in this group were otherwise treated the same as group 1 control dogs (60 minutes of occlusion followed by 60 minutes of reperfusion) with the one exception; 99mTc-labeled neutrophils for these dogs were also incubated with a saturating concentration (10 μg/ml) of R15.7 for 15 minutes before reinfusion.

**Determination of Regional Myocardial Blood Flow**

Regional myocardial blood flow was determined with radiolabeled microspheres (10–15 μm diameter, 3M, St. Paul, Minn.) using the reference withdrawal method^ as previously described. Injections of microspheres labeled with 85Sr, 95Nb, or 46Sc were given in each experiment, as described above.
Evaluation of Neutrophil Localization in Myocardial Tissue

Canine neutrophils were isolated from anticoagulated blood by dextran sedimentation followed by Ficoll-Hypaque gradient separation in a modification of the method described by Rossen et al.6 Before surgery, 40 ml peripheral venous blood was drawn into citrate-phosphate-dextrose buffer (0.14 ml/ml blood) and then sedimented for 1 hour with 6% dextran in saline (1 ml/10 ml blood). The resulting leukocyte-rich plasma was removed and centrifuged at 300g for 5 minutes at 22°C. The cell button was resuspended in Dulbecco’s phosphate buffered saline (DPBS), pH 7.4, containing 0.2% glucose and then layered over a two-step Ficoll-Hypaque gradient. The gradient was prepared by placing 4 ml Ficoll-Hypaque, specific gravity 1.095, into the bottom of a 15-ml conical polystyrene tube and then carefully layering on top 2 ml Ficoll-Hypaque, specific gravity 1.055. Five milliliters of the leukocyte-rich suspension was then layered on top of the upper Ficoll-Hypaque layer. In each case, great care was taken to preserve the interface. After centrifugation at 600g for 22 minutes at 22°C, the DPBS layer and upper Ficoll-Hypaque layer, including the band rich in mononuclear cells, were discarded. The neutrophils were retrieved from the top of the lower Ficoll-Hypaque layer, above and well separated from the RBC button in the bottom of the tube. They were washed with DPBS and collected by centrifugation at 300g for 5 minutes at 22°C. The cells were resuspended in DPBS at 107 cells/ml. The final suspension contained 99% neutrophils of which 95% were viable by trypan blue dye exclusion. 99mTc labeling of the cells was begun immediately afterward using a modification of the method described by Moon et al.9 Cells were incubated with a 2% solution of stannous chloride in acid-citrate-dextrose buffer (0.1 ml/1 ml cell suspension) for 10 minutes at 22°C. The cells were centrifuged (600g for 5 minutes), and the supernatant was discarded. The cells were then washed twice and resuspended in DPBS. 99mTc sodium pertechnetate (10 mCi, Syncon Corp., Houston) was added to the cell suspension, and the suspension was incubated for 10 minutes at 22°C. The labeled cells were washed four times with DPBS, resuspended, and counted. Radioactivity in the supernatant of the last wash was also counted to determine labeling efficiency and the specific activity of the labeled cells. Cells isolated and labeled by the above techniques were examined for evidence of activation as determined by 1) a change in cell morphology under phase-contrast microscopy indicative of activation, 2) increased expression of CD11/CD18 surface glycoproteins using flow cytometry (see below), and 3) Boyden chamber chemotaxis in response to zymosan-activated dog serum.

Assessment of 99mTc Activity in Fractionated Whole Blood Samples

To establish that 99mTc remained neutrophil bound when cells were reinjected into the circulation, 15-ml samples of whole blood were removed at 5, 15, 30, and 45 minutes after 99mTc-labeled WBC infusion. The whole blood samples were sedimented with dextran, and subsequently, the leukocyte-rich plasma was removed and centrifuged at 300g for 5 minutes at 22°C. The cell-free plasma was removed, and the cell button was resuspended in an equal volume of DPBS. Equal volumes of sedimented RBCs, resuspended WBCs, and plasma were then counted for 99mTc activity.

Monoclonal Antibody R15.7

R15.7 is an immunoglobulin G1 murine monoclonal antibody that recognizes a functional epitope of the CD18 adherence glycoprotein on both human and canine neutrophils as previously described.10 Pharmaceutical grade R15.7 monoclonal antibody was supplied by Boehringer-Ingelheim Pharmaceuticals, Inc., Ridgefield, Conn., and was purified from aseptically collected murine ascites by ammonium sulfate precipitation and protein A affinity chromatography. Endotoxin was removed by passage over a polymyxin B column with an endotoxin concentration in the final preparation of less than 1 endotoxin unit/ml. Antibody was sterile-filtered, diluted to a final concentration of 4.4 mg/ml in DPBS, and stored at −80°C until use.

Detection of R15.7 Monoclonal Antibody Binding to Canine Neutrophils

At 1 hour, 3 hours (preocclusion), and 4 hours (reperfusion) after antibody infusion, 5-ml venous blood samples were withdrawn from the test dog into citrate-phosphate-dextrose buffer for detection of excess antibody in plasma and for detection of antibody binding to circulating neutrophils, performed by immunofluorescence staining. To assess the binding of plasma antibody to circulating cells 90 μl whole blood was incubated for 15 minutes at 22°C in the presence of a saturating concentration of fluorescein-conjugated rabbit anti-mouse F(ab)’2 (Zymed Laboratories, Inc., South San Francisco). The cells were washed, depleted of erythrocytes by lysis (FACS lysing solution, Becton Dickinson Immunocytometry Systems, San Jose, Calif.), and fixed in 1% paraformaldehyde. Antibody binding to dog neutrophils was then assessed by flow cytometry (FACscan, Becton Dickinson Laboratory, Lincoln Park, N.J.) after selective gating on the myeloid cell population. The fluorescence intensity of 5,000 cells/determination was used as a quantitative measure of antibody binding. In addition, to assess the number of potential R15.7 binding sites present on circulating neutrophils, a second 90-μl aliquot of whole blood was incubated with a saturating concentration of added R15.7 for 15 minutes at 22°C, washed, and then incubated with fluorescein-conjugated antibody and processed as above. Finally, to document the presence of R15.7 monoclonal antibody in plasma in sufficient quantity to produce antibody excess, the remainder of the venous blood in each of the samples indicated above was centrifuged at 800g for 5 minutes.
at 22°C, and the plasma was removed for indirect immunofluorescence analysis. The cell button from 90 μl whole blood drawn from a separate donor dog was suspended and incubated with either a saturating concentration of R15.7 antibody or with serum from the test dog for 15 minutes at 22°C. After washing, the cells were incubated with a saturating concentration of fluorescein-conjugated rabbit anti-mouse F(ab’)2 and analyzed for antibody binding by flow cytometry as described above.

Sectioning of Hearts for Isotope Counting

After removal of the heart from the test dog, the great vessels and atria (including all valvular tissue) and the right ventricular free wall were removed from the left ventricule and septum. As depicted in Figure 1, the resulting cone of tissue was sectioned from base to apex into four or five transverse rings approximately 1 cm in thickness. Each ring was then sectioned into six pieces corresponding to the anterior free wall, the anterior papillary muscle, the lateral free wall, the posterior papillary muscle, the posterior free wall, and the septum. Each of these pieces was then subdivided equally into four transmural layers from the epicardium to the endocardium. Each tissue sample was weighed and placed in a separate plastic vial with 10% buffered formalin for subsequent determination of radioactivity. Tissue samples ranged in size from 0.25 to 1.2 g.

Statistical Analysis

For purposes of analysis and data presentation, both occlusion myocardial blood flow and neutrophil accumulation were standardized. For each dog, occlusion blood flow in each tissue sample was standardized as a percentage of the highest blood flow measured in the nonischemic region during occlusion in that dog. Neutrophil accumulation was expressed as the ratio of 99mTc-labeled neutrophils observed in a tissue sample relative to the minimum, or background, number observed in any one sample.

The relation between neutrophil localization and occlusion blood flow was examined in each transmural layer for each dog. Because of the curvilinear relation between the two seen in plots of the data in the control dogs, a polynomial regression model with linear and quadratic terms was fit. This model fits a half parabola to the data. If the p value associated with the quadratic component is statistically significant, it indicates that the curvilinear relation between flow and localization is present in a given layer. The simultaneous significance of the linear component merely changes the shape of the fitted parabola. If the linear component is significant and the quadratic is not, a straight line relation is indicated. Neither being significant indicates no apparent relation between flow and localization. Because four regressions were fit for each dog, values of p<0.0125 were regarded as statistically significant, and values of p=0.02–0.03 were regarded as strongly suggestive of a relation.

The coefficient of determination (R², the proportion of variability in the data explained by the model) provides a descriptive comparison among group 1 (control), groups 2–4 (2-, 3-, and 4-hour reperusions), and group 5 (monoclonal antibody–treated) dogs. The R²s are reported as percentages and are adjusted for having two independent variables. Informally, R²>90% is considered an excellent fit for the model. Values ranging from 70% to 90% are considered moderate to good fits; those less than 50% are not generally regarded as indicating a reasonable fit.

To compare the neutrophil accumulation among the different groups of dogs, blood flow was categorized into four regions: 1) 0–10%, 2) 11–30%, 3) 31–50%, and 4) greater than 50%. The total number of 99mTc-labeled neutrophils was calculated for each category for each dog. The neutrophil accumulation for each of the three lower flow categories was then expressed as a ratio relative to the fourth category. The mean neutrophil accumulation in each flow category for each 2-, 3-, and 4-hour reperfusion group and for the monoclonal antibody–treated group was compared with the control group using a one-factor analysis of variance (ANOVA) with Dunnett’s multiple comparison procedure. The analysis was run for each of the four regions (endocardium, midmyocardium 1 and 2, and epicardium) separately. Because of the multiple analyses, only values of p<0.005 for ANOVA were considered statistically significant. Values of p=0.01–0.02 were regarded suggestive of differences among the groups.

Results

99mTc Distribution in Whole Blood Fractions

Figure 2 demonstrates the relative amount of 99mTc activity in WBC, RBC, and plasma fractions taken from peripheral venous blood drawn 5 minutes after 99mTc-labeled WBC infusion. Similar results were obtained at 15, 30, and 45 minutes after infusion (data not shown). The data at 5 minutes indicate that
at least 65% of the $^{99m}$Tc activity remained with the WBCs. The balance of the $^{99m}$Tc activity measured was either free in the plasma and RBC bound or represented $^{99m}$Tc-labeled WBC contamination of these fractions. From these data we subsequently assumed $^{99m}$Tc activity measured in our myocardial tissue samples to represent neutrophil localization.

**Neutrophil Localization During the First Hour of Reperfusion**

Neutrophil localization during the first hour of reperfusion was carefully characterized in dogs from group 1 to form a basis for comparison with the additional groups. The accumulation of $^{99m}$Tc-labeled neutrophils was compared with regional myocardial blood flow at rest (before occlusion), during occlusion, and during reperfusion for each dog. Figure 3 demonstrates this relation for a single dog from group 1. Panel A demonstrates preocclusion myocardial blood flow. Panel B clearly demonstrates areas of reduced myocardial blood flow during occlusion, and in Panel C, $^{99m}$Tc neutrophil accumulation (during reperfusion) is clearly evident in areas of reduced flow during occlusion. Panel D demonstrates reperfusion myocardial blood flow and demonstrates some degree of hyperemia in the previously ischemic regions of this dog.

To further characterize neutrophil localization in the 1-hour reperfusion group, neutrophil localization was examined for each dog in each individual tissue sample as a function of occlusion myocardial blood flow, and then tissue samples were segregated as to the transmural layer of myocardium from which they originated. Figure 4 demonstrates in a single representative dog the pattern of neutrophil localization typical of all the dogs in this 1-hour reperfusion group. As demonstrated in the figure, neutrophil localization occurred preferentially within the subendocardial region and was inversely related to flow. Significant neutrophil accumulation also occurred within the midmyocardial region next to the endocardium but demonstrated a greater variability among tissue segments and a greater variability from dog to dog. Neutrophil accumulation near the subepicardial region, however, was markedly diminished, regardless of flow. The results of the quadratic polynomial regression models for each of the four transmural layers for each of the 1-hour reperfusion control dogs are shown in Table 1. The polynomial regression model fit at least moderately well (adjusted $R^2$, 69.1–90.0%) in the endocardial region for every dog with only one value of $p>0.03$ for the quadratic component. Moving transmurally, the overall fit was progressively worse in each layer, with four of eight dogs having a reasonable fit in the first midmyocardial layer, only one of eight in the second layer, and none in the epicardium.

**Neutrophil Localization During Hours 2–4 of Reperfusion**

A similar analysis of neutrophil localization was performed in each of the dogs from groups 2–4. Relative to 1 hour of reperfusion, neutrophil localization during hours 2–4 was less likely to be associated with myocardial blood flow during occlusion. Table 2 shows the regression results for the endocardial layer for the 2-, 3-, and 4-hour reperfusion groups. In the 2-hour group, the model fit well for four of five dogs, with the presence of the quadratic component being at least suggestive for the same four dogs. The quadratic component was significant in only 1 dog in the 3-hour group and none in the 4-hour group, with reasonable fits ($R^2$) in only one dog in each.

Dogs from the 1-, 2-, 3-, and 4-hour reperfusion groups were also analyzed as to the rate of neutrophil accumulation within the ischemic region over time. Because $^{99m}$Tc-labeled cells were given only during the last hour of reperfusion in each of the groups, they reflect neutrophil accumulation during that 1-hour time period only. As demonstrated in Figure 5, neutrophil accumulation across all regions of the myocardium was greatest within the first hour of reperfusion. Within the very low flow region of the subendocardium, the rate of neutrophil accumulation was significantly higher in the 1-hour group when compared with any of the groups with longer reperfusion times (ANOVA, $p=0.002$; Dunnett’s test, $p<0.01$). No other comparisons had $p$ values lower than 0.005 for the ANOVA model; however, in the very low flow category, both midmyocardial layers showed suggestive differences between the 1-hour group and the 4-hour reperfusion group (as well as the 3-hour group in the second midmyocardial layer). In the subendocardial region, a suggestive difference was also seen in the moderate flow category for the 1-hour group compared with the 4-hour reperfusion group.

**Systemic Infusion of R15.7 Monoclonal Antibody**

Pharmacokinetic data was obtained on the systemic administration of the R15.7 monoclonal antibody in all the study animals from group 5. Figure 6A

![Figure 2](http://circ.ahajournals.org/)

**Figure 2.** Bar graph showing technetium-$^{99m}$ activity distributed in whole blood fractions 5 minutes after left atrial infusion. Values were determined in six dogs from groups 1 and 5 and are presented as the mean±1 SD. WBC, white blood cell; RBC, red blood cell.
Figure 3. Bar graphs showing relation of neutrophil localization to regional myocardial blood flow (MBF) in single dog from group 1. In each panel, individual bars are used to represent individual pieces of tissue dissected from left ventricle as depicted in Figure 1. Rings of tissue taken from left ventricle are arranged consecutively from base to apex along x axis from left to right, and for each ring, segments of tissue are arranged consecutively in counterclockwise fashion beginning with anterior free wall. In addition, there are four layers of myocardium arranged transmurally from epicardium to endocardium for each tissue segment. Resting myocardial blood flow (ml/min/g tissue) is depicted in panel A; occlusion myocardial blood flow, in panel B; and reperfusion myocardial blood flow, panel D. Neutrophil (PMN) accumulation is represented in panel C and is expressed as 1,000 technetium-99m counts/g tissue. 99mTc neutrophil accumulation (during reperfusion) into areas of flow reduction during occlusion is clearly evident in panel C. A, anterior free wall; AP, anterior papillary muscle; L, lateral free wall; PP, posterior papillary muscle; P, posterior free wall; S, septum.
Neutrophil seven of not to group body-treated in endocardial and neutrophils control values R15.7, reaching the throughout both time, over sites on from the infusion of antibody. Excess antibody R15.7 or near the flow demonstrates the flow cytometry analysis of isolated neutrophils from a donor dog using serum from the test dog as a source of R15.7 and demonstrates that excess antibody was present in the serum at saturating or near saturating levels throughout the study protocol.

Figure 6B demonstrates flow cytometry analysis of R15.7 binding measured directly on peripheral blood neutrophils from the test dogs, expressed as the percent of total available binding sites bound by serum antibody. Panel B demonstrates that after the infusion of R15.7, the number of available binding sites on peripheral blood neutrophils appeared to be downregulated. Despite fewer binding sites present over time, and despite the availability of excess antibody in the serum, available sites on peripheral blood neutrophils were only about 50% saturated throughout the occlusion and reperfusion protocols.

Peripheral WBC and absolute neutrophil counts during the study protocol are demonstrated in Figure 6C. Both decreased in parallel after the infusion of R15.7, reaching approximately 60% of preinfusion control values by the onset of occlusion, and both began to show recovery by the time of reperfusion.

Neutrophil Localization: Control Versus R15.7-Treated Group

The regression results for the monoclonal antibody–treated group are shown in Table 3 for the endocardial and first midmyocardial layers. The tendency in the antibody-treated dogs was for neutrophil localization not to be as well associated with flow as in the control group. The model fit well in only three of seven dogs in the endocardial layer, with the presence of the quadratic component being statistically significant in only one and suggestive in the other two. In the first midmyocardial layer, the model fit well in only one dog, with the quadratic component being significant as well.

Figure 7 compares the extent of neutrophil accumulation in the control (1-hour) and antibody-treated groups in a format similar to that of Figure 5. Note that, in the subendocardial region, R15.7 treatment significantly attenuated neutrophil accumulation in the very low flow region (ANOVA, \( p = 0.0002 \); Dunnett's test, \( p < 0.01 \)). In the relatively low flow area (11–30%) of the subendocardium, a suggestive difference was seen (ANOVA, \( p = 0.01 \); Dunnett's test, \( p < 0.05 \)). In addition, R15.7 appeared to affect a similar reduction in neutrophil accumulation in the midmyocardium; however, presumably because of a greater initial variability in neutrophil accumulation relative to flow in the control group, this reduction did not reach statistical significance when considered alone. When considered transmurally, however, the effect of R15.7 was to reduce neutrophil accumulation by about 50%.

Finally, Figure 8 compares the clearance of circulating \(^{99m}Tc\)-labeled WBCs from the blood after their infusion in the R15.7-treated and control groups. The plasma half-life of circulating \(^{99m}Tc\)-labeled WBCs was significantly prolonged in the R15.7-treated group.

Discussion

The data from this study demonstrate that \(^{99m}Tc\)-labeled neutrophils had a propensity to localize in areas of the myocardium made ischemic by a 1-hour coronary artery occlusion and that the degree of neutrophil accumulation appeared inversely proportional to regional myocardial blood flow during ischemia. Further-
more, neutrophil accumulation in each dog was greatest in the subendocardial region and diminished transmurally from endocardium to epicardium, even in tissue with comparable blood flows. The rate of neutrophil accumulation was greatest in the first hour of reperfusion and diminished thereafter during periods of reperfusion up to 4 hours. In dogs given the anti-CD18 monoclonal antibody R15.7, neutrophil accumulation was significantly attenuated during the first hour of reperfusion relative to controls. These data support the concepts that rapid neutrophil accumulation within regions of myocardial ischemia occurs preferentially within the subendocardial region early after the initiation of reperfusion and that localization is, in part, CD18 dependent.

Methodological Considerations

Two concerns regarding methodology should be addressed in interpreting the results of this study. First, the question of whether the $^{99m}$Tc activity observed in the ischemic myocardium was truly reflective of neutrophil localization must be answered. $^{99m}$Tc preferentially binds to RBCs when present, and great care was taken in the neutrophil isolation technique to eliminate RBC contamination. $^{99m}$Tc-labeled neutrophils in suspension also demonstrated the tendency to leach technetium from the cells into the medium. However, when aliquots of blood were taken at multiple time points during reperfusion after the infusion of the radiolabeled cells, fractioned samples revealed that the $^{99m}$Tc activity remained primarily in the WBC fraction, with little activity noted in the RBC and plasma fractions. Consideration must also be given to the fact that the neutrophils used in this study were isolated from whole blood, tagged with a radioactive label, and subsequently reinfused into the whole animal. Cells handled in such a way invariably undergo some degree of activation, and the extent to which these cells reflect the activity of normal cells must be questioned. However, when compared with unlabeled cells from the same dog, cells labeled with $^{99m}$Tc demonstrated no significant change in cell morphology indicative of activation nor did they demonstrate a significant increase in the surface expression of CD18 by flow cytometry. Furthermore, labeled cells retained chemotactic responsiveness to zymosan-activated serum when examined by the Boyden chamber method (data not shown). Thus, insofar as in vitro tests could determine, these cells were normally responsive to physiological stimuli before their return to the vascular compartment.

Neutrophil Localization

The accumulation of neutrophils in ischemic myocardium during reperfusion has been previously documented by myeloperoxidase assay$^{12,13}$ as well as by using cells labeled with $^{111}$In.$^{14-16}$ The preferential localization of neutrophils at the endocardial surface

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**TABLE 1. Comparison of Quadratic Polynomial Regression Models for Neutrophil Localization Between Transmural Layers in 1-Hour Reperfusion Control Group**

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<th>Layer</th>
<th>Linear</th>
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<td>&lt;0.001</td>
<td>0.001</td>
<td>67.1</td>
</tr>
<tr>
<td></td>
<td>0.31</td>
<td>0.65</td>
<td>28.0</td>
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<tr>
<td></td>
<td>0.020</td>
<td>0.059</td>
<td>24.6</td>
</tr>
<tr>
<td>Epicardium</td>
<td>0.47</td>
<td>0.57</td>
<td>0.0</td>
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<tr>
<td></td>
<td>0.24</td>
<td>0.21</td>
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<td>0.022</td>
<td>0.013</td>
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<td>0.99</td>
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<tr>
<td></td>
<td>0.086</td>
<td>0.26</td>
<td>25.5</td>
</tr>
<tr>
<td></td>
<td>0.024</td>
<td>0.086</td>
<td>30.4</td>
</tr>
<tr>
<td></td>
<td>0.49</td>
<td>0.58</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>0.40</td>
<td>0.72</td>
<td>14.7</td>
</tr>
</tbody>
</table>

**TABLE 2. Comparison of Quadratic Polynomial Regression Models for Neutrophil Localization in Endocardial Region for Different Times of Reperfusion**

<table>
<thead>
<tr>
<th>Reperfusion</th>
<th>Linear</th>
<th>Quadratic</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 Hour</td>
<td>0.012</td>
<td>0.11</td>
<td>49.8</td>
</tr>
<tr>
<td></td>
<td>&lt;0.001</td>
<td>0.014</td>
<td>73.4</td>
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<tr>
<td></td>
<td>&lt;0.001</td>
<td>0.004</td>
<td>96.1</td>
</tr>
<tr>
<td></td>
<td>&lt;0.001</td>
<td>0.001</td>
<td>85.4</td>
</tr>
<tr>
<td></td>
<td>&lt;0.001</td>
<td>0.039</td>
<td>70.3</td>
</tr>
<tr>
<td>3 Hour</td>
<td>0.77</td>
<td>0.60</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>58.5</td>
</tr>
<tr>
<td></td>
<td>0.56</td>
<td>0.84</td>
<td>30.1</td>
</tr>
<tr>
<td></td>
<td>0.001</td>
<td>0.18</td>
<td>79.6</td>
</tr>
<tr>
<td>4 Hour</td>
<td>0.14</td>
<td>0.55</td>
<td>36.2</td>
</tr>
<tr>
<td></td>
<td>0.34</td>
<td>0.25</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>0.003</td>
<td>0.10</td>
<td>76.1</td>
</tr>
<tr>
<td></td>
<td>0.29</td>
<td>0.50</td>
<td>63.6</td>
</tr>
</tbody>
</table>
in ischemic myocardium has also been noted. This study expands on those previous observations with a more detailed accounting of the transmural distribution of neutrophil localization during reperfusion. This study also provides a new observation regarding the accelerated rate of neutrophil accumulation in the first hour of reperfusion, which subsequently diminishes over the first 4 hours. Two possibilities exist to explain this observation. First, neutrophil accumulation might be related to reperfusion blood flow to the ischemic zone. Five of eight dogs in the 1-hour reperfusion controls (group 1) demonstrated a modest degree of hyperemia (60–100% increase in flow to tissue samples from the previously ischemic zone relative to the nonischemic region) at the time of 99mTc-labeled neutrophil infusion. One dog demonstrated a 200% increase in flow, and two dogs demonstrated no hyperemia. Dogs in the longer reperfusion groups demonstrated no hyperemia when labeled neutrophils were infused. However, two of five dogs in group 2, two of four dogs in group 3, and four of four dogs in group 4 demonstrated a modest reduction in flow to tissue samples from the ischemic region, indicative of no reflow to at least a portion of the capillary bed from those samples. However, when reperfusion blood flow was analyzed as an independent variable in our model of neutrophil localization, it significantly improved the relation between neutrophil localization and occlusion blood flow in only two of eight dogs in group 1, one of five dogs in group 2, one of five dogs in group 3, and none of the four dogs in group 4 (data not shown). Thus, the effect of reperfusion blood flow on neutrophil localization appeared to be quite modest in our model.

The second possibility exists that neutrophil accumulation may be related to neutrophil chemotactic activity present in the ischemic zone. The accelerated neutrophil accumulation noted in the first hour of reperfusion demonstrates a close temporal relation to our previous observation of peak chemotactic activity released into the cardiac lymph during reperfusion after a coronary artery occlusion of similar duration. The reduction in the rate of neutrophil accumulation at 4 hours of reperfusion also occurs simultaneously with a marked reduction in chemotactic activity in cardiac lymph over that time period.

Although the cumulative localization of neutrophils may be substantial over time, the "critical mass" of neutrophils necessary to cause myocardial injury is unknown. However, our observation of significant neutrophil accumulation within the first hour of reperfusion may indicate the need for anti-inflammatory measures to be in effect early in the postischemic period to have a measurable therapeutic benefit.

R15.7 Administration

Potential effector mechanisms for neutrophil-mediated myocardial injury include vascular plugging and endothelial cell or myocyte damage through
oxygen free radical release or the release of proteolytic enzymes. Previous data\textsuperscript{10,17-19} suggest that each of these processes is dependent on neutrophil adher-

ence and that each is, at least in part, dependent on the CD18 adherence glycoprotein family. Our previous data\textsuperscript{5} have also demonstrated an upregulation of CD18 on the cell surface of neutrophils isolated from cardiac lymph during reperfusion. Thus, an anti-CD18 monoclonal antibody with demonstrated efficacy at blocking canine neutrophil adherence reactions in vitro was chosen for this experiment.

Data from several pilot animals demonstrated that the peak amount of monoclonal antibody binding to neutrophils in vivo did not occur for several hours after administration. This prompted the administration of antibody before occlusion to give the antibody an opportunity to bind to circulating neutrophils, allowing us to assess its efficacy from a mechanistic standpoint rather than to attempt administration at reperfusion, which might more closely approximate its therapeutic use.

The observation that not all available binding sites on circulating neutrophils were occupied by antibody was true for every dog in this study, despite the presence of excess antibody in the serum in sufficient quantity to saturate cells in vitro throughout the study protocol. A similar observation was made by Simpson et al.,\textsuperscript{20} who used the anti-CD11b antibody 904. We have no clear explanation for this observation. The lack of binding site saturation by R15.7 in the model might be explained by a difference in the avidity of this antibody for CD18 in vivo when compared to in vitro results. In each study dog, the presence of antibody in serum also appeared to have a modulating effect on the number of available CD18 binding sites on circulating neutrophils. Surface CD18 appeared to decrease as the study protocol progressed. One could speculate that surface CD18 expression in this setting is dynamic, with antibody-associated CD18 being internalized and new non-bound CD18 being expressed. The extent of surface

\begin{table}[ht]
\centering
\caption{Comparison of Quadratic Polynomial Regression Models for Neutrophil Localization Between First Two Transmural Layers in Monoclonal Antibody–Treated Group}
\label{tab:regression_models}
\begin{tabular}{lccc}
\hline
Layer & Linear & Quadratic & $R^2$ \\
\hline
Endocardium & 0.31 & 0.44 & 1.2 \\
 & <0.001 & 0.001 & 72.8 \\
 & 0.64 & 0.90 & 11.0 \\
 & 0.001 & 0.03 & 67.5 \\
 & 0.002 & 0.02 & 39.4 \\
 & 0.02 & 0.11 & 39.9 \\
 & 0.006 & 0.11 & 64.2 \\
Midmyocardium 1 & 0.97 & 0.97 & 0.0 \\
 & 0.10 & 0.35 & 38.1 \\
 & 0.81 & 0.92 & 0.0 \\
 & <0.001 & <0.001 & 84.4 \\
 & 0.81 & 0.75 & 0.0 \\
 & 0.48 & 0.53 & 0.0 \\
 & 0.64 & 0.78 & 0.0 \\
\hline
\end{tabular}
\end{table}
CD18 that must be antibody bound for the adhesiveness of the neutrophil to be reduced is also unknown.

Clearly though, an antibody effect was observed in this model. R15.7 administration in this study inhibited neutrophil localization in the ischemic regions of the myocardium and prolonged the half-life of circulating 99mTc-labeled cells. These results strongly suggest a role for CD18-mediated adhesion in the inflammatory process associated with reperfusion. A previous study in rabbits by Seewaldt-Becker et al\textsuperscript{21} demonstrated that systemic administration of an anti-CD18 monoclonal antibody reduced infarct size after reperfusion. More recently, however, two canine studies\textsuperscript{22,23} reported an inhibition of neutrophil localization with other anti-CD18 monoclonal antibodies but reported equivocal results regarding the reduction in infarct size.

The reasons for these discrepant results are unclear but could be related to differences in the antibodies used or to technical differences in the models used. The present study suggests both a time frame for neutrophil localization and a region of myocardium for which neutrophil-mediated injury may be greatest after an ischemic interval of 60 minutes. Future investigations of myocardial salvage with anti-inflammatory interventions may need to be designed with these considerations in mind. However, certain questions remain: 1) How many neutrophils are necessary to cause damage to the myocardium? 2) To what extent must neutrophil localization or other neutrophil functions be altered to effect neutrophil-mediated injury? 3) After how long an occlusion interval might an anti-inflammatory mechanism be expected to prevent additional myocardial injury? Additional studies are necessary to address these interesting questions.

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References


KEY WORDS • reperfusion injury • chemotaxis • neutrophils • monoclonal antibodies
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W J Dreyer, L H Michael, M S West, C W Smith, R Rothlein, R D Rossen, D C Anderson and M L Entman

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