Neutrophil Depletion Limited to Reperfusion Reduces Myocardial Infarct Size After 90 Minutes of Ischemia

Evidence for Neutrophil-Mediated Reperfusion Injury

Marc R. Litt, MD, Richmond W. Jeremy, MD, PhD, Harlan F. Weisman, MD, Jerry A. Winkelstein, MD, and Lewis C. Becker, MD

Reperfusion of ischemic myocardium may accelerate necrosis of injured myocytes. To determine the role of neutrophil leukocytes in this process, we examined whether neutrophil depletion during reperfusion could modify infarct size in anesthetized dogs. The proximal circumflex coronary artery was occluded for 90 minutes and then reperfused for 2 hours via an extracorporeal circuit with either whole blood (n=11) or with blood depleted of neutrophils by leukocyte filters (n=11). The leukocyte filters caused near-total neutropenia in blood reperfusing the ischemic myocardium (7±7 neutrophils/μl compared with 2,551±317/μl in controls, mean±SEM; p<0.001. Infarct size was measured by planimetry of myocardial slices stained with triphenyltetrazolium chloride (TTC), and the accuracy of TTC for identifying necrotic myocardium was verified by electron microscopy. The size of the ischemic risk region was the same in the control (41.6±1.0%) and neutropenic (41.8±2.1%) groups. Collateral blood flow to the risk region was the same in control (0.15±0.03 ml/min/g) and neutropenic (0.13±0.03 ml/min/g) groups. Among dogs with collateral flow less than 0.2 ml/min/g, infarct size was reduced in the neutropenic group (27.7±6.7% of risk region, n=8), compared with control dogs (52.5±5.7%; n=7; p=0.02). Multiple linear regression described the relation between infarct size, risk region size, and collateral flow in the control group, and the same regression relation was used to predict infarct size for the neutropenic group. Mean predicted infarct size in the neutropenic group (n=11) was 16.8±3.4% of left ventricle, whereas mean observed infarct size was 9.6±3.1% (p<0.01). The extent of the no-reflow zone (absence of thioflavin-S-fluorescence) was also less in the neutropenic than the control group (2.2±0.8% vs. 8.1±2.7% of the risk region, p<0.05). Neutropenia limited to the reperfusion period was associated with significant reductions in the extent of the infarct and no-reflow zones after 90 minutes of ischemia. These findings support the hypothesis that reperfusion necrosis occurs after prolonged myocardial ischemia and indicate that neutrophil leukocytes are important mediators of such reperfusion injury. (Circulation 1989;80:1816–1827)

Early reperfusion of ischemic myocardium after coronary occlusion can reduce the extent of necrosis1–3 and is associated with improved left ventricular function and prognosis.4–6 There is, however, increasing evidence that these benefits may be limited by reperfusion-induced necrosis of ischemic but potentially viable myocytes.7,8 Reperfusion injury has been attributed in part to a burst of oxygen free radical formation with subsequent lipid peroxidation and membrane damage.9 This hypothesis is supported by studies that demonstrate that infarct size can be reduced by treatment with free radical scavengers administered during ischemic and early reperfusion periods.10–15 Neutrophil leukocytes are a potential source of oxygen free radicals, which can be generated by the nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase reaction.16–18 Although only a few neutrophils are present in normal or acutely

From the Department of Medicine, Division of Cardiology, and the Department of Pediatrics, Eudowood Division of Immunology, The Johns Hopkins Medical Institutions, Baltimore, Maryland.

Supported by US Public Health Service grants 17655-13 (SCOR in Ischemic Heart Disease), RO1-33360, and AI-19278 from the National Institutes of Health, Bethesda, Maryland.

R.W.J. is an Overseas Research Fellow of the National Heart Foundation of Australia and recipient of a Telecommunications Traveling Fellowship of the Royal Australasian College of Physicians.

H.F.W. is the recipient of a Clinician-Scientist Award from The Johns Hopkins Medical Institutions.

Address for correspondence and reprints: Lewis C. Becker, MD, Halsted 500, The Johns Hopkins Medical Institutions, 600 North Wolfe Street, Baltimore, MD 21205.

Received April 17, 1989; revision accepted August 3, 1989.
ischemic myocardium, increasing numbers can be identified within the first few hours after reperfusion.\textsuperscript{19} In humans, who have low levels of endothelial xanthine oxidase,\textsuperscript{20} neutrophils may well represent the principal source of oxygen free radicals in postischemic myocardium. In addition, neutrophils may also contribute to myocardial necrosis by plugging capillaries, resulting in persistent ischemia and the "no-reflow" phenomenon.\textsuperscript{21-24}

A role of neutrophils in mediating myocardial necrosis during ischemia, reperfusion, or both is supported by previous studies showing that either neutrophil depletion\textsuperscript{25,26} or pharmacologic inhibition of neutrophil chemotaxis and function\textsuperscript{27-33} during ischemia can reduce infarct size. The extent of the no-reflow zone also appears to be reduced by neutropenia during ischemia and reperfusion.\textsuperscript{22,33}

Despite these findings, the role of neutrophils in mediating reperfusion injury, as distinct from injury occurring during the ischemic period, remains unknown, since in nearly all previous studies the antineutrophil intervention was given before or during ischemia. Such a distinction is clinically important. Although neutrophil depletion during ischemia might be beneficial in the laboratory, the requirement for pretreatment is clinically impractical. If, however, neutrophils are responsible for extension of necrosis during reperfusion, reduction of neutrophil numbers or activity might indeed represent a feasible adjunct to thrombolytic therapy. We, therefore, examined the effects of neutrophil depletion, confined to the reperfusion period, on infarct size and the extent of the no-reflow zone after 90 minutes of coronary occlusion in anesthetized dogs.

Methods

Instrumentation

Thirty-one mongrel dogs of either sex, weighing 20–25 kg, were anesthetized with intravenous sodium thiamylal (12.5 mg/kg) and intramuscular \(\alpha\)-chloralose (14 mg/kg) in urethane (136 mg/kg) and ventilated with air and supplemental oxygen. Arterial blood gases were monitored to confirm that arterial oxygen saturation remained above 90%. Catheters were placed in the left femoral vein for maintenance infusion of fluids, the descending aorta via the left femoral artery for pressure measurement (Statham P23Db) and microsphere reference sampling, and the right internal carotid for subsequent extracorporeal perfusion of the circumflex coronary artery.

The heart was exposed by a thoracotomy in the fifth left intercostal space and suspended in a pericardial cradle. A polyvinyl catheter was placed in the left atrium for pressure measurement and microsphere injection. The circumflex artery was then exposed for 10 mm proximal to the first large marginal branch, and a black silk suture was loosely placed around the artery. Lead II of the electrocardiogram was monitored throughout the study, and

![Figure 1. Schematic of the extracorporeal circuit used for controlled reperfusion of circumflex artery. In the neutropenic group, four leukocyte filters were included in the perfusion line between the roller pump and the pressurized arterial reservoir. The sampling port was used to obtain serial blood samples for hematology measurements. Circumflex flow was measured by an electromagnetic flow probe (EMF), and perfusion pressure was measured at the circumflex cannula tip.](http://circ.ahajournals.org/doi/abs/10.1161/01.cir.93.4.625?journalCode=circ)
land) before entering the circumflex artery via a short stainless-steel cannula (3.0 mm ID, 3.1 mm OD). The perfusion pressure to the circumflex artery was measured at the tip of the cannula by a fluid-filled catheter (0.6 mm ID, 0.8 mm OD) inserted through a side arm into the lumen and connected to a pressure transducer (Statham P23Db). Mean circumflex perfusion pressure was adjusted to match mean aortic pressure during the experiment by regulating air pressure within the reservoir, and extracorporeal perfusion was maintained for the next 2 hours.

Neutrophil depletion in the blood reperfuasing the circumflex territory was achieved by pumping the blood through four leukocyte filters (Imugard IG500, Terumo Co, Japan) before its entry into the pressurized arterial reservoir. The control dogs had no filters in the extracorporeal circuit. Serial measurements of systemic and circumflex arterial blood hemoglobin, leukocyte, and platelet counts were made in each dog before coronary occlusion, just before coronary reperfusion, and after 5 and 120 minutes of reperfusion.

Measurement of Infarct Size

After 2 hours of reperfusion, a solution of the dye thioflavin-S (0.2 g/10 ml) was injected into the circumflex perfusion line to delineate regions of no-reflow at the end of reperfusion. Because thioflavin-S fluoresces yellow-green under ultraviolet light, regions of myocardium that did not exhibit fluorescence represented areas of no-reflow. An additional 3 minutes of coronary reperfusion was allowed before the roller pump was stopped and the perfusion line occluded, and 20 ml monastral blue dye was injected into the left atrium to define the ischemic risk region. The heart was then arrested by intra-atrial injection of potassium chloride and excised, and the atria, right ventricular free wall, and epicardial fat and valvular tissues were removed.

Each heart was sectioned into five transverse slices parallel to the atrioventricular groove, and the slices were weighed and traced on clear acetate sheets under white light to visualize the risk region (absence of monastral blue) and ultraviolet light to visualize the no-reflow area (absence of thioflavin-S). The slices were then incubated in a 2% solution of 2,3,5, triphenyl-tetrazolium chloride (TTC) for 20 minutes at 37°C. Regions that failed to demonstrate brick-red staining with TTC were considered to represent infarcted myocardium. Each slice was photographed in color to record infarct size. Myocardial samples (0.5–1.5 g) were taken from normal, ischemic, and infarcted regions of each slice and placed in a well spectrometer (Packard model 5986) for gamma counting and calculation of regional blood flow.

Histopathology

To confirm the validity of the TTC staining, a total of 27 samples (weighing 50–100 mg) were taken from the endocardial and midwall zones of the risk region in four dogs (two control and two neutrophil depleted). These samples were immediately placed in cold fixative (phosphate-buffered solution of 4% formalin and 1% glutaraldehyde) for subsequent electron microscopy. The site of each myocardial sample was marked with a colored pin, and after TTC staining, the samples were retrospectively labeled as having come from either TTC-positive or -negative areas. After washing with cold phosphate buffer (0.1 M), the samples were post-fixed with cold 1% osmium tetroxide, dehydrated in alcohol and propylene oxide, and embedded in epoxy resin. Ultrathin (75 nm) sections (four to 12 per sample) were prepared from tissue blocks, mounted on copper grids, stained with lead citrate and uranyl acetate, and examined and photographed with a Hitachi H600 electron microscope.

Subsequently, the electron microscope images of each section were examined by two blinded observers and graded according to the degree of structural damage present (Table 1). The scores from individual sections were averaged and rounded off to the nearest whole integer to obtain an overall injury score for each tissue sample. According to the grading system, grade 1 injury was deemed to represent reversible injury, but all other grades, characterized by moderate to severe mitochondrial swelling and intramitochondrial amorphous densities, were judged to represent irreversible injury. The pathologic classification of the samples was then compared with the findings from TTC staining.

Complement and Myeloperoxidase Assays

Immediately before reperfusion, arterial blood samples were simultaneously withdrawn from the
systemic circulation and the distal circumflex perfusion line in nine control and eight neutropenic dogs. The samples were promptly centrifuged, and the serum removed and frozen at −70°C for later analysis of titers of complement components C3 and C5 by standard hemolytic assays.37,38

After TTC staining of six of the hearts (three control and three neutrophil-depleted), multiple biopsies (50–150 mg) were taken from both the infarcted and noninfarcted areas of the risk region and frozen at −70°C. Subsequently, the activity of the neutrophil specific enzyme, myeloperoxidase, was measured by previously described methods.39 Briefly, the biopsies were pulverized under liquid nitrogen, homogenized and sonicated, and then freeze-thawed to release the myeloperoxidase into the supernatant. Myeloperoxidase activity was measured by adding 0-dianisidine hydrochloride and hydrogen peroxide and then observing the change in light absorbance at a wave length of 460 nm during a period of 2 minutes. Results were calculated as units myeloperoxidase/100 mg wet tissue wt.

Data Analysis

Color slides of the transverse heart slices were projected and traced on clear acetate sheets without knowledge of their assignment to control or neutrophin groups. The areas of the ischemic risk region and infarcted myocardium were determined by digitized planimetry of the corresponding areas on these tracings. The extent of the no-reflow region was determined by planimetry of the tracings made under ultraviolet light immediately after excision of the heart. Infarct size and the size of the no-reflow region were then standardized as a fraction of the risk region for each heart.

Hematologic and hemodynamic parameters during ischemia and reperfusion in each group were compared by repeated-measures analysis of variance. Comparisons of infarct size in the control and neutropenic groups were made by Student’s t test for both whole groups and specifically for the 15 dogs with more severe ischemia (defined as mean transmural blood flow of less than 0.20 ml/min/g). The relation between the dependent variable of infarct size as a percentage of the left ventricle, and the independent variables of risk region size and collateral blood flow during ischemia was determined by multiple linear regression analysis.40 The effect of neutropenia on infarct size was examined by calculating a predicted infarct size, based on the multiple linear regression of the control group, for each heart in the neutropenic group. The predicted and observed infarct sizes in the neutropenic group were then compared by analysis of covariance.41 Results are given as mean±SEM, and a p value of less than 0.05 is regarded as significant.

Results

Study Group

During the period of coronary occlusion, nine dogs developed ventricular fibrillation, of which three were resuscitated. Among the 25 dogs surviving the ischemic period, 12 had been assigned to the neutropenic group and 13 to the control group. During the early reperfusion period, 11 dogs developed ventricular fibrillation (six neutropenic and five control), of which one neutropenic and two control dogs could not be resuscitated. Data are, therefore, reported for 11 neutropenic and 11 control dogs.

Neutrophil Depletion

The hematocrit levels, platelet counts, and neutrophil counts were similar in the two groups during basal conditions before coronary occlusion (Table 2). Passage of blood through the extracorporeal circuit did not significantly alter neutrophil, monocyte, eosinophil, or leukocyte counts in the control group, but there was a mild reduction in platelet count. However, in the filter group there was near total neutropenia (7±9/μl, p<0.001 vs. control) and severe thrombocytopenia (6±1×10³/μl, p<0.001 vs. control) in blood perfusing the circumflex region.

<table>
<thead>
<tr>
<th>Table 2. Hematologic Parameters in Control and Neutrophin Groups During Circumflex Artery Occlusion and Reflow</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Hematocrit (%)</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>Neutropenic</td>
</tr>
<tr>
<td>Leukocytes (/μl)</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>Neutropenic</td>
</tr>
<tr>
<td>Neutrophils (/μl)</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>Neutropenic</td>
</tr>
<tr>
<td>Platelets (/nl)</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>Neutropenic</td>
</tr>
</tbody>
</table>

Values are given as mean±SEM.
*p<0.05 vs. basal.
†p<0.01 vs. control.
The filters also markedly reduced monocyte, eosinophil, and lymphocyte counts. Severe neutropenia and deficiency of other leukocytes persisted throughout the 2-hour reperfusion period in the filter group, although there was a small increase in neutrophil and other leukocyte levels by 2 hours. Significant systemic neutropenia was also observed in the filter group throughout the reperfusion period. The hematocrit was similar in the two groups during the study, but there was a fall in hematocrit in both groups during reperfusion, reflecting requirements for maintenance intravenous infusion of dextran and saline to preserve stable hemodynamic conditions during extracorporeal coronary perfusion.

In biopsies taken from infarcted areas (TTC-negative), mean myeloperoxidase activity was reduced in the neutropenic dogs (0.105±0.02 units/100 mg) compared with controls (0.287±0.08 units/100 mg, p<0.05). Myeloperoxidase activity was also reduced in biopsies taken from noninfarcted (TTC = positive) tissue within the risk region in neutropenic dogs (0.075±0.03 units/100 mg) compared with controls (0.308±0.10 units/100 mg, p<0.05). Mean myeloperoxidase activity in nonischemic myocardium averaged 0.029±0.006 units/100 mg.

In the control group, there was no reduction in C3 titer during passage of blood through the perfusion circuit, with 96±9% of the systemic C3 titer being present in blood entering the circumflex artery. In contrast, after passage through the neutrophil filters, the C3 titer was only 53±5% of systemic levels (p=0.001 vs. control). Similarly, the C5 activity in blood perfusing the circumflex artery tended to be lower in the neutrophil filter group (88±14% vs. 67±9%, p=NS).

**Hemodynamics**

Hemodynamic parameters during the occlusion and reperfusion periods are summarized for the control and neutropenic groups in Table 3. There were no significant changes in heart rate during the experiment in either group. However, in both groups there was a decline in mean aortic pressure during coronary occlusion and a further decrease during reperfusion. Mean aortic pressures at each stage of the experiment were similar in the control and neutropenic groups. Mean circumflex perfusion pressure did not differ between the two groups, except during initial reperfusion when a lower proximal coronary pressure and higher circumflex flow were observed in the neutropenic dogs. Circumflex coronary flow remained slightly higher in the neutropenic group throughout the reperfusion period, but the differences were not statistically significant.

During occlusion of the circumflex artery, mean collateral blood flow to the risk region was 0.15±0.03 ml/min/g (range, 0.02–0.35 ml/min/g) in the control group and 0.13±0.03 ml/min/g (range, 0.01–0.35 ml/min/g) in the neutropenic group (NS).

**Ultrastructural Correlation of TTC Staining**

In both groups, the TTC-positive areas of myocardium within the risk region showed either normal ultrastructure (grade 0) or intact myocytes with mild cellular edema, mild mitochondrial swelling, and moderate loss of glycogen (grade 1 injury) (Figures 2 and 3). The sarcolemma was intact and the myofibrils were normal in all specimens, consistent with reversible ischemic injury. Only one of the 11 samples from TTC-positive areas demonstrated more severe ultrastructural changes, with mitochondrial amorphous dense bodies (grade 2 injury) consistent with irreversible necrosis (Figures 2 and 3). In contrast, in the TTC-negative areas of the risk region, the samples generally exhibited numerous mitochondrial densities and marked sarcolemmal disruption and myofibrillar contraction bands consistent with irreversible ischemic injury (Figure 2). Injury was judged as grade 3 or 4 in nine of 16 samples and grade 2 in four of 16 (Figure 3). Only three of 16 samples exhibited the less-severe grade 1 structural changes (p=0.0002, χ²=13.60 vs. TTC positive).

---

**TABLE 3. Hemodynamic Parameters in Control and Neutropenic Groups During Circumflex Artery Occlusion and Reperfusion**

<table>
<thead>
<tr>
<th></th>
<th>Occlusion (min)</th>
<th>Reperfusion (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal</td>
<td>5</td>
</tr>
<tr>
<td>Heart rate (/min)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>137±7</td>
<td>134±6</td>
</tr>
<tr>
<td>Neutropenic</td>
<td>132±7</td>
<td>128±5</td>
</tr>
<tr>
<td>Mean aortic pressure (mm Hg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>104±5</td>
<td>92±7</td>
</tr>
<tr>
<td>Neutropenic</td>
<td>107±8</td>
<td>88±7</td>
</tr>
<tr>
<td>Ptc (mm Hg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>79±5</td>
<td>73±5</td>
</tr>
<tr>
<td>Neutropenic</td>
<td>62±6†</td>
<td>69±5</td>
</tr>
<tr>
<td>Qlc (ml/min)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>85±13</td>
<td>62±12</td>
</tr>
<tr>
<td>Neutropenic</td>
<td>92±19</td>
<td>83±15</td>
</tr>
</tbody>
</table>

Values are given as mean±SEM.
Ptc, mean perfusion pressure of circumflex artery; Qlc, mean flow to circumflex artery.

*p<0.05 vs. basal, †p<0.05 vs. control.
Infarct Size

The proportion of the left ventricle involved in the ischemic risk region was the same in the control (41.6±1.0%) and neutropenic (41.8±2.1%) groups (Figure 4, upper panel). Comparison of the whole groups indicated that the percentage of the risk region exhibiting infarction was less in the neutropenic group (21.4±5.8%) than in the control group (36.7±7.6%), but the difference was not significant (p=0.12). However, four control and three neutropenic dogs had relatively high collateral flows (>0.2 ml/min/g) and as result exhibited little infarction (typically less than 5% of the left ventricle and confined to small subendocardial regions). In those dogs with collateral flow of less than 0.2 ml/min/g (Figure 4, lower panel), infarct size in the neutropenic group (27.7±6.7% of risk region, n=8) was significantly less than that in the control group (52.5±5.7%, n=7, p=0.02). In these dogs, infarct size was 12.4±3.0% of the left ventricle in the neutropenic group compared with 22.4±2.9% in the control group (p<0.05). Neutrophil depletion during the reperfusion period was, thus, associated with an approximate 40% mean reduction in infarct size.

Infarct size was inversely related to collateral blood flow in both control and neutropenic groups (Figure 5). The overall relation between collateral flow and infarct size appeared curvilinear, and a greater proportion of the variance in the relationship was accounted for by a log-linear regression model (control, r2=0.82; neutropenic, r2=0.72) than by a linear regression model (control, r2=0.73; neutropenic, r2=0.56). Over a wide range of collateral flows, the neutropenic group exhibited a lesser degree of infarction than did the control group, and the difference was most evident for dogs with low collateral flows. Analysis of covariance indicated that the relation between infarct-risk and collateral flow in the neutropenic group (y=−16.5 ln x−17.5%, r=−0.85) differed significantly from that of the control group (y=−24.2 ln x−20.8%, r=−0.91, p=0.03).

The extent of the no-reflow region was also less in the neutropenic group than in controls (2.2±0.8% vs. 8.1±2.7% of risk region, p<0.05). The extent of the no-reflow region was directly related to infarct size. However, the size of the no-reflow zone for a given infarct size in the neutropenic group (y=0.11x−0.09, SEE=1.6%, r=0.81) was significantly less than in the control group (y=0.28x−2.24, SEE=5.6%, r=0.80, difference in slopes p=0.03).

Predictors of Infarct Size

Multiple linear regression analysis confirmed that risk region size and collateral blood flow during proximal circumflex artery occlusion were the major predictors of infarct size in the control group. The overall relation was y=0.27x1−23.8 ln x2−19.2%, r=0.94, p<0.005, where y=infarct/LV, x1=risk/left ventricle, and x2=collateral blood flow. This regression equation was used to calculate a predicted infarct size for each animal in the neutropenic group (Figure 6). Covariance analysis confirmed that the relation between predicted and observed infarct sizes in the neutropenic group differed from the control group (p=0.05). The mean predicted infarct size in the neutropenic group was 16.8±3.4% of left ventricle, which was similar to the observed value of 15.6±3.4% in the control group. For the dogs in the neutropenic group, the observed infarct size was less than the predicted size, although the amount of the reduction was variable (Table 4). Neutropenia was associated with a mean salvage of 17.5±3.9% of the risk region, with a range of 1.8% to 41.0%. The dog with the least preservation of myocardium (dog 8) was the only dog to have any detectable neutrophils in the circumflex arterial blood at the moment of reperfusion (80/μl). One other dog (dog 4) exhibited little reduction in infarct size, and this dog had high levels of myeloperoxidase activity in both the infarcted and noninfarcted risk region (59% of the level in the control animals and 3.5-fold higher than the levels in the other two neutrophil-depleted dogs examined).

Discussion

Although early coronary reperfusion can salvage jeopardized myocardium after prolonged myocardial ischemia, there is evidence that reperfusion may also have deleterious consequences and may cause necrosis of ischemic but reversibly injured myocytes.7,8 The present study, which shows that infarct size is reduced significantly by neutrophil depletion limited to the early reperfusion period, strongly suggests that “reperfusion necrosis” is indeed a significant contributor to final infarct size and that neutrophil leukocytes are important mediators of this phenomenon.

Many studies have examined the role of neutrophils in the pathogenesis of myocardial infarction. During the first 24–48 hours after reperfusion, there is considerable accumulation of neutrophils in the infarct region.21,22 Treatment with anti-inflammatory agents before ischemia reduces this neutrophil accumulation in infarcted myocardium and is associated with up to 40% reduction in infarct size.29–32 This beneficial effect appears to be related to inhibition of the lipooxygenase pathway of arachidonic acid metabolism, resulting in reduced neutrophil activation.29 Pretreatment with prostacyclin, or its analogue, iloprost, also reduces leukocyte accumulation and infarct size.36,42 Other studies, with rabbit antineutrophil serum to produce neutropenia in dogs, demonstrated a mean reduction in infarct size of 39–43% after 90 minutes of coronary occlusion29,36 but no significant effect after 3 hours33 or 4 hours46 of occlusion. Neutrophils, thus, appear to mediate necrosis of jeopardized myocytes after coronary occlusions of medium duration. However, after longer coronary occlusions, neutrophil depletion

Litt et al Neutrophils and Reperfusion Injury 1821
seems to make no difference to infarct size, probably because of the severity of myocyte injury related to prolonged ischemia.\textsuperscript{43,44}

The important difference between these previous reports and the present study is that we limited neutropenia to the reperfusion period. Throughout the preocclusion period and the entire 90 minutes of ischemia, circulating neutrophil levels were similar in the control and treatment groups. It seems reasonable to assume that neutrophil levels in the myocardium were also similar in the two groups during the period of coronary occlusion. Despite this, the reduction in infarct size observed in the present study is similar to that documented in the
prominent i-bands (i) and intracellular edema. The components of the sarcolemma are not readily distinguishable (curved brackets), and breaks in the sarcolemma are seen (arrow). The sarcoplasmatic reticulum (sr) network remains intact (original magnification, ×25,000). Bottom middle panel: Injury grade 3: In two adjacent cells, zones of hypercontracted sarcomeres alternate with zones of overstretched sarcomeres, giving the characteristic appearance of contraction bands (cb). The sarcolemma is lifted away from the cell surface by edema, forming a large bleb (arrow), and there is separation at the intercalated disc cell junctions (ij) (original magnification, ×3,750). Top right panel: Injury grade 3: Higher magnification of upper cell in bottom right panel, showing total loss of intracellular organization. The myofilaments (mf) are pulled apart from the z-lines (z). Mitochondria (m) are swollen with large matrix densities (arrows) (original magnification, ×25,500).

Figure 2. Microphotographic examples of ultrastructural injury in postischemic myocardium after 90 minutes of ischemia and 2 hours of reperfusion. Top left panel: Injury grade 0: Two adjacent normal cells from TTC-positive area within the risk region. The plasmalemma and outer glycocalyx components of the sarcolemma are intact in both cells (curved brackets), and normal subsarcolemmal pinocytotic vesicles are present (arrowheads). Both cells have normal mitochondria (m) containing densely packed cristae and matrix granules (arrows). The myofibrils (mf) and z lines (z) are in alignment, the sarcoplasmatic reticulum (sr) is intact, and there is abundant glycogen (g) within the sarcoplasm. Collagen (c) and a pericyte (p) are seen in the intercellular space between the two cells (original magnification, ×25,500). Bottom left panel: Injury grade 1: Reversible injury. In two adjacent cells, the mitochondria are swollen, and matrix granules are no longer present. There is mild edema and loss of glycogen, but sarcomere structure is normal, and the sarcolemma (between curved brackets) is intact. sr, sarcoplasmatic reticulum; z, z lines; mf, myofibrils; m, mitochondria; curved brackets, plasmalemma and outer glycocalyx components of the sarcolemma (original magnification, ×25,000). Top middle panel: Injury grade 2: Irreversible injury. The mitochondria (m) are swollen and contain amorphous densities (arrow heads). The sarcomeres are relaxed with earlier studies. This suggests that a major contribution of neutrophils to myocyte necrosis occurs during early reperfusion rather than during the ischemic period. This conclusion is supported by the findings of Mullane and Moncada, who showed that administration of the lipoxygenase and cyclooxygenase inhibitor BW755C, during the reperfusion period only, significantly reduced infarct size in anesthetized dogs.

In the present study, care was taken to account for differences in the size of the risk region and degree of collateral blood flow when comparing infarct size between the control and neutropenic groups. There is an inverse relation between collateral flow and infarct size, as observed in the control animals. In accord with a previous study from our laboratory, which examined effects of superoxide dismutase on infarct size, the limitation of infarct size in the neutropenic group was most evident in dogs with low collateral blood flow to the risk region, and similar findings have been reported by other investigators.

The use of tetrazolium staining to measure infarct size after a short period of reperfusion has been criticized on the grounds that TTC may artifically stain tissue that is irreversibly injured. If this were the case, the infarct-to-risk ratio in the control group should be less than that observed after prolonged reperfusion. However, the relation between infarct size and collateral flow in the controls was similar to that observed after 4 days reperfusion in other studies with either TTC or histologic measures of infarct size. Furthermore, the ultrastructural findings in the present study confirmed the validity of TTC in differentiating necrotic and reversibly injured myocardium after 2 hours of reperfusion.

There are several possible mechanisms by which neutrophils could cause reperfusion necrosis, including lysosomal proteolysis, oxygen free radical formation, and capillary occlusion with persistent microvascular ischemia. There is controversy regarding the role of oxygen free radicals in mediation of reperfusion necrosis. Two experimental studies, using electron paramagnetic resonance and spin-trapping techniques, have detected evidence of a burst of superoxide radicals on reperfusion of ischemic myocardium, although not all
investigators accept these techniques. Many studies have demonstrated reduced infarct size after pretreatment with either superoxide dismutase or xanthine oxidase inhibitors. The findings of these studies support the hypothesis that oxygen free radicals, derived from neutrophils or endothelial xanthine oxidase, contribute to myocardial necrosis during and immediately after prolonged ischemia. Other groups, however, have found no limitation of infarct size by oxygen free radical scavengers, particularly after prolonged periods of reperfusion of up to 4 days. One study, which specifically examined the effects of free radical scavengers administered during the reperfusion period only, found a mean reduction in infarct size of approximately 20%, but the difference between control and treatment groups did not reach statistical significance. While some of this discrepancy may reflect a short ischemic insult, other studies have found no significant salvage after 90 minutes of ischemia. In part, this may be related to the relatively short plasma half-life of free radical scavengers and the occurrence of reperfusion necrosis over an extended time frame of hours to days. A recent study with superoxide dismutase conjugated to polyethylene glycol, which has a plasma half-life of more than 30 days, demonstrated persistent myocardial salvage after 4 days of reperfusion.

Histopathologic evidence indicates that neutrophils accumulate in the ischemic myocardium, particularly after reperfusion, and can occlude significant numbers of capillaries. Such capillary obstruction contributes to the no-reflow phenomenon and may also result in persistent regional microvascular ischemia with continuing local myocyte necrosis. Other studies in our laboratory have shown that development of the no-reflow phenomenon is time dependent, consistent with progressive microvascular occlusion by neutrophils during reperfusion. The smaller no-reflow zones observed for a given level of infarct size in the neutropenic dogs in this study are consistent with this hypothesis. Pre-
vention of such microvascular occlusion may, thus, represent another mechanism by which neutrophil depletion can reduce reperfusion injury.

Neutropenia was produced in this study by mechanical filtration rather than antiserum to avoid the potentially harmful effects of neutrophil vascular margination. Although the absence of neutrophils was probably the key factor responsible for the reduced infarct size, the filters also produced significant thrombocytopenia. Platelets could theoretically contribute to reperfusion injury by releasing vasoactive and chemotactic substances and producing microvascular plugging. However, the histologic studies of Engler et al.22 found no evidence of platelet plugs in reperfused myocardium.

**Table 4.** Predicted and Observed Infarct Sizes in the Neutropenic group

<table>
<thead>
<tr>
<th>Dog</th>
<th>Predicted size (% of LV)*</th>
<th>Observed size (% of LV)</th>
<th>Salvaged myocardium (% of R)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.4</td>
<td>1.7</td>
<td>4.8</td>
</tr>
<tr>
<td>2</td>
<td>14.1</td>
<td>5.7</td>
<td>24.0</td>
</tr>
<tr>
<td>3</td>
<td>23.8</td>
<td>12.0</td>
<td>23.1</td>
</tr>
<tr>
<td>4</td>
<td>23.2</td>
<td>17.8</td>
<td>12.9</td>
</tr>
<tr>
<td>5</td>
<td>39.4</td>
<td>22.8</td>
<td>41.0</td>
</tr>
<tr>
<td>6</td>
<td>8.0</td>
<td>4.1</td>
<td>8.8</td>
</tr>
<tr>
<td>7</td>
<td>27.6</td>
<td>19.3</td>
<td>16.8</td>
</tr>
<tr>
<td>8</td>
<td>20.0</td>
<td>19.0</td>
<td>1.9</td>
</tr>
<tr>
<td>9</td>
<td>10.0</td>
<td>1.2</td>
<td>24.9</td>
</tr>
<tr>
<td>10</td>
<td>0.6</td>
<td>0.0</td>
<td>1.8</td>
</tr>
<tr>
<td>11</td>
<td>14.7</td>
<td>1.6</td>
<td>32.3</td>
</tr>
</tbody>
</table>

*Calculated according to multiple linear regression of control group; *difference between predicted and observed size normalized for risk region size.
LV, left ventricle; R, risk region.
*p<0.01 vs. predicted.

In addition, other studies have observed no reduction in infarct size after marked platelet depletion.60,61 We also found decreased levels of the complement components C3 and C5 downstream from the neutrophil filters. If this represented activation of serum complement by the filters, then the true benefit of neutrophil removal may have been underestimated because any neutrophils remaining in the blood, as well as those in the myocardial tissue, might have been more completely activated. Conversely, if complement plays a role in reperfusion injury and is removed by the filters without activation or if activated complement fragments were degraded before reaching the myocardium, the benefit of neutropenia could have been overestimated.

Many further questions are raised by the present findings, including whether other contributors to reperfusion necrosis exist and the time frame within which neutrophils exert a detrimental effect. Recent evidence suggests that reperfusion injury may occur over an extended period of time.62 Neutrophil depletion for only the first 2 hours of reperfusion may not permanently reduce infarct size because of delayed neutrophil-mediated injury. It may, therefore, be necessary to maintain neutropenia for several days to permanently limit reperfusion injury. The degree of neutropenia necessary to prevent necrosis is also uncertain, although it has been suggested that only a very few neutrophils are necessary to cause injury to postischemic myocardium.63 This is consistent with our observation that the two dogs with the least protection in the neutropenic group either had some residual neutrophils in the reperfusing blood (only 80/μl) or unexpectedly high tissue myelop-
oxidase levels. It is unknown whether a further reduction in infarct size could be achieved by additional treatment, such as combined neutropenia and free radical scavengers, or whether the approximately 40% reduction in infarct size observed in this study represents the limit of reperfusion necrosis. Finally, although mechanical filtration of neutrophils is impractical for clinical use, recent reports suggest that infarct size can be effectively reduced by inhibition of neutrophil chemotaxis with monoclonal antibodies directed against a membrane adhesion protein (Mac-1).62,64 If this approach proves to be as effective as neutrophil depletion, it may represent an important adjunctive treatment for patients with acute myocardial infarction undergoing reperfusion therapy.

Acknowledgments

We gratefully acknowledge the valuable assistance of the technicians in our research laboratory, Anthony F. DiPaola Jr., Michelle Leppo, and Alexander "Skins" Wright, in the performance of these studies, and of Christine G. Holzmueller in the preparation of this manuscript.

References


44. Reimer KA, Jennings RB: The “wavefront phenomenon” of myocardial ischemic cell death: II. Transmural progression of necrosis within the framework of ischemic bed size (myocardium at risk) and collateral flow. *Lab Invest* 1979; 40:633–644


47. Richard VJ, Murry CE, Jennings RB, Reimer KA: Therapy to reduce free radicals during early reperfusion does not limit the size of myocardial infaracts caused by 90 minutes of ischemia in dogs. *Circulation* 1988;78:473–480


54. Reimer KA, Jennings RB: Failure of the xanthine oxidase inhibitor allopurinol to limit infarct size after ischemia and reperfusion in dogs. *Circulation* 1985;71:1069–1075


56. Urrizee A, Reimer KA, Murry CE, Jennings RB: Failure of superoxide dismutase to limit size of myocardial infarction after 40 minutes of ischemia and 4 days of reperfusion in dogs. *Circulation* 1987;75:1237–1248


**KEY WORDS** • reperfusion injury • leukocytes
Neutrophil depletion limited to reperfusion reduces myocardial infarct size after 90 minutes of ischemia. Evidence for neutrophil-mediated reperfusion injury.
M R Litt, R W Jeremy, H F Weisman, J A Winkelstein and L C Becker

Circulation. 1989;80:1816-1827
doi: 10.1161/01.CIR.80.6.1816
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1989 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/80/6/1816