Neutrophil accumulation in experimental myocardial infarcts: relation with extent of injury and effect of reperfusion

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ABSTRACT The effects of reperfusion on the myocardial accumulation of neutrophils and their role in the extent of injury were investigated in a canine preparation with a 3 hr coronary occlusion followed by 21 hr of reperfusion. The left anterior descending coronary artery (LAD) was permanently occluded in group 1 and reperfused after 3 hr in four others (groups 2 to 5). All but group 5 received lidocaine (1 mg/min over 8 hr). A critical stenosis was produced and left in place at reperfusion only in group 2. In groups 1 and 2, In-labeled autologous neutrophils were injected at the time of coronary occlusion. Group 4 animals were rendered leukopenic 2 hr before the coronary ligation and throughout the experiment by injection of an antineutrophil rabbit serum. Quantification of the radioactivity by digitized scintigraphy of the heart slices revealed an 80% (p < .05) increase in neutrophil accumulation in the infarct region after reperfusion (group 2) as compared with permanent occlusion (group 1). Gamma counting of myocardial tissue samples showed that the neutrophil accumulation ratio in the subendocardial central zone of the infarct was increased five times (p < .05) by reperfusion, whereas no difference was evident in the subepicardium. Infarct size and myocardial area at risk were not statistically different among the five groups. However LAD flow in the leukopenic group (group 4) was significantly higher (p < .05) 30 min after reperfusion (40.0 ± 5 ml/min) when compared with the preocclusion value (21.7 ± 4 ml/min). In contrast, in a parallel experiment without leukopenia (group 3), LAD flow after reperfusion did not differ from the preocclusion value. We conclude that after a 3 hr occlusion, reperfusion enhances the myocardial accumulation of neutrophils and modifies their distribution in the necrotic area. Neutrophil accumulation appears not to be responsible for the lack of beneficial effect of reperfusion on infarct size in this preparation, since leukopenia failed to limit the extent of injury not otherwise modified by lidocaine. However, neutrophils may adversely affect restoration of flow by the formation of leukocyte plugs.


CORONARY REPERFUSION is the most promising treatment to limit the size of necrosis and to improve prognosis in patients with acute transmural myocardial infarction. The duration of ischemia before reperfusion is certainly one of the most critical factors that determine myocardial salvage. Another factor potentially influencing ultimate infarct size is the extent of the acute inflammatory reaction in the early course of myocardial infarction. Neutrophils are suspected to be mediators of this phenomenon because of their ability to release proteolytic lysosomal enzymes, free-radicals, and active metabolites of arachidonic acid, all being potentially detrimental for ischemic and normal myocardium. Reduction of the extent of ischemic myocardial injury has been reported in experimental preparations using anti-inflammatory as well as leukopenic and anticomplementary agents. On the other hand, some observations have suggested an increase or an acceleration of the inflammatory processes by reperfusion. Therefore reperfusion represents a paradoxical situation with both potentially beneficial and detrimental effects on the myocardium. In other words, if reperfusion enhances neutrophil accumulation, which can be detrimental for the myocardium, the beneficial effects of flow restitution may be attenuated.

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The development of techniques for labeling blood cells with $^{111}$In-oxine offers the potential for the study of the inflammatory response in vivo. Accordingly, myocardial infarcts have been imaged successfully by scintigraphy.\textsuperscript{7-8} This experimental study was designed to assess the accumulation of $^{111}$In-labeled neutrophils in reperfused and nonreperfused myocardial infarcts and to explore whether neutrophil accumulation plays an active part in the final extent of ischemic injury.

Methods

Ischemia and reperfusion. Mongrel dogs of either sex weighing 20 to 30 kg were anesthetized with sodium pentobarbital (30 mg/kg iv), intubated and ventilated mechanically. The electrocardiogram was monitored throughout the surgical maneuvers. After injecting pancuronium bromide (0.1 mg/kg), a left thoracotomy was performed in the fifth intercostal space. A catheter was located in the left ventricle through the left atrial appendage and pressure monitored continuously. The left anterior descending coronary artery (LAD) was isolated distally to the first diagonal branch and flow was measured with an electromagnetic flowmeter.

In groups 1 and 2, a critical stenosis sufficient to abolish the hyperemic response after a 10 sec occlusion but without affecting basal flow was established close to the probe 30 min before coronary occlusion. The LAD was occluded with a ligature in all groups. The occlusion was maintained in group 1 until death 24 hr later. In group 2, the occlusion was released after 3 hr to allow reperfusion. In groups 3 and 5, reperfusion was established after 3 hr in the absence of a critical stenosis. The protocol for group 4 was identical to that for group 3, with the addition of a rabbit antiserum for leukocyte depletion. After these procedures, the chest was closed and the animals were extubated and allowed to recover.

Perfusion of lidocaine (1 mg/min) was started at the time of anesthesia and maintained over 8 hr in every animal of groups 1 to 4; group 5 received saline. Dogs with ventricular fibrillation after coronary occlusion were excluded because of possible tissue damage by electrical defibrillation.

Isolation and $^{111}$In-labeling of neutrophils. After anesthesia in groups 1 and 2, two samples of blood (42.5 and 20 ml) were drawn from the cephalic vein into 7.5 ml of acid citrate dextrose and 400 U of heparin, respectively, for isolation and labeling of autologous neutrophils.\textsuperscript{23, 24}

The citrated blood was allowed to sediment for 30 min after addition of 32 ml of 4% dextran 250. The leukocyte-rich plasma was washed twice with Hanks' balanced solution and the cells were suspended in 16 ml of dextran 40 (2.4%) containing 2% bovine albumin. The cell mixture was deposited on layered double Percoll (Pharmacia Inc.) gradients of 1.072 and 1.087 g/ml. After 30 min of centrifugation (900 g), the neutrophils were collected at the interface of the gradients, washed twice with Hanks' solution, and incubated 1 min at 37°C with 500 µCi $^{111}$In-oxine (Amersham Corp.). Platelet-poor plasma prepared from the heparinized blood sample was added to bind free $^{111}$In-oxine and eliminated after centrifugation. The autologous labeled neutrophils were resuspended in 5 ml of platelet-poor plasma and injected intravenously at the time of coronary occlusion. The following viability and function tests were carried out in vitro by standard procedures on the labeled neutrophils, and the results for each dog were compared with those obtained with corresponding neutrophils in native whole blood: trypan blue test,\textsuperscript{25} tetrazolium blue reduction,\textsuperscript{26} nylon wool adherence,\textsuperscript{27} and phagocytosis.\textsuperscript{28}

Blood was collected at different times (figure 1) after the injection of the $^{111}$In-neutrophils for the study of their kinetics in the circulation and to assess whether the ratio between the circulating and injected radioactivity was similar among dogs, a prerequisite for the quantification of the neutrophils in the infarcts based on gamma spectrometry. The equation used for the determination of the ratio was:

$$\text{circulating activity} = (BS - PS) \times 75 \times \text{body weight (kg)}$$

$$\text{injected activity} = \text{total counts of injected neutrophils}$$

whereby BS is the blood sample count and PS (plasma sample counts) represents traces of free or protein-bound $^{111}$In. The blood volume was assumed to be 75 ml/kg of body weight.\textsuperscript{29}

Leukocyte depletion. Dog neutrophil rabbit antiserum was prepared according to Romson et al.,\textsuperscript{19} which after intravenous injection produces a reduction of circulating neutrophils without affecting lymphocyte and monocyte counts. Neutrophils (1 x 10$^9$) prepared as previously, were homogenized in complete Freund's adjuvant and injected intramuscularly on two occasions at an interval of 10 days. Ten days later, the rabbits were bled and the serum was inactivated at 56°C for 40 min.

After an initial blood cell count, the antiserum was injected intravenously, 4 ml at the time of anesthesia and 2 ml 1 hr later. Thereafter the antiserum was infused at a rate of 1.5 ml/hr for the next 24 hr. Surgery was initiated at the start of the infusion, and coronary occlusion was begun 2 hr after the first antiserum injection to avoid the vasodepressor response.\textsuperscript{19} Leukocytes, erythrocytes, and platelet counts were done during the first 6 hr and before death.

The protocol for administration of antiserum was established previously in control dogs and found to produce a complete neutrophil depletion (99.5%).

Infarct sizing and scintigraphy. Twenty-four hours after coronary occlusion, heparin (10,000 U) was injected intravenously and the animals were killed. The heart was rapidly excised. Cannulation of the LAD at the occlusion site and of the aorta was followed by perfusion at both regions with cold saline (0.9%) for 1 min and subsequently by perfusion of the aorta by Evans blue (EB) 0.5% and the LAD by saline (0.9%) at a pressure of 100 mm Hg.\textsuperscript{13} The left heart was then included in a polyurethane foam (kindly supplied by MIA Chemicals Montreal, a division of Canada Fiberglass, Inc.) and cut with a
commercial meat slicer in 7 mm thick transverse slices. The slices were immersed in triphenyltetrazolium (TPT) 1.5% in Tris buffer (2.4%, pH 7.8) 10 min at 37° C for delimitation of the infarcted region. The normally perfused myocardium (EB positive), the area at risk of ischemia (EB negative), and the necrotic myocardium (EB negative and TPT negative) were measured by planimetry for each slice. Infarct size was related to both the left ventricle and the area at risk.

The heart slices (groups 1 and 2) were placed side by side on a medium-energy collimator equipped with gamma camera (Technicare Model 420/550). An 8 hr acquisition was done directly on a DEC gamma-11 system. After background subtraction, a region of interest comprising the zone of elevated count density was delineated. Counts of all regions of interest were added to obtain the total infarct radioactivity. To allow comparison of activity values between different groups, radioactivity measurements were corrected by linear regression estimation for the number of 111In-neutrophils injected (covariate 1) and infarct size (covariate 2). Corrected values were pooled to obtain a mean value for each group.

**Well counting and histology.** Myocardial tissue samples were selected in the area at risk of a midventricular slice of each heart. One sample in the central infarct zone was chosen and cut into a subepicardial and a subendocardial layer, which were weighed and counted separately in a gamma counter. A tissue sample in the nonischemic posterior wall of the left ventricle was also selected, prepared, and counted in the same manner. The counts per gram of tissue of every sample were estimated. A neutrophil accumulation ratio between the ischemic and the nonischemic samples was calculated for the above layers of myocardial tissue. Neutrophil accumulation ratios of the subepicardial and subendocardial central zone were pooled on a group basis and compared.

Histologic sections were prepared from the samples used for gamma counting and stained with hematoxylin phloxin saffron. Sections were examined qualitatively for the presence or absence of neutrophils in the infarcts. Other histologic criteria included hemorrhages, arteriolar thrombosis, and leukocyte plugs.

**Statistical analysis.** Results are expressed as mean ± SE. An unpaired t test was used to compare values between control and intervention groups. An analysis of variance was performed on the hemodynamic data of all groups. For the quantification of the radioactivity in infarcts by digitized gamma scintigraphy, the final comparison between the group means was done with a t test on values adjusted for the two covariates (the number of injected neutrophils and the infarct size). The critical value for the t test was chosen at n1 + n2 - 4 degree of freedom to account for the two slopes estimated for covariate adjustment. The chosen level of significance was p < .05.

**Table 1**

<table>
<thead>
<tr>
<th></th>
<th>Heart rate (beats/min)</th>
<th>LVSP (mm Hg)</th>
<th>LVEDP (mm Hg)</th>
<th>Coronary flow (ml/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before occlusion</td>
<td>After 1 hr occlusion</td>
<td>Before occlusion</td>
<td>After 1 hr occlusion</td>
</tr>
<tr>
<td>Group 1 (n = 6)</td>
<td>131 ± 4.3</td>
<td>136 ± 9.3</td>
<td>144 ± 7.0</td>
<td>125 ± 3.4^</td>
</tr>
<tr>
<td>Group 2 (n = 6)</td>
<td>143 ± 5.4</td>
<td>150 ± 4.5</td>
<td>157 ± 13.1</td>
<td>142 ± 10.3</td>
</tr>
<tr>
<td>Group 3 (n = 7)</td>
<td>142 ± 6.2</td>
<td>137 ± 4.2</td>
<td>150 ± 5.7</td>
<td>145 ± 4.0</td>
</tr>
<tr>
<td>Group 4 (n = 6)</td>
<td>133 ± 2.1</td>
<td>136 ± 6.9</td>
<td>137 ± 8.7</td>
<td>136 ± 10.2</td>
</tr>
<tr>
<td>Group 5 (n = 6)</td>
<td>159 ± 6.5</td>
<td>164 ± 6.4</td>
<td>139 ± 6.1</td>
<td>139 ± 4.9</td>
</tr>
</tbody>
</table>

LVSP = left ventricular systolic pressure; LVEDP = left ventricular end-diastolic pressure.  
^p < .05 vs before occlusion.

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Studies of the kinetics of the labeled neutrophils in vivo showed that the ratio between circulating and 111In-labeled neutrophils decreased linearly with time at a rate of 4.13% per hour (figure 1). Variations were small among dogs at equilibrium after 2 hr and the mean disappearance half-time of the labeled cells in the circulating pool was 8.4 ± 0.7 hr.

Scintigraphy and tissue counting. Gamma scintigraphy of the slices showed a clear delineation of the infarcted region in all cases (figure 2). The sum of digitized counts for every animal calculated for each animal of groups 1 and 2 was correlated significantly with the injected number of 111In-neutrophils (r = .71, p < .01; figure 3, left). The myocardial radioactivity was corrected according to the number of labeled neutrophils injected (covariate 1). As shown in figure 3, right, the corrected myocardial radioactivity of every heart correlated with infarct size (r = .70, p < .05). The myocardial activity was adjusted further according to infarct size (covariate 2; figure 3, right). Mean myocardial radioactivity corrected for covariates 1 and 2 was significantly (p < .025) higher in group 2 (18,647 ± 2418 cpm) compared with that in group 1 (10,331 ± 1633 cpm; figure 4).

Gamma counting of myocardial tissue samples revealed that the neutrophil accumulation ratio in the infarcted region differed significantly between permanent occlusion (group 1) and reperfusion (group 2) in the subendocardial central layer (5.0 ± 2.9 vs 27.0 ± 5.8; p < .01, figure 5). In contrast, the neutrophil accumulation ratio of the subepicardial layer did not differ between these groups.

Effects of antineutrophil serum on hematologic variables. Rabbit antineutrophil serum rapidly produced a marked depletion (83 ± 6%) of blood leukocytes within the first hour after injection without affecting erythrocyte and platelet counts (figure 6). Perfusion of the antiserum maintained this leukopenia from the initiation of surgery until death.

Infarct sizing and histologic examination. The myocardial area at risk was identical among the five groups (table 3) and involved approximately one-third of the total left ventricle. Reperfusion in the presence (group 2) or absence of a critical stenosis (group 3) resulted in infarct size comparable to the one observed during permanent occlusion (group 1). Furthermore, infarct size in animals with leukopenia (group 4) was not significantly different from that in group 3, which differed in the protocol only by the absence of leukopenia, and in group 5, which did not receive lidocaine. Thus infarct size related to the area at risk was identical among the five groups (table 3).

Histologic examination revealed that interstitial hemorrhage was consistently present in reperfused infarcts. Interstitial accumulation of neutrophils was present in every infarct of groups 1 to 3 and 5 but was virtually absent in the leukopenic group (group 4). Likewise, accumulation of neutrophils in the microcirculation and referred to as “leukocytes plugs” was observed in all groups but group 4. Two dogs in group 2 showed arteriolar thrombosis in the infarcted area. This finding was not seen in the other groups.

![FIGURE 2. Gamma scintigraphy of heart slices. The radioactivity is located in the myocardial infarction.](image-url)
Discussion

The duration of 3 hr for coronary occlusion was selected primarily for two reasons: First, for comparison of the extent of leukocyte accumulation in reperfused and nonreperfused infarcts of comparable size, we wished to prevent marked salvage by reperfusion alone. A 3 hr occlusion appeared appropriate for this purpose, since several studies have demonstrated little if any limitation of infarct size by reperfusion after this interval in the absence of pharmacologic intervention. Second, we sought to determine whether leukocyte infiltration is at least in part responsible for the limitation of salvage of reperfusion after 3 hr. Identification of factors limiting the effective time for myocardial salvage by reperfusion has important clinical implications.

Labeling of neutrophils with $^{111}$In has provided a new tool for the investigation of the acute inflammatory response in myocardial infarcts. Highly pure (95%) and functional autologous labeled neutrophil preparation was obtained by the procedure used in this study. Only two dogs had to be eliminated because of abnormal quality tests on both labeled and unlabeled neutrophils in native blood. In vivo, the number of circulating labeled cells reached a steady state 2 hr after their injection. Their number decreased monoexponentially at a rate of 4.1% per hour and the mean disappearance half-time was 8.4 ± 0.7 hr, a value close to that reported by other investigators.

There was a significant correlation ($r = .70$) between total scintigraphic count rate of all slices corrected for injected activity and infarct size, indicating that the number of accumulated neutrophils increases in proportion to the necrotic mass. Analysis after correction for infarct size revealed that, 24 hr after coronary occlusion, accumulation of labeled neutrophils was augmented by 80% in reperfused infarcts. The results of this study, using radionuclide imaging, support the concept that reperfusion enhances the inflammatory response, including neutrophil accumulation in the infarcted region. Our results are in accordance with those of previous studies demonstrating histologi-

![FIGURE 3. Left, Correlation between scintigraphic myocardial activity and the number of labeled injected neutrophils (PMN) for each dog in groups 1 and 2. Right, Correlation between the corrected myocardial activity and the infarct size (% of left ventricle) for each dog in groups 1 and 2.](image)

![FIGURE 4. Myocardial activity corrected for the number of injected neutrophils and for infarct size. Comparison between group 1 (left) and group 2 (right); the radioactivity is significantly higher ($p < .025$) in the reperfused infarcts.](image)

![FIGURE 5. Neutrophil accumulation ratio (NAR) of necrotic tissue samples from area at risk to nonischemic tissue. Transmural myocardial samples selected in the central zone of myocardial infarction are divided into subendocardial and a subepicardial area. The NAR is significantly higher ($p < .01$) in the subendocardial central zone of the reperfused infarcts.](image)
corroborate findings by Reimer et al., who observed that after a 3 hr ischemic insult reperfusion associated with another anti-inflammatory intervention, the administration of ibuprofen, did not result in significant myocardial salvage. On the other hand, Lucchesi et al., observed a marked reduction of infarct size during antiserum-induced leukopenia after a 90 min coronary occlusion and 6 or 24 hr of reperfusion. The following factors may explain the discrepancy between the latter and the present study. First, antineutrophil intervention may only modify reperfusion damage of jeopardized but viable myocardium, which is virtually absent in the area at risk after a 3 hr coronary occlusion. Second, the methods used for the assessment of infarct size may account for the difference. In our study, immersion of tissue slices rather than intracoronary perfusion of tetrodol dye was used. In the latter approach regional differences in the delivery of TTC may influence the staining. Third, although leucocyte counts in our experiment were as low as 17% of normal after administration of antineutrophil serum, this may be insufficient to prevent leucocyte induced damage. However, leucocyte depletion was comparable to that in the study of Romson et al., who observed a significant reduction of the extent of ischemic injury by neutrophil depletion in the dog. Finally, lidocaine, as used in the present study, is not connected to the observed difference, since infarct size was similar among groups receiving (group 3) or not receiving (group 5) the antiarrhythmic agent. Experiments in vitro have demonstrated that lidocaine-related drugs can inhibit neutrophil function and impair their ability to release free radicals, suggesting a potential role for the drug in limitation of infarct size. In our study, scintigraphic imaging of infarcts by labeled neutrophils and quantification of the enhancement of neutrophil accumulation by reperfusion attest that lidocaine does not prevent the chemotactic ability of neutrophils in vivo. However, if lidocaine has interfered with the release by leucocytes of toxic oxygen metabolites, the effect had no measurable benefit on infarct size after a 3 hr coronary occlusion.

Recent observations have suggested that leucocyte plugging of capillaries is an early phenomenon after brief coronary occlusion. This phenomenon may contribute to incomplete recovery of perfusion after restoration of vessel patency, referred to as the "no-reflow" phenomenon. In our study, coronary flow recovered to preocclusion values 30 min after reperfusion in the presence or absence of critical coronary narrowing. However, leucocyte plugging was consistently observed in these animals. In contrast to untreated dogs,
LAD flow after reperfusion was significantly increased compared with the preocclusion values in the leukopenic dogs. In these animals, histologic examination revealed the absence of leukocyte plugging. These results suggest that leukopenia may improve early restoration of myocardial perfusion by preventing leukocyte plugging.

Critical stenosis is often implemented in reperfusion experiments to prevent reperfusion arrhythmias and to limit interstitial hemorrhage. However, the presence of residual stenosis may affect myocardial salvage by reperfusion. In this study, the presence of a residual stenosis did not influence ultimate infarct size. In some experiments with stenosis, however, arteriolar thrombosis was observed histologically in the necrotic area, suggesting that residual stenosis may promote impairment of microcirculation by mechanisms other than leukocyte plugging alone.

**Conclusion.** The effect of reperfusion on myocardial accumulation of neutrophils was assessed by autologous 111In-neutrophils and digitized scintigraphy. Reperfusion after a 3 hr occlusion of the LAD increased markedly the accumulation of labeled neutrophils compared with nonreperfused myocardium. The increase was limited to the subendocardium as revealed by gamma counting of tissue samples. Histologic examination revealed leukocyte plugging after reperfusion. Coronary flow during reperfusion was higher in the presence of leukopenia, suggesting a role of neutrophil accumulation in persistent impairment of microcirculation. However, leukopenia did not limit infarct size after a 3 hr occlusion.

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