Evaluation of the Role of Polymorphonuclear Leukocytes on Contractile Function in Myocardial Reperfusion Injury
Evidence for Plasma-Mediated Leukocyte Activation

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Background. It has been hypothesized that chemotaxis and activation of polymorphonuclear leukocytes (PMNs) occur upon reperfusion of ischemic myocardium. Questions remain, however, regarding the mechanisms by which PMNs are chemotaxed and activated and how this process causes contractile failure.

Methods and Results. Studies were performed in an isolated rat heart model in which the effects of isolated cellular or humoral factors could be studied. Isolated rat hearts were perfused by the method of Langendorff, subjected to 20 minutes of global ischemia, and reperfused with perfusate alone or with perfusate containing PMNs, plasma, PMNs plus plasma, or PMNs plus inactivated plasma (preheated to 56°C for 30 minutes to denature complement) (n=10 in each group). Left ventricular developed pressure (LVDP) was measured during 1 minute of preschemic control infusion and on reflow after a 20-minute period of global ischemia. Additional measurements of free-radical generation were also performed on the coronary effluent by electron paramagnetic resonance spectroscopy (EPR) with the spin trap 5,5'-dimethyl-1-pyrroline-N-oxide (DMPO). During control infusion, no significant alterations in LVDP were observed, and there was no measurable free-radical generation. Reperfusion with plasma or PMNs alone did not alter postischemic LVDP, whereas plasma and PMNs together caused marked injury. LVDP after 45 minutes of reflow with PMNs plus plasma was 31.9±6.1% of control compared with 60.6±9.9% with plasma, 64.5±8.8% with PMNs, and 63.6±7.2% with perfusate alone (p<0.01). With plasma, which was preheated to deplete complement, this injury was not seen; LVDP was 70.8±10.9%. EPR measurements with the spin trap DMPO in the absence of PMNs demonstrated that oxygen free-radical generation is observed only during the first 1–2 minutes of reflow. Upon reperfusion with PMNs and plasma, however, radical generation persisted for more than 10 minutes. Increased neutrophil accumulation was observed in the postischemic heart in the absence of plasma; however, plasma factors were required for neutrophil-mediated contractile failure. CSA alone did not cause significant injury, but in the presence of PMNs it effectively substituted for plasma, causing marked injury.

Conclusions. Thus, plasma factors, most likely complement, are required for neutrophil activation with oxygen free-radical generation and secondary contractile dysfunction. (Circulation 1993;87:536–546)

Key Words: • leukocytes • myocardial reperfusion injury • free radicals

Polymorphonuclear leukocytes (PMNs) have been hypothesized to either cause or amplify the process of reperfusion injury in postischemic myocardium. It has been suggested that PMNs mediate reperfusion injury by chemotaxis and activation with the generation of oxygen free radicals and the release of proteolytic enzymes and other toxic products. In addition, they also release various inflammatory and chemotactic mediators that can result in a cycle of further cellular chemotaxis and injury.1 There is increasing evidence that within reperfused myocardial tissue there is extensive margination of PMNs and capillary plugging of the coronary microvasculature, which subsequently leads to extensive myocardial damage.2–4 Several studies have demonstrated that PMN depletion can decrease infarct size and prevent myocardial stunning.5 There is recent evidence that plasma factors including complement may be involved in the process of PMN activation.6,7

With in vivo models, there are many different cellular and humoral factors that cannot be controlled; thus, it is often difficult to definitively determine the importance of isolated cellular or humoral factors in the pathogenesis of myocardial injury. Therefore, we developed an

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isolated rat heart model in which the effects of isolated cellular and humoral factors on postischemic injury could be studied. In this model, it is possible to measure contractile function, PMN adherence, and free-radical generation.

The effects of PMNs and plasma factors on postischemic injury were studied. We observed that PMNs can markedly increase the injury that occurs on postischemic reperfusion; however, plasma factors, most likely complement, are required for this PMN-mediated injury.

Methods

Isolated Heart Perfusion

Female Sprague-Dawley rats (weight, 250–500 g) were heparinized and anesthetized with intraperitoneal pentobarbital. The hearts were excised, the ascending aorta was cannulated, and retrograde reperfusion was initiated. The hearts were then perfused with Krebs bicarbonate perfusate (in mM: glucose 17, sodium chloride 120, sodium bicarbonate 25, calcium chloride 2.5, EDTA 0.5, potassium chloride 5.9, and magnesium chloride 1.2) at 37°C at a constant pressure of 80 mm Hg. The perfusate was bubbled with 95% O2/5% CO2. Two side arms in the perfusion line located just proximal to the heart cannula allowed infusion of PMNs and plasma directly into the heart (Figure 1, top panel). To assess contractile function, a latex balloon was inserted into the left ventricular cavity and connected to a pressure transducer as described previously. The balloon was initially inflated with the volume of distilled water required to produce an end-diastolic pressure between 8 and 14 mm Hg. Subsequent measurements of left ventricular developed pressures (LVDP) were calculated as the difference between the peak-systolic and end-diastolic pressures. Left ventricular pressure was recorded with a Gould RS3400 four-channel recorder. Coronary flow and heart rates were measured periodically every 5 minutes before 20-minute global ischemia and after global ischemia for 45 minutes of reperfusion.

PMN Preparation

Human neutrophils were prepared by the method of Kensler and Trush, which yields PMNs with a purity of >95%. Freshly sampled blood (50 ml) was drawn from volunteer donors in heparinized 10-ml vacuum containers and centrifuged at 500g in a Beckman TJ-6 for 10 minutes. The plasma and buffy coat were aspirated, leaving the red cell layer. The cells were then mixed with an equal volume of 6% dextran (1 g dextran MW 500,000, 5 g dextran MW 80,000, and 100 ml 0.9% normal saline), with the tubes rinsed thoroughly to secure all the cells. The mixture was then transferred to 30-ml plastic syringes, which were then inverted and incubated at 37°C for 1 hour until a clear separation was obtained. Next, the upper layer was ejected through a
16-gauge needle with a 90° bend into 50-ml plastic centrifuge tubes and spun for 10 minutes at 500g. The supernatant was discarded, and the pellet was resuspended in ice-cold ACK lysing buffer (0.155 M NH₄Cl, 0.01 M KHCO₃, and 0.1 mM EDTA at pH 7.4) and respun for 10 minutes at 500g. Finally, the pellet was washed, resuspended in Dulbecco’s phosphate-buffered saline (PBS) with 1% glucose, and spun for 5 minutes at 340g. A small volume of the cells resuspended in Dulbecco’s was counted with a hemocytometer.

Rat Plasma
Rat blood obtained by closed-chest intracardiac puncture with a 10-ml heparinized syringe was immediately centrifuged at 500g. To denature complement, the upper plasma layer was subsequently transferred to a plastic centrifuge tube and incubated in a water bath at 56°C for 45 minutes.10

Electron Paramagnetic Resonance Measurements
Hearts were isolated and perfused as described above except that no EDTA was included in the perfusate. The experimental protocol remained the same except that the spin trap 5,5'-dimethyl-1-pyrroline-N-oxide (DMPO) was infused through a third side arm located at the level of the heart with a final concentration of 40 mM. Periodic collections of the effluent were made in 20-second aliquots during the control period and also for the first 2, 5, 7.5, and 10 minutes of reperfusion. In addition, experiments were performed to detect the generation of oxygen free radicals in PMNs plus plasma with and without zymosan, an activator of complement.11 Care was taken to keep the DMPO-containing solutions covered to prevent any light-induced degradation. The DMPO purchased from Aldrich was further purified by double distillation. Electron paramagnetic resonance (EPR) spectra were recorded in an EPR flat cell at room temperature with a Bruker-IBM ER 300 spectrometer operating at X-band using a TM₁₀ cavity, a modulation frequency of 100 kHz, modulation amplitude of 0.5 G, microwave power of 20 mWatts, and microwave frequency of 9.77 GHz. Each spectral acquisition file was the sum of ten 1-minute scans. The digital Bruker spectral data were transferred to an AST 386 personal computer for analysis. Software capable of isotropic spectral simulation, developed in this laboratory, was used for component analysis of experimental spectra as described previously.12-14 Spectral simulations consisting of linear combinations of the component signals were performed to match the observed spectra. From the weighted intensities of each component in these simulations, the relative amount of each component signal was determined. The total radical concentration was then determined from the ratio of the double integral of the observed spectra to the known concentration of 2,2,6,6-tetramethylpiperidinoxyl free radical in aqueous solution as previously described.12,14

Histology
After completion of the experiments, hearts were quickly removed from the cannula, and the ventricles were sliced into sections 3–5 mm thick. The sections were immediately immersed and stored in 10% formalin at 4°C. Histological processing was done by conventional methods. Sections were stained with hematoxylin and eosin. The histological sections were examined for the extent of PMN infiltration in myocardial interstitium and around the capillaries and arterioles.

Measurement of Complement Activation
Measurement of complement activation by red cell lysis assay was performed to determine whether the mixture of human PMNs and rat plasma caused any nonspecific activation of complement. In these assays, 0.4 ml of the PMN-containing buffer and 0.1 ml plasma were added to 0.5 ml packed human blood cells and incubated at 37°C for 30 minutes. The red blood cells were then pelleted by centrifugation at 500g for 5 minutes. Spectrophotometric measurements of hemoglobin in the supernatant solution were then performed. No hemolysis was observed when the PMNs and plasma were mixed, which demonstrated that there was no nonspecific complement activation. On addition of the complement activator zymosan, 1 mg/ml, however, marked hemolysis was observed.

Chemicals
Recombinant C5a, zymosan A, and bovine copper-zinc superoxide dismutase were purchased from Sigma Chemical Co., St. Louis, Mo. Double-distilled, deionized water was used to prepare the perfusate and other solutions. DMPO, 97% pure, was purchased from Aldrich Chemical Co. and further purified by double distillation.

Experimental Protocol
After a 10–15-minute equilibration period, baseline LVDP, left ventricular end-diastolic pressure, and coronary flow were measured (Figure 1, bottom panel). Hearts were then subjected to a 1-minute preischemic control infusion with PMNs alone, plasma alone, PMNs and plasma, or PMNs and inactivated plasma, and then hearts were allowed to equilibrate again with Krebs bicarbonate buffer for a period of 10 minutes, during which time measurements of coronary flow and LVDP were noted. Hearts subsequently received a 30-second infusion of one of the requisite media before the onset of the 20-minute period of 37°C global ischemia. At the onset of ischemia, the balloon was deflated. The intra-ventricular balloon volume was then reinflated with the same volume as previously used to set the baseline end-diastolic pressure immediately after the onset of reflow. At the onset of reperfusion, the hearts were reperfused for the first 5 minutes with the requisite media for each of five different groups, after which perfusion was continued with Krebs buffer alone for a total of 45 minutes of reflow, during which time serial measurements of coronary flow and LVDP were performed every 5 minutes. One or more side-arm ports were placed just above the aortic cannula to allow administration of cells, plasma, or both (Figure 1). To determine free-radical generation, hearts were similarly perfused in each of the groups in the presence of 40 mM DMPO as described above, but without EDTA in the perfusate. Five experimental groups were studied, with 10 hearts in each group:

Group 1. Hearts were reperfused with only Krebs perfusate during preischemia and on reperfusion.
Human PMNs can be isolated in the large numbers required, 30–60 million, and can be readily purified from other blood elements by procedures that do not result in activation of the cells. In practice, 50 ml of blood is required to isolate sufficient cells for one or two experiments. To isolate this number of PMNs from the rat, complete phlebotomy of 10 animals is required, and it is difficult to purify the cells to >70% purity. Pilot studies were performed infusing human plasma into rat hearts. Plasma even at 1:20 dilution resulted in irreversible asystole. This appeared to be caused by cross-reactive human antibodies reacting with the rat antigens. Immunoabsorption of the human plasma with heart homogenate decreased but did not eliminate this toxicity of the human plasma. In contrast, upon infusion of rat plasma into rat hearts, no toxicity was seen and no alterations in LVDP occurred. To verify that the human PMNs did not activate complement or cause other adverse reactions, all hearts studied were first subjected to control preischemic infusions of both the PMNs and plasma, and it was observed in all 10 hearts studied that after infusion, LVDP and coronary flow were not significantly altered. In contrast, if complement was activated with 1 mg/ml of zymosan or PMNs were activated with 200 ng/ml of phorbol 12-myristate 13-acetate (TPA), marked sustained injury was observed with a >60% decrease in contractile function. To further validate the hypothesis that mixture of the human PMNs and rat plasma did not cause PMN activation, EPR measurements were performed on solutions of PMNs and plasma. When 300,000 PMNs per milliliter were mixed with 5% plasma in PBS in the presence of 50 mM DMPO, no signal was observed. Repeated measurements with up to 1 million PMNs per milliliter and up to 50% plasma also showed no significant signal, confirming that the rat plasma did not cause activation of an oxidative burst from the human PMNs. To further confirm that the PMNs did not induce any activation of complement, red cell lysis assays were performed as described above. These assays demonstrated that mixture of human PMNs and rat plasma did not result in any measurable complement activation. If complement was intentionally activated with 1 mg/ml of zymosan, however, marked red cell lysis and marked free-radical generation was observed. Thus, these validation experiments demonstrated that there was no baseline complement activation, neutrophil activation, or alterations in preischemic function in this model.

**Statistics**

Data are presented as mean±SEM. Comparisons between groups during preischemic control infusions as

**Table 1. Final Recovery of Physiological Parameters**

<table>
<thead>
<tr>
<th>Group</th>
<th>LVEDP (mm Hg)</th>
<th>LVDP (% recovery)</th>
<th>CF (% recovery)</th>
<th>RPP (% recovery)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control hearts</td>
<td>46±5.9</td>
<td>63.6±7.2</td>
<td>68.0±6.2</td>
<td>62.8±5.5</td>
</tr>
<tr>
<td>PMNs plus plasma</td>
<td>75.8±7.2</td>
<td>31.9±6.1</td>
<td>43.6±9.9</td>
<td>30.1±7.0</td>
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<tr>
<td>PMNs alone</td>
<td>45.7±5.7</td>
<td>64.5±8.8</td>
<td>71.2±8.8</td>
<td>66.9±7.0</td>
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<tr>
<td>Plasma alone</td>
<td>42.7±7.4</td>
<td>60.6±8.9</td>
<td>68.3±8.7</td>
<td>59.8±9.8</td>
</tr>
<tr>
<td>PMNs plus inactivated plasma</td>
<td>40.3±10.6</td>
<td>70.8±10.9</td>
<td>60.3±8.2</td>
<td>71.7±19.9</td>
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</tbody>
</table>

LVEDP, left ventricular end-diastolic pressure; LVDP, left ventricular developed pressure; CF, coronary flow; RPP, rate-pressure product; PMNs, polymorphonuclear leukocytes. n=10 in each group.
did not alter the time course or magnitude of recovery of postischemic contractile function from that observed with perfusate alone, group 1. In hearts reperfused with PMNs and plasma, however, a marked decrease in the recovery of contractile function was observed (Figure 2). After 45 minutes of reperfusion, group 4 hearts treated with PMNs and rat plasma recovered 31.9±6.1%, compared with group 1 hearts treated with perfusate alone, which recovered 63.6±7.2% (p<0.005); group 2 hearts treated with PMNs alone, which recovered 64.5±8.8% (p<0.0003); and group 3 hearts treated with rat plasma alone, which recovered 60.6±8.9% (p<0.015). Clearly the group 4 hearts treated with PMNs and rat plasma recovered poorly compared with other groups, suggesting that a plasma factor such as complement might be responsible for inducing leukocyte activation. The group 5 hearts treated with PMNs and heat-inactivated rat plasma exhibited recovery of contractile function similar to that of groups 1, 2, and 3, with a final recovered LVDP of 70.8±10.9% (p<0.009 compared with group 4). Thus, PMNs alone did not alter the recovery of contractile function, but PMNs along with plasma resulted in markedly increased injury, which could be prevented by heat inactivation of the plasma.

**Results**

**Functional Data**

**Left ventricular developed pressure.** Preschismic baseline values of LVDP did not differ significantly in the five groups studied; a mean pressure of 127±5 mm Hg was observed. Reperfusion with either PMNs alone, group 2, or plasma alone, group 3, did not alter the time course or magnitude of recovery of postischemic contractile function from that observed with perfusate alone, group 1. In hearts reperfused with PMNs and plasma, however, a marked decrease in the recovery of contractile function was observed (Figure 2). After 45 minutes of reperfusion, group 4 hearts treated with PMNs and rat plasma recovered 31.9±6.1%, compared with group 1 hearts treated with perfusate alone, which recovered 63.6±7.2% (p<0.005); group 2 hearts treated with PMNs alone, which recovered 64.5±8.8% (p<0.0003); and group 3 hearts treated with rat plasma alone, which recovered 60.6±8.9% (p<0.015). Clearly the group 4 hearts treated with PMNs and rat plasma recovered poorly compared with other groups, suggesting that a plasma factor such as complement might be responsible for inducing leukocyte activation. The group 5 hearts treated with PMNs and heat-inactivated rat plasma exhibited recovery of contractile function similar to that of groups 1, 2, and 3, with a final recovered LVDP of 70.8±10.9% (p<0.009 compared with group 4). Thus, PMNs alone did not alter the recovery of contractile function, but PMNs along with plasma resulted in markedly increased injury, which could be prevented by heat inactivation of the plasma.

**Rate-pressure product.** The product of heart rate and LVDP, the rate-pressure product, was measured as a further index of cardiac work, and a dependence similar to that for LVDP alone was observed. The group 4 hearts exhibited a markedly lower rate-pressure product than the other groups throughout reflow. Approximately a twofold difference was observed between the recovery of rate-pressure product between group 4 and

**Graph showing percent recovered coronary flow in hearts reperfused after 20 minutes of global ischemia.** Open circles: hearts infused with only Krebs bicarbonate buffer; closed circles: hearts infused with polymorphonuclear leukocytes (PMNs) alone; filled triangles: hearts infused with plasma alone; filled diamonds: hearts treated with PMNs plus plasma; open squares: hearts treated with PMNs plus inactivated plasma. Hearts treated with PMNs and plasma demonstrated a significantly decreased recovery of coronary flow compared with other groups (p<0.04).
the other groups after 45 minutes of reflow (Figure 3 and Table 1).

**End-diastolic pressure.** Baseline end-diastolic pressure was 12±1.5 mm Hg in all the groups. Final recovery of end-diastolic pressure after 45 minutes of reperfusion in group 4 hearts treated with PMNs and rat plasma was 75.8±7.2 mm Hg compared with 46±5.9 mm Hg (p<0.00004) in group 1 hearts treated with perfusate alone, 45.7±5.7 mm Hg in group 2 hearts treated with PMNs alone (p<0.001), 42.7±7.4 mm Hg in group 3 hearts treated with rat plasma (p<0.003), and 40.3±10.6 mm Hg in group 5 hearts treated with PMNs with inactivated rat plasma (p<0.008) (Figure 4). It is clear that the group 4 hearts treated with PMNs and rat plasma had significantly higher end-diastolic pressures after postischemic reperfusion than the other five groups, consistent with the conclusion that reperfusion with PMNs and plasma causes contractile failure with impaired diastolic relaxation.

**Coronary flow.** Coronary flow measured before the onset of global ischemia was 15±1.8 ml/min in all the groups. Throughout the entire 45 minutes of reperfusion, coronary flow rates were not significantly altered in group 1, group 2, and group 3 hearts. Comparison of the final recovery of coronary flow between group 1, perfusate alone, and group 4 hearts, treated with PMNs and rat plasma, demonstrated a significant decrease in flow: 68.0±6.2% versus 43.6±9.9% (Figure 5). Therefore, it was observed that neither plasma nor PMNs alone cause a significant decrease in coronary flow, whereas both together do result in significantly decreased flow. With heat inactivation of the plasma, this decrease in coronary flow was prevented, and coronary flow was indistinguishable from that of the hearts in groups 1, 2, and 3.

**PMN Accumulation**

Experiments were performed to determine whether PMN accumulation was increased after ischemia and whether this accumulation was dependent on the presence of plasma factors. PMN accumulation was measured by a differential count on perfusate solution entering the heart cannula and coronary effluent collected during control infusion before ischemia and the first 5 minutes of reflow. In group 2 hearts reperfused with PMNs alone, a 2.9-fold increase in PMN accumulation was observed after ischemia. In group 4 hearts, a similar 2.2-fold increase in PMN accumulation was observed after ischemia. In group 5 hearts, reperfused in the presence of heat-inactivated plasma and PMNs, no decrease in PMN accumulation was seen compared with the other groups. Accumulation of PMNs in all the postischemic groups was similar, suggesting that the mechanism of neutrophil accumulation was independent of plasma factors (Table 2).

**Histology**

Histological sections were examined from hearts in each of the four groups studied. In sections from groups 2, 4, and 5, PMNs were seen adherent to the endothelium of arterioles and capillaries, whereas in group 3 hearts treated with plasma alone, very few if any PMNs were seen. In the sections from group 4, PMNs were also noted to have migrated to the site of myocytes, and degranulating or degranulated PMNs were seen adjacent to both capillary endothelium and myocytes (Figure 6). Marked myocyte damage was noted in this group, with myofibrillar destruction noted in scattered regions of myocardium. In contrast, in sections from the other groups, myofibrillar structure appeared largely normal.

**Measurement of Free-Radical Generation**

Activated PMNs are known to give rise to an oxidative burst with the production of superoxide free radicals. Therefore, we performed studies in the isolated heart preparation to determine whether free-radical generation was increased in the presence of PMNs and plasma factors. Hearts were subjected to 20 minutes of ischemia and reperfusion in the presence or absence of PMNs and plasma. These hearts were perfused in the presence of a 40 mM concentration of the spin trap DMPO, and coronary effluent was sampled every 20 seconds for the first 2 minutes of reflow and again at 5, 7.5, and 10 minutes. In the absence of PMNs and plasma, a burst of radical generation is observed early, peaking in the first 20 seconds of reflow, followed by a gradual decrease until, after 2–5 minutes, no radical signals are observed (Figures 7 and 8). In the presence of PMNs and plasma, however, radical generation persisted for more than 10 minutes (Figures 7 and 9). With reperfusion of plasma or PMNs alone, no significant difference in magnitude or time course of radical generation was seen compared

### Table 2. Polymorphonuclear Leukocyte Adherence, Postischemic Infusion

<table>
<thead>
<tr>
<th></th>
<th>PMNs infused (n)</th>
<th>PMNs in effluent (n)</th>
<th>PMNs adherent (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMNs alone</td>
<td>28.2±1.4</td>
<td>10.5±2.45</td>
<td>18.7±6.0</td>
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<tr>
<td>PMNs plus plasma</td>
<td>26.4±3.5</td>
<td>2.7±0.92</td>
<td>23.7±3.4</td>
</tr>
<tr>
<td>PMNs plus inactivated plasma</td>
<td>28.45±1.6</td>
<td>6.1±2.35</td>
<td>22.2±1.7</td>
</tr>
</tbody>
</table>

PMNs, polymorphonuclear leukocytes.

### Table 3. Final Recovery of Physiological Parameters

<table>
<thead>
<tr>
<th></th>
<th>LVEDP (mm Hg)</th>
<th>LVDP (% recovery)</th>
<th>CF (% recovery)</th>
<th>RPP (% recovery)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZAP alone (200 μg/ml)</td>
<td>46±18</td>
<td>54±23</td>
<td>57±14</td>
<td>48±18</td>
</tr>
<tr>
<td>ZAP plus PMNs</td>
<td>74±14</td>
<td>30±8.5</td>
<td>41.5±18</td>
<td>33±12.4</td>
</tr>
<tr>
<td>CsA alone (500 ng/ml)</td>
<td>46±14</td>
<td>58±7.4</td>
<td>71±5</td>
<td>50±2.6</td>
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<tr>
<td>CsA plus PMNs</td>
<td>79±19</td>
<td>41.5±1.5</td>
<td>53±3.5</td>
<td>37±2</td>
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LVEDP, left ventricular end-diastolic pressure; LVDP, left ventricular developed pressure; CF, coronary flow; RPP, rate–pressure product; ZAP, zymosan-activated plasma; PMNs, polymorphonuclear leukocytes. n=3 in each group.
with that observed with buffer alone. Thus, reperfusion with PMNs and plasma resulted in a marked prolongation in the duration of radical generation.

**Effects of C5a and Activated Plasma on Contractile Function and Radical Generation**

In the above-described studies, we observed that increased myocardial injury was observed only in the presence of both plasma and PMNs. If complement is activated, one might expect that this alone could cause injury. To quantify the effect of activated complement in this model, additional experiments were performed in which hearts were infused with either C5a or zymosan-activated plasma. The experiments with C5a were designed to determine whether there was a concentration of C5a sufficient to cause PMN activation and free-radical generation but not sufficient in itself to have a significant effect on myocardial function in the normal or posts ischemic heart.

EPR studies were performed with 10⁶ PMNs per milliliter in the presence of 50 mM DMPO and 5 vol% of zymosan-activated plasma or various concentrations of C5a. With control PMNs, no detectable EPR signal was seen, whereas with 5% zymosan-activated plasma, a prominent 1:2:2:1 quartet signal with hyperfine coupling constants a₁₁/a₁₁ = 14.9 G indicative of DMPO–OOH and a smaller signal of DMPO–OOH (Figure 10). Both of these signals were quenched in the presence of 100 units/ml of superoxide dismutase, indicating that both signals were derived from the superoxide free radical. With C5a, prominent free-radical signals were also seen. As shown in Figure 10, with 500 ng/ml of C5a, a clear 1:2:2:1 quartet signal of DMPO–OH is seen.

Four additional groups of hearts were subjected to an experimental protocol similar to that of groups 1–5. **Figure 6.** Panel A, facing page: Photomicrographs showing histology of nonischemic rat myocardium subjected to perfusion with polymorphonuclear leukocytes (PMNs) and plasma. Original magnification, ×100. Note normal myocardial structure of the myocytes, sinusoids, and small arterioles. Panel B, facing page: Photomicrographs showing histology of heart tissue reperfused with PMNs and plasma. Original magnification, ×100. Note the infiltration of PMNs within the interstitium and the marked myofibrillar destruction. Panel C, above: Histology from a rat heart myocardium subjected to the same protocol as the middle panel. Original magnification, ×160. Note the presence of two PMNs that are degranulating with the interstitium of the myocardium.

**Figure 7.** Graph showing total free radical concentration measured using 40 mM 5,5‘-dimethyl-1-pyrroline-N-oxide (DMPO) and electron paramagnetic resonance spectroscopy during the first 10 minutes of reperfusion. Circles: control hearts; triangles: polymorphonuclear leukocytes plus plasma.
presented above, except that zymosan-activated plasma (5%) or C5a (500 ng/ml) was infused in the presence or absence of PMNs (Table 3). It was observed that zymosan-activated plasma alone did not significantly decrease the recovery of contractile function or coronary flow. However, zymosan-activated plasma and PMNs together induced marked injury, with more than a twofold decrease in LVDP and rate-pressure product from group 1 control hearts. Coronary flow was also significantly reduced. With C5a concentrations of 500 ng/ml, similar results were obtained, with significant injury seen only in the presence of PMNs. Thus, activated complement alone was not sufficient to cause the marked alterations in contractile function observed in this model. Marked contractile dysfunction was seen only in the presence of both PMNs and activated complement.

Discussion

Reperfusion of ischemic myocardium limits ischemic damage; however, reperfusion itself has been shown to initiate a chain of events that cause a new form of tissue damage. This damage has been called reperfusion injury, and it is characterized by a unique histological picture, with the formation of contraction bands in the contractile proteins, calcific granules within the mitochondria, and cell swelling with the disruption of sarcoplasmic and mitochondrial membranes. A number of mechanisms have been proposed to mediate reperfusion injury. These include cellular calcium loading, the formation of oxygen free radicals, and neutrophil chemotaxis and activation. The role of the neutrophil is of particular importance in that it could also cause the other mechanisms of injury. It is well known that PMNs, once activated, generate a massive oxidant burst, with the production of large amounts of the superoxide free radical. Indeed, the activated PMN is the most potent cellular source of superoxide. It is also known that free radicals such as the superoxide and hydroxyl free radicals can cause myocyte calcium overload. Thus, one can envision that the PMN could play a central role in the induction or amplification of reperfusion injury in the heart. The PMN could induce injury in several ways, including the generation of an oxidant burst, capillary plugging resulting in a no-reflow state, and release of proteolytic enzymes. It has been demonstrated that extensive PMN margination as well as capillary plugging occurs in reperfused myocardium. Several studies have demonstrated that PMN depletion can decrease infarct size and prevent myocardial stunning. In another study, Marc et al have shown a decrease in infarct size by depletion of neutrophils, which strongly suggests that neutrophils are an important mediator of reperfusion injury. There is recent evidence that plasma factors including complement may be involved in the process of PMN activation.

In this study, we developed an isolated heart model to evaluate the effects of isolated cellular or humoral factors on posts ischemic injury. In particular, this model was used to investigate the role of the PMN in reper-
fusion injury and the importance of plasma factors in this process. In this model, it is uniquely possible to define the effects of pure PMNs and of plasma factors on contractile function, coronary flow, and free-radical generation. In addition, PMN accumulation can be simply assessed from differential cell counts on the coronary effluent.

We observed that PMNs alone did not enhance reperfusion injury; however, when hearts were reperfused with PMNs in the presence of plasma, a marked enhancement of injury occurred. This injury was characterized by a more than twofold decrease in the recovery of LVDP and a marked increase in diastolic pressure. Significantly, lower coronary flows were also observed. These changes were not seen upon reperfusion with plasma alone and were totally blocked when the plasma was heat inactivated, suggesting that activation of the complement cascade is probably the factor responsible for PMN activation in these experiments. These results are consistent with those of Crawford et al., who previously demonstrated that administration of cobra venom factor, which inactivates complement, before coronary ligation decreased ischemic injury in the in vivo baboon model. Rosser et al. have demonstrated that subcellular constituents of injured myocardium can bind the first component of complement, with the subsequent activation of the complement cascade with the generation of C3a and C5a, both of which can induce PMN chemotaxis and activation. In addition, Bennett et al. demonstrated in patients with acute myocardial infarction that plasma levels of C3a and C5a are increased after initiation of reperfusion therapy with recombinant tissue plasminogen activator.

In the present study, we noted that when hearts were reperfused in the presence of both PMNs and plasma, the magnitude of free-radical generation was amplified and the duration was considerably increased from that observed in hearts reperfused in the absence of PMNs. This increased free-radical generation was associated with the subsequent markedly increased failure of cardiac contractile function. These results are consistent with the large number of studies that have demonstrated that reperfusion injury is partly caused by free-radical generation. In these studies, it has been shown that free-radical-scavenging compounds or enzymes can decrease infarct size and prevent myocardial stunning.

In postischemic hearts, PMN accumulation within the heart was increased twofold to threefold compared with that observed before ischemia. This increased PMN accumulation was observed in the presence or absence of plasma, indicating that PMN accumulation was independent of plasma factors. However, EPR studies of free-radical generation demonstrated that plasma factors were necessary to observe PMN activation with the generation of an oxidant burst. Histological studies showed that PMNs adhere to arteriole and capillary endothelium in the absence of plasma factors; however, in the presence of plasma, PMNs egress out to the myocytes, inducing myocyte injury. In the presence of plasma, PMNs were observed to be degranulating adjacent to myocytes, with myocyte necrosis and disruption of myofibrillar structure. Since PMN adherence is thought to be caused primarily by specific binding of adhesion molecule receptors, these data may suggest that independent of complement activation, adhesion molecules such as ICAM on the endothelial cell or CD-18 on the PMN are upregulated during ischemia and early reperfusion. In the presence of the chemotactic stimulation of activated complement, PMN migration out of the vasculature into the tissue may occur.

It has been suggested by Ito et al. that complement activation alone may cause a significant degree of myocardial dysfunction. They have observed that infusion of C5a into the coronary arteries of pigs can result in marked contractile dysfunction and can exacerbate ischemic injury. Therefore, we investigated the effects of activated complement, C5a, or zymosan-activated plasma. With either 500 ng/ml of C5a or 5% vol% of zymosan-activated plasma, prominent PMN activation was observed, with measurable free-radical generation. Infusion of C5a alone did not induce significant injury compared with the control group, group 1. In the presence of PMNs in addition to C5a, marked injury was observed. Since C5a resulted in PMN-mediated injury identical to that with whole plasma, these experiments suggest that the plasma factor required for PMN activation is C5a. Additional experiments in which complement was activated with zymosan to maximally activate the full complement cascade were performed and also demonstrated that the activated complement alone was not sufficient in itself to induce significant contractile failure but was sufficient in the presence of PMNs. These studies demonstrate that complement activation, although not sufficient in itself to increase postischemic injury, is sufficient to activate free-radical generation by PMNs, which in turn causes marked injury.
In summary, these studies demonstrate that PMNs in the presence of plasma can markedly amplify reperfusion injury, resulting in marked contractile failure, increased free-radical generation, and increased histological damage. The plasma factors required for PMN activation appear to be primarily complement, with C5a effectively able to substitute for whole plasma. Thus, we observe that complement is required for PMN activation in the postischemic heart with the generation of an oxidative burst and subsequent myocardial injury.

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Evaluation of the role of polymorphonuclear leukocytes on contractile function in myocardial reperfusion injury. Evidence for plasma-mediated leukocyte activation.

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