Early and Late Effects of Leukopenic Reperfusion on the Recovery of Cardiac Contractile Function

Studies in the Transplanted and Isolated Blood-Perfused Rat Heart

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Background. Since there is considerable evidence that leukocytes contribute to tissue injury during ischemia and reperfusion, the present study was designed to (1) determine whether reperfusion in vivo with leukopenic blood affords protection in a model of reversible hypothermic ischemia, (2) determine the duration of any protection, (3) characterize the relation between protection and duration of leukopenic perfusion, and (4) assess the effect of leukopenic reperfusion on myocardial glutathione content.

Methods and Results. Rat hearts (n=12 per group) were excised, immediately arrested with an infusion (2 minutes at 4°C) of St Thomas’ cardioplegic solution, and subjected to 4 hours of global ischemia (4°C). The hearts were then transplanted (1 hour additional ischemic time) into the abdomen of saline-treated or leukopenic recipients. Leukopenia was induced by intraperitoneal administration of mustine hydrochloride (2 mg/kg) 3 days before study. Hearts were then reperfused in situ for 1, 4, or 24 hours, after which they were excised and either processed for histological examination (n=4 per group) or perfused aerobically with bicarbonate-buffered saline for 20 minutes, and contractile function was assessed (n=8 per group); at the end of this period, some hearts (n=5 per group) were taken for metabolite analysis. After 1 hour of reperfusion, contractile function in the saline-treated control group was significantly reduced compared with aerobic controls that had not been subjected to ischemia (left ventricular developed pressure [LVDP], 106±2 vs 126±3 mm Hg at an end-diastolic pressure of 12 mm Hg; P<.05). However, in the hearts with leukopenic reperfusion, LVDP (119±2 mm Hg) was similar to that of aerobic controls. This benefit, however, was lost after 4 and 24 hours of reperfusion. Cardiac compliance was not influenced by leukopenia. Coronary flow recovered significantly better in the leukopenic hearts during the first 4 hours of reperfusion (11.8±0.5 vs 9.3±0.4 mL/min at 1 hour and 10.0±0.5 vs 8.0±0.4 mL/min at 4 hours, P<.05), but again this benefit was lost after 24 hours of reperfusion. The myocardial contents of reduced and oxidized glutathione after 1, 4, and 24 hours of reperfusion were similar in saline-treated and leukocyte-depleted animals. In additional studies, the period of ischemia was extended to 8 hours, and similar results were obtained, with improved recovery of contractile function and coronary flow but not cardiac compliance in the leukopenic group after 1 hour of reperfusion. In further studies with the isolated blood-perfused rat heart, ischemia was induced for 8 hours; this was followed first by reperfusion for 0, 2, 10, 30, or 60 minutes with leukopenic blood and then by perfusion with blood from saline-treated animals for 60, 58, 50, 30, or 0 minutes, respectively. Reperfusion with leukopenic blood for 2 minutes did not improve the recovery of LVDP (106±7 vs 96±10 mm Hg in controls; NS) but when continued for 10, 30, or 60 minutes resulted in significant improvements (137±5, 138±3, and 150±10 mm Hg, respectively). Although coronary flow tended to be greater in all leukopenic groups, by the end of 60 minutes of reperfusion, only those hearts reperfused with leukopenic blood for the entire reperfusion period showed a significant improvement (3.4±0.3 vs 2.5±0.2 mL/min in controls; P<.05). Histological studies revealed no intravascular aggregation of leukocytes or features of myocyte necrosis.

Conclusions. Reperfusion with leukopenic blood accelerated the rate of recovery of cardiac function after reversible myocardial injury but did not lead to a sustained increase in the eventual extent of recovery. Reperfusion with leukopenic blood for the first 10 minutes of reflow is sufficient to obtain this benefit. (Circulation 1993;88:673–683)

KEY WORDS • ischemia • reperfusion • transplantation

Numerous reports, although they are not unchallenged,1–3 suggest that leukocytes are involved in the injury that occurs during ischemia and reperfusion and that leukocyte inactivation or depletion can be protective.4–11 But even though protective effects have been observed in most studies with severe ischemia (1.5 to 3 hours) and infarction,4–9 the results after shorter periods of ischemia have been less encourag-
This raises the possibility that the extent of the benefit associated with the inactivation or depletion of leukocytes might be related to the severity of the ischemia. It has also been suggested that the duration of leukocyte depletion might influence the extent of protection. Thus, in studies in the canine heart subjected to 90 minutes of regional ischemia, Simpson et al.\(^1\) found it necessary to inhibit neutrophil activation for the first 48 hours of reperfusion in order to achieve protection.

The precise mechanism by which leukocytes injure the tissues during ischemia and reperfusion remains to be elucidated; however, it is believed that free oxygen radicals play a major role. Although several potential sources of radicals have been identified (the xanthine-oxidase system,\(^1\) free radicals,\(^9\) the mitochondrial electron transport system,\(^16\) and the cyclooxygenase pathway of arachidonic acid metabolism\(^17\)), the relative contributions of these systems and the sequence of events that they trigger remain controversial.

In the present studies, we used a model of reversible injury to mimic the situation occurring during open-heart surgery and transplantation. By exploiting the heterotopic rat heart preparation and the blood- and crystalloid-perfused isolated heart preparations, we investigated (1) whether leukocyte depletion improves the rate or final extent of recovery of cardiac function after hypothermic global ischemia, (2) the duration over which any protection persists, (3) the minimum duration of leukopenic reperfusion required to achieve protection, and (4) whether leukopenic reperfusion exerts any effect on the glutathione content of the myocardium.

**Methods**

**Animals**

Male homozygous Lewis rats weighing 300 to 350 g (donor and recipient rats in study 1 and donor rats in study 2) and 400 to 500 g (support rats in study 2) were used. All animals received humane care in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH No. 80-23, revised 1985).

**Leukocyte Depletion and Its Assessment**

Three days before surgery, recipient (study 1) and support (study 2) animals received 2 mg/kg IP of mustine hydrochloride (also known as mechlor etham in hydrochloride; 1 mg dissolved in 1 mL of saline) or an equivalent volume of saline. To prevent infection, antibiotics (sul famethoxazole plus trimethoprim) were added to the drinking water for the entire duration of the experiment in both control and leukopenic groups.

Blood samples from leukocyte-depleted and control animals were taken for leukocyte counting at various times before and after heart transplantation and during the blood perfusion of isolated hearts. This was done in Neubauer chambers after lysis of the red cells with 0.3% acetic acid containing 1% crystal violet. Differential cell counts were performed under light microscopy on fresh blood smears stained with May-Grünwald Giemsa stain (May-Grünwald Giemsa stain, 4 minutes; Giemsa freshly diluted four parts to six parts water, 10 minutes). Platelet counts and the assessment of hemoglobin and hematocrit values were performed in a Coulter counter.

**Studies Involving Heterotopic Heart Transplantation**

The experimental preparation and techniques have been previously described in detail.\(^14\) The experimental time course is shown in Fig 1.

**Excision and storage of hearts.** Rats (n=12 per group) were anesthetized with pentobarbital (60 mg/kg IP) and mechanically ventilated through a tracheotomy at a rate of 55 strokes per minute and a ventilation pressure of 12 to 14 mm Hg. The chest was opened, and the venae cavae and pulmonary veins were isolated; heparin was administered (1000 IU/kg IV). The venae cavae and pulmonary veins were then ligated, and the heart was excised and placed in cold (4°C) saline. The aorta was rapidly cannulated, and St Thomas' cardioplegic solution (containing in mmol/L: NaCl 130.0, KCl 16.0, MgCl\(_2\) 16.0, CaCl\(_2\) 1.2, NaHCO\(_3\) 10.0 at pH 7.8) was immediately infused at a constant pressure of 45 mm Hg for 2 minutes at 4°C. Hearts were then immersed in the same cardioplegic solution and stored for 4 hours at 4°C.

**Transplantation procedure.** The abdomen of an anesthetized recipient rat was opened through a midline incision, and the abdominal aorta and inferior vena cava were exposed. The donor heart, after being stored for 4 hours, was removed from the hypothermic chamber and, with a 9-0 monofilament polyamide suture, the aorta and pulmonary artery were anastomosed (end to side) to the abdominal aorta and inferior vena cava, respectively, of the recipient rat. The heart was main-
tained at 10 to 14°C during implantation by being wrapped in a wet swab that was regularly irrigated with cold saline (6 to 10°C). To minimize variability between experiments, the duration of the implantation period was standardized at 60 minutes. The hearts were then reperfused. The abdominal wall was closed in two layers, and the animals were allowed to recover for 1, 4, or 24 hours (n=12 per group); in the 24-hour reperfusion group, the rats were kept in an unrestrained state with normal feeding.

Posttransplantation in vitro assessment with crystalloid perfusion. One, 4, or 24 hours after reperfusion of the implanted hearts, 8 of each group of 12 recipient animals were taken for functional assessment. The rats were anesthetized, the right femoral vein was exposed, and heparin (1000 IU/kg) was administered. The abdomen was reopened, and the transplanted heart was excised and placed in cold (4°C) saline. Blood from the recipient animals was then taken for a white blood cell count. The aorta was rapidly cannulated, and each heart was perfused aerobically in the Langendorff mode19 at 37°C for 20 minutes with perfusion fluid20 (containing in mmol/L: glucose 11.1, NaCl 118.5, KCl 4.8, MgSO4 1.2, KH2PO4 1.2, NaHCO3 25.0, CaCl2 1.4 at pH 7.4 when gassed with 95% O2 plus 5% CO2) at a constant pressure (75 mm Hg). All hearts were paced via the right atrium at 320 beats per minute. At the end of this period, coronary flow was measured and function curves for left ventricular developed pressure (LVPD) were constructed by progressively increasing the volume of the balloon to achieve end-diastolic pressures of 0, 4, 8, 12, 16, and 20 mm Hg. The volume of the balloon was measured at each end-diastolic pressure, and the relations served as an index of cardiac compliance. After removal of the balloon, some hearts (n=5 per group) were freeze-clamped and taken for metabolite analysis. For comparative purposes, aerobic measurements of control cardiac function were also made in fresh, non-transplanted hearts (n=8) that had not been rendered ischemic.

Histological studies. The remaining 4 of the 12 transplanted hearts in each group and the heart and lungs of each of the recipient rats were excised and processed for histological examination.

Study groups with 8 hours of hypothermic ischemic storage. The protocol was identical to that for the studies with 4 hours of hypothermic ischemia with the exception that hearts were stored for 8 hours and reperfused for only 1 hour.

Studies With the Blood-Perfused Rat Heart Preparation

Experimental time course. The experimental time course is shown in Fig 2. Two support rats, one saline-treated and one leukocyte-depleted, were anesthetized with pentobarbital (60 mg/kg IP) and placed supine on a heating pad (37.0±0.5°C). The animals were allowed to breathe spontaneously a mixture of 95% O2 plus CO2, the flow rate being controlled to maintain arterial PO2 and PCO2 within the physiological range. They were anticoagulated with heparin (1000 IU/kg IV), and a femoral artery and femoral vein were cannulated for the arterial blood supply to the donor heart and the return of blood to the support animal. The support animals were used for no longer than 4 hours, and anesthesia was administered as necessary (pentobarbital, 10 mg/kg IP).

Donor rats were anesthetized, and the heart was excised and rendered ischemic at 4°C for 8 hours. Reperfusion was carried out in the Langendorff mode19 with arterial blood from a support rat delivered to the heart through a peristaltic pump. Perfusion pressure was monitored continuously and maintained at 60±1 mm Hg. The temperature of the blood was maintained at 37.0±0.5°C by a thermostatically regulated heat exchanger. Blood was infused back into the support animal through a blood filter (pore size, 200 μm). A balloon catheter attached to a pressure transducer was inserted into the left ventricle through the atrium and then inflated to achieve a constant left ventricular end-diastolic pressure (LVPD) of 4 mm Hg. LVPD was recorded throughout the experiment. Heart rate was maintained at 320 beats per minute by right atrial pacing.

Study groups. Hearts (n=8 per group) were excised and infused (2 minutes at 4°C) with St Thomas’ cardioplegic solution. They were then stored at 4°C for 8 hours before being reperfused for 60 minutes as blood-perfused preparations according to one of the following protocols: (1) 60 minutes of reperfusion with blood from a saline-treated support rat, (2) 2 minutes of reperfusion with blood from a leukocyte-depleted support rat followed by 58 minutes of perfusion with blood from a saline-treated support rat, (3) 10 minutes of reperfusion with blood from a leukocyte-depleted support rat plus 50 minutes of reperfusion with blood from a saline-treated support rat, (4) 30 minutes of reperfusion with blood from a leukocyte-depleted support rat plus 30 minutes of perfusion with blood from a saline-treated support rat, or (5) 60 minutes of reperfusion with blood from a leukocyte-depleted support rat. Postischemic recovery of LVPD was recorded at 10-minute intervals. At the end of the experiment, some hearts (n=4 per group) were processed for histological examination. The heart and lungs (n=4 per group) of support animals that had sustained the extracorporeal circuit for 4 hours but without perfusing an isolated heart were used for control histological analysis. Periodically, blood was taken

![Fig 2. Diagram of experimental time course. Hearts (n=8 per group) were excised and immediately arrested with an infusion (2 minutes at 4°C) of St Thomas’ cardioplegic solution (ST). Hearts were subjected to 8 hours of hypothermic (4°C) global ischemia and then reperfused in one of the following modes: 0, 2, 10, 30, or 60 minutes with blood from a leukocyte-depleted support rat followed by 60, 58, 50, 30, or 0 minutes, respectively, with blood from a saline-treated support rat. Cardiac function was assessed during the 60-minute reperfusion period (see text for details), and some hearts (n=4 per group) were taken for histological analysis.](http://circ.ahajournals.org/doi/10.1161/01.CIR.105.1.675)
from the circuit for monitoring of the white blood cell count.

Assessment of Myocardial Oxidant Stress

This study was performed to assess whether reperfusion with leukopenic blood is associated with changes in the myocardial antioxidant status. Frozen heart tissue was weighed and deproteinized with 6 vol of perchloric acid (3 mol/L) and then centrifuged at 6,000g for 15 minutes, after which it was neutralized with potassium carbonate (2 mol/L). The tissue contents of reduced and oxidized glutathione (GSH and GSSG) were then measured as described by Ferrari et al.21 and the tissue protein content as described by Bradford.22

Histological Studies

These studies were aimed at identifying any structural changes, intravascular aggregation of leukocytes, or development of necrosis. After fixation in 10% formaldehyde and embedding in paraffin wax, myocardial tissue sections from donor, recipient, and support animals were cut at 5-μm thickness, stained with hematoxylin and eosin, and examined by light microscopy. Similarly, sections of lung tissue from recipient and support rats were examined for evidence of intravascular aggregation of leukocytes. The interpretation of histology was performed semiquantitatively in a blinded manner by two different observers. Intravascular aggregation of leukocytes was classified as absent, mild (small aggregation), moderate (increased aggregation but without vascular occlusion), and severe (increased aggregation with vascular occlusion). Necrosis was identified by the presence of myocyte coagulation necrosis and contraction bands.

Expression of Results and Statistical Analysis

LVDP (mm Hg) was calculated as the difference between peak systolic pressure and a specified LVEDP. Coronary flow (mL/min) was measured by timed collection of coronary effluent. The white blood cell count was expressed as ×10⁹ cells/μL. All results were expressed as mean±SEM. The two-tailed unpaired Student’s t test was used for comparison between two means. ANOVA was used for comparison of more than two means. If, after a significant F value was obtained, comparisons between the untreated and each of the treated groups were carried out, the two-tailed Dunnett’s test was used. ANOVA for repeated measurements was applied when the values obtained within each group at various times were compared to the basal value. A difference was considered statistically significant when P<.05.

Results

Efficacy of Leukocyte Depletion

As shown in Table 1, the administration of mustine hydrochloride 3 days before the experiment resulted in a profound depletion of white blood cells without any effect on red cell or platelet characteristics. Total leukocytes, lymphocytes, neutrophils, and monocytes were reduced by 81%, 73%, 99%, and 97%, respectively (P<.05). Animals treated with mustine hydrochloride also exhibited a loss of body weight (20 to 40 g) and diarrhea.

| Table 1. White Blood Cell and Platelet Counts and Hemoglobin and Hematocrit Values in Saline Control Rats and Rats 3 Days After Treatment With Mustine Hydrochloride |
|-----------------|-----------------|-----------------|
|                  | Saline control  | Leukocyte-depleted |
| Leukocytes (×10⁹ cells/μL) | 8.61±0.49       | 1.60±0.1*        |
| Lymphocytes (×10⁹ cells/μL)  | 5.67±0.47       | 1.55±0.10*      |
| Neutrophils (×10⁹ cells/μL)  | 2.22±0.14       | 0.02±0.01*      |
| Monocytes (×10⁹ cells/μL)    | 0.74±0.04       | 0.02±0.01*      |
| Platelets (×10¹² cells/μL)   | 683±68          | 667±47          |
| Hemoglobin (g/dL)            | 13.8±0.2        | 14.1±0.2        |
| Hematocrit (%)               | 38.6±2.0        | 39.7±0.9        |

n=5 per group. *P<.05 compared with the saline-treated control group.

The Extent and Persistence of Any Protective Effects of Leukocyte Depletion

In the present studies, St Thomas’ cardioplegic solution was chosen for the preservation of hearts. This solution, widely used in humans for routine cardiac operations and heart transplantation, has been shown experimentally to be protective.23,24

Studies with 4 hours of hypothermic ischemic storage.

(1) Cardiac function. Fig 3 shows the pressure-volume relations obtained in isolated crystalloid perfused hearts that had been previously transplanted (after 4 hours of hypothermic storage) and reperfused in vivo for 1, 4, or 24 hours. In each instance, curves for hearts excised from saline-treated and leukopenic rats are compared. Fig 3, A also shows the pressure-volume relation for fresh hearts that had not been rendered ischemic or transplanted. It can be seen that posts ischemic cardiac function in the saline-treated group was significantly lower than in the aerobic controls. For example, LVDP at an LVEDP of 12 mm Hg was 108±5 versus 126±3 mm Hg (P<.05). In contrast, posts ischemic contractile function in hearts that had been reperfused with leukopenic blood was significantly better than with whole blood (119±2 versus 108±5 mm Hg at an LVEDP of 12 mm Hg; P<.05). In fact, there were no significant differences in the functional characteristics of nonischemic aerobic controls hearts and those reperfused with leukopenic blood at any of the left ventricular volumes. This protective effect, however, was lost when hearts were reperfused for 4 or 24 hours (Fig 3, B and C), under which circumstances both saline-treated and leukopenic groups showed identical posts ischemic recoveries of mean LVDP.

In the 1-hour reperfusion groups, in comparison with the fresh aerobic controls, there was a consistent tendency for cardiac compliance to be reduced in the saline-treated and the leukocyte-depleted groups (319±8 versus 279±13 and 309±9 μL, respectively, for left ventricular volume at an LVEDP of 12 mm Hg; NS). Cardiac compliance deteriorated further in both groups after 4 hours (244±20 and 273±23 μL, respectively; NS) and 24 hours of reperfusion (147±18 and 178±17 μL, respectively; NS). The time-dependent deterioration in the 4 hour and 24 hour reperfusion groups can be explained on the basis of the unloaded state of the heterotopically transplanted heart and the consequent reduction in left ventricular volume.
In the 1-hour reperfusion saline-treated group, coronary flow (Fig 4) recovered to 74% (9.3±0.4 mL/min) of the aerobic control values (12.6±0.5 mL/min; P<.05). Leukocyte depletion resulted in an improved coronary flow (11.8±0.5 mL/min), which returned to a value close to that of the aerobic control. In the 4-hour reperfusion groups, coronary flow had deteriorated further in both the saline-treated and the leukopenic groups, but again the rate of flow was greater in the leukopenic group (10.0±0.5 mL/min) than in the saline-treated controls (8.0±0.4 mL/min; P<.05). After 24 hours of reperfusion, mean coronary flow remained greater in the leukopenic group (11.0±0.7 mL/min) than in the saline-treated controls (9.4±0.5 mL/min); however, this difference was not statistically significant.

(2) Time course for changes in leukocyte count over 24 hours of reperfusion. White cell counts were performed at the time of reperfusion and after 1, 4, and 24 hours of reperfusion. In addition to leukocyte-depleted and nondepleted controls, sham-operated rats (anesthetized, abdomen opened, abdominal aorta and inferior vena cava dissected out, and abdomen closed without heart transplantation) were also investigated to assess the effects of anesthesia and surgery on the white cell counts. The results show that the dramatic reduction in white blood cell counts observed in mustine hydrochloride-treated rats (Table 1) persisted throughout the 24-hour reperfusion period (Table 2). In the saline-treated controls, white blood cell counts fell progressively throughout the 24 hours of reperfusion. In contrast, in sham-operated rats, after an initial decline, the white cell counts progressively rose such that by 24 hours after surgery, they were fully recovered. This indicates that, although the initial fall in white cell counts was greater in the leukopenic group compared to the saline-treated group, the effects were not statistically significant.

Table 2. Effect of Mustine Hydrochloride Administered 3 Days Before Transplantation on the Circulating Counts of White Blood Cells

<table>
<thead>
<tr>
<th>Duration of reperfusion (h)</th>
<th>Sham-operated</th>
<th>Saline-treated</th>
<th>Leukocyte-depleted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukocytes</td>
<td>(×10⁶ cells/µL)</td>
<td>(×10⁶ cells/µL)</td>
<td>(×10⁶ cells/µL)</td>
</tr>
<tr>
<td>1</td>
<td>5.63±0.80</td>
<td>5.98±0.57</td>
<td>1.25±0.07*</td>
</tr>
<tr>
<td>24</td>
<td>7.30±0.78</td>
<td>4.48±0.43*</td>
<td>2.20±0.19*</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>(×10⁶ cells/µL)</td>
<td>(×10⁶ cells/µL)</td>
<td>(×10⁶ cells/µL)</td>
</tr>
<tr>
<td>1</td>
<td>4.02±0.43</td>
<td>3.98±0.30</td>
<td>0.99±0.07*</td>
</tr>
<tr>
<td>24</td>
<td>3.23±0.24</td>
<td>3.55±0.28</td>
<td>1.00±0.06*</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>(×10⁶ cells/µL)</td>
<td>(×10⁶ cells/µL)</td>
<td>(×10⁶ cells/µL)</td>
</tr>
<tr>
<td>1</td>
<td>1.22±0.36</td>
<td>1.77±0.27</td>
<td>0.19±0.02*</td>
</tr>
<tr>
<td>24</td>
<td>3.29±0.26</td>
<td>1.14±0.25*</td>
<td>0.25±0.02*</td>
</tr>
<tr>
<td>Monocytes</td>
<td>(×10⁶ cells/µL)</td>
<td>(×10⁶ cells/µL)</td>
<td>(×10⁶ cells/µL)</td>
</tr>
<tr>
<td>1</td>
<td>0.39±0.11</td>
<td>0.25±0.08</td>
<td>0.07±0.01*</td>
</tr>
<tr>
<td>24</td>
<td>0.27±0.05</td>
<td>0.19±0.07</td>
<td>0.08±0.02*</td>
</tr>
</tbody>
</table>

n=4 or 5 per group. *P<.05 compared with the sham-operated group.
counts may be attributed to the surgical trauma, the implantation and reperfusion of the donor heart is likely to be responsible for the subsequent decline.

(3) Histology. After ischemia, transplantation, and 1 or 4 hours of reperfusion, transplanted hearts in both saline-treated and leukocyte-depleted groups showed no evidence of necrosis or intravascular leukocyte aggregation. When hearts were reperfused for 24 hours, features of acute fibrinous epicarditis were observed in both saline-treated and leukopenic groups.

Histological examination was also carried out on the hearts and lungs of the recipient rats. Hearts from both saline-treated and leukopenic groups were histologically normal; however, moderate pulmonary intravascular aggregation of leukocytes, mainly neutrophils, was seen in the saline-treated groups but not in the leukopenic groups after 1 and 4 hours of reperfusion. This accumulation of leukocytes in the lungs may account, at least in part, for the decrease of white cell counts. After 24 hours of reperfusion, no intravascular aggregation of leukocytes was observed in the lungs of either salinetreated or leukopenic recipients. These findings would exclude the lungs as a major sink for circulating leukocytes at this time.

(4) Myocardial glutathione content. After 4 hours of hypothermic ischemia and 1, 4, or 24 hours of reperfusion, the myocardial contents of the GSH and GSSG were similar in saline-treated controls and leukocyte-depleted rats (11.2±0.3, 9.0±0.4, and 11.0±0.7 nmol/mg protein versus 11.9±0.2, 10.7±0.5, and 9.9±0.6 nmol/mg protein for GSH and 0.6±0.1, 0.6±0.2, and 0.7±0.1 nmol/mg protein versus 0.7±0.1, 0.7±0.1, and 0.4±0.1 nmol/mg protein for GSSG, respectively; NS). In these studies, the tissue content of GSH after ischemia and reperfusion in all groups was within the range of aerobic, nonischemic control values reported previously by ourselves25 and by others21 in rabbit hearts. However, the content of GSSG was twofold greater than the aerobic control values observed in those studies.21,25

Studies with 8 hours of hypothermic ischemic storage and 1 hour of reperfusion. Our failure to show a major or sustained protective effect of leukopenia on contractile function may arguably be a result of the relatively small degree of injury inflicted on the heart during the 4-hour period of hypothermic ischemic storage. Thus, recovery even in the saline-treated group was very good. To overcome this limitation, the preceding studies (with 1 hour of reperfusion) were repeated in hearts that had been subjected to 8 hours of hypothermic storage.

(1) Cardiac function. When the period of ischemic storage of hearts was increased from 4 to 8 hours, we again obtained evidence that leukocyte depletion during reperfusion had a protective effect on the recovery of cardiac contractile function assessed after 1 hour of reperfusion (Fig 5, A). For example, the postischemic mean LVDP at an LVEDP of 12 mm Hg was 72±4 mm Hg in saline-treated controls, whereas it was significantly improved to 85±2 mm Hg (P<.05) in the leukopenic group.

Figure 5, B shows the results for cardiac compliance. As observed previously, function tended to be better in the leukopenic group (188±12 μL at 12 mm Hg of LVEDP) than in saline-treated controls (171±19 μL); however, the difference failed to achieve statistical significance.

The increase in the severity of the ischemia was also reflected in a poorer recovery of coronary flow after reperfusion in both the saline-treated and the leukopenic groups; however, hearts reperfused in leukopenic animals showed a greater flow (9.8±0.4 mL/min) than those reperfused in saline-treated rats (7.5±0.4 mL/min; P<.05).

(2) Leukocyte counts. In saline-treated animals, 1 hour of reperfusion resulted in a greater fall in the leukocyte count (3.75±0.50 ×10⁸ cells/μL) than was observed with only 4 hours of ischemia (5.98±0.57 ×10⁸ cells/μL). This suggests that the greater the severity of the ischemic insult, the more severe is the activation and sequestration of leukocytes in the peripheral blood.
Leukopenic rats showed a leukocyte count after 1 hour of reperfusion similar to that seen in the previous 4 hours storage group (1.44±0.32 versus 1.25±0.07 ×10⁶ cells/μL, respectively).

(3) Histology. Hearts subjected to 8 hours of ischemia, 1 hour of transplantation, and 1 hour of reperfusion in both control and leukopenic groups revealed no histological evidence of necrosis or intravascular leukocyte aggregation. Hearts from saline-treated and leukopenic recipients were histologically normal, as previously observed with 4 hours of ischemia. A moderate intravascular aggregation of leukocytes was again seen in the lungs of saline-treated recipients but not in leukopenic recipients.

Time Dependence of the Protective Effect of Leukocyte Depletion

Cardiac function. As shown in Fig 6, A, reperfusion with blood from leukopenic support rats for 2 minutes did not significantly improve LVDP (106±7 versus 96±10 mm Hg in controls), but reperfusion for only 10 minutes exerted an effect on the recovery of contractile function (137±5 mm Hg; *P<.05) similar to that seen after reperfusion for 30 and 60 minutes (138±3 and 150±10 mm Hg, respectively; *P<.05).

Although the recovery of coronary flow tended to be greater in hearts reperfused with blood from leukopenic support rats, the benefit was lost when blood from saline-treated rats was reinfused (Fig 6, B). Thus, by the end of 60 minutes of reperfusion, only hearts reperfused with leukopenic blood for the entire period of reperfusion showed a significantly greater recovery of coronary flow (3.4±0.3 vs 2.5±0.2 mL/min in hearts perfused with blood from saline-treated rats for the entire reperfusion period; *P<.05). This increase in flow cannot be attributed solely to theoretical rheological changes in the blood induced by leukocyte depletion, since the aerobic perfusion of hearts with blood from saline-treated or leukopenic support rats showed nearly identical values during and at the end of a 60-minute perfusion period (2.7±0.2 versus 2.8±0.2 mL/min, respectively). We should emphasize that although the group reperfused with leukopenic blood for the entire 60 minutes of reperfusion exhibited the greatest recovery of coronary flow at the end of 60 minutes of reperfusion (even above the aerobic, nonischemic controls), the flow in the other groups (with or without reperfusion with leukopenic blood) recovered fully and was not significantly different from that in the aerobic controls.

Histology. As observed in the previous studies, hearts reperfused for 1 hour showed no histological evidence of necrosis or intravascular leukocyte aggregation. The hearts from support animals were also histologically normal. However, a moderate intravascular leukocyte aggregation was observed in the lungs of saline-treated support animals but not in leukopenic rats. The maintenance of extracorporeal blood circulation for 4 hours (without perfusing any heart) in saline-treated hearts also resulted in pulmonary vascular aggregation of neutrophils, suggesting that the exposure of blood to foreign surfaces in the perfusion circuit might be a principal contributor to the effect. This would be supported by the demonstration of a progressive decline in the entire leukocyte population over a period of 4 hours of continuous extracorporeal circulation (Fig 7, A to D).

Discussion

The present studies demonstrate the following. (1) Reperfusion of rat hearts that have been subjected to long-term hypothermic storage with leukocyte-depleted...
blood is associated with short-term enhancement of the recovery of systolic function and coronary flow. However, this is not sustained, as the period of reperfusion is extended. (2) The first 10 minutes of reperfusion appears to represent a critical period during which leukopenia can induce these effects. Leukopenic reperfusion of shorter duration is not associated with any benefit. (3) Leukopenic reperfusion does not influence changes in GSH or GSSG induced by ischemia and reperfusion.

**Involvement of Leukocytes in Reperfusion-Induced Injury**

These studies, in which a model of reversible hypothermic global ischemia was used, show that reperfusion with leukocyte-depleted blood improved the recovery of systolic cardiac function but did not have an effect on diastolic function. Furthermore, recovery of coronary flow was enhanced. However, the role of leukocytes as mediators of reperfusion-induced injury is not clear. Some investigators have shown a reduction of myocardial infarct size with inactivation or depletion of leukocytes after regional ischemia for periods of up to 3 hours, whereas others have been unable to show protection after longer periods of ischemia (ie, 3 or 4 hours). Adding to the controversy, depletion or inactivation of leukocytes in experiments with very short periods of ischemia (10 or 15 minutes) has been shown both to improve and to have no effect on the recovery of cardiac function. Apart from possible differences in the experimental models (eg, global versus regional ischemia, hypothermic versus normothermic ischemia, inability to distinguish the effects of leukocytes during ischemia and reperfusion), it appears that the involvement of leukocytes in the cardiac dysfunction seen during reperfusion may depend on the extent of injury sustained during the preceding period of ischemia.

**Time Dependence for the Protective Effect of Reperfusion With Leukopenic Blood**

Two important questions relate to the protective effects of leukopenic reperfusion. What is the minimum duration of leukocyte depletion that is required to achieve a protective effect? What happens to this protection when the leukocytes are eventually readmitted to the tissue?

The present studies are the first to reveal that to improve the recovery of cardiac contractile function by reperfusion with leukocyte-depleted blood, the duration of the leukopenic reperfusion can be as short as 10 minutes. However, reperfusion with leukopenic blood for 2 minutes failed to protect the heart, whereas reperfusion for 30 or 60 minutes afforded similar protection to that observed in the 10-minute group. It should be noted that by 10 minutes of reperfusion, hearts in all groups exhibited a similar recovery of contractile function and that it was only after that time that cardiac function improved in the groups reperfused with leukopenic blood for at least the first 10 minutes. It would appear that leukocytes exert their deleterious effect during the first 10 minutes of reperfusion, but the consequence of that action is not evident until a later time.

Hall et al have shown, however, that in lungs subjected to 24 hours of hypothermic preservation, the readmission of leukocytes after 5, 15, or 30 minutes of leukopenic reperfusion caused progressive functional loss, whereas readmission after 1 hour produced no measurable injury. In addition, Westlin and Mullane have demonstrated that although leukocyte depletion in the canine heart subjected to 15 minutes of regional ischemia improved the recovery of contractile function, the benefit was progressively lost with the return of the leukocytes. In canine studies, Simpson et al have shown that in hearts subjected to 90 minutes of irreversible myocardial regional ischemia, a sustained reduction of
myocardial infarct size requires that the duration of intervention (iloprost or neutrophil antibodies) to inhibit neutrophil activation has to be as long as 48 hours. The apparent disagreement between the present and other studies may be explained by differences in the severity of the myocardial ischemia. Supporting this possibility are the findings of the present study and those of others, showing that reversible ischemia fails to promote leukocytic infiltration (as assessed by histology, electron microscopy, or indium labeling), whereas irreversible injury is accompanied by neutrophil infiltration. Therefore, it appears that myocellular damage is necessary to provoke neutrophil infiltration. Our findings of neutrophil aggregation in epicardial vessels and of epicardial neutrophil infiltration, although preventable by reperfusion with leukopenic blood, are most likely to represent the consequences of the surgical manipulation of the heart, since neutrophils activated by ischemia accumulate preferentially in the subendocardium.

It is unlikely that mustine hydrochloride itself had a direct effect on the postischemic recovery of cardiac function, because 3 days after administration, control and mustine-treated animals exhibited identical hemodynamic profiles (data not reported). Furthermore, preliminary studies showed a similar contractile function and coronary flow in hearts from controls and mustine-treated rats (data not reported).

Duration of the Protective Effect of Reperfusion With Leukopenic Blood

Although reperfusion with leukopenic blood has already been reported to improve the recovery of function after long-term hypothermic ischemia in the neonatal piglet heart, the present studies are the first to show that the protection does not last for more than 4 hours. Thus, we would conclude that, at best, leukopenic reperfusion simply accelerates the rate of recovery (ie, overcomes myocardial stunning) without exerting any sustained or long-term effects on the extent of recovery of function. This conclusion might nevertheless be limited to recovery after mild or reversible ischemia. More severe ischemia might respond differently, and the result might be a reduction in the ultimate extent of myocardial injury. Support for such a possibility comes from studies in which the injury induced by 90 minutes of regional ischemia was still reduced after 72 hours when reperfusion was carried out with inactivated leukocytes. This finding might suggest that chemotactic factors persist during irreversible injury and, therefore, protection might require more extended periods of leukocyte inactivation or depletion to obtain maximum benefit.

Mechanisms of Leukocyte-Mediated Injury

There is evidence that leukocytes contribute to the pathogenesis of postischemic myocardial cell injury; however, the precise mechanisms and extent of their involvement are not fully understood. It has been suggested that leukocytes might induce postischemic damage by (1) plugging the microvasculature and contributing to the no-reflow phenomenon and (2) liberating reactive oxygen metabolites, hydrolytic enzymes, and eicosanoids, which would lead to microvascular and parenchymal cell injury.

Despite evidence from the canine myocardium that leukocytes can cause microvascular plugging and significantly decrease subendocardial blood flow, the present studies show that, in situations of reversible ischemia, plugging of leukocytes does not occur. This is in agreement with the finding that leukocytes are not essential for the development of myocardial no-reflow in isolated ischemic hearts after perfusion with a cell-free buffer, although their presence can exacerbate it.

Since it has been shown in in vitro and in vivo studies that the production of oxygen free radicals peaks during the first 2 to 10 minutes of reflow and that stimulated neutrophils can produce large quantities of hydrogen peroxide, a possible explanation for the protective effect of reperfusion with leukopenic blood for the first 10 minutes may be suppression of this production. Arguing against this hypothesis is our finding that the glutathione status of the myocardium was not significantly modified; however, it might be that glutathione is not a good indicator of oxidative injury with the modest degree of injury imposed in the present study.

Another explanation for the beneficial effect of reperfusion with leukopenic blood might be the suppression of hypochlorous acid production by activated neutrophils. This highly toxic oxidant, formed from hydrogen peroxide in a reaction catalyzed by the enzyme myeloperoxidase, has been shown to cause a decline in mechanical function, block of endothelium-mediated coronary dilation, extensive damage to proteins and inactivation of enzymes, rise in cytosolic calcium, and inactivation of the sarcolemmal Na⁺,K⁺-ATPase. Some of these changes have been associated with alterations in protein thiol redox status. Furthermore, we and others have used the decrease in the tissue content of GSH and the increase of GSSG as an index of tissue oxidative stress. In the present studies, however, we were unable to show significant changes in the tissue content of glutathione. This would argue against the possibility that a reduction of the oxidant stress caused by the removal of leukocytes from the reperfusion medium might result in a sparing effect on antioxidant defenses. It should be acknowledged that the degree of injury inflicted in our study was not sufficient to cause significant changes in the myocardial antioxidant status, and undoubtedly, this would have reduced the scope for any improvement after reperfusion with leukopenic blood.

Alternatively, other factors such as elastase or leukotrienes may be involved in the injury induced by leukocytes. However, their role has also been contested, and further investigations will be required to define their place in ischemia/reperfusion-induced injury.

Mustine hydrochloride depresses all types of leukocytes; however, it is only the neutrophils that have been implicated in myocardial injury. It is conceivable (although unlikely in the short term) that some of the other white cell types might be beneficial in terms of tissue protection (eg, mononuclear cells release cytokines that modulate endothelial activity) and that the loss of these cells offsets some of the protection produced by neutrophil depletion.
Limitations of the Present Study

The heterotopic heart transplant provides a means with which to study the recovery of globally ischemic hearts over a 24-hour period of reperfusion with blood. This contrasts with the majority of isolated preparations in which reperfusion is carried out for relatively short periods of time. In this preparation, however, the heart is denervated and unloaded, which leads to a time-dependent mechanical deterioration and atrophy.\textsuperscript{18,26} Nevertheless, we have previously shown that major deterioration does not occur until at least 24 hours after implantation.\textsuperscript{18} The present studies do not exclude the possibility that some deterioration may have influenced our results, and this should be taken into account when attempting any extrapolation to the human heart.

The isolated blood-perfused preparation was also used in the present studies. By using two different support animals (leukocyte-depleted and nondepleted), we were able to study the time dependency of the protection given by leukopenic reperfusion. The exposure of blood to foreign surfaces in this preparation may have important consequences\textsuperscript{37-61} on the activation of plasma proteins (eg, coagulation, fibrinolysis, complement) and on the activation and degranulation of neutrophils. Although these factors could have influenced our results, it should be stressed that all groups were subjected to identical protocols and the procedure in many ways mimicked the conditions during open-heart surgery and transplantation, in which exposure of blood to foreign surfaces also occurs. In these studies, the use of heparin (as is required during open-heart surgery) may have resulted in inhibition of the activation of the complement.\textsuperscript{62-64} Since complement activation may be necessary for the interaction of neutrophils with the tissues, the inactivation of the complement may have decreased the response to the activation of neutrophils.\textsuperscript{65} This effect would have been more pronounced in the control group, which had a normal white blood cell count, than in the mustard-treated groups. However, this complication is unlikely to occur in our transplantation studies, since heparin was given to the donor heart before explantation and was not administered to the recipient rat. In this way, transplanted hearts were not exposed to heparin during the entire reperfusion period.

Finally, our studies have not determined whether the aggregation and plugging of leukocytes in the lungs exerts any effect on the reperfusion-induced injury seen in the ischemic myocardium. Clarification of this point would require further investigations.

Concluding Comments

The present studies demonstrate that the use of leukocyte-depleted blood during the first 10 minutes of reperfusion protects against reperfusion-induced injury by improving the rate of recovery of cardiac function. This protection, however, is not sustained.

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