Sustained Limitation of Myocardial Reperfusion Injury by a Monoclonal Antibody That Alters Leukocyte Function

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Pentobarbital anesthetized dogs were subjected to 90 minutes of left circumflex coronary artery (LCCA) occlusion followed by 72 hours of reperfusion. Control or anti-Mo1 (904) F(ab')2 fragments of monoclonal antibodies were administered intravenously at a dose of 1 mg/kg beginning 45 minutes after occlusion and at a dose of 0.5 mg/kg at 12, 24, 36, and 48 hours after reperfusion. Myocardial infarct size expressed as a percentage of the area at risk (IN/AR) measured postmortem after 72 hours of reperfusion was significantly reduced by 904 F(ab')2 (21.6±2.8%, n=8) compared with control F(ab')2 (37.4±5.8%, n=8; p<0.025). There were no significant differences between groups in heart rate, mean arterial blood pressure, rate-pressure product, or LCCA blood flow that could account for a reduced infarct size. Regional myocardial blood flow (RMBF) was determined with 15-μm radiolabeled microspheres. Transmural blood flows (ml/min/g) within the region of myocardium at risk were not statistically different between treatment groups. Infarct size in both groups was related to regional myocardial blood flow, and the relation was shifted downward in the group treated with the anti-Mo1 F(ab')2 antibody (analysis of covariance, p=0.01). Thus, anti-Mo1 F(ab')2 produces a sustained limitation of myocardial infarct size compared with controls under similar hemodynamic conditions and a similar degree of myocardial ischemia as determined by RMBF. These data suggest that inhibition of neutrophil adhesive interactions (as suggested by the inhibitory effect of anti-Mo1 on canine neutrophil aggregation) may be an effective mechanism for protection against myocardial injury secondary to myocardial ischemia and reperfusion. (Circulation 1990;81:226–237)

It is now well recognized that the process of myocardial infarction with reperfusion of the previously ischemic myocardium involves components of a typical inflammatory reaction. There is elaboration of inflammatory mediators as well as infiltration of blood cells such as polymorphonuclear leukocytes (neutrophils).1 Abundant evidence exists to indicate that neutrophil infiltration and activation in the previously ischemic region result in an extension of myocardial injury. Neutrophil depletion before induction of regional ischemia2–3 or pharmacologic suppression of neutrophil activation2–8 results in a limitation of myocardial infarct size. The potential for immunologic suppression of neutrophil activation during inflammatory processes such as myocardial infarction9 and hemorrhagic shock10 has become possible with the use of specific antibodies directed against neutrophil plasma membrane adhesion molecules.

Mo1 (CD11b/CD18; Mac-1) is a heterodimeric glycoprotein expressed on the plasma membrane of neutrophils and monocytes.11 Monoclonal antibodies directed against this glycoprotein inhibit neutrophil aggregation, adhesion and spreading on natural and artificial substrates, and chemotaxis in vitro.12–17 Anti-Mo1 antibody reduced myocardial infarct size9 when administered to anesthetized open-chest dogs that were subjected to 90 minutes of regional myocardial ischemia and 6 hours of reperfusion. The mechanism of infarct size reduction was attributed to inhibition...
of adhesive (including inhibition of aggregation) interactions of neutrophils within the myocardium after reperfusion.

The results of a previous study\(^4\) suggested that by blocking neutrophil adhesive interactions, anti-Mol could be useful as a therapeutic agent to reduce myocardial infarct size. However, several questions remained unanswered: Can anti-Mol administration provide for effective myocardial protection after a longer period of reperfusion (e.g., 72 hours)? Does the apparent beneficial effect of anti-Mol occur because of a specific blockade of Mol-mediated adhesive interactions or because of non-specific Fc receptor-mediated clearance of activated neutrophils from the circulation? Thus, the present study was undertaken to determine whether the administration of a F(ab\(^\prime\))\(_2\) fragment of anti-Mol could reduce the extent of myocardial ischemia-reperfusion injury that results when the acute inflammatory response is fully expressed (72 hours). The data provide additional evidence to support the concept that an alteration in neutrophil function can effectively reduce the extent of tissue injury associated with myocardial reperfusion.

**Methods**

**Materials**

The generation of the murine IgG\(_1\) monoclonal antibodies (anti-Mol, clone 904, and control antibodies) has been described previously.\(^9,15\) The anti-Mol antibody binds to an epitope on the CD11b, 155-kD \(\alpha\)-polypeptide of the Mol glycoprotein expressed by human\(^15\) and dog\(^9,18\) neutrophils. The control antibody was raised against the idiotype determinant of a T-cell receptor expressed by a human T-cell hybridoma. Thus, it displays no reactivity with human or canine cells. Sterile, pyrogen-free F(ab\(^\prime\))\(_2\) fragments prepared by pepsin digestion of whole antibody and purified by chromatographic techniques were supplied by Coulter Immunology (Hialeah, Florida). All other reagents were supplied by Sigma Chemical (St. Louis, Missouri) unless otherwise noted.

**Surgical Preparation**

Adult male, mongrel dogs (12–17 kg) were anesthetized with 30 mg/kg sodium pentobarbital and ventilated with room air (tidal volume, 30 ml/kg at a rate of 12/min). Aseptic surgical methods were used. A left thoracotomy was performed, and the proximal left circumflex coronary artery (LCCA) was isolated and instrumented for continuous blood flow measurement with a calibrated electromagnetic flow probe. Arterial blood pressure, heart rate, the standard limb lead II electrocardiogram, and LCCA blood flow were recorded continuously. Regional myocardial ischemia was produced by occluding the LCCA for 90 minutes followed by reperfusion (see protocol scheme, Figure 1) in the presence of a critical stenosis. The critical stenosis consisted of a silk ligature placed around the vessel and tightened just enough to reduce the reactive hyperemia by 60% of the prestenotic value after a 10-second mechanical occlusion. This stenosis prevents the development of hemorrhagic infarction and reduces the incidence of reperfusion-induced ventricular fibrillation. After 1 hour of reperfusion, the critical stenosis was removed. The thoracotomy incision was closed after 1 hour of reperfusion, and the animals were allowed to recover from surgical anesthesia. Seventy-two hours after initiating reperfusion, the animals were reanesthetized, the chest was reopened, and the heart was fibrillated electrically.

**Experimental Protocol**

Dogs were assigned in a random and blinded fashion to either an anti-Mol F(ab\(^\prime\))\(_2\)-treatment group or a control F(ab\(^\prime\))\(_2\)-treatment group.

**Anti-Mol group.** The anti-Mol group consisted of eight dogs that received 1 mg/kg i.v. of the antibody 45 minutes after coronary artery occlusion and 0.5 mg/kg i.v. infusion (over 5 minutes) at 12, 24, 36, and 48 hours after reperfusion. The dosing schedule was chosen because a previous study\(^9\) indicated that it is necessary to inhibit neutrophil function for 48 hours after reperfusion to observe a beneficial effect of neutrophil suppressive therapy at 72 hours of reperfusion.

**Control group.** The control group consisted of eight dogs that received control murine monoclonal F(ab\(^\prime\))\(_2\), which lacks specific binding activity against the neutrophil. The F(ab\(^\prime\))\(_2\) was administered at the same dosage and schedule as indicated above for the anti-Mol antibody.
Infarct Size

Infarct size was assessed by the ex vivo dual perfusion histochemical method. This histochemical assessment of viable and irreversibly injured myocardium involves perfusion of the cannulated LCCA with a 1.5% triphenyltetrazolium chloride (TPT) solution buffered with 20 mM potassium phosphate (pH 7.4), while simultaneously perfusing the remainder of the coronary circulation with an aqueous blue dye (0.25%) introduced into the aorta. Both solutions were delivered to the respective vascular distributions under a constant pressure of 100 mm Hg and at a temperature of 39°C for a period of 5 minutes. The hearts were cut into five to six transverse sections, 1 cm in thickness; infarct size, region at risk, and total left ventricular mass were determined planimetrically. The method of quantitating infarct size with TPT has been validated by many investigators and shown to demarcate viable from nonviable myocardial tissue as determined by the histochemical reaction between TPT and myocardial dehydrogenase enzymes.

Histologic Assessment of Myocardial Tissue

Midventricular transmural sections from hearts in each of the two treatment groups were examined by light microscopy. The relative degree of neutrophil infiltration was assessed on hematoxylin and eosin-stained sections of left ventricle in a semiquantitative manner by two pathologists who were blinded with respect to the treatment regimens.

Determination of Regional Myocardial Blood Flow

Regional myocardial blood flows were determined at three time points during each experiment (before occlusion, 5 minutes after occlusion, and 80 minutes after occlusion) with tracer-labeled microspheres (15-μm diameter, New England Nuclear) by the reference withdrawal method as previously described. Injections of microspheres (labeled with cerium 141, ruthenium 103, or scandium 46) were made in each experiment, with the order of the isotopes randomized. Each bottle of microspheres was placed in an ultrasonic bath with subsequent vortex agitation before injection to ensure that adequate dispersal of the microsphere suspensions was achieved before being administered. Reference arterial blood samples were obtained simultaneously from both the femoral and carotid arteries beginning immediately before the injection of microspheres into the left atrium and ending 2 minutes later. The reference sample counts were averaged for calculation of myocardial blood flow. If the reference sample counts varied by more than 15%, the data were discarded.

Tissue samples weighing 0.5–1.0 g were dissected from the subepicardium, midmyocardium, and subendocardium of the perfusion bed supplied by the LCCA and from the nonischemic region. At least three sections from each heart were used so that blood flows to each region represent the average of three or four samples for each experiment. Regional myocardial blood flow was calculated by the formula: Qm = Qt × Cm/Cr where Qm is myocardial blood flow (ml/min), Qt is reference blood flow (ml/min), Cm is number of counts per minute in myocardial sample, and Cr is number of counts per minute in reference blood sample. Adjustments were made in blood flow to the ischemic region for apparent microsphere loss due to tissue edema.

Detection of Residual Anti-Mo1 Antibody in Serum of Treated Dogs

To document the administration of anti-Mo1 (904) F(ab')2 or control IgG, F(ab')2 sufficient to produce antibody excess, samples of serum were analyzed for the presence of free residual antibody. This was assessed in a semi-quantitative ELISA assay in which the binding of free 904 F(ab')2 or control F(ab')2 in undiluted serum to microtiter wells (3912 Microtest III; Falcon Plastics, Oxnard, California) precoated with goat anti-mouse immunoglobulin (100 μg/ml; 0211-0231, Cooper Biomedical, Malvern, Pennsylvania) was detected by the subsequent binding of alkaline-phosphatase–conjugated goat anti-mouse IgG (A-4656; Sigma Chemical, St. Louis, Missouri). The plate was developed by the addition of p-nitrophenyl phosphate substrate (Sigma; 1 mg/ml in 10% diethanolamine, pH 9.8). After incubation at 25°C for 30 minutes, the yellow reaction color was quantitated on a Dynatech MR700 ELISA reader (Chantilly, Virginia) at 410 nm. The colorimetric recordings from serum samples were compared to parallel wells containing known concentrations of F(ab')2.

In addition to this semi-quantitative assessment of circulating F(ab')2 concentrations, the detection of immunologically active 904 F(ab')2 was assayed by indirect immunofluorescence analysis in which 106 Mo1-positive test cells (calcium ionophore A23187-stimulated human neutrophils) were incubated with dog serum (½ final dilution) for 30 minutes at 4°C. After washing in buffer containing a saturating concentration of fluorescein-conjugated goat anti-mouse Ig (Coulter Immunology) for an additional 30 minutes at 4°C, antibody binding to test cells was quantitated by flow cytometric analysis as described previously. Relative F(ab')2 reactivity (%) in serum samples was determined by comparing the mean channel fluorescence intensity (linear scale) of 5,000 test cells exposed to serum samples relative with the mean channel fluorescence intensity of cells exposed to a saturating quantity of 904 F(ab')2.

Ex Vivo Analysis of Neutrophil-904 Binding by Flow Cytometry

Neutrophils from the blood of each animal were isolated before the initial bolus infusion of 904 (t=0) or control antibody and then at 1, 24, 48, and 72 hours thereafter (including both preadministration and postadministration of additional bolus infusions of 904 or control (F(ab')2) given at 24 and 48 hours). In this analysis, we isolated a leukocyte-enriched fraction of EDTA-anticoagulated whole blood by
ammonium chloride lysis of erythrocytes and then subjected these cells to immunofluorescence staining for any bound 904 by a 30-minute incubation with FITC-labeled goat anti-mouse Ig (which detects the binding of 904 to activated human or dog PMNs). When the postinfusion samples were compared with neutrophils obtained before the initial 904 infusion (a suitable negative control baseline), there was minimal and variable staining above background (an observation consistent with the variable degree of intact IgG bound to the PMNs of 904-treated dogs in our earlier study). 

Evaluating of Neutrophil Accumulation in Myocardial Tissue: Myeloperoxidase (MPO) Assay and Histology

Samples of myocardium (50–200 mg) were taken from the central infarct region, the noninfarcted tissue within the area at risk, the endocardial to epicardial border zone between infarct region and area at risk, and normal noninfarcted and unstained myocardium. The tissue samples were homogenized and assayed for MPO content as described by Bradley and coworkers.29 The assays involve the extraction of MPO by homogenization (Polytron) the tissue in buffer containing 50 mM phosphate with 0.5% hexadecyltrimethylammonium bromide and 5 mM EDTA (pH 6.0). After two freeze-thaw cycles and sonication, the samples were then centrifuged, and the supernatants were assayed in 50 mM phosphate (pH 6.0) with 0.167 mg/ml O-dianisidine and 0.0005% hydrogen peroxide. The rate of decomposition of hydrogen peroxide by MPO was determined by measuring the change in absorbance at 460 nm. One unit of MPO activity is defined as the amount of enzyme that decomposes 1 μmol/min hydrogen peroxide at 25°C under the conditions of the assay. The myocardial content of MPO activity has been correlated with histologic evidence of neutrophil infiltration.6–8,30

Histologic assessment of neutrophil accumulation was performed in a blinded fashion separately by two pathologists (J.C.F. and K.A.R.) on hematoxylin and eosin–stained sections from the myocardium in the region of infarcted myocardium and at the border region between infarcted and uninjured tissue. A relative infiltration score of 0 to 4 was assigned to each heart based on the presence of neutrophil and monocyte infiltration.

Neutrophil Aggregation

Aggregation of neutrophils was performed as previously described.18 Briefly, canine neutrophils were isolated from citrated venous blood by ficoll-hypaque gradient separation followed by dextran gravity sedimentation. Erythrocytes were lysed with hypotonic (0.27%) sodium chloride. Neutrophils were then resuspended in Hank's balanced salt solution (107 neutrophils/ml) and allowed to equilibrate 1 minute at 37°C in aggregation cuvettes. Aggregation was induced with the addition of phorbol myristate acetate (PMA) (125 ng/ml) and was recorded as a percent change in light transmittance by a platelet aggregometer. Control F(ab')2 (117 μg/ml final concentration), 904 F(ab')2 (105 μg/ml final), or intact 904 monoclonal antibody (100 μg/ml final) was preincubated with the neutrophil suspension for 1 minute before the addition of the PMA.

Exclusion Criteria

To ensure that all treatment groups were comparable with respect to the degree of regional myocardial ischemia, predetermined exclusion criteria were established. Animals were excluded from the final data analysis if they manifested failure to demonstrate electrocardiographic signs of regional ischemia (i.e., <0.1 mV ST-segment change on coronary occlusion), ventricular fibrillation that was not converted with fewer than four attempts at low-amperage DC defibrillation (<30 J applied directly to the heart surface), or the presence of heartworms postmortem.

Statistical Analyses

All data are expressed as mean±1 SEM. Data were compared between treatment groups by Student's t test (one-tailed). The evaluation of the data from histologic sections pertaining to neutrophil accumulation was tested with Wilcoxon's two-sample rank-sum test (Mann-Whitney test).31 All other data were compared with a respective control group by Student's t test using Bonferroni's method to control for experimental α-error when making multiple comparisons with the same control group.

The size of the myocardial risk region and the degree of collateral blood flow are two important determinants in the extent of ischemic myocardial injury and tissue necrosis. Infarct size was assessed in relation to collateral blood flow measured in the outer two thirds of the central ischemic zone. An analysis of covariance was performed in which collateral blood flow was the independent variable with the object of determining whether there existed a statistically significant difference in the calculated infarct size between the two groups when the influence of collateral blood flow was controlled.

Results

Twenty-six dogs were used in this study comparing the effects of 904 monoclonal F(ab')2 and control monoclonal F(ab')2 on the size of myocardial infarct that develops after 90 minutes of ischemia and 72 hours of reperfusion. A total of 10 dogs was excluded from the final data analysis because one dog died due to ventricular fibrillation during coronary occlusion before treatment with antibody, two dogs were excluded because of failure to develop objective signs of ischemia during coronary occlusion, and seven dogs died because of ventricular fibrillation that was not converted with fewer than four attempts at low-energy cardioversion (<30 J with internal paddles) (three control dogs, three dogs that were treated with 904, and one that died before treatment with antibody). In addition, one dog that had been treated with 904 died during the first night, presumably due
to sudden arrhythmic death. Regarding ventricular fibrillation, of the remaining control dogs, one dog fibrillated once and one dog fibrillated twice; of the 904 antibody–treated dogs, one dog fibrillated once and two dogs fibrillated twice.

Myocardial Infarct Size

There was a significant limitation in the size of myocardial infarct that developed in the 904 F(ab')2–treatment group (42% reduction) compared with the control group (Figure 2). The infarct size was 21.6±2.8% (n=8) of the area at risk of infarction (infarct size/area at risk=IN/AR) in the 904 group compared with 37.4±5.8% (n=8) of the area at risk in the control group (p<0.025). The amount of myocardium that was ischemic (area at risk/left ventricle=AR/LV) was similar: 38.0±2.1% (n=8) in the control group compared with 43.2±1.1% (n=8) in the 904 group (p>0.05).

Hemodynamic Effects of Antibody Treatments

Hemodynamic values (Table 1) were obtained to determine whether the protective effect might be attributed to reduced blood pressure or heart rate. Moreover, the rate-pressure product (systolic arterial blood pressure multiplied by the heart rate divided by 100) is used as an index of myocardial oxygen consumption.33 Mean arterial pressure was only slightly higher at baseline in the 904-treatment group (p<0.025) than control. Nonetheless, blood pressure subsequent to this point, and other parameters (heart rate and rate-pressure product) were similar between the two treatment groups. LCCA blood flow (Table 1) was also similar between groups at baseline and during the first hour of reperfusion. However, when within-group values were compared, the control group had significantly less flow at 60 minutes of reperfusion compared with baseline values (p<0.025, paired Student's t test). Thus, the only observable difference in hemodynamic values that could account for a limitation in ultimate infarct size was a possible effect of antibody treatment on preventing the attenuation of reflow that sometimes accompanies myocardial reperfusion injury. Furthermore, there were no observable changes in hemodynamics associated with intravenous injection of the antibodies.

Regional Myocardial Blood Flow

There were no differences between the two treatment groups in myocardial blood flow to the ischemic myocardium (Table 2) before occlusion or 5 or 80 minutes after LCCA occlusion. The myocardial blood

<table>
<thead>
<tr>
<th>Table 1. Hemodynamic Measurements</th>
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<tbody>
<tr>
<td>Mean arterial pressure (mm Hg)</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>F(ab')2</td>
</tr>
<tr>
<td>Baseline</td>
</tr>
<tr>
<td>Occlusion (min)</td>
</tr>
<tr>
<td>30</td>
</tr>
<tr>
<td>60</td>
</tr>
<tr>
<td>90</td>
</tr>
<tr>
<td>Reperfusion (min)</td>
</tr>
<tr>
<td>30</td>
</tr>
<tr>
<td>60</td>
</tr>
</tbody>
</table>

Values are given as mean±SEM.

* p<0.025 compared with control at same time point, Student's t test with Bonferroni adjustment.
† p<0.025 compared with baseline in same group, paired Student's t test. All other values were not significantly different between groups.

Baseline values were obtained before LCCA occlusion.

Rate-pressure product=systolic blood pressure x heart rate/100.
flows to the nonischemic regions (Table 3) were also similar between groups. Transmural blood flows within the ischemic region of the myocardium were similar between treatment groups. Before occlusion, transmural blood flow was \(0.61 \pm 0.05\) ml/min/g in the control group and \(0.78 \pm 0.10\) ml/min/g in the 904-treatment group. Early occlusion (5 minutes after occlusion) transmural blood flow was \(0.08 \pm 0.01\) ml/min/g in the control group and \(0.08 \pm 0.02\) ml/min/g in the 904-treatment group. Late occlusion (80 minutes after occlusion) transmural blood flow averaged \(0.11 \pm 0.02\) ml/min/g in the control group and \(0.09 \pm 0.03\) in the 904-treatment group.

When infarct size (IN/AR) is compared with the amount of collateral blood flow, there is generally an inverse linear relation between the two parameters in untreated animals.\(^{26,32}\) This relation can be observed in the control group of the present study (Figure 3). There was a good correlation between the severity of the ischemia as determined by regional myocardial blood flow and the infarct size that eventually developed \(r=0.84\). In contrast, for a given severity of ischemia, the infarct size was smaller in the 904-treatment group than in the control group \(r=0.46\).

Using an analysis of covariance in which the mean collateral blood flow to the outer two thirds of the ischemic myocardium was a covariate, infarct size expressed as a percentage of the area at risk was smaller for the anti-Mo1 (904) F(ab')\(_2\) monoclonal antibody treatment group \((F\) ratio=9.751; \(p=0.008\)). The protective effect of the anti-Mo1 treatment was not due to a difference in ischemic collateral blood flow or to an increase in collateral blood flow to the ischemic myocardium.

**Circulating Neutrophil Counts**

There were no differences between the two treatment groups in circulating neutrophil counts at baseline (0 hours; Figure 4). When determined at 6, 24, 48, and 72 hours after the initial antibody administration, the neutrophil counts were similar between groups and showed the expected increase over baseline values.

**Myocardial MPO Content**

Three myocardial regions were assayed for myeloperoxidase activity as an indicator of neutrophil accumulation (Table 4). There were no significant differences between the two groups in MPO in any of the three myocardial regions (center of infarct, noninfarcted myocardium within the risk region, or tissue that lies between the infarcted and noninfarcted tissue within the area at risk).

**Histologic Assessment**

There were no differences between the two treatment groups in the accumulation of inflammatory cells (neutrophils and monocytes) as observed with

### Table 2. Regional Myocardial Blood Flow in the LCCA Distribution (ml/min/g)

<table>
<thead>
<tr>
<th></th>
<th>Endo</th>
<th>Mid</th>
<th>Epi</th>
<th>Endo/epi ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control F(ab')(_2) group ((n=8))</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>0.66±0.05</td>
<td>0.64±0.05</td>
<td>0.54±0.05</td>
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<td>Early occlusion</td>
<td>0.05±0.01</td>
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<td>0.12±0.02</td>
<td>0.36±0.05</td>
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<tr>
<td>Late occlusion</td>
<td>0.06±0.02</td>
<td>0.08±0.02</td>
<td>0.19±0.02</td>
<td>0.27±0.06</td>
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<tr>
<td>Anti-Mo1 F(ab')(_2) group ((n=8))</td>
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<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>0.82±0.10</td>
<td>0.78±0.10</td>
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<td>1.17±0.09</td>
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<tr>
<td>Early occlusion</td>
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<td>0.07±0.01</td>
<td>0.12±0.03</td>
<td>0.43±0.06</td>
</tr>
<tr>
<td>Late occlusion</td>
<td>0.05±0.02</td>
<td>0.07±0.02</td>
<td>0.16±0.05</td>
<td>0.35±0.06</td>
</tr>
</tbody>
</table>

Baseline, before occlusion; early occlusion, 5 minutes; late occlusion, 80 minutes after LCCA occlusion; epi, subepicardial region; mid, midmyocardial region; endo, subendocardial region.

There were no significant differences between the two treatment groups at any time point or in any myocardial region.

### Table 3. Regional Blood Flow in the Nonischemic Myocardium (ml/min/g)

<table>
<thead>
<tr>
<th></th>
<th>Endo</th>
<th>Mid</th>
<th>Epi</th>
<th>Endo/epi ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control F(ab')(_2) group ((n=8))</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>0.65±0.04</td>
<td>0.64±0.04</td>
<td>0.48±0.03</td>
<td>1.35±0.08</td>
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<td>Early occlusion</td>
<td>0.68±0.05</td>
<td>0.67±0.06</td>
<td>0.52±0.06</td>
<td>1.38±0.12</td>
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<tr>
<td>Late occlusion</td>
<td>0.81±0.06</td>
<td>0.82±0.07</td>
<td>0.62±0.09</td>
<td>1.42±0.14</td>
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<tr>
<td>Anti-Mo1 F(ab')(_2) group ((n=8))</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>0.78±0.10</td>
<td>0.78±0.10</td>
<td>0.60±0.08</td>
<td>1.33±0.08</td>
</tr>
<tr>
<td>Early occlusion</td>
<td>0.83±0.11</td>
<td>0.79±0.10</td>
<td>0.59±0.09</td>
<td>1.47±0.10</td>
</tr>
<tr>
<td>Late occlusion</td>
<td>0.90±0.12</td>
<td>0.91±0.12</td>
<td>0.71±0.12</td>
<td>1.37±0.11</td>
</tr>
</tbody>
</table>

Baseline, before occlusion; early occlusion, 5 minutes; late occlusion, 80 minutes after LCCA occlusion; epi, subepicardial region; mid, midmyocardial region; endo, subendocardial region.

There were no significant differences between the two treatment groups at any time point or in any myocardial region.
Relation Between Myocardial Infarct Size and Regional Myocardial Blood Flow (90 minutes LCX Occlusion / 72 hours of Reperfusion) Anti-Mol-F(ab')2 Treatment

Myocardial Infarct Size (% Area at Risk) vs. Myocardial Blood Flow to Outer 23% of Ischemic Region

CONTROL n=8
ANTIBODY n=8

y = -350.5 + 70.1 x + 0.84

y = -67.9 + 23.6 x + 0.46

FIGURE 3. Infarct size versus collateral blood flow. Plot of regional myocardial blood flow versus infarct size. Infarct size is normalized as a percent at risk and is plotted against myocardial blood flow in the outer two thirds of the ischemic left ventricle. Collateral blood flow (outer two thirds) was calculated by averaging the subepicardial and midmyocardial blood flows within the ischemic region. Each point on the graph represents one animal. There is an inverse relation between infarct size and collateral blood flow, and the regression line that describes the relation for the control group is displaced downward in the anti-Mol 904–treated group. Least-squares linear regression estimates for the control group were y = 70.1 - 350.5 x; r = 0.84 and y = 27.6 - 67.9 x; r = 0.46 for the anti-Mol1 (904) group. The data indicate that for a given severity of ischemia, the infarct size that developed in the anti-Mol1 group was significantly smaller. Analysis of covariance indicated a significant difference between the two treatment groups (F ratio = 9.751, p = 0.008). Thus, the mechanism of infarct size limitation by anti-Mol1 (904) is not due to differences in collateral blood flow to the ischemic myocardium or to an increase in collateral blood flow.

CIRCULATING NEUTROPHIL COUNTS

CONTROL n=8
ANTIBODY n=8

FIGURE 4. Circulating neutrophil counts. Blood samples were obtained before antibody (0 hours) and at 6, 12, 24, 48, and 72 hours after initial antibody administration. Neutrophil counts were determined by counting with a hemocytometer to obtain total white blood cell counts and then differential staining of blood smears. There were no significant differences between the two groups at any time point.

TABLE 4. Myocardial Myeloperoxidase Activity (milliunits/g tissue)

<table>
<thead>
<tr>
<th></th>
<th>Center of infarct region</th>
<th>Endocardial border region</th>
<th>Noninfarcted tissue within the risk region</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control F(ab')2</td>
<td>11±3</td>
<td>8±2</td>
<td>7±2</td>
</tr>
<tr>
<td>Anti-Mol F(ab')2</td>
<td>10±3</td>
<td>7±1</td>
<td>5±1</td>
</tr>
</tbody>
</table>

There were no significant differences between groups in myocardial myeloperoxidase activity.

Serum F(ab')2 Concentrations Determined by ELISA

Serum F(ab')2 concentrations were determined for each dog in both treatment groups as an indication of circulating antibody excess (Table 5). Baseline values for F(ab')2 concentrations in animals (before antibody administration) were below detectable limits (<0.019 μg/ml). Antibody concentrations during the course of the 72 hours of reperfusion indicate that there is circulating antibody excess in the blood in most dogs during the initial 48 hours after reperfusion.

Serum F(ab')2 Concentration Determined by Flow Cytometry

In addition to determining serum antibody concentration by ELISA, serum samples were allowed to

FIGURE 5. Relative accumulation of inflammatory cells. Sections of infarcted myocardial tissue were processed by histologic methods and assessed by blinded experts for the relative degree of inflammatory cell (neutrophils and monocytes) infiltration. A semiquantitative grade of 0–4 was assigned to each heart. Values represent scores for individual experiments. There was no difference between the two groups as evaluated with the Wilcoxon rank sum test.
Table 5. Serum F(ab')2 Determined by ELISA (μg/ml)

<table>
<thead>
<tr>
<th>Experiment</th>
<th>5 min</th>
<th>1 hr</th>
<th>6 hr</th>
<th>12 hr†</th>
<th>12 hr§</th>
<th>24 hr†</th>
<th>24 hr§</th>
<th>36 hr†</th>
<th>36 hr§</th>
<th>48 hr†</th>
<th>48 hr§</th>
<th>72 hr</th>
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<td>Control F(ab')2 dogs (all values at t=0 were &lt;0.019 μg/ml)</td>
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<td>0.9</td>
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<td>1.0</td>
<td>0*</td>
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<td>3.5</td>
<td>1.9</td>
<td>1.5</td>
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<td>0*</td>
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<tr>
<td>904 F(ab')2 dogs (all values at t=0 were &lt;0.019 μg/ml)</td>
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</tr>
</tbody>
</table>

*0≤0.019 μg/ml (lower limit of detection of ELISA was 0.019 μg/ml).
**Values above 20 μg/ml were not titrated further.
†Values represent samples taken before injection of F(ab')2.
§Values represent samples taken 10 minutes after injection of F(ab')2.

Canine neutrophils aggregated readily on stimulation with PMA as detected by the increase in light transmittance with a platelet aggregometer. For each preparation of neutrophils, the amount of change in light transmittance when cells were activated with 125 ng/ml PMA was considered 100%. The results were then expressed as a percent of maximal possible aggregation. The control F(ab')2 did not inhibit PMA induced aggregation (91.8±7.6%, n=6, Figure 7). The anti-Mol F(ab')2 (904) inhibited aggregation to 36.3±3.6% (n=4; p<0.0005, compared with control F(ab')2). The intact anti-Mol antibody (whole 904) limited aggregation to only 11.3±4.5% of PMA-induced aggregation (n=3; p<0.0005 compared with control F(ab')2). The n values in parentheses indicate the number of separate preparations of canine neutrophils. Assays for each n were run in triplicate or duplicate and averaged. Thus, both the intact anti-Mol and the F(ab')2, anti-Mol were effective inhibitors of canine neutrophil aggregation in vitro.

**Discussion**

The present study reports evidence that ultimate myocardial infarct size can be reduced with the intravenous administration of F(ab')2 fragments of a monoclonal antibody directed against the leukocyte adhesion-promoting molecule Mo1 (CD11b/CD18). In a previous study from this laboratory, the intact monoclonal antibody (904) limited the development of myocardial necrosis after 90 minutes of coronary artery occlusion and a short period (6 hours) of reperfusion. The present study confirms and extends these observations to show that F(ab')2 fragments were as effective as the whole antibody and protection was observed after a prolonged period of reperfusion (72 hours).
There is much evidence to suggest that the neutrophil plays a major, deleterious role during reperfusion of ischemic and infarcting tissue. Neutrophil depletion, inhibitors of fatty acid lipoygenase, or other agents that inhibit the activation of neutrophils are effective in reducing experimental myocardial injury during reperfusion by reducing the extent of neutrophil accumulation, activation, or both within the previously ischemic myocardium.

What Is the Relevant Mechanism of Protection by Anti-Mo1 F(ab')2 in Protecting Against Myocardial Reperfusion Injury?

The mechanism of the observed protective effect of anti-Mo1 F(ab')2 may be related to an inhibition of neutrophil adhesive function. This conclusion is based on the results of previous in vitro experiments in which monoclonal antibodies specific for the α-subunit of Mo1 (CD11b epitopes) or the β-subunits common to Mo1, LFA-1, and p150,95 (CD18 epitopes) blocked neutrophil aggregation in response to chemotactic factors; neutrophil (and monocyte) adhesion and spreading to a variety of substrates including glass, plastic, and monolayers of endothelial cells; and neutrophil-mediated vascular endothelial cell and epithelial cell injury (monolayers and perfused lungs). The contribution of CD11/CD18 molecules to neutrophil migration and tissue destruction in the inflammatory response is further suggested by in vivo experiments in which the intravenous administration of antibodies specific for CD11b or CD18 epitopes blocked the recruitment of neutrophils to sites of inflammatory stimulation in rabbit skin, mouse peritoneum, and dog coronary arteries; attenuated ischemia-reperfusion injury in cat intestine; and enhanced survival of rabbits subjected to hypovolemic shock (with diminished vascular endothelial injury as assessed histologically). With regard to the specific effects of 904 anti-Mo1 antibody on neutrophil function, this specific antibody has been reported to inhibit the spreading of human neutrophils to plastic substrates and to retard chemotaxis in response to f-MLP.

With the granulocyte, the role of the CD11/CD18 subunits in adhesion to endothelium varies and is dependent on the “activation states” of the granulocyte and of the endothelial cell. A recent report by Arnaout et al emphasizes the fact that different sets of adhesion molecules are involved in leukocyte–endothelial cell binding. The PMA-induced adhesion of granulocytes to endothelial cells was mediated primarily by Mo1 and not inhibited by monoclonal antibodies against LFA-1 or p150.95. In contrast, Smith et al (Reference 43 and personal communication) have shown that 904 (like other anti-CD11b antibodies) does not block the adhesion of unstimulated human or canine neutrophils to interleukin-1 (IL-1)–stimulated endothelial cells. However, pretreatment of endothelial cells with recombinant IL-1β (i.e., “activated endothelium”) results in granulocyte adhesion that is dependent on all three subunits of the CD11/CD18 complex family. Thus, each subunit may interact with a specific ligand on the endothelial cell. More recently, Smith et al have demonstrated that unstimulated neutrophils adhere to human umbilical vein endothelium (HUVE) in vitro via LFA-1 (CD11a/CD18)–dependent attachment to intercellular adhesion molecule-1 (ICAM-1), whereas neutrophils stimulated with chemokactant (f-MLP) adhere to HUVE via a Mo1 (CD11b/CD18)–dependent attachment to ICAM-1. Thus, differences between in vitro and in vivo findings with respect to adhesion of neutrophils to endothelial cells or the vessel wall suggest that in vitro studies may not reflect the activation processes that occur in vivo during ischemic damage to the endothelial cell as well as the processes that result in local activation of the neutrophil. The role of cytokines released during the inflammatory process must be considered as potentially important regulators of cell-cell interactions. The in vivo studies with the F(ab')2 fragments of the Mo1 antibody may provide additional insight into the mechanism by which reperfusion may extend the damage to previously injured myocardium.

Anti-Mo1 F(ab')2 may reduce reperfusion injury by virtue of its ability to prevent neutrophil aggregation and subsequent obstruction (“plugging”) of the coronary microvasculature. The 904 antibody is reported to have little inhibitory effect on static adhesion of unstimulated human or dog neutrophils to monolay-
ers of human or canine vascular endothelial cells (Reference 43 and personal communication). The possibility remains that 904 exerts its major inhibitory effect on neutrophil inflammatory function in vivo by preventing neutrophil aggregation and subsequent microvascular plugging, thereby leading to the reflow state. The F(ab')2 fragment of 904 blocks the aggregation of canine neutrophils in response to PMA (Figure 7) and human neutrophils in response to f-MLP (R.F.T., unpublished observations). However, in the present study, the relevant mechanism of protection by 904 via a direct inhibitory effect of the antibody on endothelial cell-neutrophil interaction or on neutrophil chemotaxis and diapedesis to sites of inflammation has not been excluded. Indeed, such an effect has been shown with an inhibitory effect on indium 111-labeled neutrophil adhesion in dog coronary arteries in vivo by 904 F(ab')2.40

Anti-Mo1 F(ab')2, may reduce reperfusion injury by virtue of its ability to prevent release of neutrophil-derived oxidants from adherent cells. Shappell and colleagues have shown that the 904 F(ab')2 does block the respiratory burst (release of H2O2) from activated neutrophils adherent to canine endothelial cells. Because Nathan has shown that adherence is a prerequisite for priming for maximal release of oxidants from neutrophils that are triggered by soluble inflammatory mediators, the 904 antibody may interfere with some component of adherence that is necessary for this priming phenomenon. The release of toxic oxidative intermediates by activated adherent neutrophils is a potential mechanism by which the inflammatory cells may exert tissue injury. The inhibitory effect of 904 on the neutrophil oxidative response represents another possible mechanism by which the antibody may modify ultimate infarct size in ischemically injured tissue subjected to reperfusion.

The data on neutrophil accumulation in the myocardium from the present study that used two separate techniques (histology, Figure 5; and myeloperoxidase activity in myocardium Table 4) may indicate that there was no attenuation of inflammatory cell infiltration. However, whereas MPO activity is an acceptable indicator of neutrophil content after short periods of reperfusion, neutrophil autoxidation and autooxidation of MPO at the inflammatory site may obscure differences in neutrophil accumulation after longer periods of reperfusion (e.g., 72 hours). These data are consistent with our earlier observations with the administration of iloprost in the same animal model of myocardial infarction: Iloprost reduced infarct size and neutrophil accumulation when canine hearts were reperfused for 6 hours and then examined after sacrifice, but after 72 hours of reperfusion (in which iloprost still limited infarct size).19 Measurements of MPO activity in heart muscle and histologic assessment indicated that the neutrophil influx had resolved. The resolution of the acute inflammatory process by 72 hours of reperfusion was also observed by Reimer and coworkers. Nonetheless, it is possible that anti-Mo1 (904) may be protecting without reducing the accumulation of neutrophils in the heart.

The increase in circulating neutrophil counts (Figure 4) that accompanies the development of a myocardial infarct in this study is similar to previous reports. Several interesting observations can be made with respect to neutrophil counts. First, the circulating neutrophil counts did not decrease significantly when exposed to the anti-Mo1 F(ab')2, indicating that the antibody-bound neutrophils are not being cleared from the circulation. Second, the anti-Mo1 F(ab')2 did not prevent the leukocytosis that accompanies the inflammatory reaction to surgical intervention and acute myocardial infarction. This is to be expected because humans who have a genetic deficiency in the Mo1 glycoprotein expression have defects in leukocyte adhesion and demonstrate a persistent elevation in circulating leukocyte counts.

Because both groups of dogs developed similar degrees of myocardial ischemia when measured by regional myocardial blood flow (Table 3 and Figure 3), the mechanism of protection can not be attributed to baseline differences in regional blood flow or to an increase in collateral blood flow to the ischemic region. In addition, there were no differences in hemodynamics that could have accounted for a myocardial protective effect. Thus, the myocardial protection evidenced in these experiments is best explained by the effect of anti-Mo1 antibody on neutrophil function.

In conclusion, the administration of F(ab')2 fragment of the monoclonal antibody directed against Mo1 significantly limits the extent of myocardial injury that results after 72 hours of reperfusion. The results indicate that inhibition of leukocyte adhesion, aggregation, or oxidant production by anti-Mo1 antibodies may prove to be useful adjunctive therapy in conjunction with thrombolysis or angioplasty in reducing the amount of myocardium that becomes irreversibly injured after reperfusion.

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The authors would like to thank Dr. Kenneth H. Kortright and his staff at Coulter Immunology, Hialeah, Florida, for providing 904 and control F(ab')2 reagents. The authors also gratefully thank Dr. Keith A. Reimer and Dr. Charles Murry, Duke University Medical School, for assistance in the histological assessment of myocardial tissue, and Mr. Thomas McClanahan, Mr. Paul T. Hoff, Dr. Jan M. Kitzen, Mr. Mathew D. Adams, Ms. Lucinda R. Tripp, and Ms. Clare E. Rogers for their technical support.

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**KEY WORDS** • neutrophils Mo1/Mac-1 • monoclonal antibodies • F(ab′)₂
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P J Simpson, R F Todd, 3rd, J K Mickelson, J C Fantone, K P Gallagher, K A Lee, Y Tamura, M Cronin and B R Lucchesi

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