F(ab')₂ Fragments of Anti-Mol (904) Monoclonal Antibodies Do Not Prevent Myocardial Stunning


To determine if inhibition of leukocyte adhesion and aggregation could improve posts ischemic ventricular dysfunction ("stunning"), a monoclonal antibody (904) that binds to the adhesion-promoting Mol glycoprotein on the cell surface of leukocytes was administered intravenously (0.5 mg/kg) to open-chest dogs before a 15-minute coronary occlusion. Ultrasonic crystals placed in ischemic and control myocardium were used to measure systolic wall thickening during a 15-minute occlusion of the left anterior descending artery and for 3 hours after reperfusion. Myocardial blood flow was measured with tracer-labeled microspheres before occlusion, after 10 minutes of occlusion, 3 minutes of reperfusion, and at 1 and 3 hours after reperfusion. Six animals receiving anti-Mol antibody had antibody excess demonstrated with immunofluorescence techniques at 5 minutes and 3 hours of reperfusion; this finding indicated saturation of binding sites. Five animals served as controls and received an antibody (murine immunoglobulin G) that does not influence neutrophils. The two groups did not differ hemodynamically during ischemia and reperfusion. Risk areas and myocardial blood flow were also not significantly different between the two groups. The main parameter used to define regional myocardial stunning, percentage systolic wall thickening in the ischemic/reperfused area, did not differ significantly between the two groups. Specimens from nonischemic myocardium were compared with ischemic specimens for myeloperoxidase content. There were no significant differences within or between groups. These data indicate that the anti-Mol monoclonal antibody (904) is not effective in improving the profound myocardial dysfunction that persists for 3 hours of reperfusion after 15 minutes of ischemia. (Circulation Research 1989;65:1112–1124)

Reperfusion after 15 minutes of coronary artery occlusion results in regional dysfunction that may persist for hours or even days, despite absence of cellular necrosis; this condition has been identified as the stunned myocardium. A substantial literature has developed focusing on this phenomenon. A variety of potential causes for stunning have been proposed; these include high energy phosphate depletion, sarcomplasmic reticular dysfunction, reduction of sympathetic responsiveness, uncoupling of energy sources from contractile machinery, and depressed calcium activation of contraction. No clear-cut mechanism for stunning has emerged as yet, but oxygen free radicals have been repeatedly demonstrated to contribute to the stunning process.

Since neutrophils represent a potentially important source of oxygen radicals in reperfused myocardium, Engler and Covell evaluated the effects of neutrophils in terms of myocardial stunning. After 15 minutes of occlusion, they reperfused the left anterior descending coronary artery with arterial blood that had been passed through Leukopak filters. Reperfusion with virtually neutrophil-free blood completely eliminated myocardial stunning; this result lead to the conclusion that neutrophils play a key role in the stunning phenomenon. Additional support for this view was recently provided by the preliminary report of Westlin and Mullane, who made similar observations by use of a com-

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parable experimental preparation. For the human patient, however, removal of all circulating neutrophils (to minimize the functional consequences of reperfusion) is not practical except in special circumstances.

In the present study, we examined another potentially more practical alternative that involved modification of neutrophil function with an anti-Mo1 (904) monoclonal antibody F(ab')2 fragment. The Mo1 glycoprotein is expressed on the plasma membrane of neutrophils, monocytes, and certain macrophages. Monoclonal antibodies directed against Mo1 glycoproteins have been documented to inhibit chemotaxis by spreading on natural and artificial surfaces; they also inhibit neutrophil aggregation and adhesion.

Recently, an anti-Mo1 (904) monoclonal antibody was used successfully to decrease acute infarct size in anesthetized dogs. Preliminary results also demonstrated a sustained decrease in acute infarct size in anesthetized dogs. Since these findings suggested that neutrophil function could be successfully modified in vivo to minimize reperfusion injury after a prolonged ischemic period, the present study was performed to test the hypothesis that alteration of neutrophil function with anti-Mo1 F(ab')2 fragments could improve regional function in reversibly injured or stunned myocardium that was reperfused after 15 minutes of occlusion. The severity of functional stunning was determined by the effect of ischemia and reperfusion on systolic wall thickening measured with sonomicrometers.

Materials and Methods

Materials

The generation of hybridoma clone 904 that produces an immunoglobulin G1, × murine monoclonal antibody specific for Mo1 (CD11b) has been described elsewhere. The F(ab')2 fragments of clone 904 anti-Mo1 and control immunoglobulin G1 monoclonal antibody (unreactive with canine neutrophils), which were prepared by 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.

Experimental Preparation

Mongrel dogs weighing 23–30 kg were anesthetized with diazepam (50 mg/kg) and sodium pentobarbital (10 mg/kg), and mechanically ventilated by a respirator (Harvard Apparatus, South Natick, Massachusetts) with oxygen-supplemented room air. Arterial blood gases were measured to verify that ventilatory parameters were within normal limits. The heart was exposed through a left fifth thoracotomy and suspended in a pericardial cradle. The left anterior descending artery was gently dissected free of surrounding tissue immediately distal to the first diagonal branch, and a nylon suture was placed loosely around it. During occlusion, the artery was elevated with the suture, and a small vascular clamp was used to occlude the vessel.

A micromanometer (model PC350, Millar, Houston, Texas) was passed into the left ventricle via the left carotid artery for measurement of left ventricular pressure. Tygon catheters (Norton Performance Plastics, Akron, Ohio) were placed in the left atrium (for injection of microspheres) and femoral artery (for continuous recording of arterial pressure). Catheters were also placed in the other femoral artery to obtain reference arterial samples for calculation of myocardial blood flow. An intramyocardial temperature probe (Sarns, Ann Arbor, Michigan) was placed in the basal left ventricle, and myocardial temperatures were recorded throughout the experiment. Temperature was maintained at approximately 38° C by placing the dogs on a continuously circulating water blanket.

Regional myocardial function was measured with sonomicrometers that were aligned to continuously measure transmural wall thickness. Sonomicrometers were implanted in the central ischemic area perfused by the left anterior descending artery and in the nonischemic (or control) area perfused by the left circumflex artery (Figure 1). In some of the dogs, a third pair of sonomicrometers was implanted in the ischemic myocardium close to the anticipated location of the perfusion boundary produced by occlusion of the left anterior descending artery (Figure 1). One crystal of each wall thickness pair was inserted tangentially through the myocardium to the endocardium. The other crystal, attached to a Dacron patch, was sewn to the epicardium with shallow sutures after locating the position of least distance between the crystals by monitoring the signals on an oscilloscope. The signals from the transducers were processed with a sonomicrometer (model 120, Triton Technology, San Diego, California). Data were not used if crystals were improperly aligned. Location of the inner crystals within the subendocardial third of the myocardial wall and correct alignment of the crystals were confirmed at the time of necropsy.

Experimental Protocol

Dogs were randomized to receive either the anti-Mo1 F(ab')2 antibody fragment (0.5 mg/kg) or a murine immunoglobulin G control antibody that does not bind to canine neutrophils. All experimenters were blind to treatment, and the same experimental protocol was followed in each experiment. The code was broken only after the completion of data analysis and reduction. Baseline hemodynamics and regional function were measured, and the antibody was administered intravenously. After 15 minutes, microspheres were injected to obtain a
baseline measurement of myocardial blood flow, and then the left anterior descending artery was occluded. After 10 minutes of occlusion, a second injection of microspheres was made to measure ischemic levels of perfusion. At 15 minutes of occlusion, the vascular clamp was released to reperfuse the vascular bed supplied by the left anterior descending artery. No lidocaine was used in any of the experiments. Microspheres were injected after 3 minutes, 1 hour, and 3 hours of reperfusion while continuously monitoring regional wall thickness and hemodynamics. Arterial blood samples were taken before the administration of antibody and then again at 5 minutes of reperfusion and at 3 hours of reperfusion for white blood cell counts and assessment of serum anti-Mo1 F(ab')\_2 fragment excess. A baseline hematocrit determination was compared with that at 3 hours of reperfusion.

**Myocardial Blood Flow Measurements**

Regional myocardial blood flow was measured with tracer-labeled microspheres (15-\(\mu\)m diameter, New England Nuclear, Boston, Massachusetts) by the reference withdrawal method.\(^{32}\) Five injections of microspheres labeled with \(^{141}\)Ce, \(^{113}\)Sn, \(^{103}\)Ru, \(^{95}\)Nb, or \(^{46}\)Sc for each flow determination were made in every experiment. The order of isotopes was randomized. Approximately two million microspheres were injected into the left atrium for measuring blood flows. Reference arterial samples were obtained from one of the femoral arteries at a constant rate (9.2 ml/min) with a withdrawal pump (Harvard Apparatus); withdrawals were initiated before the injection of microspheres and completed 2 minutes later.

At the end of the experiment, the dog was killed with a bolus of potassium chloride, and the heart was excised. After infusion of triphenyl tetrazolium chloride (TTC) and removal of samples for myeloperoxidase assays (see below), the heart was fixed in formalin. One slice of left ventricular tissue (approximately 2–3 cm wide) was cut perpendicular to the perfusion boundary defined by the TTC. Included in the slice of tissue were the sonomicrometers used to measure wall thickness (Figure 1). The left ventricular slice was divided into 11–13 transmural tissue blocks, which were cut into subepicardial, midmyocardial, and subendocardial thirds. Samples near the perfusion boundary (delineated approximately by the margin of TTC staining) were cut to be 3–4 mm wide at the endocardial surface to delineate the perfusion boundary between ischemic and nonischemic myocardium as clearly as possible. The position of the crystals was carefully noted at this time, and each piece of tissue was then weighed and assayed for radioactivity using a gamma counter (model 1185, TM Analytic, Elk Grove Village, Illinois). Regional myocardial blood flow was calculated with the equation\(^{32}\): \(Q_m = \frac{(C_m \times Q_r)}{C_r}\), where \(Q_m\) is myocardial blood flow (ml/min), \(C_m\) is counts per minute in tissue samples, \(Q_r\) is withdrawal rate of the reference arterial sample (ml/min), and \(C_r\) is counts per minute in the reference arterial sample. Flow data were normalized per gram of myocardium by dividing flow in each sample by its weight. Using this approach enabled construction of blood flow maps (examples of which are shown in Figure 2) that were used to distinguish ischemic and nonischemic samples.

**Risk Area Calculation**

Immediately after removal of the heart from the thorax, the left anterior descending artery was cannulated at the point of occlusion and TTC was infused. The TTC-stained myocardium was separated from the rest of the left ventricle and weighed to enable estimation of the size of the risk region as a percentage of the left ventricle. Infusion of TTC also allowed gross evaluation of the tissue in terms of myocardial infarction. Since TTC was infused alone, there was a tendency for the TTC to leak or diffuse beyond the margins of the ischemic area, which resulted in overestimation of risk area size. The same method was used in both experimental...
FIGURE 2. Graphs showing myocardial blood flow (MBF, ml/g/min) distribution and wall thickening (dWT, mm) during baseline conditions, during occlusion of the left anterior descending coronary artery, at 3 minutes of reperfusion, and at 1 hour of reperfusion in one of the control experiments. Subendocardial blood flow (ENDO BF) is shown with the solid symbols; subepicardial blood flow (EPI BF) is shown with the open symbols. Blood flow data are referenced to the right y axis in each graph. Wall thickening data, shown with the solid bars, are referenced to the left y axis. The numbers on the x axis refer to the 11 tissue samples obtained in this experiment. Coronary occlusion drastically altered the distribution of MBF, enabling us to define the ischemic (IS), nonischemic (NIS), and transition zone (TZ) samples. The transition zone refers to those samples that included variable amounts of ischemic and nonischemic tissue. Note that the sonomicrometer within the transition zone was characterized by severe dysfunction (akinesia) but that it recovered rapidly after reperfusion, in contrast to the ischemic sonomicrometer only a few centimeters away.

Myeloperoxidase Measurement

Myeloperoxidase assays were performed on tissue samples to provide an estimate of neutrophil accumulation in the myocardium. The entire heart was placed at -20° C for approximately 30 minutes after excision and TTC infusion. Transmural samples of tissue (0.5–1.5 gm) were cut from the central ischemic region in the anterior wall and from a control region laterally. The samples were from approximately the same positions in each heart. The myeloperoxidase assay was performed according to the methods of Bradley et al. The tissue was homogenized in a buffer containing 50 mM phosphate with 0.5% hexadecyltrimethylammonium bromide and 5 mM EDTA, pH 6.0, and sonicated after two freeze-thaw cycles. The samples were 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 myeloperoxidase decomposition was measured by the change in absorbance at 460 nm. One unit of myeloperoxidase activity is the amount of enzyme that decomposes one micromole of hydrogen peroxide per minute at 25 degrees.

Detection of Antibody Excess

Samples of serum were obtained in each experiment to determine if anti-Mol F(ab')2 fragments were present free in serum at 5 minutes and at 3 hours of reperfusion to document if sufficient levels had been achieved to saturate all neutrophil binding sites. Persons performing the assay were blinded to treatments. Indirect immunofluorescence for the detection of antibody was done with 1x10^6 Mol-positive test cells (calcium ionophore A23187-stimulated human neutrophils) that were incubated in buffer containing serum at a 1:2 final dilution for 30 minutes at 4° C. Cells were washed and then incubated in a second buffer containing a saturating concentration of fluorescein-conjugated goat antimouse immunoglobulin for an additional 30 minutes at 4° C. A flow cytometer (Epics C, Coulter Immunology) was used to measure the fluorescence intensity of the test cells relative to the intensity of cells exposed to a saturating quantity of 904 F(ab')2.
TABLE 1. Hemodynamics

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Coronary occlusion</th>
<th>3-Minute</th>
<th>60-Minute</th>
<th>180-Minute</th>
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<tbody>
<tr>
<td><strong>HR (beats/min)</strong></td>
<td></td>
<td></td>
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<tr>
<td>Control</td>
<td>130±16</td>
<td>138±17</td>
<td>135±19</td>
<td>129±18</td>
<td>140±23</td>
</tr>
<tr>
<td>Ab</td>
<td>132±10</td>
<td>137±11</td>
<td>137±10</td>
<td>134±18</td>
<td>157±20</td>
</tr>
<tr>
<td><strong>LVSP (mm Hg)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>Control</td>
<td>159±12</td>
<td>147±20</td>
<td>143±18</td>
<td>146±16</td>
<td>146±23</td>
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<tr>
<td>Ab</td>
<td>147±20</td>
<td>148±18</td>
<td>150±22</td>
<td>152±14</td>
<td>155±16</td>
</tr>
<tr>
<td><strong>MLAP (mm Hg)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Control</td>
<td>7.7±0.7</td>
<td>9.8±2.4</td>
<td>8.7±3.0</td>
<td>6.8±1.6</td>
<td>8.9±2.7</td>
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<tr>
<td>Ab</td>
<td>8.8±1.6</td>
<td>11.3±1.6</td>
<td>9.8±1.9</td>
<td>10.1±2.0</td>
<td>10.7±2.4</td>
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<td><strong>MAP (mm Hg)</strong></td>
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<tr>
<td>Control</td>
<td>127±8</td>
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<td>120±8</td>
<td>117±16</td>
<td>122±16</td>
</tr>
<tr>
<td>Ab</td>
<td>119±21</td>
<td>123±18</td>
<td>124±19</td>
<td>125±13</td>
<td>129±19</td>
</tr>
</tbody>
</table>

Values are mean±SD. HR, heart rate; LVSP, left ventricular systolic pressure; MLAP, mean left atrial pressure; MAP, mean arterial pressure; Control, control antibody-treated group (n=5); Ab, anti-Mol antibody-treated group (n=6). There were no significant differences between control and Ab groups by unpaired t tests or across conditions within each group by repeated measures ANOVA.

White Blood Cell Counts

White blood cell counts were performed after red cell lysis with a hemocytometer (Unopette, Becton-Dickinson Labware, Lincoln Park, New Jersey). Monolayered smears for differentials were prepared with a slide spinner and automatically stained with Wright-Giemsa. One hundred cells were counted for each differential. The percentage of neutrophils (including band forms) was multiplied by the white blood cell count to obtain the absolute number of neutrophils per milliliter of blood.

Data Analysis

Recordings of blood pressure and regional wall thickness were made continuously during each experiment on a pressurized-ink recorder (model 2800S, Gould, Cleveland, Ohio). Wall thickness and hemodynamic data were averaged from ten cardiac cycles. Data were analyzed manually from recordings made at a paper speed of 100 mm/sec. Variables analyzed were end-diastolic wall thickness (defined at the onset of peak positive dP/dt), end-systolic wall thickness (defined at 20 msec before peak negative dP/dt), extent of wall thickening (difference between end-systolic and end-diastolic wall thickness), percentage wall thickening (extent of thickening divided by end-diastolic wall thickness), peak systolic pressure, mean arterial pressure, mean left atrial pressure, and heart rate.

All data are reported as mean±1 SD. Statistical comparisons between treatment groups were made with unpaired t tests. For comparisons of hemodynamic, functional, and blood flow data across time, repeated-measures ANOVA was used. When the ANOVA was significant, paired t tests (corrected with the Bonferroni inequality adjustment) were used for multiple comparisons within each group.

Results

Fourteen animals were randomized to receive Mo1 monoclonal antibody or control antibody. Two control dogs were excluded: one animal for failure to develop dyskinesia during ischemia (indicating significant native collateral investment) and another for intractable arrhythmias that made wall thickening data impossible to interpret. One animal receiving the anti-Mo1 F(ab')2 fragment was excluded because of unacceptably poor contractile function (wall thickening less than 10% of end-diastolic thickness) during baseline conditions. Data from five dogs were used in the control group; data from six dogs were used in the anti-Mo1 F(ab')2 fragment group. In all 11 dogs, there were no instances of ventricular fibrillation at any time during the experiment.

Hemodynamic Data

Hemodynamic data are summarized in Table 1. There were no differences between the two groups at any time period. Likewise, there were no significant differences within groups across time periods. Infusion of the anti-Mo1 F(ab')2 fragment exerted no significant effects on hemodynamics. The data shown in Table 1 during baseline antibody exerted no significant differences between treatment groups by unpaired t tests or across conditions within each group by repeated measures ANOVA.

Area at Risk

The areas at risk produced by the proximal left anterior descending artery occlusions were not significantly different; they averaged 30.2±2.9% (range, 27.8–35.1%) in the control group and 34.2±2.8% (range, 29.7–37.0%) in the anti-Mo1 F(ab')2 fragment group. There was no gross indication of infarction (by TTC staining) in any of the dogs.
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CONmOLDOS
Mo1 ABmOLDOS
NON-ISCHEMIC ISCHEMIC

FIGURE 3. Bar graph showing myeloperoxidase activity in tissue samples from ischemic and nonischemic myocardium. There were no significant differences between the control group and the anti-Mol (Mol AB) group or between ischemic and nonischemic samples within groups. Because the myeloperoxidase data can be used as an index of neutrophil accumulation, the results shown here indicate that there was no neutrophil accumulation in stunned myocardium beyond that observed in normal myocardium.

White Blood Cell Counts

Circulating neutrophil counts were not significantly different between the groups during baseline conditions or early or late reperfusion. They averaged 5,068±2,035/μl and 4,796±1,622/μl (p=NS) during baseline conditions in the control and anti-Mol groups, respectively. At early reperfusion, little change in neutrophil counts was observed (control group, 5,163±2,687/μl; anti-Mol group, 4,600±1,549/μl), but a significant trend (by ANOVA) for an increase was evident 3 hours after reperfusion (control group, 7,931±5,600/μl; anti-Mol group, 9,603±3,280/μl).

Myeloperoxidase

The ischemic and nonischemic areas were assayed for myeloperoxidase activity as an indicator of neutrophil accumulation (Figure 3). In the control group, myocardial myeloperoxidase activity averaged 0.14±0.08 units/g tissue in the nonischemic area and 0.13±0.07 units/g tissue in the ischemic area (p=NS). In the Mol antibody group, activity was 0.21±0.12 units/g tissue in the nonischemic area and 0.16±0.07 units/g tissue in the ischemic area (p=NS). There were no significant differences between the groups in the nonischemic and ischemic areas; this finding indicated there were no differences in terms of neutrophil accumulation. The average values were quite similar to those observed in normal noninfarcted tissue in a previous study.

Antibody Excess

To document the administration of a dose of anti-Mol F(ab')2 antibody sufficient to saturate all Mol receptor sites in vivo, serum samples were tested for the presence of free immunoreactive antibody. In each of the anti-Mol treated animals, free anti-Mol F(ab')2 antibody was detected in serum taken at 40 and 220 minutes after antibody administration; antibody was measured by the binding of immunoreactive antibody to human neutrophil target cells.

TABLE 2. Myocardial Blood Flow in Ischemic Areas

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Coronary occlusion</th>
<th>Reperfusion</th>
<th>Reperfusion</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Coronary occlusion</td>
<td>3-Minute</td>
<td>60-Minute</td>
</tr>
<tr>
<td>Endo (ml/g/min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Control</td>
<td>1.43±0.54</td>
<td>0.03±0.02*</td>
<td>7.06±0.76*</td>
<td>0.76±0.44*</td>
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<tr>
<td></td>
<td>p NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Ab</td>
<td>1.03±0.39</td>
<td>0.05±0.04*</td>
<td>6.58±2.40*</td>
<td>0.68±0.16*</td>
</tr>
<tr>
<td></td>
<td>1.43±0.35</td>
<td>0.02±0.04*</td>
<td>7.43±0.63*</td>
<td>0.76±0.44*</td>
</tr>
<tr>
<td></td>
<td>p NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Mid (ml/g/min)</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Control</td>
<td>1.37±0.16</td>
<td>0.11±0.10*</td>
<td>4.83±1.61*</td>
<td>0.99±0.35*</td>
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<td>&lt;0.05</td>
<td>NS</td>
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<tr>
<td>Ab</td>
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<td>3.10±1.25*</td>
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<td>1.41±0.32</td>
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<td>6.21±0.70*</td>
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<tr>
<td></td>
<td>p NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Mean (ml/g/min)</td>
<td></td>
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</tr>
<tr>
<td>Control</td>
<td>1.41±0.32</td>
<td>0.06±0.05*</td>
<td>6.21±0.70*</td>
<td>0.84±0.40*</td>
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<tr>
<td></td>
<td>p NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Ab</td>
<td>1.14±0.34</td>
<td>0.14±0.09*</td>
<td>4.95±1.73*</td>
<td>0.89±0.25</td>
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</table>

Values are mean±SD. Endo, subendocardial third; Mid, midmyocardial third; Epi, subepicardial third; Mean, average transmural value; Control, control antibody–treated group (n=5, except for 180-minute postreperfusion values in which n=4); p, probability of difference between control and Ab groups by unpaired t test; Ab, anti-Mol antibody–treated group (n=6); NS, not significant.

*Significantly different from baseline value by repeated-measures ANOVA followed by Bonferroni corrected paired t test.
There were no significant differences between groups in the early (3-minute) reperfusion phase. Group during occlusion (Table 2).

Blood flows were substantially elevated in both groups. Because of technical difficulties with the withdrawal sample, data from one of the control dogs could not be used at this time point. Data from the remaining four dogs are included in Table 2 set off with parentheses to indicate that these data were not included in the control group ANOVA.

Myocardial blood flow data in control (nonischemic) areas are presented in Table 3. These data also represent pooled values for all nonischemic samples since blood flows in individual samples containing the nonischemic sonomicrometers were not different from the remaining nonischemic samples. There were no differences between groups or across conditions within each group.

**Regional Systolic Wall Thickening**

Ischemic wall thickness data are presented in Table 4. Examples of recorded tracings from one of the experiments (the same experiment shown in Figure 2) in the control group are shown in Figure 4 to demonstrate the effects of coronary occlusion and reperfusion on wall thickening.

In Figure 5 are presented the relative changes in ischemic wall thickening (expressed as a percentage of baseline values) in the control and anti-Mo1 groups. There were no significant differences between groups in terms of absolute wall thickness parameters (Table 4). Systolic thickening was replaced by thinning during coronary occlusion, consistent with the marked flow reduction produced in the ischemic area (Table 2). There was a slight rebound in thickening early after reperfusion, but regional function thereafter was mildly dyskinetic in both groups;
TABLE 4. Ischemic Wall Thickness Data

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Coronary occlusion</th>
<th>Reperfusion</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>3-Minute</td>
<td>60-Minute</td>
</tr>
<tr>
<td><strong>EDWT (mm)</strong></td>
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<td></td>
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<tr>
<td>Control</td>
<td>8.23±0.90</td>
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<tr>
<td>Ab</td>
<td>8.85±0.72</td>
<td>8.91±1.01</td>
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<tr>
<td><strong>ESWT (mm)</strong></td>
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</tr>
<tr>
<td>Control</td>
<td>10.26±1.13</td>
<td>8.47±1.74*</td>
<td>7.98±2.15*</td>
</tr>
<tr>
<td>Ab</td>
<td>10.96±1.70</td>
<td>9.15±1.29</td>
<td>8.00±0.72*</td>
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<td><strong>dWT (mm)</strong></td>
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<tr>
<td>Control</td>
<td>2.03±0.33</td>
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<tr>
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<tr>
<td><strong>%dWT</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>24.8±3.6</td>
<td>-3.9±4.4*</td>
<td>-2.5±5.6*</td>
</tr>
<tr>
<td>Ab</td>
<td>23.3±9.9</td>
<td>2.8±8.8</td>
<td>-2.2±5.6*</td>
</tr>
</tbody>
</table>

Values are mean±SD. EDWT, end-diastolic wall thickness; ESWT, end-systolic wall thickness; dWT, ESWT minus EDWT; %dWT, (dWT/EDWT)×100; Control, control antibody-treated group (n=5); Ab, anti-Mol antibody-treated group (n=6). There were no significant differences between control and Ab groups within conditions by unpaired t test.

*Significantly different from baseline condition data by repeated-measures ANOVA.

this finding indicated that both groups were characterized by equally intense myocardial stunning.

Nonischemic wall thickness data are summarized in Table 5, and relative changes are summarized in Figure 6. There were no differences between groups or within groups. Although trends
for augmented thickening during occlusion and decrease in wall thickening late in the experimental period were observed, these changes were not statistically significant.

In four of the dogs (two from the control group and two from the anti-Mol group), sonomicrometers were located near the perfusion boundary produced by left anterior descending artery occlusion, as well as in the homogeneously ischemic and nonischemic myocardium. The location of one such sonomicrometer is plotted in a blood flow map in Figure 2, and examples of analog tracings from the same sonomicrometer are shown in the third row (labeled TZ WT) of Figure 4. Although they exhibited substantial dysfunction during coronary occlusion, these sonomicrometers subtended tissue samples with a mixture of ischemic and nonischemic blood supplies when their location was plotted in the blood flow maps. Since they did not represent homogeneously ischemic or nonischemic measurements, they were assigned to a "transition zone" category.

The relative changes in wall thickening observed in the four dogs with transition zone, as well as ischemic and nonischemic, wall thickness are presented in Figure 7. During baseline conditions, transition zone percentage wall thickening averaged 24.4±2.7%, values similar to those derived from the sonomicrometers approximately 2-4 cm away in the area that would become the central ischemic zone (23.5±2.6%). During occlusion, wall thickening was nearly completely eliminated in the transition zone (0.5±5.0%). The transition zone sonomi-

**TABLE 5. Nonischemic Wall Thickness Data**

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Coronary occlusion</th>
<th>3-Minute</th>
<th>60-Minute</th>
<th>180-Minute</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDWT (mm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>11.75±1.59</td>
<td>11.52±1.46</td>
<td>11.83±1.64</td>
<td>11.68±1.84</td>
<td>12.00±1.76</td>
</tr>
<tr>
<td>Ab</td>
<td>11.80±1.40</td>
<td>11.26±1.42</td>
<td>11.71±1.38</td>
<td>11.58±1.26</td>
<td>11.99±1.44</td>
</tr>
<tr>
<td>ESWT (ram)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>14.06±1.70</td>
<td>13.87±1.71</td>
<td>14.12±1.67</td>
<td>13.46±2.19</td>
<td>13.73±1.92</td>
</tr>
<tr>
<td>Ab</td>
<td>13.87±1.37</td>
<td>13.72±1.05</td>
<td>14.04±1.27</td>
<td>13.69±1.34</td>
<td>13.82±1.62</td>
</tr>
<tr>
<td>dWT (mm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.07±0.26</td>
<td>2.47±0.74</td>
<td>2.30±0.28</td>
<td>1.78±0.70</td>
<td>1.73±0.97</td>
</tr>
<tr>
<td>Ab</td>
<td>2.30±0.30</td>
<td>2.40±0.54</td>
<td>2.33±0.51</td>
<td>2.11±0.51</td>
<td>1.82±0.89</td>
</tr>
<tr>
<td>%dWT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>19.8±2.9</td>
<td>20.9±3.8</td>
<td>19.7±3.6</td>
<td>15.3±6.1</td>
<td>14.8±9.0</td>
</tr>
<tr>
<td>Ab</td>
<td>17.8±3.4</td>
<td>22.6±8.7</td>
<td>20.2±5.6</td>
<td>18.4±8.4</td>
<td>15.4±7.6</td>
</tr>
</tbody>
</table>

Values are mean±SD. EDWT, end-diastolic wall thickness; ESWT, end-systolic wall thickness; dWT, ESWT minus EDWT; %dWT, (dWT/EDWT) x 100; Control, control antibody-treated group (n=4); Ab, anti-Mol antibody-treated group (n=5). There were no significant differences across conditions in either the control or Ab groups by repeated-measures ANOVA or between the control and Ab groups within conditions by unpaired t test.
crometers were in tissue samples in which mean transmural blood flow was reduced from 1.35±0.42 ml/g/min to 0.34±0.30 ml/g/min during coronary occlusion, whereas blood flow was reduced from 1.60±0.52 ml/g/min to 0.09±0.08 ml/g/min in the central ischemic samples. During reperfusion, in contrast to the central ischemic measurements, transition zone sonomicrometers exhibited considerable recovery (or less stunning) with percentage thickening of 15.4±5.4% (61±16% of baseline) and 10.7±4.3% (43±17% of baseline) at 60 and 180 minutes of reperfusion, respectively. The counterpart values in the ischemic dimensions were significantly lower (Figure 6) with percentage thickening of 2.5±4.4% and −4.3±6.7% at 60 and 180 minutes.

These data suggest that functional measurements made in heterogeneously perfused myocardium may display exaggerated recovery from stunning. The shape of the risk region and irregular transmural course of the perfusion boundary produced by occlusion of the left anterior descending artery can make accurate delineation of the ischemic area difficult. Therefore, we suggest that particular attention be paid to verifying the location of functional measurements relative to the perfusion boundary to avoid obscuring interpretation of results by including data on flow and function in heterogeneously perfused myocardium.

Discussion

The objective of this study was to test the hypothesis that an antibody directed against Mo1 receptors on neutrophils could modify the severity of myocardial stunning after 15 minutes of coronary occlusion. The rationale for the study was based on the demonstration by Engler and Covell24 that stunning could be eliminated by filtering all neutrophils from blood used to reperfuse ischemic myocardium. Although their findings suggest that neutrophils play a crucial role in myocardial stunning, it is obvious that elimination of circulating neutrophils is a practical alternative for human patients only in very restricted circumstances. Antibodies directed against neutrophils or specific aspects of neutrophil function, however, may represent a practical means of modifying the effects of neutrophils in ischemic or reperfused myocardium.

Therefore, in the present study we tested the efficacy of an Mo1 antibody on regional function in postischemic (stunned) myocardium. Clone 904 anti-Mo1 antibody has been demonstrated to inhibit neutrophil function, particularly in terms of adhesion and aggregation, in vitro25,36 and has also been shown to reduce myocardial infarct size in dogs subjected to 90 minutes of coronary occlusion followed by reperfusion. The latter studies are notable because they documented that it is possible to modify neutrophil function in vivo and, thereby, alter the consequences of relatively prolonged myocardial ischemia.

The main finding of our study, however, is that use of the Mo1 antibody did not change regional function in reperfused myocardium after 15 minutes of ischemia. Myocardial stunning was just as severe in the group treated with the anti-Mo1 F(ab')2 fragment as in the control group. The failure to demonstrate improvement with the Mo1 antibody cannot be attributed to differences in hemodynamics, baseline regional function, circulating neutrophil counts, neutrophil accumulation in the ischemic area, or intensity of ischemia (measured as reduction in blood flow with tracer-labeled microspheres), which were not significantly different between the two groups. Although subepicardial blood flow was significantly higher during occlusion in the Mo1 antibody group (Table 2), a disparity favoring recovery in this group, there was no difference in wall thickening between the two groups at any time point in the protocol (Table 4).

Why didn't the Mo1 antibody work? One possibility relates to the specificity of the Mo1 antibody that we used in this study. Whereas clone 904 anti-Mo1 has been shown to block neutrophil aggregation and the adherence and spreading of neutrophils to artificial substrates in vitro, it has minimal inhibitory effect on neutrophil adhesion to endothelial cell monolayers.37 Neutrophil-endothelial cell adhesion during inflammation may instead be mediated by other leukocyte adhesion receptors such as LFA-1 (CD11a/CD18).38 Thus, if 904 anti-Mo1 antibody exerts its effect on neutrophil function in vivo by inhibiting aggregation without blocking neutrophil-endothelial cell adhesion and the accompanying

**FIGURE 7.** Graph showing relative changes in wall thickening from four of the experiments that included measurements of "transition zone" wall thickness. Two of the dogs were in the anti-Mo1 group, and two were from the control group. Ischemic wall thickening data are shown with solid circles (mean±SD), nonischemic wall thickening data are shown with open circles, and transition zone data are shown with stippled circles. Note that the transition zone data, obtained from sonomicrometers in tissue samples with a heterogeneous blood supply, demonstrate a level of recovery in the reperfusion phase that appears substantially better than the ischemic measurements, despite severe dysfunction (akinesia) during the occlusion, whereas blood flow was reduced from 1.35±0.42 ml/g/min to 0.34±0.30 ml/g/min during coronary occlusion, whereas blood flow was reduced from 1.60±0.52 ml/g/min to 0.09±0.08 ml/g/min in the central ischemic samples. During reperfusion, in contrast to the central ischemic measurements, transition zone sonomicrometers exhibited considerable recovery (or less stunning) with percentage thickening of 15.4±5.4% (61±16% of baseline) and 10.7±4.3% (43±17% of baseline) at 60 and 180 minutes of reperfusion, respectively. The counter- part values in the ischemic dimensions were significantly lower (Figure 6) with percentage thickening of 2.5±4.4% and −4.3±6.7% at 60 and 180 minutes.

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release of reactive oxidative intermediates and proteases, it may not significantly influence the severity of myocardial stunning.

Although the 904 monoclonal antibody has not been documented to inhibit neutrophil-endothelial adherence in vivo, it did attenuate reperfusion injury resulting from 90-minute occlusions of the left circumflex coronary artery followed by 6 hours of reperfusion, providing the rationale for its use in this study. In a follow-up to that study, in which the same protocol was modified to include a 72-hour reperfusion period, a similar reduction in infarct size was demonstrated with the same F(\text{ab}')\text{2} fragment used in the present study.

A second possible explanation relates to the assumption that neutrophils are important in the process of stunning. Successful modification of myocardial stunning with interventions targeted at oxygen radicals has been repeatedly demonstrated, but whether neutrophils are the source of oxygen radicals remains to be definitively established. We observed no difference in myeloperoxidase activity between the control and the anti-Mol groups or even between ischemic and nonischemic zones within each group. Therefore, we find it difficult to attribute the effects of stunning to neutrophils because neutrophil accumulation was the same in nonischemic myocardium as in the reperfused myocardium (Figure 3) despite drastic differences in regional function between the two zones. It could be argued that flushing the ischemic area with TTC after excising the hearts may have washed out neutrophils and, thereby, masked an accumulation of neutrophils in both groups. Perfusing with TTC, however, would not have flushed out more neutrophils from the control group than from the Mol group. Consequently, it seems unlikely that our conclusions about relative neutrophil content were erroneous due to perfusion with TTC at necropsy.

Recently, Go et al measured neutrophil accumulation in reperfused myocardium with neutrophils labeled with indium 111. They observed fewer neutrophils in myocardium reperfused after 12 minutes of coronary occlusion than in nonischemic myocardium; this observation lends support to the view that neutrophils do not accumulate in transiently ischemic myocardium. In a preliminary report, O'Neill et al used goat antisera to deplete circulating neutrophils in dogs to approximately 10% of control levels. They observed no effect on the intensity of myocardial stunning and concluded that neutrophils do not contribute importantly to myocardial stunning. Jeremy and Becker also failed to demonstrate modification of stunning when the left anterior descending artery was reperfused with blood rendered extremely neutropenic by filtration. It should be acknowledged, however, that Jeremy and Becker perfused only the left anterior descending artery, in contrast with Engler and Covell and Westlin and Mullane, who perfused the entire left coronary system. Therefore, neutrophils delivered through coronary collateral connections may have influenced regional function in Jeremy and Becker's experiments. The minimum number of neutrophils potentially required to do myocardial damage, however, remains undetermined and as controversial as the importance of neutrophils in stunning.

The results of Engler and Covell suggest that neutrophils are almost singlehandedly responsible for stunning since filtering them out of reperfused blood completely eliminated stunning. This is a striking finding, especially given that no other intervention designed to decrease the effects of oxygen radicals has been as successful. Although significant improvement has been shown repeatedly with many different interventions, rapid and total restoration of function to baseline levels, similar to that reported in the study by Engler and Covell, has been observed previously only when inotropic agents or calcium were infused after reperfusion or when the intensity of ischemia during occlusion was relatively mild.

If the intensity of ischemia was less striking in the study by Engler and Covell compared with our data, it is possible that the rapid recovery they observed was not due to manipulation of neutrophil levels alone. Bolli et al recently showed that the intensity of stunning is directly related to the severity of ischemia during occlusion. Engler and Covell reported that subendocardial blood flow was reduced to approximately 0.2 ml/g/min in their leukopenic animals, whereas the counterpart values in our study were substantially lower (Table 2). The control group animals in the study of Engler and Covell were also characterized by much more substantial recovery than that observed in nearly all previous studies on stunning. We propose that differences in severity of ischemia may also have contributed to the disparity between our results and those of Engler and Covell in terms of functional recovery.

In conclusion, the administration of a monoclonal antibody that inhibits adhesion-dependent neutrophil function was not effective in preventing myocardial stunning. Since the same anti-Mol antibody failed to attenuate infarct size resulting from longer periods of ischemia and reperfusion, its failure to influence myocardial stunning raises questions about the contribution of neutrophils to this process. However, since we cannot exclude the involvement of Mol-independent mechanisms of neutrophil-mediated injury, our results do not eliminate the possibility that neutrophils play a role in the pathophysiology of myocardial stunning. Whether the failure to modify stunning was due to inadequate alteration of neutrophil function or to the possibility that neutrophils do not cause stunning will require additional investigation.

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KEY WORDS • sonomicrometer • myocardial blood flow • coronary occlusion • reperfusion injury • neutrophil function
F(ab')2 fragments of anti-Mo1 (904) monoclonal antibodies do not prevent myocardial stunning.

R J Schott, B S Nao, T B McClanahan, P J Simpson, M C Stirling, R F Todd, 3rd and K P Gallagher

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