Inhibition of Mannose-Binding Lectin Reduces Postischemic Myocardial Reperfusion Injury

James E. Jordan, PhD; Michael C. Montalto, PhD; Gregory L. Stahl, PhD

Background—Complement consists of a complex cascade of proteins involved in innate and adaptive immunity. The cascade can be activated through 3 distinct mechanisms, designated the classical, alternative, and lectin pathways. Although complement is widely accepted as participating in the pathophysiology of ischemia-reperfusion injury, the specific role of the lectin pathway has not been addressed.

Methods and Results—Monoclonal antibodies (mAbs; P7E4 and 14C3.74, IgG1κ isotypes) were raised against rat mannose-binding lectin (rMBL). Both mAbs recognized rMBL-A by Western analysis or surface plasmon resonance. P7E4, but not 14C3.74, exhibited a concentration-dependent inhibition of the lectin pathway, with maximal effect at 10 μg/mL. In vivo, rats were subjected to 30 minutes of left coronary artery occlusion and 4 hours of reperfusion. Complement C3 deposition was greatly attenuated in hearts pretreated with P7E4 compared with 14C3.74-treated hearts. Pretreatment with P7E4 (1 mg/kg) significantly reduced myocardial creatine kinase loss (48%), infarct size (39%), and neutrophil infiltration (47%) compared with 14C3.74-treated animals. In addition, P7E4 pretreatment significantly attenuated the expression of proinflammatory genes (intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and interleukin-6) after ischemia-reperfusion.

Conclusions—The lectin complement pathway is activated after myocardial ischemia-reperfusion and leads to tissue injury. Blockade of the lectin pathway with inhibitory mAbs protects the heart from ischemia-reperfusion by reducing neutrophil infiltration and attenuating proinflammatory gene expression. (Circulation. 2001;104:1413-1418.)

Key Words: ischemia ■ infarction ■ antibodies ■ cell adhesion molecules ■ inflammation

Reperfusion, although necessary for the preservation of reversibly injured tissue, is associated with additional injury mediated largely by neutrophils.1,2 In addition, the restoration of blood flow to ischemic tissue initiates a cascade of inflammatory-like events, including endothelial dysfunction, neutrophil sequestration, and complement activation, that contributes to postischemic injury. Although neutrophil and endothelial cell functions after ischemia have been extensively studied, the role of complement is less understood.

The complement system is a complex cascade of proteins that are activated in 3 distinct ways, designated the classical, alternative, and lectin pathways. The classical pathway is initiated by C1q binding to antigen-antibody complexes, whereas activation of the alternative or lectin pathway is antibody-independent.3 The alternative pathway is activated by spontaneously cleaved C3 or by C3b generated by one of the other pathways.3 The lectin pathway is activated by mannose-binding lectin (MBL) interacting with its ligand, leading to activation of 2 MBL-associated serine proteases.4

Human MBL is found in a range of high-molecular-weight oligomers composed of various numbers of monomers, with each monomer consisting of three 32-kDa peptide subunits.5 In contrast to human MBL, rat MBL (rMBL) exists as 2 distinct isoforms, MBL-A and -C.6 MBL-A appears to be the human MBL homologue, because it forms higher-order oligomers, associates with mannose-binding lectin–associated serine protease-1 or -2, fixes complement, and is an acute-phase protein.7,8 Recent work in mice, however, suggests that mouse MBL-C also forms high-order oligomers and fixes complement.8 Thus, the specific roles of rMBL-A and -C in experimental models need to be carefully evaluated.

A large body of literature documents the activation of the complement system after ischemia and reperfusion. Studies have demonstrated localization of various complement components within ischemic tissue9–11 and protection from ischemia-reperfusion injury by complement depletion.12 Anti-C5 monoclonal antibodies (mAbs), soluble complement receptor 1 (sCR1), and C1-esterase inhibitor (C1-INH) are cardioprotective in models of ischemia-reperfusion.11,13,14 No studies, however, have assessed the specific role of the individual pathways in activating complement after ischemia-reperfusion, mainly because of the lack of specific complement inhibitors to the individual pathways.

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Recently, MBL depletion, use of natural inhibitors of MBL, and anti-human MBL mAbs established that the lectin pathway initiates complement activation after hypoxia-reoxygenation of human endothelial cells.\textsuperscript{13} Complement has also been shown to play an important role in mediating tissue injury after myocardial ischemia-reperfusion in rats.\textsuperscript{13} Furthermore, in this model, MBL and C3 are colocalized within the myocardium, suggesting that the lectin pathway may be activated.\textsuperscript{15} The specific action of the lectin pathway in mediating tissue injury after myocardial ischemia-reperfusion, however, is unknown. Therefore, the role of the lectin complement pathway in vivo was investigated by use of novel mAbs against MBL-A in a model of myocardial ischemia-reperfusion.

Methods

Immunization and Antibody Production

Purified rMBL was isolated as previously described.\textsuperscript{16} Antibodies directed against rMBL were produced by standard hybridoma technology as described.\textsuperscript{17} Hybridomas recognizing rMBL were identified by antibody-capture ELISA, cloned by limiting dilution, and isotyped.\textsuperscript{18}

Antibody Characterization

Western blots using purified recombinant rMBL-A and -C (gift from Dr Russell Wallis, Oxford, UK) were performed to determine mAb specificity. Briefly, native and reduced recombinant MBL proteins (1 \( \mu \)g each) were separated by SDS-PAGE, transferred to nitrocellulose, blocked, and probed with 10 \( \mu \)g/mL of anti-MBL antibody. After washing, a horseradish peroxidase (HRP–coupled goat anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories, Inc) was used for detection. The membranes were developed by use of chemiluminescence and film.

To confirm the Western blot data, the interaction of purified recombinant rMBL-A and rMBL-C with the mAbs was determined by surface plasmon resonance. A biosensor CMS (BIAcore Inc) chip was activated with N-hydroxysuccinimide and N-ethyl-N'-\( \text{dimethylaminopropyl} \)-carbodiimide according to the manufacturer’s instructions. Anti-mouse Fcy (75 \( \mu \)g/mL in acetate buffer, pH 4.0) was coupled to experimental and control flow cells (ie, 1000 resonance units). Anti-rMBL mAbs (40 \( \mu \)g/mL P7E4 and 14C3.74, diluted with PBS containing calcium and magnesium) were captured on the experimental flow cell. Purified recombinant rMBL-C (10 \( \mu \)g/mL) was then passed over both flow cells, followed by rMBL-A (10 \( \mu \)g/mL), and the data were collected. The difference in response between the experimental and control flow cells is reported as the specific interaction between the anti-MBL antibodies and MBL-A.

Hemolytic assays were performed as described using rat sera treated with mAb at 100 \( \mu \)g/mL.\textsuperscript{13}

MBL-Dependent C3 Deposition ELISA

An MBL-dependent complement-activation ELISA was used as described, with minor modifications.\textsuperscript{19} Briefly, N-acetyl-\( \alpha \)-d-glucosamine (GlcNAc) was conjugated to BSA (Sigma) as previously described\textsuperscript{17} and plated onto microtiter plates. Rat serum was incubated with (1) veronal-buffered saline with calcium and magnesium (VBS 1:1); (2) various concentrations of mAb; or (3) various concentrations of \( \alpha \)-mannose. These mixtures (100 \( \mu \)L/well) were then added to the BSA-GlcNAc plates and incubated at 37°C for 30 minutes. The plates were washed and incubated with HRP-conjugated goat anti-rat C3 antibody (1:2000; Cappel). After washing, C3 deposition was quantified with ABTS and a microtiter plate reader. Background optical density (VBS 1:1 only) was subtracted from experimental wells.

Myocardial Ischemia and Reperfusion Studies

The myocardial ischemia-reperfusion protocol was performed as previously described.\textsuperscript{13} Adult, male Sprague-Dawley rats (300 to 400 g) were anesthetized with sodium pentobarbital (20 mg/kg IP) and ketamine HCl (60 mg/kg IP). Jugular vein and trachea were cannulated, and ventilation was maintained with 100% oxygen by a rodent ventilator (CWE, Inc) adjusted to maintain exhaled CO\(_2\) between 3.5% and 5%.

A left thoracotomy was performed, and a suture was placed 3 to 4 mm from the origin of the left coronary artery. Five minutes before ischemia, animals were randomly given P7E4 (0.05 or 1 mg/kg) or 14C3.74 (1 mg/kg). Ischemia (30 minutes) was initiated by tightening of the suture around the coronary artery and was followed by 4 hours of reperfusion. Sham-operated rats were prepared identically, except that the suture was not tightened.

Infarction Studies

After the reperfusion period, infarct size was determined by differential staining with patent blue violet (5%) and triphenyl tetrazolium chloride (TTC) as described previously, with minor modifications.\textsuperscript{13} Briefly, the coronary ligature was retightened, and an intravenous injection of patent blue violet was given to stain the normally perfused regions of the heart. The heart was then removed and bathed in ice-cold saline before removal of the atria, great vessels, and right ventricle. The left ventricle was sliced into thin sections, and the unstained area at risk (AAR) was separated from the normally perfused blue sections, cut into 1- to 2-mm\(^3\) pieces, and incubated with TTC. With a dissecting microscope, the necrotic areas (AN, pale) were separated from the TTC-positive (brick red–staining) areas. All areas of the myocardium were then weighed individually, and infarct size was calculated (AN/AAR \( \times \) 100).

Tissue Cytosol Kinase and Myeloperoxidase Activity

Rats were subjected to the same ischemia-reperfusion protocol as outlined above. After reperfusion, the hearts were removed and rinsed in ice-cold saline, the septum was separated from the left ventricular free wall, and both samples were frozen. Frozen samples were analyzed for creatine kinase (CK) activity (Sigma) and myeloperoxidase (MPO) activity as described.\textsuperscript{13}

Immunohistochemical Analysis of Complement C3 Deposition

Samples for immunohistochemistry were obtained from the central region of the AAR, fixed for 3 hours in 4% paraformaldehyde, cryoprotected in 15% sucrose overnight, frozen in OCT (Tissue-Tek), and stored at −80°C until processed. Tissue sections (10 \( \mu \)m) were mounted onto slides and stored at −20°C. Sections were fixed for 10 minutes with ice-cold acetone and allowed to dry. Endogenous peroxidases were inhibited with 3% hydrogen peroxide in PBS, blocked with 10% goat serum containing 2% BSA, and incubated with an HRP-conjugated goat anti-rat C3 antibody (Cappel). After washing, 3,3'-diaminobenzidine (DAB) peroxidase substrate (Vector) was used to develop the slides before counterstaining with hematoxylin. Controls consisted of slides incubated without primary antibody and resulted in no nonspecific cellular staining.

Semiquantitative Reverse Transcription–Polymerase Chain Reaction

Total RNA was extracted from frozen left ventricular free wall tissue subjected to myocardial ischemia-reperfusion by the acid guanidinium-thiocyanate extraction procedure as described.\textsuperscript{18} RNA was treated with DNase, extracted with a phenol-chloroform mixture, and ethanol-precipitated. Concentrations were determined by optical density (\( A_{260}/A_{320} \)) and agarose gel electrophoresis, cDNA was synthesized with random primers by the Reverse Transcription System (Promega). Amplification products (Table 1) were resolved by electrophoresis on 1.8% agarose gels containing 0.06 \( \mu \)g/mL of ethidium bromide. Bands were digitized and net band intensity
normalized to GAPDH and plotted as arbitrary units. To ensure that amplification was in the linear range, the number of polymerase chain reaction cycles for each gene was titrated, and the optimal cycle number was chosen. Water samples and RNA samples containing no reverse transcriptase were amplified in parallel to ensure that no contaminating DNA was present.

Statistical Methods
All data are presented as mean ± SEM. Statistical comparisons of groups were made with 1-way ANOVAs. Where appropriate, a Student-Newman-Keuls post hoc analysis for multiple comparisons was performed to determine group differences. Differences were considered statistically different at a value of $P \leq 0.05$.

Results

Recognition of rMBL by mAbs
Western blots were performed to determine which rMBL isoform(s) was recognized by mAbs 14C3.74 and P7E4. Under reducing conditions, a single band of $\approx 29$ kDa, consistent with reduced MBL-A ($A^-$), was recognized by both mAbs. Under nonreducing conditions, both mAbs recognized multiple higher-order oligomers of rMBL-A but not rMBL-C (Figures 1A and 1B). A polyclonal antibody raised against native rMBL recognized reduced and nonreduced rMBL-A or -C on the membranes (data not shown). These data suggest that mAbs 14C3.74 and P7E4 recognize only rMBL-A.

Surface plasmon resonance was performed to demonstrate that the mAbs recognized and bound MBL in the fluid phase. Figure 1C demonstrates that mAb P7E4 does not recognize rMBL-C, whereas P7E4 does recognize rMBL-A in the fluid phase. Similar results were observed for mAb 14C3.74 (data not shown). These data confirm that both mAbs recognize and bind rMBL-A.

Inhibition of MBL-Dependent Complement Activation
Complement activation and C3 deposition via the lectin pathway were assessed in vitro by C3 ELISA. D-Mannose decreased C3 deposition in a concentration-dependent manner (Figure 2A), with maximal inhibition at 100 mmol/L. In contrast, mAb 14C3.74 did not inhibit C3 deposition at concentrations up to 100 $\mu$g/mL (Figure 2C). P7E4 inhibited C3 deposition in a concentration-dependent manner (Figure 2B), however, with maximal inhibition ($\approx 80\%$) at 10 $\mu$g/mL P7E4. Furthermore, neither antibody attenuated the hemolytic activity of rat sera in sensitized chicken red blood cells. These data demonstrate that these mAbs specifically bind MBL-A, that P7E4 inhibits the lectin pathway, and that neither mAb inhibits the classical pathway or forms immune complexes with MBL to deplete complement.

Complement Deposition in Ischemic-Reperfused Myocardial Tissue
Frozen sections of rat myocardium demonstrated substantial C3 staining (brown) in the myocardium after ischemia and reperfusion (Figure 3B) compared with sham-operated animals (Figure 3A). Hearts treated with 14C3.74 (Figure 3B) demonstrated more C3 deposition than P7E4-treated (Figure 3C) hearts. Similar amounts of C3 deposition were observed in PBS-treated animals and 14C3.74-treated rats after ischemia-reperfusion (data not shown). These data suggest that the lectin complement pathway is activated after ischemia-reperfusion and that anti-MBL mAb reduces C3 deposition in vivo.

Infarct Size
As a measure of ischemia-reperfusion injury, the amount of infarcted tissue after 30 minutes of ischemia and 4 hours of

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tbody>
<tr>
<td>GAPDH</td>
<td>TGCACCACCAACTGCTTA</td>
<td>GGATGCAGGGATGATGTTC</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>ATGGCTTCAACCCTGCCAGGC</td>
<td>TCAGGAGGCGGGGCTTGTAC</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>ATGGCTTGAGGATGTCGCC</td>
<td>CAGGAGCAGAACCTGAGCC</td>
</tr>
<tr>
<td>TNF-α</td>
<td>TACTGAACTTCGGGATTTCTC</td>
<td>CAGCCCTTGGCCTGGAGAGACC</td>
</tr>
<tr>
<td>IL-1α</td>
<td>CTAGAAGACTTCCACATCCCGACG</td>
<td>CTGGAATAAAAAACACTGAGGAGGG</td>
</tr>
<tr>
<td>IL-6</td>
<td>CTGCCGACCCATTGCTCCTC</td>
<td>CAGGAGCATTGGAAGTGGGG</td>
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TNF indicates tumor necrosis factor. All primers are written 5’ to 3’.
reperfusion was assessed. The AAR of left ventricle was 42±3%, 43±2%, and 37±4% for the 14C3.74 (n=11), the 0.05 mg/kg (n=7), and the 1 mg/kg (n=6) P7E4 groups, respectively. These values were not statistically different from each other, indicating that all of the groups were exposed to similar ischemic stress. Infarct size (Figure 4) was significantly reduced (~40%) by 1 mg/kg P7E4 compared with 14C3.74. No additional protection was seen in animals treated with 10 mg/kg (data not shown). The lower dose of antibody (0.05 mg/kg) reduced infarct size by nearly 10% but was not significantly different from 14C3.74-treated hearts. These data demonstrate that inhibition of rMBL-A decreases infarct size after myocardial ischemia and reperfusion.

CK Activity
To confirm the infarct size results, residual left ventricular free wall CK activity was determined in an independent cohort of animals subjected to ischemia-reperfusion. Rats pretreated with 14C3.74 (n=7) had reduced CK activity in the left ventricle compared with sham controls (n=6; Figure 5A). Treatment with 1 mg/kg P7E4 (n=8) significantly attenuated the loss of CK activity, suggesting tissue protection. These data are in agreement with the infarct size data and confirm the cardioprotective effects of P7E4.

Left Ventricular MPO Activity
To assess neutrophil accumulation within the ischemia-reperfused myocardium, MPO activity was measured in the left ventricular free wall (Figure 5B). Sham-operated animals (n=6) exhibited very low MPO activity, whereas the 14C3.74-treated hearts (n=7) had significantly elevated activity. MPO activity was significantly reduced by P7E4 (n=8) to a level not statistically different from sham-operated hearts. These data demonstrate that inhibition of MBL-A reduces neutrophil accumulation within the ischemia-reperfused myocardium and may represent one mechanism by which anti-MBL treatment is cardioprotective.

mRNA Expression
Because neutrophil infiltration into ischemia-reperfused tissue is regulated by cytokines and the expression of adhesion molecules, the mRNA expression of several proinflammatory genes was examined. After ischemia and reperfusion, mRNA levels of intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, interleukin (IL)-6, IL-1α, and tumor necrosis factor-α increased significantly compared with sham-operated animals (Table 2). Hearts pretreated with P7E4 attenuated the expression of these genes, with the reductions in ICAM-1, VCAM-1, and IL-6 reaching statistical significance. Thus, MBL inhibition in rat myocardial ischemia/reperfusion attenuates the expression of several...
It is generally accepted that complement plays an important role in ischemia-reperfusion injury. The importance of the late complement components (C5a and C5b-9) for causing injury was demonstrated by use of anti-C5 mAbs. Soluble complement receptor 1, which inhibits the C3 and C5 convertases, has provided similar protective effects. Finally, complement receptor 1, which inhibits the C3 and C5 components, has provided similar protective effects.11

Complement can be activated by any of the 3 pathways: classical, alternative, or lectin. This study did not address the roles of either the classical or the alternative pathways. In light of the fact that C1-INH also provides protection from ischemia-reperfusion injury in similar models, it is possible that the classical pathway also mediates complement activation after ischemia. Because C1-INH also inhibits the lectin pathway, however, it is currently impossible to fully evaluate the potential molecular mechanisms for tissue protection.

**Discussion**

It is generally accepted that complement plays an important role in ischemia-reperfusion injury. The importance of the late complement components (C5a and C5b-9) for causing injury was demonstrated by use of anti-C5 mAbs. Soluble complement receptor 1, which inhibits the C3 and C5 convertases, has provided similar protective effects.11 Finally, the importance of classical pathway activation has been demonstrated with C1-INH. The present study specifically evaluates the role of the lectin pathway after ischemia-reperfusion. This study shows that selective inhibition of the lectin pathway attenuated C3 deposition in the heart, decreased infarct size and tissue injury (CK), limited neutrophil infiltration into ischemic-reperfused heart. *P*<0.05 vs Sham; †P<0.05 vs 14C3.74.

**TABLE 2. Inflammatory Gene Expression in Hearts Subjected to Sham Operation or Ischemia-Reperfusion**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sham</th>
<th>14C3.74</th>
<th>P7E4</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICAM-1</td>
<td>0.12±0.04</td>
<td>0.79±0.12*</td>
<td>0.29±0.10†</td>
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<tr>
<td>VCAM-1</td>
<td>0.20±0.07</td>
<td>1.00±0.21*</td>
<td>0.38±0.10†</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.29±0.14</td>
<td>2.19±0.31*</td>
<td>0.90±0.24†</td>
</tr>
<tr>
<td>IL-1α</td>
<td>0.04±0.02</td>
<td>0.33±0.10*</td>
<td>0.15±0.07</td>
</tr>
<tr>
<td>TNF-α</td>
<td>0.23±0.10</td>
<td>1.94±0.63*</td>
<td>1.16±0.42</td>
</tr>
</tbody>
</table>

TNF indicates tumor necrosis factor. Values are arbitrary intensity units normalized for GAPDH expression.

*P*<0.05 vs Sham; †P<0.05 vs 14C3.74.
the role of the classical pathway in vivo. To delineate the absolute involvement of each of these pathways, future studies will have to use specific inhibitors of the classical pathway. The present study, however, clearly demonstrates a role for the lectin pathway in the pathophysiology of myocardial ischemia and reperfusion. Specifically, MBL inhibition decreased infarct size, neutrophil accumulation, C3 deposition, CK loss, and expression of ICAM-1, IL-6, and VCAM-1.

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