Complement Gene Expression by Rabbit Heart
Upregulation by Ischemia and Reperfusion


Abstract—Activation of the complement system has been implicated in the pathogenesis of myocardial ischemia/reperfusion injury. It has always been assumed that liver is the primary source of complement components. In the present study, we used the reverse-transcriptase polymerase chain reaction technique to establish that the mRNAs for complement proteins C3 and C9 are expressed in rabbit heart. Rabbit liver, brain, spleen, and kidney were also shown to express C3 and C9 mRNAs. We used Western blotting to establish that these mRNAs in heart are translated into the corresponding proteins. We further established that dramatic upregulation of the mRNAs occurred in Langendorff-perfused isolated hearts subjected to ischemia and reperfusion. C3 mRNA was always expressed at higher levels than was C9 mRNA, but C9 mRNA showed greater upregulation under stress. Compared with levels in control hearts subjected to 5 minutes of normoxic perfusion, hearts subjected to 0.5 hours of ischemia followed by 1 hour of reperfusion had a 4.72-fold increase in C3 mRNA and a 19.5-fold increase in C9 mRNA. By contrast, C3 mRNA in hearts subjected to 3.5 hours of normoxic perfusion showed no change, and those subjected to 3.5 hours of ischemia showed only a 1.72-fold increase, whereas C9 mRNA levels increased by 5.17-fold after 3.5 hours of normoxic perfusion and 12.5-fold after 3.5 hours of ischemia. The results of this study demonstrate for the first time that heart tissue is capable of expressing genes and proteins of the complement system, although it is not yet known which cell types are responsible. They further demonstrate that ischemia and reperfusion of the heart promotes a rapid upregulation of the mRNAs encoding the complement proteins C3 and C9 and that these abnormal levels considerably exceed those of normal liver. These observations are consistent with the hypothesis that local production of complement proteins may contribute significantly to the degree of ischemic injury to the myocardium and that complement expression is augmented by reperfusion. (Circ. Res. 1998;82:1224-1230.)

Key Words: myocardial infarction ■ complement C3 ■ complement C9 ■ liver ■ spleen ■ brain

The pathogenesis of reperfusion injury is a complex process, characterized, in part, by an inflammatory response.1 Multiple aspects of inflammation, including infiltration of neutrophils into the ischemic zone, have been demonstrated to be involved in reperfusion injury. Recently, attention has focused on the role of the complement system in mediating cell damage, particularly after the restoration of coronary artery perfusion flow.

Activation of the complement system can produce direct tissue injury through formation of the MAC (C5b-9), followed by its insertion into host cell membranes.2 Rapid cell death can occur through disruption of cellular integrity.3 However, even when formed in sublytic quantities, the MAC may significantly alter normal cell functioning by modulating the transcription of genes encoding proinflammatory mediators.4-6

Components of the complement system such as C3, C4, C5, and the MAC have been identified in experimentally infarcted tissue as well as in human ischemic and infarcted myocardium.7-9 C5b-9 accumulates rapidly in ischemic myocardium during reperfusion.10 In animal models of myocardial ischemia, treatment with interventions that impede the complement system reduce the extent of injury. These include C1 esterase inhibitor,11-13 antibodies to C5a,14 and the soluble form of CR1.7,15,16 Additional evidence for the role of the complement system in mediating ischemia and reperfusion injury is derived from rabbits deficient in the complement protein C6. Animals deficient in C6 have been demonstrated to have a reduced infarct size compared with C6-sufficient rabbits.17 The concept that reperfusion plays a critical role in mediating complement deposition is demonstrated by the observation that in the absence of reperfusion, MAC accumulation occurred only as a late event.10 However, in the presence of reperfusion, the complement activation occurs rapidly, suggesting an important role of reperfusion in mediating the activation of complement.

Such results in model systems are consistent with what is known about human myocardial infarction. Deposition of the MAC and other indicators of complement activation have been noted in areas of myocardial injury while the surround-
ing normal tissue remains relatively free of complement components.\textsuperscript{18–22} Taken together, these results indicate that activation of the complement pathway in the ischemic/reperfused heart leads to deposition of the MAC and subsequent myocardial injury and that inhibition of the complement cascade limits that injury.

We hypothesized that local production by the heart might be a major source of injurious complement components. In the present study, we sought to determine whether the mRNAs encoding the complement proteins C3 and C9 occurred in organs other than the liver, whether the mRNAs were being transcribed into proteins, and whether mRNA levels in the heart were affected by ischemia and/or reperfusion.

Materials and Methods

Guidelines for Animal Research

The procedures used in the present study were in accordance with the guidelines of the University of Michigan Committee on the Use and Care of Animals. Veterinary care was provided by the University of Michigan Unit for the Laboratory Animal Medicine. The University of Michigan is accredited by the American Association for Accreditation of Laboratory Animal Care, and the animal care use program conforms to the standards in the Guide for the Care and Use of Laboratory Animals (publication No. [NIH] 86-23).

Langendorff-Perfused Heart

Male New Zealand White rabbits (1.8 to 2.2 kg) were rendered unconscious by cervical dislocation. Heart, liver, brain, spleen, and kidney were excised quickly. Hearts were then mounted on a Langendorff apparatus. The preparation has been described in detail previously.\textsuperscript{23} The heart was gassed continuously with a mixture of 95% O\textsubscript{2}/5% CO\textsubscript{2} to maintain normal tissue and was maintained at 37°C throughout the experiment by enclosing the heart in a temperature-regulated double lumen glass chamber.

Experimental Protocol

Isolated hearts were stabilized under normoxic conditions for 15 to 20 minutes before the induction of global ischemia. Induction of total global ischemia was accomplished by stopping the flow of perfusate to the heart. Reperfusion of the heart was conducted by turning on the pump to the original flow rate. Four experimental groups were studied: group 1 consisted of normal hearts that had been perfused for 5 minutes with buffer and then removed from the apparatus; group 2 consisted of hearts subjected to 3.5 hours of normoxic perfusion; group 3 consisted of hearts subjected to 3.5 hours of global ischemia only; and group 4 consisted of hearts exposed to 0.5 hours of global ischemia followed by 0.5, 1, 2, or 3 hours of reperfusion. The number of hearts (n) was 3 for each condition. Functional parameters were recorded every 10 minutes during the reperfusion period until termination of the protocol. A constant temperature of 37°C was maintained throughout the periods of ischemia and reperfusion.

Western blots were performed as reported previously\textsuperscript{24} on the cytosolic fraction of homogenates of rabbit heart and liver and were compared with human serum. Tissue samples were homogenized in 5× (vol/wt) extraction buffer (0.02 mol/L Tris-HCl, pH 7.5) containing the protease inhibitors phenylmethylsulfonyl fluoride (100 μg/mL) and aprotinin (10 μg/mL), along with 1 mmol/L EDTA. Homogenates were centrifuged at 18,000 g for 30 minutes. The protein content of the supernatants was determined according to the Lowry method.\textsuperscript{25} They were then diluted in SDS sample buffer (60 mmol/L Tris [pH 6.8], 2.5% SDS, and 5% β-mercaptoethanol) to a final protein content of 1 mg/mL and were boiled for 3 minutes. The normal human serum was diluted 1:500 in a similar manner. For C3 determination, 4 μL of the samples was loaded onto a 7.5% acrylamide minigel; for C9 determination, 15 μL was loaded. Life Technologies high-range prestained standards were used as molecular weight markers. After 45 minutes of electrophoresis (200 V), the proteins were transferred onto nitrocellulose membranes (Immobilon P, Millipore Corp) at 7 V for 45 minutes using a semidyblotter. Membranes were blocked in 5% skim milk for 2 hours before they were incubated with sheep anti-rabbit C3 antibody (1:5000, Biodesign International) or goat anti-human C9 (1:5000, Quidel) for 2 hours at room temperature. The immunoblots were then treated for another 1 hour at room temperature with anti-goat IgG labeled with horseradish peroxidase (1:5000, Sigma Immunochemicals). Immunoactive bands were visualized by incubation with Supersignal CL–HRP chemiluminescent substrate (Pierce Chemical Co). After they were drained and wrapped in a layer of plastic film, the membranes were exposed to x-ray film (Hyperfilm ECL, Amersham Life Science) for appropriate lengths of time.

mRNA Preparation and RT-PCR

Total RNA from ~500 mg of each tissue sample was extracted by the acid guanidinium thiocyanate–phenol–chloroform method. The extracted RNA was quantified by scanning spectrophotometry. The fluorescence ratio (fluorescence at 260 nm/280 nm) for all preparations was >1.8. To avoid contamination of the RNA with genomic DNA, all the samples were treated with 10 U of RNase-free DNase (Pharmacia) for 60 minutes at 37°C in 25 μL of 1× reverse transcriptase buffer (50 mmol/L Tris-HCl, 75 mmol/L KCl, and 3 mmol/L MgCl\textsubscript{2}) containing 40 U of RNase inhibitor (Pharmacia) and 1 mmol/L dithiothreitol, followed by an incubation at 85°C for 5 minutes to inactivate the enzyme. To verify the absence of genomic DNA contamination of RNA, an aliquot (~200 ng) of each sample was subjected to PCR amplification without the RT step.

For semiquantitative RT-PCR amplification, single-strand cDNA synthesis was first performed on 5 μg of total RNA. The reaction mixture consisted of the RNA sample, 25 μL of 1× RT buffer containing 1 μg random hexamer primers (pD\textsubscript{6}N, Pharmacia), 1 mmol/L deoxynucleotides (GIBCO BRL), 5 mmol/L dithiothreitol,
40 U of RNase inhibitor (Pharmacia), and 500 U of RT (Superscript TMII RT, GIBCO BRL). Duplicate assays were carried out at 42°C for 90 minutes, followed by heat inactivation of the enzyme (65°C for 10 minutes).

The resultant cDNA (1 μL), covered with 50 μL of mineral oil, was amplified in a 50-μL reaction buffer containing 67 mmol/L Tris-HCl (pH 8.8), 16.6 mmol/L ammonium sulfate, 10 mmol/L 2-mercaptoethanol, 200 μmol/L dNTPs, 2 mmol/L MgCl₂, 40 pmol of each specific oligonucleotide primer, and 2.5 U of Taq DNA polymerase (GIBCO BRL). The thermal profile used on a Fisher Scientific programmable thermal controller consisted of a denaturation step of 94°C for 1 minute, an annealing step of 55°C for 30 seconds, and an extension step of 72°C for 1 minute. The extension step in the first cycle was for 3 minutes at 72°C. All samples were initially denatured for a total of 5 minutes (94°C).

In preliminary studies, we found that the amount of PCR product increased exponentially from 20 to 29 cycles for cyclophilin and from 25 to 37 cycles for C3 and C9. A plateau phase due to the accumulation of PCR byproducts began at 30 cycles for cyclophilin and at 35 cycles for C3 and C9. Accordingly, each cDNA sample was treated by the PCR procedure, with the cyclophilin product being amplified for 27 cycles and the C3 and C9 products being amplified for 35 cycles. Each PCR reaction product was electrophoresed through a 6% polyacrylamide gel, and the products were visualized by incubation for 10 minutes in a solution containing 10 ng/mL ethidium bromide. Resulting gel bands were imaged using a GDS 6700 image analyzer (Ultra Violet Products). The relative intensities of the bands, expressed as optical density values, were analyzed, as well as values relative to cyclophilin mRNA analysis to provide an internal standard. Direct quantitative analysis of the bands using NIH image software 1.61.

Statistical Analysis
Data were expressed as mean±SE. Differences between control and experimental groups were checked for statistical significance (P<0.05) with 1-way ANOVA and Student t test for unpaired observations, as appropriate.

Results
Hearts exposed to 0.5 hours of ischemia began to show significant changes in functional properties by the first hour of reperfusion. The left ventricular developed pressure (difference between the left ventricular systolic pressure and left ventricular end-diastolic pressure) decreased while the coronary perfusion pressure increased (Table 2). These functional alterations corresponded in time with the changes in complement C3 and C9 mRNAs as detailed below.

To determine whether C3 and C9 mRNAs in heart were being transcribed into proteins, Western blots were run on protein extracts of human serum, rabbit liver, and reper-

**TABLE 1. PCR Primer Sequences of C3, C9, and Cyclophilin Genes**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Position in Sequence*</th>
<th>Sequence of Primer</th>
<th>Product Size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3</td>
<td>Forward: 1031–1056</td>
<td>5′-CAATACCAACCCGATGCTGACC-3′</td>
<td>298</td>
</tr>
<tr>
<td></td>
<td>Reverse: 1329–1304</td>
<td>5′-CATCCTGCGTTCTACGTCCTC-3′</td>
<td></td>
</tr>
<tr>
<td>C9</td>
<td>Forward: 376–401</td>
<td>5′-GGGGAATCATGCTGAGACCTTT-3′</td>
<td>202</td>
</tr>
<tr>
<td></td>
<td>Reverse: 578–553</td>
<td>5′-TCCCATCAAACAGGTCACAGAG-3′</td>
<td></td>
</tr>
<tr>
<td>Cyclophilin</td>
<td>Forward: 15–40</td>
<td>5′-ATGGTCAACCGTCACGTCACCGAC-3′</td>
<td>205</td>
</tr>
<tr>
<td></td>
<td>Reverse: 220–195</td>
<td>5′-GTGTGAACTCACCACCTGACACA-3′</td>
<td></td>
</tr>
</tbody>
</table>

*Numbers represent nucleotide base pair position within the respective gene sequence.
fused rabbit heart for comparison. Human serum was chosen as the overall reference source, since the primary antibodies used to detect the C3 and C9 proteins were originally made against human complement. Rabbit liver was chosen as the rabbit reference source, since liver is believed to be the main source of complement proteins. Rabbit heart made ischemic for 0.5 hours followed by reperfusion was chosen as a serum-free source with upregulated C3 and C9 mRNAs. Rabbit C3 has 79% homology 28 and rabbit C9 has 78% similarity 29 to the human counterparts. Figure 1 shows that similar bands for both C3 and C9 were obtained from all 3 extracts. On C3 blots, human serum demonstrated strong bands at 115 and 75 kDa, as previously reported. 24 These bands correspond to the C3α- and β-chains, respectively. 24 The corresponding bands for rabbit heart and liver were at the slightly higher molecular weights of 120 and 80 kDa. On C9 blots, all extracts demonstrated a distinct band at 80 kDa. An additional band was demonstrated by rabbit heart and liver at 100 kDa and by human serum at 60 kDa. These data establish that C3 and C9 proteins are synthesized by rabbit heart and rabbit liver.

Protein synthesis in tissue requires the presence of appropriate mRNAs. RT-PCR amplification from total RNA extracts was used to establish the presence of mRNAs for C3 and C9 in heart tissue. Results of a typical RT-PCR experiment are shown in Figure 2. The C3 primers yielded a product corresponding to the calculated size of 298 bp (Figure 2A). Treatment of the product with Hin2 resulted in cleavage of the product into the 2 expected fragments of 253 and 45 bp. The C9 primers
yielded a product corresponding to the calculated size of 202 bp (Figure 2B). Treatment of the product with BamH I resulted in cleavage of the product into the 2 expected fragments of 125 and 67 bp. The cytoplasmic primers yielded a product corresponding to the calculated size of 205 bp (Figure 2C). The intensity of the C3 and C9 bands increased from those observed under normoxic conditions (Figure 2A and 2B, lanes 2 and 3) to those observed after ischemia or ischemia followed by reperfusion (lanes 4 to 8). Cyclophilin mRNA levels, as anticipated, were unaffected by the various treatments (Figure 2C, lanes 2 to 8).

Composite results of replicate experiments are presented in Figure 3 and Table 3. Figure 3 shows that C3 and C9 mRNA levels were relatively low in normal hearts perfused for either 5 minutes or 3.5 hours. There were increases after 0.5 and 3.5 hours of ischemia, but these were considerably less than those observed after only 0.5 hours of ischemia followed by various periods of reperfusion. A sharp increase occurred between 0.5 and 1 hour of reperfusion, with a slower increase occurring after 2 and 3 hours of reperfusion. Corresponding cyclophilin mRNA levels were almost constant (Figure 3B).

Quantitative data are given in Table 3. Compared with levels in control hearts subjected to 5 minutes of normoxic perfusion, hearts subjected to 0.5 hours of ischemia followed by 1 hour of reperfusion had a 4.72-fold increase in C3 mRNA and a 19.5-fold increase in C9 mRNA. By contrast, C3 mRNA in hearts subjected to 3.5 hours of normoxic perfusion showed no change, and those subjected to 3.5 hours of ischemia showed only a 1.72-fold increase, whereas C9 mRNA levels increased by 5.17-fold after 3.5 hours of normoxic perfusion and 12.5-fold after 3.5 hours of ischemia.

Figure 4 shows the relative levels of C3 and C9 mRNAs in various rabbit organs. Both mRNAs were observed in all organs examined, ie, heart, brain, liver, spleen, and kidney, with C3 always being expressed more abundantly than C9. The highest levels in normal tissue were observed in the liver. However, in hearts subjected to 0.5 hours of global ischemia followed by 1 hour of reperfusion, the expression of C3 mRNA was higher than in the liver by 2.6-fold (P<0.001) and that of C9 mRNA was higher by 1.3-fold (P=0.01). There were no significant differences in the cyclophilin mRNA levels in these organs (Figure 4B).

**Discussion**

These data establish for the first time that myocardium can express both the proteins and mRNAs for the complement components C3 and C9. It has not yet been determined which cell types are responsible, but cardiomyocytes and endothelial cells are obvious candidates. Since sequences are not yet known for all rabbit complement genes, it is not possible at this stage to determine whether rabbit heart expresses all complement genes. However, we have recently identified in human heart the mRNAs for all the classical complement genes (authors’ unpublished data, 1998). In the present study, we have shown that the mRNAs for C3 and C9 are produced in all the organs tested, ie, heart, liver, brain, spleen, and kidney. Although the liver produces the highest levels under normal conditions, the ischemic and reperfused heart produces substantially higher levels of C3 than the liver (see Figure 4).

Evidence is now accumulating that local production may be the principal source of complement proteins in many tissues. For example, it has recently been shown that pyramidal neurons in the brain express the genes and proteins for all classical complement components. In culture, endothelial cells, astrocytes, microglia, gliomas, neuroblastomas, monocytes, fibroblasts, and other types of cells have been shown to produce one or more of the complement mRNAs, with translation into the protein products taking place. In Alzheimer’s disease, a chronic inflammatory state exists around the lesions, and there is upregulation of complement mRNAs and complement proteins. Activated fragments of the pathway, including the MAC, are deposited

### Table 3. Relative C3, C9, and CP mRNA Levels

<table>
<thead>
<tr>
<th>Treatment</th>
<th>C3</th>
<th>C9</th>
<th>CP</th>
<th>100 C3/CP</th>
<th>100 C9/CP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normoxic perfusion</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 min</td>
<td>651±66</td>
<td>18±7</td>
<td>7394±36</td>
<td>8.8±0.9</td>
<td>0.2±0.08</td>
</tr>
<tr>
<td>3.5 h</td>
<td>507±14</td>
<td>93±5</td>
<td>7142±17</td>
<td>7.1±0.1</td>
<td>1.3±0.05†</td>
</tr>
<tr>
<td>Ischemia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 h</td>
<td>674±37</td>
<td>139±6</td>
<td>7331±31</td>
<td>9.2±0.7</td>
<td>1.9±0.21†</td>
</tr>
<tr>
<td>3.5 h</td>
<td>1119±140</td>
<td>225±9</td>
<td>7220±23</td>
<td>15.5±1.1*</td>
<td>3.1±0.15†</td>
</tr>
<tr>
<td>Ischemia/reperfusion</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 h/0.5 h</td>
<td>1511±33</td>
<td>231±14</td>
<td>7109±60</td>
<td>21.2±0.6†</td>
<td>3.2±0.18†</td>
</tr>
<tr>
<td>0.5 h/1 h</td>
<td>3076±70</td>
<td>351±11</td>
<td>7052±76</td>
<td>43.6±0.8†</td>
<td>4.9±0.12†</td>
</tr>
<tr>
<td>0.5 h/2 h</td>
<td>3353±25</td>
<td>358±6</td>
<td>7296±49</td>
<td>45.9±0.2†</td>
<td>4.9±0.08†</td>
</tr>
<tr>
<td>0.5 h/3 h</td>
<td>3464±52</td>
<td>385±6</td>
<td>7168±37</td>
<td>48.3±0.9†</td>
<td>5.3±0.08†</td>
</tr>
</tbody>
</table>

The optical density of each electrophoresed band from rabbit heart total RNA samples (see Figure 3 as an example) was measured and analyzed as described in “Materials and Methods.” For calculation of statistical significance, the ratio of C3 and C9 mRNA optical density values were taken relative to that of cyclophilin (CP) mRNA in the same sample. In each case, values (mean±SEM) represent relative levels of mRNA expression in independent experiments from 3 rabbits.

*P<0.02 and †P<0.001 vs 5-minute normoxic perfusion.
in lesioned areas. Upregulation of complement components also occurs when monocytes and macrophages are stimulated.

In the present study, ischemia alone was only a weak stimulant for upregulation of the mRNAs for C3 and C9. However, reperfusion was a potent and rapid inducer. Within an hour, levels of the mRNAs for C3 and C9 increased 4.72- and 19.5-fold (Table 3). Clearly, it is important to determine the precise signals for this dramatic and rapid upregulation.

It has previously been established that activated complement components, including the MAC, are deposited on ischemic/reperfused hearts. In one study, MAC deposition was observed after only 15 to 30 minutes of ischemia if reperfusion took place, but not after 5 hours of ischemia alone. Such data are consistent with the mRNA upregulation observed in the present study.

These results may provide insight into the sequence of events that occur in human myocardial infarction. It is well established that human infarcted tissue is richly decorated with activated components of the complement cascade and, especially, the MAC. This clearly indicates that nontraditional activation of the complement pathway takes place, perhaps initiated by mitochondrial derived factors or C-reactive protein, and that the MAC contributes in a substantial way to cardiac damage. The data from the present and previous studies suggest that ischemia itself, extending over several hours, may not be particularly damaging to cardiac myocytes. It is the reperfusion stimulus, acting at least in part to upregulate complement biosynthesis, that paves the way for subsequent destructive events. Identifying the signals that cause local upregulation of complement production could be important, since blocking them might be effective in reducing cardiac damage after restoration of blood flow. To participate in an activated complement cascade, complement proteins must be secreted from their intracellular sites of production. It is completely unknown what mechanisms govern this process. Identifying the factors involved, as well as those that cause subsequent activation of complement, could be very important. Moving further down the chain of events and identifying agents that will inhibit one or more of the steps in the complement cascade could be valuable. C1 esterase inhibitor has already been shown to reduce reperfusion injury in animal models. Similarly, LU51198, a highly sulfated low molecular weight heparin derivative that inhibits MAC formation, has also been shown to be effective. Antibodies to C5a, which presumably inhibit granulocyte recruitment (and soluble CR1) by the binding of C3b/C4b to prevent activation of either the classical or the alternative pathway, are also effective in reducing reperfusion injury. Thus, interference with any of the stages of full complement activation and responses to that activation are beneficial. The system is complex, offering many potential sites where intervention might limit damage to the myocardium.

In summary, the present study points to the crucial role of endogenous complement production by heart in the dramatic effects of reperfusion injury. Exploration of ways of inhibiting the complement cascade may provide new therapeutic approaches in the protection of the ischemic/reperfused myocardium.

Acknowledgments
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References


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