Human Heart Generates Complement Proteins That Are Upregulated and Activated After Myocardial Infarction

Koji Yasojima, Claudia Schwab, Edith G. McGeer, Patrick L. McGeer

Abstract—In human heart, we detected mRNAs and proteins for C1q, C1r, C1s, C2, C3, C4, C5, C6, C7, C8, and C9 with the use of reverse transcriptase–polymerase chain reaction, Western blotting, and immunohistochemical techniques. We found an upregulation of both mRNAs and proteins in areas of recent and old myocardial infarctions. In both situations, the classical complement pathway was activated, with C4d, C3d, and the membrane attack complex (C5b-9) being deposited on damaged cardiac myocytes. These activated complement components were also identified on Western blots of infarcted tissue. Complement mRNAs in infarcted heart tissue were higher than those in liver, and liver complement mRNAs were not upregulated in cases with infarcted hearts. Our results establish that (1) complement proteins are endogenously produced by human heart; (2) the classical complement pathway is fully activated after myocardial infarction; (3) complement activation is directly involved in myocardial damage after ischemic insults; and (4) damage from complement activation may be chronically sustained. These data suggest that inhibition of the complement system should be effective in treating myocardial infarction. (Circ Res. 1998;83:860-869.)

Key Words: complement gene expression ■ classical pathway ■ postmortem delay ■ immunohistochemistry ■ Western blotting ■ liver complement

There have been many reports on association of complement proteins with myocardial damage. These include human myocardial infarcts, as well as damaged hearts in animal models of ischemia. Activation of both the classical and alternative complement pathways has been reported. Evidence that such activation is not an epiphenomenon, but contributes to tissue injury, comes directly from postmortem examination of human heart in which the membrane attack complex (MAC, C5b-9) has been identified on damaged muscle fibers. It comes indirectly from animal models in which ischemic myocardial damage is ameliorated by interference with complement activation. Intervention by administration of C1 esterase inhibitor, antibodies to C5a, 22 and the soluble form of CR-1 23,24 has reduced myocardial damage. The particular role of the MAC (C5b-9) comes from evidence that rabbits deficient in the complement protein C6 have a reduced infarct size in cardiac ischemia-reperfusion models compared with C6-sufficient rabbits.

It has traditionally been assumed that liver is the source of complement proteins that participate in these events. But we have recently shown that complement proteins are produced in several organs of the body, including brain and heart. We have also shown that production of C3 and C9 mRNAs and their protein products is sharply upregulated in isolated rabbit heart after reperfusion injury, and that the production by heart in this circumstance substantially exceeds that of normal liver. In brain, we have shown that all proteins of the classical complement pathway are produced by neurons, and that this production is upregulated in Alzheimer disease, a condition characterized by a chronic neuroinflammatory state.

In the present study, we report that human heart expresses the mRNAs and proteins for all of the components of the classical complement pathway. We also report that this production is upregulated in areas of myocardial infarct, and that the classical complement pathway is fully activated on injured myocardial tissue. Most importantly, we report that continuing activation and damage occur in previous myocardial infarcts.

Materials and Methods

Cases Studied

Heart tissues from 12 autopsied subjects were used in the present study. Details of the cases are provided in Table 1. Subjects ranged in age from 25 to 94 years, with postmortem delays varying from 17 to 132 hours. Five of the subjects had heart disease as described below.

Case 1 was a 94-year-old man known to have ischemic heart disease and chronic renal failure. The patient suffered a myocardial infarct 2 months before death. Five days before death, he was admitted to hospital after a seizure. He was discharged after 3 days but readmitted to another hospital. At this time, elevated cardiac enzymes and ECG changes were noted. He died 2 days later. On autopsy, the heart was enlarged, with atherosclerotic occlusions in the right coronary artery, the left anterior descending artery, and the left circumflex artery. Areas of recent and old infarcts were clearly visible. Tissue was taken for analysis from a healthy, well-perfused area, a sclerotic area indicative of a previous infarct, and a mottled area characteristic of a recent infarct. In this case, and subsequent

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From the Kinsmen Laboratory of Neurological Research, University of British Columbia, Vancouver.
Correspondence to Dr Patrick L. McGeer, Kinsmen Laboratory of Neurological Research, University of British Columbia, 2255 Wesbrook Mall, Vancouver, BC, V6T 1Z3, Canada. E-mail mcgeerpl@unixg.ubc.ca
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cases, the heart samples were thoroughly washed to remove all residual blood except that retained within capillaries.

Case 2 was a 78-year-old man with previous severe angina, admitted for a quadruple coronary bypass operation. The patient suffered an episode of cardiac arrest in the operating room. He deteriorated and died 3 days later. On autopsy, an extensive, recent myocardial infarct, who suddenly lost consciousness and died of cardiac arrest en route to the hospital. On autopsy, the heart was subendocardial hemorrhage Normal 0 10 13 9 32 34 11 31 36

8. 60 M 22 Brain tumor None Normal 0 18 30 4 42 42 7 0 12 0 0

9. 68 M 27 Alzheimer disease None Normal 0 12 20 10 44 46 40 23 11 31 36

10. 90 F 41 Lung cancer None Normal 0 19 15 4 60 60 0 0 0 0

11. 43 F 43 Diabetes, sepsis None Normal 0 16 16 13 60 8 0 10 0 0

12. 65 F 16 Alzheimer disease None Normal 0 21 0 16 8 0 17 16 28 28

100/CP indicates that each value has been multiplied by 100 relative to the level of cyclophilin mRNA in the same extract. mRNA levels are given in optical density units detected by ethidium bromide fluorescence (see Materials and Methods). MI indicates myocardial infarct; rMI, recent MI; and oMI, old MI.

### Specific Complement Primers and Preparation of Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR) Products

The DNA sequences of the genes of interest were obtained from the GenBank database. The specific PCR primers were designed on the basis of published human cDNA sequences. For all but C1r, genomic DNA data were also available. Primers were therefore chosen to span intron sequences, reducing the possibility of genomic DNA contamination of the RT-PCR products. Cyclophilin was selected as the reference standard, because it is expressed in virtually all types of tissues and has been highly conserved during mammalian evolution. The primer sequences are listed in Table 2.

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× reverse-transcriptase buffer containing 1 μg random hexamer primers (pDN6, Pharmacia), 2 μmol/L deoxynucleotides ( Gibco BRL), 5 mmol/L dithiothreitol, 40 U of RNase inhibitor (Pharmacia), and 500 U of reverse transcriptase (Superscript TM1 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 mixture 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 μmol/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 denatur-
In preliminary studies, we found that the amount of PCR product increased exponentially from 20 cycles to 29 cycles for cyclophilin and from 25 cycles to 37 cycles for complement products. A plateau phase was reached after 29 and 37 cycles, respectively. Accordingly, each cDNA sample was treated by the PCR procedure, with the cyclophilin product being amplified for 27 cycles and the complement products for 35 cycles. Each PCR product was electrophoresed through a 6% polyacrylamide gel, and the product was visualized by incubation for 10 minutes in a solution containing 10 ng/mL of 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 units, were quantitatively analyzed using NIH image software 1.61. Each complement mRNA analysis was made in parallel with a cyclophilin mRNA analysis to provide an internal standard. Values were analyzed relative to cyclophilin. Cyclophilin values were almost constant from sample to sample. Most were within 1% of each other, with the range less than 4%. Polaroid photographs of the gels were taken.

### Restriction Digest Analysis

The PCR products were purified by the ethanol precipitation procedure. Unique restriction sites and restriction enzymes were selected using the DNA strider computer program. The restriction enzyme used for each PCR product is shown in Table 2. Each restriction digestion reaction was carried out for 2 hours at 37°C. The digested

<table>
<thead>
<tr>
<th>Gene</th>
<th>Position in Sequence</th>
<th>GenBank Accession No.</th>
<th>Sequence of Primer (5’ to 3’)</th>
<th>Product Length, bp</th>
<th>Enzyme</th>
<th>Fragment, bp</th>
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<tbody>
<tr>
<td>C1q (1 intron)</td>
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<td>Forward</td>
<td>91–114</td>
<td>KO 3430</td>
<td>CCCAGGGATAAAGGAGAGAAAGG</td>
<td>358</td>
<td>Nco I</td>
<td>46, 71, 241</td>
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<td>448–425</td>
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<td>GGCCTGTAGATGTAAGTAGAG</td>
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<td>C1r</td>
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<td>Forward</td>
<td>1793–1817</td>
<td>M 14058</td>
<td>GCCTCCTGTACACGTTACCTCTCTCTA</td>
<td>216</td>
<td>HaeIII</td>
<td>90, 126</td>
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<td>CTGCCTGTTATAGGTGGGTGTTCC</td>
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<td>860–864</td>
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<td>Forward</td>
<td>1710–1734</td>
<td>KO 4481</td>
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<td>215</td>
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<td>1924–1900</td>
<td>KO 1236</td>
<td>TAAGGTATGTTGCAATTTCC</td>
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<td>C3 (2 introns)</td>
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<tr>
<td>Forward</td>
<td>509–533</td>
<td>KO 2765</td>
<td>TCACCGTCAACACGCTGCTACC</td>
<td>186</td>
<td>HaeIII</td>
<td>29, 31, 126</td>
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<td>TTTCATAGTAGGCTGACCACC</td>
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<tr>
<td>C4 (2 introns)</td>
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<td>Forward</td>
<td>3228–3252</td>
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<td>ATGTTCTCTATGTGCTTTCGTG</td>
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<td>GCGATGGTCACAAAGGCTGAGGTG</td>
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<td>C5 (3 introns)</td>
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<tr>
<td>Forward</td>
<td>1810–1834</td>
<td>M 57719</td>
<td>GTGGCATAGAGCGAGACTGAGATG</td>
<td>315</td>
<td>Alu I</td>
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<td>Reverse</td>
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<td>GCAGGCTCCATCGTAAACACATTCC</td>
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<tr>
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<td>Forward</td>
<td>1652–1676</td>
<td>JO 5064</td>
<td>TGCAGTGCAAAAAAGCAAAACCTCT</td>
<td>338</td>
<td>Alu I</td>
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<td>1989–1965</td>
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<td>TGCACTGTTCTTCTCTGCTC</td>
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<td>C7 (1 intron)</td>
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<td>Forward</td>
<td>455–479</td>
<td>JO 3507</td>
<td>GGAACAGAGTCAATACCAAAAG</td>
<td>248</td>
<td>Hincl</td>
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<tr>
<td>Reverse</td>
<td>702–678</td>
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<td>ACTGGCGTGAGAGATGAGATGAGAT</td>
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<td></td>
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<tr>
<td>C8 (1 intron)</td>
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<tr>
<td>Forward</td>
<td>76–100</td>
<td>M 17999</td>
<td>GACTCGGACCCCTCTCTGCTGCTGTC</td>
<td>258</td>
<td>Alu I</td>
<td>32, 226</td>
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<tr>
<td>Reverse</td>
<td>333–309</td>
<td></td>
<td>TTTGGAAGTACTGACAGCCATG</td>
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</tr>
<tr>
<td>C9 (1 intron)</td>
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<tr>
<td>Forward</td>
<td>128–152</td>
<td>KO 2766</td>
<td>GAATGAGCCTGGTGAATGTTGTC</td>
<td>180</td>
<td>Sau3AI</td>
<td>32, 148</td>
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<tr>
<td>Reverse</td>
<td>307–283</td>
<td></td>
<td>CATTCCGACGTCATCTCAGCATC</td>
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<tr>
<td>Cyclophilin (3 introns)</td>
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<tr>
<td>Forward</td>
<td>15–39</td>
<td>YO 0052</td>
<td>ATGTCGAACCCACCGGCTTTCTCG</td>
<td>206</td>
<td>HaeIII</td>
<td>53, 153</td>
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<tr>
<td>Reverse</td>
<td>220–196</td>
<td></td>
<td>CGTGTGAAGTCAACACCTCGACACA</td>
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</table>
PCR products were analyzed by electrophoresis on a 6% nondenaturating polyacrylamide gel. In each case, the correct number of products was detected of the expected size (Table 2).

Western blots were performed on extracts of the soluble fraction of homogenates of normal and infarcted human heart tissue and compared with normal human serum and serum activated by aggregated IgG. Heart tissue samples were homogenized in 50 mM Tris-HCl, pH 7.5 containing the protease inhibitors PMSF (100 μg/mL) and aprotinin (10 μg/mL) and 1 mM EDTA. Homogenates were centrifuged at 18,000 g for 30 minutes. The protein content of the supernatants was determined according to the Lowry method. The samples were then diluted in SDS sample buffer (60 mM Tris [pH 6.8]; 2.5% SDS, 5% β-mercaptoethanol) to a final protein content of 1 mg/mL and were boiled for 3 minutes. Samples containing 20 μg of protein were loaded onto 7.5% acrylamide minigels.

Normal human serum taken from a 44-year-old male volunteer was diluted 1:20 in veronal buffer. A 2-mL aliquot of the diluted serum was mixed with 50 μL of a solution of 2 μg/mL heat-aggregated human IgG (Sigma). The mixture was incubated at 37°C for 1 hour. Aliquots of the normal and IgG-activated serum were then diluted in 2 volumes of SDS buffer and boiled for 3 minutes. Samples containing 20 μg of protein were loaded onto 7.5% acrylamide minigels.

Because of the high molecular weight of the MAC, modifications of the electrophoresis and protein transfer steps were required. A 3% acrylamide minigel with 0.02 mol/L Tris-HCl, pH 7.5 as the gel buffer was diluted 1:20 in veronal buffer. A 2-mL aliquot of the diluted serum was mixed with 50 μL of a solution of 2 μg/mL heat-aggregated human IgG (Sigma). The mixture was incubated at 37°C for 1 hour. Aliquots of the normal and IgG-activated serum were then diluted in 2 volumes of SDS buffer and boiled for 3 minutes. Samples containing 20 μg of protein were loaded onto 7.5% acrylamide minigels.

High-range prestained standards (Life Technologies) were used as molecular weight markers. After 45 minutes of electrophoresis (200 V), the proteins were transferred onto nitrocellulose membranes (Immobilon P, Millipore) at 7 V for 45 minutes with use of a semidry blotter.

Because of the high molecular weight of the MAC, modifications of the electrophoresis and protein transfer steps were required. A 3% polyacrylamide gel was used, and separation was carried out for 11 hours at 25 V in a cold room with the apparatus surrounded by ice. The transfer to membranes was then carried out at 50 V for 11 hours in the cold.

Membranes were blocked in 5% low-fat milk for 2 hours. The immunoblots were then treated for 2 hours at room temperature with a primary anti-complement antibody, followed by treatment for 1 hour with an appropriate secondary antibody labeled with horseradish peroxidase. The primary antibodies were the same as those used for immunohistochemistry as described below. The appropriate secondary antibodies used were anti-rabbit HRP-conjugated IgG, anti-rabbit HRP-conjugated IgG, and anti-mouse HRP-conjugated IgG. All were from Sigma and were used at a 1:5000 dilution. Immunoreactivity was visualized by incubation with Supersignal CL-HRP chemiluminescent substrate (Pierce Chemical Co). After washing, the membranes were exposed to X-ray film (AR, Eastman Kodak).

Optical density units refer to ethidium bromide fluorescence (see Materials and Methods). Values are mean ± SE. Numbers in parentheses indicate the number of samples analyzed. The ratio MI/Normal is the average of all infarct samples divided by the average of all normal samples. Two-way ANOVA (pathology × mRNA) gave P < 0.0001 for normal vs old or recently infarcted tissue, whereas a nonsignificant P = 0.38 was found for old vs recently infarcted tissue. *Significantly different (P < 0.05) from the value in normal tissue by ANOVA followed by 2-tailed t-tests with use of the Holm’s stepdown procedure for multiple comparisons. The level of each mRNA in each sample was expressed as a percentage of the level of cyclophilin mRNA in the same sample. Cyclophilin levels were almost constant. MI indicates myocardial infarct; Cyc, cyclophilin.

TABLE 3. Average Levels of Each Complement mRNA in Optical Density Units in Tissue Types Examined

<table>
<thead>
<tr>
<th>Heart mRNA</th>
<th>Normal (10)</th>
<th>Old MI (3)</th>
<th>Recent MI (3)</th>
<th>MI/Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1q</td>
<td>2.0±2.0</td>
<td>43.3±7.3*</td>
<td>39.7±5.8*</td>
<td>20.7</td>
</tr>
<tr>
<td>C1r</td>
<td>10.0±1.2</td>
<td>30.0±4.0*</td>
<td>33.0±4.3*</td>
<td>3.15</td>
</tr>
<tr>
<td>C1s</td>
<td>11.3±2.3</td>
<td>43.0±2.1*</td>
<td>45.7±6.0*</td>
<td>3.92</td>
</tr>
<tr>
<td>C2</td>
<td>7.7±1.7</td>
<td>20.0±3.5</td>
<td>24.3±1.7*</td>
<td>2.82</td>
</tr>
<tr>
<td>C3</td>
<td>53.6±2.0</td>
<td>61.3±2.0</td>
<td>67.3±4.7*</td>
<td>1.20</td>
</tr>
<tr>
<td>C4</td>
<td>56.5±3.0</td>
<td>121±5.2*</td>
<td>131±1.2*</td>
<td>2.23</td>
</tr>
<tr>
<td>C5</td>
<td>8.3±2.2</td>
<td>33.0±2.1*</td>
<td>35.7±4.9*</td>
<td>4.14</td>
</tr>
<tr>
<td>C6</td>
<td>3.9±2.1</td>
<td>21.3±0.9*</td>
<td>21.3±0.3*</td>
<td>5.46</td>
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<tr>
<td>C7</td>
<td>9.8±0.9</td>
<td>39.7±0.3*</td>
<td>42.7±1.3*</td>
<td>4.54</td>
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<tr>
<td>C8</td>
<td>9.1±4.6</td>
<td>38.0±3.6</td>
<td>50.7±4.4*</td>
<td>4.52</td>
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<tr>
<td>C9</td>
<td>9.8±5.0</td>
<td>39.7±3.9</td>
<td>47.3±3.7*</td>
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<tr>
<td>Cyc/100</td>
<td>99.9±0.4</td>
<td>100.9±0.2</td>
<td>100.5±0.3</td>
<td>1.01</td>
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</tbody>
</table>

Optical density units refer to ethidium bromide fluorescence (see Materials and Methods). Values are mean ± SE. Numbers in parentheses indicate the number of samples analyzed. The ratio MI/Normal is the average of all infarct samples divided by the average of all normal samples. Two-way ANOVA (pathology × mRNA) gave P < 0.0001 for normal vs old or recently infarcted tissue, whereas a nonsignificant P = 0.38 was found for old vs recently infarcted tissue. *Significantly different (P < 0.05) from the value in normal tissue by ANOVA followed by 2-tailed t-tests with use of the Holm’s stepdown procedure for multiple comparisons. The level of each mRNA in each sample was expressed as a percentage of the level of cyclophilin mRNA in the same sample. Cyclophilin levels were almost constant. MI indicates myocardial infarct; Cyc, cyclophilin.
draining, the membranes were covered in clear plastic wrapping and exposed to x-ray film (Hyper film ECL, Amersham Life Science) for 0.3 to 2 minutes, depending on the strength of the signal.

**Statistical Analysis**

Data are expressed as mean±SE. Significance of the differences between the data for normal and infarcted tissue was assessed initially by 2-way ANOVA (pathology and mRNA type as the main factors) for global differences and then for differences in the individual mRNAs, by 1-way ANOVAs, followed by 2-tailed t tests with the use of Holm’s stepdown procedure for multiple comparisons. Analysis was done using the Macintosh StatView 512+ (Brain Power Inc). A value of *P*<0.05 was taken as indicating a significant difference.

**Immunohistochemistry**

Myocardial tissue samples for immunohistochemical and histochemical analysis were fixed for 2 to 3 days in 4% paraformaldehyde and were then transferred to a 15% phosphate buffered sucrose solution (pH 7.6). Immunohistochemistry was performed as previously reported. Briefly, 30-μm sections were cut on a freezing microtome. To reduce endogenous peroxidase activity, free-floating sections were treated for 30 minutes with 0.3% H2O2 solution in 0.01 mol/L PBS (pH 7.4) containing 0.3% Triton X-100. The sections were incubated overnight at room temperature with primary antibodies. The sections were then washed and treated with appropriate biotinylated secondary antibodies for 2 hours at room temperature, followed by incubation in avidin-biotinylated horseradish peroxidase complex (ABC Elite, Vector Laboratory) for 1 hour at room temperature.

The primary antibodies and the dilutions used were as follows (where 2 figures for dilution are given, the first was used in immunohistochemistry and the second in Western blotting): goat anti-C1q (Quidel, 1:50 000, 1:5000); sheep anti-C1r (Binding Site, 1:5000); goat anti-C1r (ICN Biochemicals, 1:500, 1:10 000); goat anti-C1s (Quidel, 1:10 000, 1:2500); goat anti-C2 (Quidel, 1:10 000, 1:2000); goat anti-C3 (Calbiochem, 1:100 000, 1:10 000); goat anti-C4 (Chemicon, 1:100 000, 1:10 000); goat-antiC5 (Quidel, 1:50 000, 1:3000); goat anti-C6 (Calbiochem, 1:50 000, 1:2000); goat anti-C7 (Quidel, 1:10 000, 1:3000); goat anti-C8 (Calbiochem, 1:50 000, 1:5000); goat anti-C9 (Quidel, 1:50 000, 1:5000); rabbit anti-C3d (Quidel, 1:100 000, 1:5000); mouse anti-C4d (Quidel, 1:10 000, 1:3000); mouse anti-neoC4d (Quidel, 1:5000); mouse anti-sC5b-9 (Quidel, 1:1000); mouse anti-C5b-9 (DAKO, 1:1000, 1:1000); and rabbit anti-neoC5b-9 (Advanced Research Technologies, 1:2000). The secondary antibodies used were anti-goat biotin-conjugated IgG, anti-rabbit biotin-conjugated IgG, and anti-mouse biotin-conjugated IgG. All 3 were from Vector and were used at 1:1000 dilution. The detection kit used was the Vectastain ABC HRP Elite (Vector, 1:2000). Peroxidase labeling was visualized by incubation of the sections in 0.01% 3,3’-diaminobenzidine (Sigma) containing 0.6% nickel ammonium sulfate and 0.00015% H2O2 in 0.05 mol/L Tris-HCl buffer (pH 7.6). When a dark purple color developed, sections were washed, mounted on glass slides, and coverslipped with a gelatin solution in distilled water. Controls were performed by omitting the primary antibody.

For histochemical detection of collagen, the Gomori trichrome method was used. Slide-mounted sections were washed in water, rinsed in 1% acetic acid, stained in Gomori’s trichrome solution (containing Chromotrope 2R 0.6 g, Fast green FCF 0.3 g, phosphotungstic acid 0.6 g, and glacial acetic acid 1 mL per 100 mL of distilled water) for 2 to 3 minutes. After staining, the sections were washed in 1% acetic acid, dehydrated, cleared in xylene and coverslipped in Entellan (Merck kGaA).

**Results**

The postmortem delay for heart tissue used in the present study varied from 6 to 132 hours. To determine whether postmortem delay significantly influenced the results, multiple tests were done to determine the quality of total RNA extracted and of the RT-PCR products obtained from each sample. Gels were run on every RNA extract, and the bands for ribosomal RNA and transfer RNA were examined. These RNA species are highly sensitive to enzymatic degradation. There was little smearing of bands and little loss of intensity associated with the longer postmortem intervals, either of which would have been indicative of postmortem degradation.

To test directly the postmortem stability of total RNA and the mRNAs being studied, the heart of case 9 was sampled at 24 hours and the remainder stored at 4°C. At 144 hours postmortem, an adjacent sample was then taken, and the quality of total RNA extracted from the 2 samples was compared. Results are illustrated in Figure 1. The 28S and 18S ribosomal bands and the transfer RNA band appeared highly similar when run on the same gel (Figure 1A). Gels were also run of the RT-PCR products. Only very minor declines were observed for cyclophilin mRNA and C4 and C3 mRNAs (Figure 1B). These data indicate that, for the time interval involved, degradation of RNA was not a major factor in the results obtained.

**Detection of mRNAs**

RT-PCR products for each of the complement mRNAs were identified in multiple samples from human hearts and liver.
Figure 2 illustrates such identification in an electrophoretic gel. The RT-PCR products were of the predicted size. Each product was purified and subjected to digestion with a specific endonuclease (Table 2). The fragments were purified and separated by electrophoresis. In all cases, the endonuclease digestion gave the expected number of fragments, and the expected bands were visible in the inactivated heart sample, as well as for normal heart tissue and complement-activated serum samples. The only strong band in the normal heart sample was for C3, with weak bands for C3d, C4, C4d, and C5 being detected. Note that the activated complement fragments C3d, C4d, and C5b-9 are strongly detected only in the inactivated heart and complement-activated serum sample. For details, see Materials and Methods.

Figure 4. Western blots from heart tissue and serum demonstrating the presence of complement proteins and their activation products. Lane 1, sample from an infarcted heart area of case 1. Lane 2, Sample from a normal heart area of case 1. Lane 3, Serum from a 44-year-old healthy man. Lane 4, Same serum as in lane 3 after activation of the complement system by aggregated IgG. Positions of molecular weight markers are shown by the arrows on the right; the estimated molecular weights of the main bands detected are shown by arrows on the left. Note that strong bands for all the complement proteins are visible in the inactivated heart sample, as well as for the normal heart tissue and complement-activated serum samples. The only strong band in the normal heart sample was for C3, with weak bands for C3d, C4, C4d, and C5b-9 being detected. Note that the activated complement fragments C3d, C4d, and C5b-9 are strongly detected only in the inactivated heart and complement-activated serum sample. For details, see Materials and Methods.

The quantitative levels of the RT-PCR complement products in heart varied according to the pathology. Cyclophilin mRNAs in heart were independent of cardiac pathology and independent of postmortem delay. Values for the RT-PCR products, in units relative to cyclophilin, are given in Table 1 for each complement mRNA in each heart sample. Analysis by 2-way ANOVA gave a P<0.0001 for the difference in complement mRNAs between normal tissue and recently infarcted tissue, as well as between normal tissue and old infarcted tissue. By contrast, a nonsignificant P value of 0.38 was obtained for the difference in recently infarcted tissue and old infarcted tissue. Results of the statistical tests for the effect of pathology on individual mRNAs are indicated in Table 3.

The quantitative levels of the RT-PCR complement products in liver did not vary with pathology or postmortem delay. Cyclophilin mRNA from liver was also independent of both postmortem delay and cardiac pathology. All of the complement mRNAs were easily detectable in all samples of infarcted heart tissue, whether recent or old (Table 1). In contrast, complement mRNAs in heart were low or undetectable in samples taken from subjects who died without a history of cardiac problems and with no gross abnormality visible in the postmortem heart (cases 6 to 12). C4 and C3 mRNAs were expressed at the highest levels in normal heart and liver, consistent with the relatively high levels of their translation products in normal serum.

Table 3 summarizes the relative levels of complement and cyclophilin mRNAs for normal, old myocardial infarcts, recent myocardial infarcts, and for samples from liver. The table shows that dramatic and highly significant increases in the mRNAs for almost all complement components occurred in old or recently infarcted heart tissue compared with normal heart tissue. The increases in infarcted tissue ranged from >20-fold for C1q to 1.2-fold for C3. Most increases were in the 3- to 5-fold range. These increased levels were higher than those typically found in liver. In contrast to heart, liver complement mRNAs were not elevated when the heart was infarcted. Cyclophilin levels in heart and liver were remarkably constant from sample to sample.

Figure 3 shows relative levels of complement and cyclophilin mRNAs in a normal area, a previously infarcted area, and a recently infarcted area of the heart of case 1. Large increases in complement mRNAs occurred in the damaged areas of heart compared with the undamaged areas, but there was no change in cyclophilin level. The data from this single heart were similar to the averages for all cases, as shown in Table 3.

Immunoblots

The results of Western blot experiments are shown in Figure 4. Lanes 1 and 2 are extracts from the recently infarcted sample and the normal sample of case 1. Lane 3 is from the normal serum sample, whereas lane 4 is from the same sample but after the complement system had been activated by aggregated human IgG. Bands were detected for all of the complement proteins from infarcted tissue (lane 1), with bands comigrating with those from normal serum (lane 3) and IgG-activated serum (lane 4). From the normal heart tissue extract, only bands for C3 and C4 and their degradation products were clearly visible (lane 2). Bands corresponding to full-length proteins were recognized by antibodies to C1r, C1s, C2, C4d, C6, C7, C8, and C9. The antibody to C1q recognized a strong band at approximately 35 kDa, corresponding to the molecular weight of the α chain. The C3 antibody recognized strong bands at approximately 115 and 75 kDa.
corresponding to the α and β chains, respectively. An additional band at 35 kDa, corresponding to C3d, was weakly recognized in the infarcted heart extract and in the IgG-activated serum, indicating that the polyclonal antibody was recognizing an epitope in the C3d region. The C3d antibody strongly recognized this 35-kDa band. The C4 antibody detected a prominent band corresponding to the β chain. The C4d antibody recognized a strong band in infarcted tissue and IgG-activated serum close to the 48-kDa value reported for C4d. The C5 antibody detected a band corresponding to the reported value for the β chain, whereas the C8 antibody identified a band corresponding to the α chain. The antibody for C5b-9 (MAC) identified a strong band in the infarcted heart and complement-activated serum of a very high molecular weight, extrapolated to about 800 kDa, which is the reported molecular weight for the MAC.

The presence of C3d and C4d indicates that, in infarcted tissue as well as in IgG-activated serum, the complement cascade has been activated past the opsonization phase, with the presence of the MAC establishing full activation of the pathway by assembly of the terminal components into the final product.

Immunocytochemistry
Each of the antibodies clearly stained cardiomyocytes in infarcted tissue (Figure 5). Such staining was in contrast to that of adjacent normal cardiomyocytes. These results were consistent with those of Western blots (compare lane 1 versus lane 2 in Figure 5) in which strong positive bands were detected in extracts of infarcted tissue for all complement proteins. C3 showed the weakest contrast immunohistochemically between normal and infarcted cardiomyocytes, consistent with the relatively small upregulation in mRNA (Table 3) and protein (Figure 4) in damaged myocardium. Thus, diffuse C3 immunostaining of normal cardiomyocytes may have reduced the contrast, with damaged cardiomyocytes visible with immunostaining for other complement proteins.

Figure 6 shows a much more dramatic increase between damaged and normal myocardium when immunohistochemistry was carried out using antibodies specific for complement activation. This is illustrated in serial sections taken from an acute infarct in case 2 and a chronic infarct in case 1. The area examined from case 2 contains no collagenous scar tissue and therefore represents an acute infarct (Figure 6A). The area of damage is clearly outlined by immunostaining for C4d, C3d, and C5b-9 in Figure 6B, 6C, and 6D, respectively. To verify that full activation of the classical complement pathway had taken place, 2 antibodies against C4d and 3 antibodies against C5b-9 were tested. The 2 antibodies for C4d and the 3 antibodies for C5b-9 gave equivalent immunostaining.

Figure 6E through 6H shows a previously infarcted area of case 1. The Gomori stain indicates surviving cardiomyocytes intermingled with extensive collagen deposits from old scarring (Figure 6E). Staining of serial sections showed intensive staining for C3d, C4d, and C5b-9 (Figure 6F through 6H), indicating activation of the full classical complement pathway in these cardiomyocytes. In this case, the intensity of C3d staining relative to the surrounding tissue was much stronger than for the acute infarct (compare Figure 6G with 6C), indicating a lack of C3 production in the contiguous collagenous areas.

Discussion
Postmortem stability is always a factor to consider in studies of human autopsy material. In this investigation, we examined tissues with postmortem delays varying from 17 to 144 hours. We found remarkably little difference in the quality of the total RNA extracted or in the levels of cyclophilin and C3 and C4 mRNAs detected by RT-PCR (see Figure 1). Moreover, the liver cyclophilin and complement mRNA levels did not vary with postmortem delay. These results illustrate that tissues that are kept in the cold, as is the case in most autopsy rooms, may have less postmortem deterioration than is
commonly believed. Postmortem delay did not appear to be a factor in the results reported in the present study.

The present study demonstrates that in human heart tissue, all of the mRNAs for complement proteins are expressed, and in areas damaged by infarction, they are strongly upregulated (Tables 1 and 3). The complement mRNA levels in damaged heart exceed those of liver. Because liver mRNA levels are not increased in cases with cardiac damage, it is suggested that heart itself, and not liver, is the source of the complement proteins found in damaged tissue.

The source of tissue complement proteins has traditionally been assumed to be liver, with serum acting as the delivery vehicle. Previously, we showed that isolated rabbit heart, perfused only with oxygenated buffer, was capable of synthesizing C3 and C9 mRNAs and their protein products. In this serum-free milieu, there was a 4.72-fold increase in C3 mRNA and a 19.5-fold increase in C9 mRNA after only 0.5 hours of ischemia followed by 1 hour of reperfusion. This indicates that increases in heart complement mRNAs can be induced rapidly. In hearts that had been subjected only to ischemia, without the insult of reperfusion, large increases in the mRNAs were not observed, and the protein products were not detected. The fact that the protein products appeared in heart that had been perfused only with oxygenated buffer and was free of serum indicated that heart muscle, and not serum, was the source of the proteins. Furthermore, such ischemia-reperfused hearts expressed higher levels of these proteins than did control rabbit liver.

In the results of the present study, a contribution from serum to heart complement proteins cannot be ruled out. However, if residual serum were the main source of the complement proteins, comparable levels should have been observed in normal and infarcted areas of the same heart. This was not the case. The levels in infarcted areas were much higher than in normal areas (eg, Figure 4, lane 1 versus lane 2). This is consistent with the previous observations on isolated perfused rabbit hearts, indicating that complement proteins can be locally produced.

C4 mRNA is the most highly expressed of the complement mRNAs in both normal and infarcted heart tissue. C4 is a pivotal component of the classical pathway, because cleavage by activated C1 exposes a thiol ester group on the fragment C4b, through which irreversible, covalent attachment to target tissue takes place. In this case, the target tissue is damaged cardiomyocytes. The attached C4b, complexed with C2a, cleaves C3, exposing a thiol ester group on C3b, which forms another covalent attachment to tissue near C4b2a. Formation of the trimolecular complex C2aC4bC3b completes the opsonization process and also cleaves C5, paving the way for formation of the MAC (C5b-9). The C4b and C3b degrade to form C4d and C3d. Thus, C4d and C3d are excellent markers for activation of the classical complement pathway, because they are amplified products of C1 activation that have become covalently attached to tissue. The presence of C5b-9 demonstrates that full activation of the classical complement pathway has taken place on damaged cardiomyocytes, indicating that these cells are being subjected to cytolytic as well as phagocytic processes. This occurs not only in acutely damaged tissue but also in surviving cardiomyocytes interspersed with collagen in previously infarcted tissue (Figure 6). These results provide evidence that the complement system is exacerbating rather than assisting to resolve the pathological process and that a chronic, autodestructive phenomenon may continue on a
long-term basis, Case 5 illustrates that this can extend for at least 3 years after an initial insult.

The immunohistochemical data are in line with previous reports on components specific for complement activation, C4d, C3d, and C5b-9 appearing on damaged myocardium after infarction. Activation of the complement system is reported to be initiated within 2 hours of coronary artery obstruction. Large increases in serum levels of the anaphylotoxins C3a and C5a, which are released after full activation of either the classical or alternative pathways, have been observed 16 hours after the onset of an acute myocardial infarction. It therefore appears that complement deposition can appear on cardiomyocytes within a few hours of an insult. Previously, we showed that isolated rabbit heart exposed to antibodies to C5a, and heparin-like compounds, which inhibit complement, can appear on cardiomyocytes within a few hours of an insult. Reports on components specific for complement activation, C4d, C3d, and C5b-9 appearing on damaged myocardium after infarction. Activation of the complement system is reported to be initiated within 2 hours of coronary artery obstruction. Large increases in serum levels of the anaphylotoxins C3a and C5a, which are released after full activation of either the classical or alternative pathways, have been observed 16 hours after the onset of an acute myocardial infarction. It therefore appears that complement deposition can appear on cardiomyocytes within a few hours of an insult. Previously, we showed that isolated rabbit heart exposed to antibodies to C5a, and heparin-like compounds, which inhibit complement, can appear on cardiomyocytes within a few hours of an insult.

The complement system cannot be activated unless the forces driving activation overcome a multiplicity of endogenous inhibitors. For the classical pathway, these include C1 esterase inhibitor, decay accelerating factor, C4 binding protein, membrane cofactor protein, C8 binding protein, vitronectin, clusterin, and protectin. C1q binding initiates the classical complement cascade. We found C1q mRNA to be the most strongly upregulated (>20-fold) of all the complement mRNAs in infarcted tissue. In general, the upregulation was higher for those genes and proteins normally expressed at lower levels, again consistent with the requirement of overcoming inhibitory factors.

Overall, these data suggest that complement activation exacerbates cardiac damage after infarction and that inhibition of the complement system should contribute significantly to effective treatment. Support for this conclusion comes from numerous studies in animal models in which complement inhibitors have been shown to limit cardiac damage after ischemia. These include C1 esterase inhibitor, CR-1, antibodies to C5a, and heparin-like compounds, which inhibit multiple stages of complement activation. Obviously, further investigation of the complement system in human cardiac disease and animal models of ischemic cardiac damage is strongly warranted.

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