Effects of Complement Activation in the Isolated Heart
Role of the Terminal Complement Components

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The mechanisms of the complement-mediated myocardial injury associated with ischemia and reperfusion have not been elucidated fully. Complement activation may directly mediate injury through actions of the anaphylatoxins C3a and C5a or generation of the membrane attack complex C5b-9. A model was developed to examine the direct effects of complement activation on heart function, assess myocardial tissue damage, and determine which complement components mediate tissue injury. Isolated rabbit hearts were perfused with Krebs-Henseleit buffer by using a modified Langendorff apparatus. Human plasma was added to the perfusate as a source of complement. Rabbit tissue activates human complement. Treatment with 6% normal plasma resulted in complement activation as assessed by the generation of Bb, C5a, and SC5b-9. Functional changes in cardiac performance became apparent 7–15 minutes after plasma addition and developed fully over the next 20–30 minutes. The effects were dependent on the complement titer and included 1) an increase in the end-diastolic pressure, 2) a decrease in the developed pressure, 3) an increase in the coronary perfusion pressure, and 4) an increase in lymphatic fluid formation. These effects were not elicited when an inhibitor of complement activation (FUT-175) was present or when heat-inactivated plasma was used. The effects of complement activation on myocardial function could not be reproduced by treatment with recombinant human C5a, zymosan-activated plasma, or plasma selectively depleted of C8. Myocardial tissue accumulated sodium and calcium and lost potassium as a result of complement activation. Activation caused the release of creatine kinase from myocytes and an increase in the radiolabeled albumin space of the hearts. The data demonstrate that complement activation caused decrements in myocardial function and increased the coronary perfusion pressure and lymphatic fluid flow rate. The effects were not mediated by the anaphylatoxins but were dependent on the distal complement component C8, suggesting that C5b-9 was responsible for the physiological changes. Complement activation directly mediated tissue injury in a manner consistent with plasma membrane disruption as a result of C5b-9 formation. The data suggest that the C5b-9 complex, which is known to form under conditions of ischemia, may contribute directly to myocardial cell injury. (Circulation Research 1992;71:303–319)

KEY WORDS • reperfusion injury • myocardial ischemia • myocardial infarction • membrane attack complex • FUT-175

Studies to elucidate mechanisms of myocardial reperfusion injury have focused primarily on the respective roles of toxic oxygen metabolites and the neutrophil. Activation of the complement system, however, can contribute significantly to myocardial tissue damage induced by ischemia and reperfusion. Depletion of circulating complement components reduces ischemia-induced myocardial tissue injury and is associated with reduced neutrophil infiltration into the ischemic tissue.1-4 Membrane-bound complement receptor 1 (CR1) is a recognized endogenous inhibitor of complement activation. When administered as the soluble CR1, it was demonstrated to reduce infarct size in the rat heart subjected to ischemia and reperfusion.5

The initial suggestion that complement activation was involved in myocardial tissue injury was made in 1970 by Hill and Ward,1 who showed that ischemic myocardial tissue released a protease responsible for the formation of a chemotactic molecule by cleavage of component C3. Depletion of C3 before ischemia prevented generation of the chemotactic molecule and blocked neutrophil infiltration into the ischemic tissue. Since then, activation of the complement system in the setting of myocardial ischemia and reperfusion has been confirmed, and the mechanism of activation has been studied in more detail. Pinckard et al6 demonstrated that human heart mitochondrial membranes bind C1q in the absence of anti-heart autoantibodies and thereby cause complement activation. Mitochondrial membrane–mediated complement activation was shown to occur through both the classical and alternative pathways.7,8 Similarly, Rossen et al9 demonstrated the pres-
ence of C1q in ischemic and reperfused canine myocardium and indicated that lymphatic fluid from ischemic myocardial tissue contains subcellular constituents rich in mitochondrial membranes that fix C1q and activate complement.\textsuperscript{10,11} They proposed that myocardial ischemia results in the release of subcellular constituents that bind C1q and activate complement, thereby generating the anaphylatoxins and stimulating infiltration of neutrophils, which may exacerbate tissue injury.

Further evidence of complement activation is provided by studies that localized complement components to injured myocardial tissue. Deposition of C3 was demonstrated on swollen and infarcted baboon myocytes after 4 hours of ischemia\textsuperscript{3} and was localized to the contractile elements and subcellular membranes of myocytes and vascular smooth muscle cells after 24 hours of ischemia.\textsuperscript{12} Components C4 and C5 were colocalized on myocytes positively stained for C3.\textsuperscript{3,4,12} These components were not found in normal myocardium. In addition, plasma concentrations of C4d\textsuperscript{13} and the C1r/C1s–C1 inhibitor complex\textsuperscript{14} were increased after acute myocardial infarction in patients, providing further evidence for classical pathway activation. Similarly, plasma concentrations of Bb\textsuperscript{13} and the C3bBbP\textsuperscript{14} complex also were elevated, providing evidence that alternative pathway activation is associated with myocardial infarction. These findings are consistent with the hypothesis that myocardial injury causes local activation of the complement system.

Because activation of the early portion of the complement system occurs in response to myocardial injury, it is likely that subsequent generation of the membrane attack complex C5b-9 also occurs. Using antibodies to neoantigens of the C5b-9 complex, investigators have localized the complex to infarcted human myocardium.\textsuperscript{15,16} An increase in the concentration of the soluble form of the membrane attack complex, SC5b-9, in the plasma of patients with acute myocardial infarction has been demonstrated.\textsuperscript{13,14,17} Immunocytochemical staining for C5b-9 in the myocardium was found to be specific and sensitive, allowing for the detection of single cell necrosis. Subsequently, quantitative measurement of SC5b-9 and C5b-9 in myocardial tissue by enzyme-linked immunosorbence assay documented an increase in these complexes in infarcted tissue that was primarily due to the C5b-9 (the membrane bound, cytolytic) form. These findings document activation of the complete complement system with generation of the potentially cytotoxic membrane attack complex. The formation of the membrane attack complex provides an important neutrophil-independent mechanism by which complement-mediated myocardial tissue damage may occur in response to activation of the system.

Complement-mediated tissue damage can occur in either a direct or an indirect manner. Direct tissue damage occurs as a result of the activated complement components themselves. Indirect damage occurs as the result of an intermediate event triggered by the activated complement components. For example, the anaphylatoxins mediate neutrophil infiltration and activation.\textsuperscript{18–21} As a result, the production of toxic oxygen metabolites and release of proteases lead to tissue damage. Thus, complement activation indirectly mediates tissue damage by the neutrophil. Similarly, opsonization of myocytes or endothelial cells leads to neutrophil-mediated damage of these cells. Many of the deleterious aspects of the myocardial inflammatory response are indirectly mediated by complement. Direct tissue damage is mediated by the membrane attack complex and possibly by direct vasocostructor properties of C5a.\textsuperscript{22,23} Insertion of the C5b-9 complex into a myocyte or endothelial cell membrane may lead directly to cell necrosis, whereas vasocostriction exacerbates ischemia.

The work described herein examines the hypothesis that complement activation can cause myocardial tissue damage in the absence of neutrophil recruitment and infiltration. It seeks to investigate more fully the mechanisms of complement-mediated tissue injury. The effects of complement activation on the functioning heart are examined, and an effort is made to define a role for the various complement components, especially the membrane attack complex, in mediating the observed complement-dependent effects. The experiments were designed to investigate the direct effects of in situ complement activation.

**Materials and Methods**

*Guidelines for Animal and Human Research*

The procedures followed in this study were in accordance with the guidelines of the Internal Review Board of the University of Michigan and with the regulations of the US Department of Health and Human Services for the Protection of Human Research Subjects (part 46 of title 45 of the “Code of Federal Regulations,” as amended) and the University of Michigan Committee on the Use and Care of Animals. Veterinary care was provided by the University of Michigan Unit for Laboratory Animal Medicine. The University of Michigan is accredited by the American Association for Accreditation of Laboratory Animal Care, and the animal care and use program conforms to the standards set by the National Institutes of Health in “The Guide for the Care and Use of Laboratory Animals” (Department of Health, Education, and Welfare publication No. [NIH] 86-23).

*Langendorff Preparation*

Hearts were removed quickly from male New Zealand White rabbits (1.8–2.4 kg) stunned by cervical dislocation. The hearts were attached to a modified Langendorff perfusion apparatus at the aortic stump and perfused in a retrograde manner. The time from interruption of normal blood flow until the establishment of retrograde perfusion was less than 90 seconds. The perfusion medium consisted of a modified Krebs-Henseleit buffer of the following composition (mM): NaCl 117.0, KCl 4.0, MgCl\textsubscript{2}·6H\textsubscript{2}O 1.2, KH\textsubscript{2}PO\textsubscript{4} 1.1, NaHCO\textsubscript{3} 25.0, CaCl\textsubscript{2}·2H\textsubscript{2}O 2.6, glucose 5.0, L-glutamate 5.0, and pyruvic acid 2.0. In addition, the perfusate contained bovine serum albumin (BSA, 0.25% [wt/vol]), insulin (10 units/l), and heparin (14 units/ml). The perfusion medium was prepared from double-distilled water. The solution was gassed continuously with a mixture of 95% O\textsubscript{2}–5% CO\textsubscript{2} at varying rates to achieve the desired oxygen partial pressure (Micro13 pH/blood gas analyzer, Instrumentation Laboratory, Lexington, Mass.). The pH was adjusted to 7.44, and the temperature of the perfusate was monitored to maintain the
heart between 35.8° and 36.2°C. The perfusate was filtered through an AP-25 prefilter (Millipore Corp., Milford, Mass.) before addition to the Langendorff apparatus. Heparinized human plasma as a source of the complement components was added to the perfusate as described below. Retrograde perfusion was performed at a constant flow (28–33 ml/min) using a Masterflex peristaltic pump and a recirculating perfusate volume of 350 ml. The hearts were paced electrically (165 impulses per minute, 5-msec duration, 4 V) using a stimulator (model SD-5, Grass Instrument Co., Quincy, Mass.) with bipolar platinum electrodes attached to the right atrium.

Various physiological parameters were measured to assess the function of the heart preparation. The aortic perfusion pressure was measured with a pressure transducer (Statham-P23 ID, Gould, Oxnard, Calif.) connected to a side arm of the aortic cannula. Isovolumic left ventricular pressure measurements were obtained with a fluid-filled latex balloon inserted into the left ventricle via the left atrium and connected to a pressure transducer. The balloon was filled to achieve an end-diastolic pressure of 12–15 mm Hg. The first derivative (±dp/dt) of the left ventricular pressure pulse was derived electronically and recorded. A small shunt and a thermistor were also placed into the left ventricle to allow drainage of any accumulated fluid and temperature monitoring, respectively. All measurements were recorded continuously on a polygraph (model 79D, Grass) and stored on a digital archive and analysis system (Po-Ne-Mah HD-4, Storrss, Conn.).

To promote complement activation in the isolated heart, the perfusate was gassed to achieve a state of relative hypoxia (PО₂, 225–275 mm Hg). All of the experimental results reported were obtained under these conditions. Subsequent experiments were performed using a PО₂ of 545–590 mm Hg. Under these conditions the baseline function of the hearts was improved, but complement activation still occurred, and similar results were obtained.

Protocol

**Effects of complement activation on the isolated perfused rabbit heart.** Several experimental treatments were used to determine the effects of complement activation on the functional parameters of the isolated perfused rabbit heart. The hearts were instrumented, and their function was allowed to equilibrate using relatively hypoxic normal buffer (PО₂, 225–275 mm Hg) as the perfusion medium. The hearts then were subjected to one of the following five treatments: 1) continued perfusion with buffer, 2) addition to the buffer of 6% heparinized normal rabbit plasma, 3) addition of 6% heparinized heat-inactivated human plasma (HiHP), 4) addition of 6% heparinized normal human plasma (NHP), or 5) addition of 6% heparinized NHP plus 18 μM complement inhibitor 6-amidino-2-naphthyl p-guanidinobenzoate dimethanesulfonate (FUT-175).

Hearts were excised and instrumented as described, and their function was allowed to stabilize for 20–25 minutes with plasma-free buffer perfusion medium. Baseline functional parameters were recorded (0-minute time point), immediately after which 2% (vol/vol) NHP was added to the reservoir containing the perfusion medium. Additional NHP (2% per time point) was added at 6 and 13 minutes of the protocol to achieve a final concentration of 6% NHP in the perfusion medium. Functional parameters were recorded at the 6-, 13-, and 20-minute time points and every 10 minutes thereafter through the end of the protocol. When 3% NHP was used instead of 6%, all of the plasma was added at time 0 instead of in three separate aliquots at 0, 6, and 13 minutes. For experiments in which FUT-175 was used, the agent was added to the perfusate simultaneously with the first addition of human plasma. At time 0 and every 10 minutes thereafter, 1.0-ml samples of pulmonary effluent and lymphatic fluid were collected, frozen in liquid nitrogen, and stored at −70°C until analyzed for their content of activated complement components or creatine kinase.

**Inhibitor of complement activation.** In some experiments, FUT-175 (Tori and Co., Tokyo) was used. FUT-175 is a protease inhibitor with potent activity against the Clr and Cls subunits of Cl of the classical pathway, as well as factors B and D of the alternative pathway.24–26 FUT-175 prevents complement activation in various in vivo experimental models.24,27,28 It was determined in the hemolysis assay that 140 μM FUT-175 completely prevented alternative pathway–mediated red blood cell hemolysis. However, this concentration of the drug caused a slow increase in the diastolic pressure of isolated hearts over the time course of the protocol. Therefore, the lower concentration of 18 μM FUT-175 was used in the experiments to minimize the direct cardiac effect of the inhibitor drug. For use, FUT-175 was dissolved in 1.5 ml distilled water and then added to the perfusate.

**Preparation of normal, heat-inactivated, and zymosan-activated plasma.** Human blood was obtained with informed consent by venipuncture from fasted donors. The blood was placed into tubes containing heparin (14 units/ml final concentration, Sigma Chemical Co., St. Louis, Mo.) and mixed. The blood was centrifuged at 2,000g for 10 minutes, and the plasma was collected. NHP was stored at −20°C until used (not more than 48 hours). Immediately before use, the plasma was thawed, centrifuged (40 minutes at 27,200g, 4°C), and filtered through a C-18 cartridge (Millipore). HiHP was prepared by heating at 56°C for 40 minutes immediately after collection. The plasma then was centrifuged and frozen as for NHP. Immediately before use, HiHP was thawed and filtered.

Rabbit blood was obtained by cardiac puncture. Rabbits were sedated with xylazine (15 mg/kg i.m.) and ketamine (15 mg/kg i.m.) and anesthetized with sodium pentobarbital (30 mg/kg i.v.). A midsternal thoracotomy was performed, and blood was withdrawn into a heparinized syringe (14 units/ml). Normal rabbit plasma was prepared using the method to prepare NHP.

Zymosan-activated plasma was prepared by obtaining human plasma and processing it as for NHP above. A modification of the method of Hugli et al29 for the generation of the anaphylatoxins and the subsequent purification of the intact molecules was used. Immediately before use, the plasma was thawed, and 2-mercaptoethyl-3-guanidinoethylthiopropionic acid (Plummer’s inhibitor, 2.0 mM) was added to inhibit carboxypeptidase N.30 The mixture was incubated with zymosan (20 g/l) at 37°C for 60 minutes, centrifuged for 30 minutes at 4°C and 27,200g, and immediately used in...
an experiment. Plasma samples were obtained just before addition of the carboxypeptidase N inhibitor and zymosan and just after centrifugation and frozen at −70°C for later analysis of anaphylatoxin content using the described method. Zymosan was prepared by boiling a 10% suspension of commercially available yeast cake in saline for 45 minutes. The mixture was transferred into 50-ml conical tubes and washed with saline eight times using centrifugation (8 minutes at 1,400g, room temperature). The washed zymosan was divided into single-use aliquots and frozen until used (−20°C).

Collection of lymphatic fluid and pulmonary artery effluent. Preparation of the heart included cannulation of the pulmonary artery to direct the pulmonary effluent to the reservoir for gassing and recirculation. The left atrial appendage incision was closed around the shunt, thermistor, and balloon tubing to prevent fluid leakage. The pulmonary veins were ligated. Therefore, fluid collecting on the surface of the heart represented “lymphatic” fluid from severed lymphatic channels at the base of the heart and from beads of fluid forming on the heart surface. The lymphatic fluid was collected into FUT-175 (18 µM final concentration), as it trickled off the apex of the heart, to prevent further activation of the complement system. The mixture was frozen in liquid nitrogen and stored at −70°C for later analysis. The rate (milliliters per minute) at which the lymphatic fluid dripped from the heart was monitored. Samples of the pulmonary artery effluent were collected into FUT-175, frozen, and stored in the same manner.

Measurement of activated complement components C3a, C5a, Bb, and SC5b-9. The concentrations of the anaphylatoxins C3a and C5a (including their des-Arg forms) were measured in the pulmonary effluent using a radioimmunoassay commercially available from Amersham Corporation, Arlington Heights, III. The appearance of activated component Bb and of the soluble form of the membrane attack complex, SC5b-9, in the lymphatic fluid was monitored using enzyme-linked immunoassays commercially available from Quidel, San Diego, Calif. It is important to emphasize that these assays use antibodies directed against human complement components, thus allowing for assessment of activation of the human complement added to the perfusate. Cross-reactivity of the anti–C3a des-Arg to C4a des-Arg, C5a des-Arg, C3a, and C3 is 0.89%, <0.22%, 100%, and 100%, respectively. Measurement of C3 is prevented by removing it with a specific precipitation step. C3a des-Arg can be accurately determined over the range of 1–25 ng per sample. Cross-reactivity of the anti–C5a des-Arg to C3a des-Arg, C5a des-Arg, C5a, and C5 is <0.22%, <0.375%, 100%, and 100%, respectively. Measurement of C5 is prevented by removing it with a specific precipitation step. C5a des-Arg can be accurately determined over the range of 0.256–10 ng per sample. The Bb enzyme-linked immunosorbent assay uses a monoclonal antibody directed against the Bb cleavage product of factor B as described by Kolb et al.21 A standard curve from 0.0 to 0.22 µg Bb was linear (r²=0.98) and was used for Bb determinations in appropriately diluted samples. The SC5b-9 enzyme-linked immunosorbent assay uses a monoclonal antibody directed against a neoantigen of SC5b-9. A standard curve from 0.0 to 153.0 ng SC5b-9 was linear (r²=0.99) and was used for SC5b-9 determinations in appropriately diluted samples.

Creatine kinase enzyme assay. The creatine kinase activity of pulmonary effluent or lymphatic fluid samples was assessed using the procedure developed by Oliver,22 as modified by Szasz et al.23 to include N-acetyl cysteine. The enzyme assay was purchased in kit form (procedure 47-UV) from Sigma. The principle of the assay is based on an increase in the absorbance of the reaction mixture at 340 nm as a result of the equimolar reduction of NAD to NADH. The rate of change in absorbance is directly proportional to the creatine kinase activity. One unit is defined as the amount of enzyme that produces 1 µmol NADH/min under the conditions of the assay procedure.

Determination of myocardial water, sodium, potassium, and calcium content. At the termination of the experiment, the heart was removed from the Langendorff perfusion apparatus and sectioned parallel to the atrioventricular groove. The apical portion, approximately 5 mm thick, was discarded. For the determination of myocardial water content, the next most apical section, 3–4 mm thick, was blotted dry, weighed, and dried to a constant weight in a 70°C oven.

For the determination of tissue electrolyte content, the second most apical section, weighing 0.5–0.8 g, was frozen at −20°C until all of the specimens were collected. The samples were thawed, minced, and placed into preweighed glass vials. The samples were dried at 150°C for 72 hours, and the dry tissue weight was determined. Five milliliters of HNO₃ acid was added to each vial, and the tissue was digested for 4 days, after which the samples were diluted to 10.0 ml in volumetric flasks (stock samples). Sodium and potassium determinations were performed using a model 143 flame photometer (Instrumentation Laboratory, Boston). The test samples consisted of 0.5 ml stock solution diluted to 10.0 ml using the appropriate dilution medium for the instrument. Calcium determinations were performed using an atomic absorption spectrophotometer (model AA375, Varian, Sunnyvale, Calif.). Stock samples were filtered to remove any particulate matter and appropriately diluted with distilled water.

Radioabeled albumin studies. Radiolabeled albumin was used to assess vascular integrity and to measure the albumin space of the hearts. The normal perfusate that contained 0.25% BSA was supplemented with 125I-BSA (0.025 µCi/ml; specific activity, 3.81 µCi/µg; NEN Du Pont, Boston) added to the reservoir concomitantly with the first plasma addition. Therefore, the 125I-BSA was present in the perfusate for the entire 70-minute protocol. The perfusate reservoir was sampled after 125I-BSA addition, and the lymphatic fluid and perfusate were sampled every 10 minutes thereafter. At the end of the protocol, the heart was perfused for 5 minutes with unlabeled perfusate to clear the vasculature of 125I-BSA, blotted dry, and weighed. The radioactivity of each sample and the whole heart was quantitated using a gamma counter (model 5550, Packard Instrument Co., Inc., Meriden, Conn.). The albumin space was calculated using the 125I-BSA content of the perfusate at 70 minutes and the following formula: (cpm/g heart)/(cpm/ml perfusate)=ml/g heart. The 125I-BSA was prepared the day it was received in the laboratory, diluted into appropriate single-use aliquots, and frozen at
&lt;20°C until used. All experiments were performed within 28 days of purchase to minimize possible degradation and liberation of free [22].

**Treatment with recombinant human CSa.** Recombinant human CSa (rhCSa, Sigma) was reconstituted into phosphate-buffered saline (pH 7.4) containing 0.25% BSA to prevent adsorptive loss. Single-use aliquots were frozen in liquid nitrogen and stored at &lt;20°C until used. Hearts were treated with rhCSa by addition of the peptide to the perfusate reservoir.

**Plasma C8 depletion by immunoaffinity column.** A C8 immunoaffinity column was prepared and used to deplete human plasma of C8. Goat anti-human C8 was obtained from Quidel. The antiserum possessed the following characteristics. Anti-C8 antiserum was judged to be monospecific when tested at various concentrations against normal human serum/plasma by double immunodiffusion, one-dimensional immunoelectrophoresis, and rocket immunoelectrophoresis. The antiserum cross-reacts strongly with C8 of baboon, dog, rabbit, and guinea pig. It cross-reacts weakly with C8 of horse, cat, hamster, and mouse. There is no cross-reactivity with C8 of cow, sheep, or chicken.

Antiserum against human C8 (2 ml at 0°C) was diluted to 4.0 ml with distilled water and saturated to 50% with ammonium sulfate. The solution was centrifuged (27,200g for 40 minutes, 4°C), and the supernatant was discarded. The immunoalbumin G/protein pellet was dissolved into 2.0 ml Dulbecco’s phosphate-buffered saline (DPBS, pH 7.4) and added to a 50% solution containing 17 ml washed 1.1-carboxyldiimidazole–activated agarose (Sigma) in DPBS. The solution was mixed at 4°C for 36 hours. Protein binding to the agarose was assessed by monitoring the absorbance at 280 nm of supernatant samples. After binding, the agarose was degassed and poured into a column (15 mm in diameter). Unbound sites were quenched with 1 M glycine treatment (3.5 hours, 4°C). The column was equilibrated with 1 M NaCl and stored until use or equilibrated with running buffer (DPBS with no added calcium or magnesium and containing 4.0 mM EDTA, pH 7.4).

Plasma to be depleted of C8 was obtained from human blood anticoagulated with EDTA (4.0 mM) by centrifugation at 2,000g for 10 minutes. The immunoaffinity column procedure was performed at 4°C. Chilled EDTA-plasma (9.5 ml) was applied to the column, recirculated over the column for 3 hours at a flow rate of 20 ml/hr, and collected from the column into a final volume of approximately 20 ml. Aliquots of the plasma were taken before and after treatment on the column to test for C8 depletion using the hemolysis assay.

A plasma sample depleted of C8 was used immediately in an isolated rabbit heart experiment or was frozen in liquid nitrogen overnight and used the next morning immediately before use. Plasma samples were treated with heparin (15 units/ml), calcium (8.0 mM), and magnesium (0.5 mM) to saturate the EDTA and to prevent fibrin formation as a consequence. As were NHP and HIHP samples, C8-depleted samples were filtered through a C-18 cartridge on addition to the perfusate.

**Plasma C8 depletion by anti-human C8 antibody in solution.** EDTA-plasma was obtained as described for the immunoaffinity column method. Antiserum to C8 was added to the chilled plasma (1:10 antiserum to plasma), incubated for 1 hour on ice, and used immediately. The samples were treated with heparin, calcium, and magnesium, as described, and filtered on addition to the perfusate. Aliquots of the plasma were taken before and after treatment with the C8 antiserum for testing in the red blood cell hemolysis assay.

**Hemolysis assay.** Complement-mediated red blood cell hemolysis was assessed using a turbidometric technique similar to those described previously. Two milliliters of blood was collected from the central artery of a rabbit ear into a heparinized syringe and then centrifuged (500g for 6 minutes, room temperature). The platelet-rich plasma anduffy coat layers were discarded, and the red blood cells were washed three times in 5 ml DPBS, pH 7.4. A solution of 10% erythrocytes was prepared in assay buffer (DPBS containing 0.25% BSA, pH 7.4). The assay was performed in a FAP-4 platelet aggregometer (Bio/Data Corp., Hatboro, Pa.) using micromolecule cuvettes and a stir speed of 1,200 rpm. One hundred percent light transmission was set using a solution of 0.5% red blood cells lysed with diluted plasma (1:1 in assay buffer). Plasma was diluted appropriately with assay buffer to a volume of 300 μl and warmed to 37°C. The assay was initiated by the addition of 15 μl of the 10% red blood cell solution (0.5% final dilution). Red blood cell lysis resulted in an increase in light transmission that was monitored for up to 16 minutes.

Plasma samples treated with EDTA for use in the C8 depletion studies were handled specially for use in the lysis assay. Before use, the EDTA-anticoagulated (4.0 mM) plasma was treated with heparin (15 units/ml), calcium (8.0 mM), and magnesium (0.5 mM). Heparin was added to prevent fibrin clot formation on addition of the calcium and magnesium needed to saturate the EDTA, thereby providing these necessary ions for complement activation. In addition, sample recirculation on the C8 affinity column resulted in dilution of the plasma. Therefore, dilution of the plasma in the lysis assay was adjusted so that comparable amounts of plasma proteins were present before and after treatment on the column.

**Statistical Analysis**

The data presented in the texts, figures, and tables represent the mean ± SEM for the indicated number of determinations. All comparisons of parameters measured over time were performed using two-way repeated-measures analysis of variance to test for group differences over time. If significance was detected (p ≤ 0.05 for the associated F value), comparisons among groups at each individual time point were performed to determine at which time points differences existed. If more than two groups were compared with a control group, the analysis was performed using Dunnett’s t test for the appropriate number of comparisons. If only two groups were compared, the nonpaired Student’s t test was used. Therefore, statistical significance as denoted by an asterisk represents a value of p ≤ 0.05 for Dunnett’s or Student’s t test after a significant repeated-measures analysis of variance. Similarly, comparison of parameters not determined over time were performed using a factorial analysis of variance followed by Dunnett’s t test if more than two groups
Results

Effects of Complement Activation on the Isolated Perfused Rabbit Heart

Effect of complement activation on the systolic and diastolic pressures of the heart. The systolic and diastolic pressures of the hearts perfused with buffer alone (Figure 1A) were characteristic for hearts subjected to the degree of hypoxia that was used. With the intraventricular balloon inflated to achieve an end-diastolic pressure of 12–15 mm Hg, the systolic pressures generated were typically 60–70 mm Hg. The positive and negative values for dP/dt were generally 750–900 and 375–475 mm Hg/ sec, respectively (Table 1). Under the hypoxic conditions, hearts could be maintained for 100 minutes with little deterioration in the recorded parameters.

Complement activation occurs when NHP is added to the perfusate (see below). Therefore, the ideal control is treatment with HIHP. Data from the other treatments were compared with those obtained with HIHP. When the hearts perfused with buffer alone were compared with those treated with HIHP, no differences in the diastolic or systolic pressures (Figure 1A) or in the rates of contraction or relaxation (Table 1) were noted. Although hypoxia was maintained throughout the 70-minute protocol, the diastolic and systolic pressures and the rates of contraction and relaxation (Table 1) remained essentially unchanged for the duration of the experiment. Four experimental treatments involved the addition of 6% heparinized plasma to the perfusate (three additions of 2% each at 0, 6, and 13 minutes). Regardless of the type or species of plasma or the presence or absence of an inhibitor of complement activation, each 2% addition typically caused a transient increase in the developed pressure (3–10 mm Hg) and an associated increase in the positive and negative dP/dt that resolved within 3–4 minutes. Addition of the plasma often caused the diastolic pressure to decrease.

Figure 1. Graphs showing diastolic and systolic pressures recorded from the isolated rabbit heart perfused with the indicated medium. FUT-175, an inhibitor of complement activation. Panel A: Data from hearts perfused with buffer alone and with heat-inactivated human plasma. The triangles represent data from hearts perfused with buffer alone (no plasma, n=6). The circles represent data from hearts perfused with 6% heat-inactivated human plasma (n=21). This treatment served as the control, to which the functional parameters of all other treatments were compared. There were no differences vs. 6% heat-inactivated human plasma at any time point. Panel B: Data from hearts perfused with 6% normal rabbit plasma (n=4). With the exception of the baseline diastolic pressure, there were no differences vs. 6% heat-inactivated human plasma. Panel C: Data from hearts perfused with 6% normal human plasma (n=24). The diastolic and systolic pressures increased, and the developed pressure decreased starting at the 20-minute time point. Panel D: Data from hearts perfused with 6% normal human plasma + 18 μM FUT-175 (n=6). The inclusion of FUT-175 in the perfusate inhibited the increase in diastolic and systolic pressures and the decrease in developed pressure induced by 6% normal human plasma. *p≤0.05 vs. 6% heat-inactivated human plasma at the same time point.
The systolic and diastolic pressures and positive and negative dP/dt of hearts perfused with 6% normal rabbit plasma (Figure 1B, Table 1) generally were not different from those of hearts perfused with 6% HIHP (Figure 1A, Table 1). The diastolic pressure was higher in the hearts treated with 6% normal rabbit plasma at time zero but not at any later time point. No difference in the systolic pressure existed between the groups at any time point. The preparations remained relatively stable but exhibited a trend toward an increase in the end-diastolic pressure near the end of the protocol. These data indicate that treatment with homologous plasma was largely without effect on the function of isolated rabbit hearts, even in the presence of relative hypoxia.

Hearts treated with 6% HIHP served as the control group. Addition of 6% HIHP did not affect the systolic and diastolic pressures of the hearts. The end-diastolic pressure remained constant throughout the protocol as did the systolic pressure (Figure 1A). The positive and negative dP/dt both increased slightly throughout the course of the experiment (Table 1). Hearts treated with 6% HIHP exhibited the same general profile of systolic and diastolic changes as hearts perfused with normal buffer. The addition of 6% heterologous (human) HIHP was without adverse effects on the function of the isolated perfused rabbit heart.

The changes in the systolic and diastolic pressures of the isolated perfused heart in response to treatment with 6% NHP are depicted in Figure 1C. In contrast to the previous data, perfusion with 6% NHP led to significant changes in the functional parameters of the hearts. Approximately 7–12 minutes after adding the final 2% NHP, the end-diastolic pressure began to rise sharply, reaching a maximum of 83 mm Hg by the 50-minute time point. There was a concomitant rise in the systolic pressure, which reached a maximum of 101 mm Hg also at the 50-minute time point. The increase in the systolic pressure can be attributed to the fixed fluid volume of the intraventricular balloon and the rising end-diastolic pressure. Importantly, as the end-diastolic pressure increased, the developed pressure decreased from a maximum of 55 mm Hg at 13 minutes to a minimum of 15 mm Hg at 70 minutes. In addition, the rates of contraction and relaxation decreased to approximately one third of their maximal values by the end of the protocol (Table 1). Perfusion of the isolated rabbit heart with 6% NHP resulted in a decrement of function characterized by an increase in myocardial stiffness and a state of partial contracture. A representative recording is presented in Figure 2.

It has been documented that rabbit tissue activates the human complement system.36-37 Therefore, the possibility was investigated that the effects caused by adding 6% NHP were a result of complement activation. The protease inhibitor and known inhibitor of complement activation, FUT-175, was used to prevent complement activation. Hearts treated with 6% NHP in the presence of 18.0 μM FUT-175 did not exhibit a decline in function (Table 1). With the exception of the baseline systolic pressure, none of the functional parameters differed from those of hearts treated with 6% HIHP (Figure 1D). However, the end-diastolic pressure increased slightly during the last 20 minutes of the protocol. The data suggest that complement activation in hearts treated with 6% NHP was responsible for the observed decline in the functional parameters. Complement activation and the resulting effects can be blocked by the inhibitor FUT-175.
Effect of complement activation on coronary perfusion pressure. The perfusion pressure was measured as an assessment of coronary vasomotor tone (Figure 3). In any treatment group, except the 6% NHP group, the perfusion pressure at any time point was not different from that of the 6% HIHP group. The perfusion pressure of hearts treated with 6% NHP began to rise approximately 5–7 minutes after the final 2% plasma addition and continued to rise through the end of the protocol, increasing threefold by 70 minutes. An increase in the perfusion pressure represents an increase in vasomotor tone of the coronary arteries. The inhibitor of complement activation, FUT-175, countered the increase in perfusion pressure, suggesting that the increase on coronary artery vasomotor tone was the result of complement activation.

Effect of complement activation on the rate of lymphatic fluid formation. The rate at which lymphatic fluid dripped from the apex of the hearts was monitored (Figure 4). In hearts treated with 6% NHP, lymphatic fluid flow increased concomitantly with the decline in functional parameters and the increase in perfusion pressure. Lymphatic flow was increased approximately fivefold from the baseline value of 1.3±0.1 ml/min by the 70-minute time point. Lymphatic flow in the remaining treatment groups was not different from that of hearts treated with HIHP. The protease inhibitor FUT-175 was effective at preventing the increase in lymphatic fluid flow as compared with control hearts.

Effect of the Complement Titer on the Response to Complement Activation

The effect of the complement titer of the perfusate was examined by treating hearts with 3% NHP. At this concentration, the complement titer was too low to facilitate pathological changes similar to those observed with 6% NHP. Four of the five hearts underwent little change in the diastolic, systolic, or perfusion pressures (Figure 5). However, one heart underwent changes that were characteristic of and similar in magnitude to those observed with 6% NHP. Thus, the grouped data show a small increase in the mean diastolic and perfusion pressures, with relatively large standard errors. The values for the positive dP/dt were not different at any time point from those of hearts treated with 6% HIHP (Table 1). In comparison with the same group, the values for the negative dP/dt were slightly decreased at the 60- and 70-minute time points. The lymphatic fluid flow rate was measured in only two hearts treated with 3% NHP. The mean flow rate increased from 1.6 ml/min at baseline to 2.2 ml/min at the 70-minute time point.

Assessment of Complement Activation in the Isolated Perfused Rabbit Heart

Generation of the anaphylatoxins C3a and C5a. The concentrations of the anaphylatoxins C3a and C5a (and their breakdown products C3a des-Arg and C5a des-Arg) generated as a result of complement activation...
were monitored in the pulmonary artery effluent of isolated hearts treated with 6% NHP or 6% HIHP. In hearts treated with 6% NHP, generation of C3a des-Arg occurred by the 20-minute time point. Continued C3a generation through the remainder of the protocol was evident as indicated by a further increase in the C3a des-Arg concentration at the 40- and 70-minute time points (Figure 6, left panel). In contrast, a low concentration of C3a des-Arg was detected at the 20-minute time point in hearts treated with 6% HIHP, and no increase in the C3a des-Arg concentration occurred thereafter. The time course of C5a generation in hearts treated with 6% HIHP was similar to that observed for C3a. Significant C5a des-Arg generation was evident by the 20-minute time point, and the concentration continued to rise during the remainder of the protocol (Figure 6, right panel). In hearts treated with 6% HIHP, a low concentration of C5a des-Arg was present at 20 minutes of the protocol, and it did not increase thereafter.

**Generation of the activated complement component Bb.** The Bb molecule is released into solution on degradation of the alternative pathway C3 convertase. Appearance of the Bb molecule was monitored in the lymphatic fluid collected from the cardiac apex (Figure 7, left panel). Appropriate corrections were made for variations in the lymphatic fluid flow rate among the groups. In hearts treated with 6% NHP, the appearance of Bb was evident by 30 minutes, and its formation was increased further by the 70-minute time point. In contrast, no generation of Bb over time was measured in hearts treated with 6% HIHP. The addition of 18.0 μM FUT-175 to the perfusate containing 6% NHP blocked generation of Bb. The formation of Bb in hearts treated with 6% NHP documents alternative pathway complement activation in those hearts but does not rule out classical pathway activation as well.

**Generation of the complement component SC5b-9.** Formation of the soluble form of the membrane attack complex, SC5b-9, is indicative of activation of the entire complement system and assembly of the terminal components. The appearance of SC5b-9 was assessed in the lymphatic fluid collected from the hearts (Figure 7, right panel). Appropriate corrections were made for variations in the lymphatic fluid flow rate among the groups. An increase in SC5b-9 formation was measured in lymphatic fluid from hearts treated with 6% NHP at both the 30- and 70-minute time points. No generation of SC5b-9 was detected in hearts treated with 6% HIHP. Treatment of the perfusate that contained NHP with 18.0 μM FUT-175 resulted in the inhibition of formation of the SC5b-9 complex. Formation of the SC5b-9 complex in hearts treated with NHP indicates the likelihood of simultaneous membranolytic C5b-9 complex formation in those hearts.

**Tissue Damage Mediated by Complement Activation**

**Tissue electrolyte content.** Several additional studies were performed to understand more fully the mechanisms of complement-mediated myocardial damage. Tissue injury was assessed by measuring the electrolyte content of the cardiac tissue at the end of the 70-minute protocol. Hearts treated with buffer or 6% HIHP had similar tissue contents of total sodium, potassium, and calcium. Treatment with 6% NHP resulted in accumulation of sodium and calcium and a loss of tissue potassium. Calcium accumulation was proportionally larger than either the accumulation of sodium or the loss of potassium. The addition of FUT-175 to the perfusate attenuated but did not prevent the changes in electrolyte content resulting from treatment with NHP. While FUT-175 only partially inhibited the electrolyte alterations, it almost prevented the deterioration of
function resulting from treatment with NHP, indicating that another mechanism is partially responsible for the loss of systolic and diastolic function. The tissue electrolyte content is summarized in Table 2.

The observed changes in electrolyte content were not due to the accumulation of edema fluid. The total water content of the samples was 85.2±0.3%, 83.9±0.3%, 86.1±0.6%, and 85.7±0.3% for the normal buffer (n=6), 6% HIHP (n=10), 6% NHP (n=8), and 6% NHP plus FUT-175 (n=6) groups, respectively. The values for all four groups were similar, and the magnitude of the electrolyte alterations cannot be explained by accumulation of edema fluid by the myocardial tissue.

Myocardial creatine kinase release. The release of creatine kinase from the myocytes into the pulmonary artery effluent was monitored as a marker of damage to the plasmalemma of myocytes. Treatment with 6% NHP resulted in increased release of creatine kinase from the myocytes when compared with hearts treated with 6% HIHP. The increase began between 20 and 30 minutes of treatment and continued to rise for the remainder to the protocol. The creatine kinase activity in pulmonary artery fluid from hearts treated with 6% HIHP remained very low throughout the protocol but did begin to increase slightly at the 60- and 70-minute time points (Table 3).

Radionabeled albumin extravasation and sequestration. Radionabeled albumin was used to assess the integrity of the vasculature and the myocytes. Labeled albumin was added to the perfusate of hearts treated with 6% HIHP or 6% NHP, and its equilibration into the lymphatic fluid was

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**Figure 6.** Graphs showing generation of the anaphylatoxins C3a and C5a. Left panel: Appearance of activated complement component C3a des-Arg in the pulmonary effluent of isolated perfused hearts. The C3a des-Arg concentration was elevated by 20 minutes and continued to rise thereafter in hearts treated with 6% normal human plasma. *p≤0.05 vs. 6% heat-inactivated human plasma at the same time point. Right panel: Appearance of activated complement component C5a des-Arg in the pulmonary effluent of perfused isolated hearts. The C5a des-Arg concentration was elevated by 20 minutes and continued to rise thereafter in hearts treated with 6% normal human plasma. *p≤0.05 vs. 6% heat-inactivated human plasma at the same time point.

**Figure 7.** Graphs showing appearance of activated complement components Bb and SC5b-9 in the lymphatic effluent of isolated perfused hearts. The Bb generation (left panel) and SC5b-9 generation (right panel) were corrected for dilution due to lymphatic flow differences among groups. Bb and SC5b-9 generation in hearts treated with 6% normal human plasma began by 30 minutes, increased greatly by 70 minutes, and was inhibited by the addition of protease inhibitor FUT-175. *p≤0.05 vs. 6% heat-inactivated plasma at the same time point.
The labeled albumin equilibrated rapidly into the lymphatic fluid. By the 10-minute time point in both treatment groups, the lymphatic fluid contained nearly 90% of the perfusate content of labeled albumin. At the later time points, this figure rose to above 95% in both treatment groups, but the labeled albumin had a slightly higher access to the lymphatic fluid in hearts treated with 6% NHP. Values for the ratio of lymphatic fluid to perfusate were (for 6% NHP versus 6% HIHP) 0.89±0.03 versus 0.86±0.01 cpm/ml, 0.93±0.01 versus 0.91±0.01 cpm/ml, 0.96±0.004 versus 0.92±0.01 cpm/ml (p<0.05), 0.97±0.004 versus 0.92±0.02 cpm/ml (p<0.05), 0.98±0.005 versus 0.92±0.02 cpm/ml (p<0.05), 0.96±0.17 versus 0.91±0.02 cpm/ml, and 0.97±0.005 versus 0.92±0.02 cpm/ml (p<0.05) at 10, 20, 30, 40, 50, 60, and 70 minutes, respectively. Based on the albumin content (in counts per minute per milliliter) and the rate of lymphatic fluid formation (in milliliters per minute), the rate of albumin extravasation was calculated. The rate of extravasation in hearts treated with 6% NHP rose sharply from 29.8±5.2 to 286.1±53.2 and 305.3±71.2 (cpm×10^3/min) at 10, 40, and 70 minutes, respectively. In contrast, the rate in hearts treated with 6% HIHP remained unchanged throughout the protocol: 23.6±2.2, 17.2±5.0 (p<0.05 versus 6% NHP), and 23.4±3.7 (p<0.05 versus 6% NHP) cpm×10^3/min at 10, 40, and 70 minutes, respectively. The data suggest that the increased rate of albumin extravasation was due primarily to a general increase in lymphatic fluid formation and not an increase in the selective permeability to the labeled albumin.

In addition, the total albumin space was calculated for each heart at the end of the protocol. The albumin space for hearts treated with 6% HIHP was 0.19±0.01 ml/g wet wt and was significantly increased to 0.37±0.03 ml/g wet wt in hearts treated with 6% NHP. The increase in the total albumin space cannot be accounted for by the presence of edema fluid or an increase in interstitial fluid that is due to a relative lack of contractile function. Two hearts treated with 6% HIHP and electrically fibrillated at 30 minutes to prevent rhythmic contraction and allow for interstitial fluid accumulation had a mean albumin space of 0.14 ml/g wet wt.

### Table 2. Myocardial Tissue Electrolyte Content

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>[Na+] (μmol/g dry wt)</th>
<th>[K+] (μmol/g dry wt)</th>
<th>[Ca²⁺] (μmol/g dry wt)</th>
</tr>
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<tbody>
<tr>
<td>Buffer alone</td>
<td>6</td>
<td>504±14</td>
<td>198±7</td>
<td>15.6±3.0</td>
</tr>
<tr>
<td>6% HIHP</td>
<td>10</td>
<td>498±16</td>
<td>190±10</td>
<td>18.4±2.6</td>
</tr>
<tr>
<td>NHP</td>
<td>9</td>
<td>666±46*</td>
<td>118±9*</td>
<td>47.3±10.8*</td>
</tr>
<tr>
<td>NHP+FUT-175</td>
<td>6</td>
<td>624±27*</td>
<td>152±7*</td>
<td>38.1±4.9*</td>
</tr>
</tbody>
</table>

n, Number of samples; HIHP, heat-inactivated human plasma; NHP, normal human plasma; FUT-175, a complement inhibitor. *p<0.05 vs. 6% HIHP.

### Table 3. Creatine Kinase Activity in Pulmonary Effluent From Isolated Rabbit Heart

<table>
<thead>
<tr>
<th>Time points (minutes)</th>
<th>n</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
<th>60</th>
<th>70</th>
</tr>
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<tbody>
<tr>
<td>NHP CK activity (units/l)</td>
<td>5</td>
<td>31±12</td>
<td>59±33</td>
<td>151±61</td>
<td>547±246</td>
<td>1,012±391</td>
<td>1,684±586*</td>
<td>2,502±814*</td>
<td>2,927±800*</td>
</tr>
<tr>
<td>6% HIHP CK activity (units/l)</td>
<td>5</td>
<td>22±6</td>
<td>35±9</td>
<td>55±16</td>
<td>84±28</td>
<td>113±40</td>
<td>141±51</td>
<td>209±78</td>
<td>277±108</td>
</tr>
</tbody>
</table>

n, Number of samples; NHP, normal human plasma; CK, creatine kinase; HIHP, heat-inactivated human plasma. *p<0.05 vs. 6% HIHP CK activity.
major role in the generation of the complement-dependent effects in this model.

Effects of C8-depleted plasma on the isolated perfused rabbit heart. The contribution of the membrane attack complex to the genesis of the complement-dependent effects in the rabbit heart was investigated using human plasma deficient in C8. Activation of the complement system in human plasma specifically depleted of C8 results in generation of the anaphylatoxins, however, but prevents formation of the C5b-9 complex.37,38 Human plasma was depleted specifically of C8 using a C8 immunoaffinity column or by C8 absorption to an anti-human C8 antibody in solution. C8 depletion was assessed using a hemolysis assay (Figure 9). A loss of hemolytic activity due to C8 depletion and the ability to reconstitute hemolytic activity by the addition of purified C8 were determined for each experiment. Reconstitution of the hemolytic activity by the addition of purified C8 demonstrated that the remainder of the complement system remained intact through the C8-depletion process.

The systolic and diastolic pressures of hearts treated with 6% C8-depleted human plasma (Figure 9) were similar to those treated with 6% HIHP (Figure 1A).

**Figure 8.** Left panel: Graph showing effects of 6% zymosan-activated plasma (ZAP) + 2.0 mM Plummer's inhibitor on the isolated perfused rabbit heart (n=6). The addition of 6% ZAP caused a transient increase in the perfusion pressure and developed pressure and had no effect on the diastolic pressure. The prolonged increase in the diastolic and perfusion pressures and decrease in developed pressure observed on treatment with 6% normal human plasma (see Figure 1C) were not replicated. There were no differences vs. 6% heat-inactitated human plasma at any time point. Right panel: Graph showing effects of recombinant human C5a (rhC5a) on the isolated perfused heart (n=3). At a concentration of 6.4 nM, rhC5a did not alter the coronary perfusion pressure, left ventricular end-diastolic pressure, or systolic pressure from baseline values.

**Figure 9.** Left panel: Graph showing effects of 6% C8-depleted plasma on the isolated perfused rabbit heart (n=6). The data are combined from experiments in which C8 depletion was obtained by using an immunoaffinity column (n=1) or by treatment with anti-C8 antibody in solution (n=5). See text for details. Treatment with 6% C8-depleted plasma did not affect the diastolic, developed, or perfusion pressures in a manner similar to that of 6% normal human plasma (see Figures 1C and 3). *p≤0.05 vs. 6% heat-inactitated plasma at the same time point. Right panel: Tracings showing the results obtained with human complement-mediated rabbit red blood cell hemolysis. Tracing A shows hemolysis mediated by control plasma at dilutions ranging from 1:20 to 1:2. Tracing B shows hemolysis mediated by plasma depleted of C8 using a C8 immunoaffinity column. Panel C shows hemolysis mediated by C8-depleted plasma reconstituted with purified human C8 to 2 μg/ml.
Only the systolic pressure at the 60- and 70-minute time points was significantly elevated. No increase in the diastolic or perfusion pressures was noted. The positive dP/dt values increased slightly from 902±47 mm Hg/sec at baseline to 1,000±31 mm Hg/sec at 70 minutes. The negative dP/dt values increased slightly from 519±20 mm Hg/sec at baseline to 723±29 mm Hg/sec at 70 minutes. The values for positive or negative dP/dt were not decreased at any time point when compared with those of hearts treated with 6% HIHP (Table 1). The rate of lymphatic fluid formation decreased from 0.6±0.1 ml/min at baseline to 0.3±0.04 ml/min at 70 minutes. The increase in lymphatic fluid flow obtained on treatment with 6% NHP plasma (Figure 3) was not reproduced by treatment with 6% C8-depleted plasma. The lack of complement-mediated effects observed on treatment with C8-depleted human plasma implicates a role for the membrane attack complex in the genesis of the functional changes observed on perfusion with 6% NHP.

Discussion

Summary of the Observations Demonstrating Complement-Mediated Myocardial Tissue Injury

The direct effects of the activated complement components on the function of the heart and the associated myocardial tissue injury that occurs as a result of complement activation were investigated. Treatment with 6% normal autologous plasma or 6% heterologous (human) plasma in which the complement system had been destroyed by heat inactivation had no effect on the functional parameters, the coronary perfusion pressure, or the rate of lymphatic fluid flow of the hearts. In contrast, treatment with 6% heterologous (human) plasma containing an intact complement system resulted in a delayed alteration of the measured parameters. Seven to 15 minutes after the third addition of 2% plasma (to achieve 6% total), the end-diastolic pressure rose sharply over a 25–30-minute period. The developed pressure decreased concomitantly with the rise in diastolic pressure. The changes in the systolic and diastolic pressures were associated with, but not preceded by, an increase in the coronary perfusion pressure and an increase in the rate of lymphatic fluid formation. Because similar alterations were not evident when the protease inhibitor FUT-175 (18 μM) was included in the perfusion medium, the complement system was strongly implicated in the genesis of the responses to 6% NHP. Demonstration of complement activation was achieved by measuring the generation of activated complement components Bb, C3a, C5a, and SC5b-9. In hearts treated with 6% NHP, generation of all four of the activation products occurred with a similar profile over time. The inhibitor of complement activation, FUT-175, prevented Bb and SC5b-9 generation. Activated complement components were not detected when hearts were exposed to 6% HIHP.

Complement activation resulted in alterations in the total tissue electrolyte content. An accumulation of sodium and calcium and a loss of potassium were measured in tissue from hearts treated with 6% NHP. The complement inhibitor FUT-175 partially attenuated the changes in tissue electrolyte content. Complement activation in hearts treated with 6% NHP resulted in the release of creatine kinase from the myocytes into the pulmonary artery effluent. In addition, the radiolabeled albumin space of hearts treated with 6% NHP was approximately twice that of hearts treated with 6% HIHP. The results are in agreement with the concept that complement activation causes the loss of membrane function and/or integrity, allowing for the transmembrane movement of electrolytes and some proteins. The findings are consistent with the hypothesis that complement activation directly mediates myocardial tissue damage through generation of the cytolytic form of the membrane attack complex.

To further investigate the potential role of the membrane attack complex as a mediator of myocardial tissue injury, several experiments were performed to differentiate the effects of the anaphylatoxins from those of the membrane attack complex. If the associated formation of anaphylatoxins caused the complement-mediated effects, then the observed changes should be reproduced on administration of the anaphylatoxins. The administration of 6% zymosan-activated human plasma did not elicit effects similar to those observed with 6% NHP. Zymosan activation of the human complement was performed in the presence of the carboxypeptidase N inhibitor (Plummer’s inhibitor, 2.0 mM). This compound prevents degradation of the generated anaphylatoxins. Similarly, the administration of 55 ng/ml (6.4 nM) of rhC5a did not elicit the effects mediated by complement activation. The results suggest that the human anaphylatoxins play a minimal role in the genesis of the complement-mediated effects in the isolated rabbit heart.

The role of the membrane attack complex was investigated further by administering 6% C8-depleted human plasma. Depletion of the C8 component blocked the formation of the membrane attack complex but left the earlier portion of the complement system functional. This was demonstrated using a hemolysis assay. The functional effects caused by treatment with 6% NHP were not reproduced on treatment with 6% C8-depleted human plasma, suggesting a primary role for the membrane attack complex in their genesis. The findings are consistent with the recognition that C8 binding to C5b-7 completes the C5b-8 complex. The latter inserts into the cell membrane and acts as a receptor for C9 as well as a catalyst for C9 polymerization to yield the highly effective C5b-9 cytolytic membrane attack complex. The membrane attack complex is composed of C5b-8(C9)n, in which n may range from 1 to 18. The extent of the membrane lesion is dependent on the availability of the monomeric C9 and the number of individual transmembrane channels that form.38

Pathway, Site, and Extent of Complement Activation

Activation of the complement system in human plasma by contact with rabbit myocardial tissue is not unexpected. Platts-Mills and Ishizaka36 demonstrated that human serum caused the hemolysis of unsensitized rabbit red blood cells as the result of activation of the alternative pathway of complement. The ability of rabbit lung tissue to activate the human complement system was demonstrated by Seeger et al.,37 who used isolated, ventilated, blood-free perfused rabbit lungs in a model very similar to the heart model used for the present
studies. Activation of the human complement system in the heart model used here was documented by measuring the generation of Bb, C3a, C5a, and SC5b-9. The Bb molecule is released into solution from the alternative pathway C3 convertase on its degradation by factor H, decay accelerating factor, or CR1.38,39 The generation of large amounts of the Bb molecule indicates that the alternative pathway of the human complement system was activated by contact with rabbit cardiac tissue. In contrast to guinea pig models of complement-mediated myocardial anaphylaxis,40,41 the present study did not use previous sensitization of the hearts with antiserum or challenge with antigen to promote activation of the classical pathway. However, simultaneous activation of the classical pathway of complement cannot be excluded. Preliminary data (not shown) indicated an appearance in the lymphatic fluid of low concentrations of the C4 breakdown product, C4d, a component of the complement system generated on activation of the classical pathway. Such activation of the classical pathway may be due to the presence of low levels of heterophilic antibodies to rabbit antigens found in most human serum. It is likely that human complement activation in the isolated perfused rabbit heart model occurs primarily through the alternative pathway, but with some contribution from the classical pathway. Regardless of the extent of their individual contribution, activation of either pathway of the complement system results in the formation of C3a, C5a, and the membrane attack complex. The data support the contention that it is the latter component of complement activation that results in myocardial damage as assessed in the present study.

Activation of the human complement system by rabbit red blood cells46 and rabbit lung tissue37 suggests that the phenomenon can be elicited by a variety of, if not all, rabbit tissues. In the model described, complement activation would be expected to occur initially on the luminal surface of the endothelium. Early intravascular activation of human complement was documented by the appearance of C3a and C5a in the pulmonary artery effluent at the 20-minute time point. The loss of myocyte integrity and the resulting release of creatine kinase implicates complement activation in the interstitial fluid at the myocyte surface. The appearance of the SC5b-9 macromolecular complex in the lymphatic fluid at the 30-minute time point supports this premise. SC5b-9 formed as a result of intravascular complement activation would be excluded from the lymphatic fluid unless extensive damage to the vascular wall was present at the early time point. In addition, preliminary studies (data not presented) with immunohistochemical staining for the membrane attack complex document the presence of the complex in the vascular wall and also show extensive localization on the myocytes.

The extent of complement activation in the isolated heart system can only be estimated. By using published values38,42,43 for the plasma concentration of C3 and the molecular weights of C3 and C3a and assuming that the C3a concentration measured at 70 minutes was equilibrated with the total perfusate volume because of recirculation, it can be calculated that approximately 15% of the human C3 had been converted to C3a (or C3 des-Arg; the antibody used in the radioimmunoassay reacts with both forms of the molecule) by the end of the protocol. This also assumes that all of the C3a des-Arg generated remains in circulation and that there is no significant further proteolysis of C3a des-Arg.

Mechanisms of Complement-Mediated Tissue Damage

The mechanisms by which complement activation led to altered heart function have been only partially defined by these studies. Several experiments were performed to demonstrate that generation of the anaphylatoxins C3a and C5a did not lead to the observed changes in the systolic and diastolic pressures. No increase in the diastolic pressure or decrease in the developed pressure could be replicated by treatment with 6% zymosan-activated human plasma despite the inclusion of 2.0 mM Plummer's inhibitor30 to prevent degradation of the highly active anaphylatoxins to their less active des-Arg forms. The half-lives of the anaphylatoxins in the absence of the inhibitor is measured in seconds,44 but 2.0 mM Plummer's inhibitor protects against their degradation. Similarly, treatment with 6% C8-depleted human plasma did not induce systolic and diastolic pressure alterations. Reconstitution of hemolytic activity to the C8-depleted plasma by adding purified C8 indicates that the early components of the complement system were left intact during the depletion process. Therefore, it is plausible that activation of the early portion of the cascade and anaphylatoxin generation occurred when hearts were treated with the C8-depleted plasma. Finally, rabbit hearts were treated with 55 ng/ml (6.4 nM) rhC5a to verify further the lack of efficacy of the human anaphylatoxins. The concentration of C5a was 14-fold greater than the C5a concentration generated endogenously by the end of the 70-minute protocol and 27-fold greater than the C5a concentration at 20 minutes, when the changes in the systolic and diastolic pressures actually were occurring. The antibody used in the C5a radioimmunoassay also binds C5a des-Arg. Thus, the measured C5a concentrations generated on treatment with 6% NHP may be composed of substantial concentrations of C5a des-Arg, accentuating the differences between the concentrations of the added recombinant C5a and the C5a actually present when the measurement (that also detects C5a des-Arg) was performed.

The lack of response of the isolated rabbit heart to the human anaphylatoxins might appear incongruous in view of the well-described spasmodenic activity of the anaphylatoxins,45,46 However, our findings are not inconsistent with those of previous studies. Human C3a22,23 and C5a have been reported to cause coronary vasoconstriction and a negative inotropic effect in isolated buffer-perfused guinea pig hearts,29 whereas other investigations have reported a positive inotropic effect of the human anaphylatoxins on isolated guinea pig atria.47 Martin et al20 demonstrated that human C5a has no effect on isolated porcine coronary arteries20 and, along with Ito et al,21 that vasopressor and negative inotropic effects of C5a administered in vivo to the porcine heart is dependent on the presence of circulating neutrophils, platelets, or both. Circulating cellular blood elements were not present in the model used for our studies. Administration of the human anaphylatoxins to the canine heart in vivo has no effect until relatively high doses (6–12.5 μg) of C3a or C5a are
used, at which time a vasodilation is observed independent of any inotropic effect.\textsuperscript{48} Human C3a (0.5 μg/ml) causes relaxation of isolated precontracted canine coronary vascular strips.\textsuperscript{48} Thus, the effects of the human anaphylatoxins vary, depending on the species and the experimental model used. In the isolated rabbit pulmonary artery, human C5a induces vasoconstriction or vasodilation depending on the resting tension of the vascular segment.\textsuperscript{49,50} Furthermore, C3a or C5a may have no effect, depending on which portion of the pulmonary artery is used.\textsuperscript{51} Hugli and Marceau\textsuperscript{50} demonstrated that human C3a was inactive on rabbit vascular strips and that human C5a caused only minimal relaxation of these tissues.

The human anaphylatoxins did not alter the measured functional parameters or contribute to tissue injury in the isolated rabbit heart model used in these studies. This observation does not imply that the anaphylatoxins are without in vivo effects. The present experimental model is one in which circulating neutrophils are absent and is designed to examine the direct effects of complement activation on myocardial function. On the other hand, autologous C5a and zymosan-activated serum administered into the coronary circulation of the in vivo porcine heart cause an ischemic event, cardiac dysfunction, and intracardiac granulocyte sequestration.\textsuperscript{29} Therefore, it is likely that generation of the anaphylatoxins in the setting of ischemia and reperfusion\textsuperscript{20,52} contributes to myocardial tissue injury in vivo. It is possible, however, that the effects of the anaphylatoxins may not be fully expressed in present experiments because of insufficient concentrations being formed at the time of complement activation or because of insufficient amounts of rhC5a being added to the perfusion medium. It may not be possible to definitely conclude that anaphylatoxins did not play an important role in these studies.

Membrane attack complex–mediated lysis of nucleated cells exhibits multiple-hit characteristics.\textsuperscript{53} Nucleated cells have the ability to destroy or remove complement channels from their plasma membrane and can thereby recover from limited complement attack.\textsuperscript{53,54} Myocardial tissue damage would require sufficient formation of lytic membrane attack complexes, within the time frame of the experiment, to overcome the reparative mechanisms of the cells. The relative lack of functional damage observed, within the length of the protocol, on treatment with 3% NHP may be explained by these mechanisms.

**Membrane Attack Complex and In Vivo Myocardial Tissue Damage**

Several studies have demonstrated formation of the membrane attack complex in association with myocardial ischemia and necrosis. The plasma concentration of the SC5b-9 complex (noncytolytic form) was increased up to 32-fold 16 hours after acute myocardial infarction\textsuperscript{14} and subsequently increased again on reinfarction during hospitalization. Yasuda et al\textsuperscript{13} reported similar findings and demonstrated a correlation between the SC5b-9 concentration and the peak plasma creatine kinase concentrations. Immunohistochemical staining methods have localized the C5b-9 complex (membrane-bound form) to infarcted human heart\textsuperscript{15,16,55} or rat myocardial tissue.\textsuperscript{5} The results of the present study demonstrate the significance of these findings. The deleterious contributions that result from formation of the membrane attack complex in the setting of myocardial ischemia and reperfusion are poorly understood. Whether the membrane attack complex contributes to myocardial injury or merely localizes to previously irreversibly injured myocytes has been a point of uncertainty. The present findings suggest that formation of the C5b-9 complex on the myocardial cell causes the loss of myocyte integrity, leading to disturbances of the electrolyte balance, which include calcium accumulation, and also causes the release of intracellular constituents, which include creatine kinase, into the interstitial fluid. These events were associated with the development of a state of contracture. The functional and cellular changes associated with activation of the complement system resemble those that occur with ischemia and reperfusion. Thus, the membrane attack complex may play a significant role in the development of tissue injury, thereby providing a potential site for pharmacological interventions for the purpose of protecting the ischemic/reperfused heart.

The current investigation demonstrates that the membrane attack complex can contribute directly to the induction of myocardial tissue injury. Additional damage in vivo, in the presence of whole blood, may be mediated by the anaphylatoxins and by indirect damage as a result of a complement-stimulated inflammatory cell response. Formation of the membrane attack complex resembles, in several respects, the calcium-induced polymerization of perforin.\textsuperscript{38} The latter is a 70-kd monomer that binds to phosphatidyicholine residues in target cell membranes. Perforin, in the presence of calcium, polymerizes to form 16-nm pores in the membrane of the target cell, thereby causing cell lysis.\textsuperscript{56,57} The role of perforin in the cytosis that occurs during cardiac transplantation rejection\textsuperscript{58} and cowpoxvirus B3–induced acute myocarditis\textsuperscript{59} is of current interest. Like the membrane attack complex, perforin produces structural and functional lesions in target cells. The membrane attack complex and perforin are considered to be both structurally and functionally related\textsuperscript{60}; both molecules are capable of disrupting host cells. Consideration of the events leading to the assembly of these molecules may be of significance in the design of appropriate interventions for the protection of cells against irreversible injury.

**Critique of the Experimental Model**

The isolated rabbit heart model developed for these experiments was particularly suitable for studying the direct effects of complement activation on the heart for the following reasons: 1) In situ complement activation occurred without the need for exogenous activators such as cobra venom factor or endotoxin, which may cause undesirable effects themselves. 2) The cellular elements of the blood were absent from the perfusate, eliminating their interactions with complement and allowing for a more accurate assessment of the direct effects of complement activation on the physiological and biochemical parameters of the heart. 3) The anti-complement antibodies and purified components necessary for complement studies are readily available only for the human components.
Interpretation of the experimental results must consider that heterologous (human) plasma was used as a source of the complement components. Several groups have demonstrated the presence of proteins termed homologous restriction factor\(^5\),\(^6\) and protectin\(^5\),\(^6\) on the surface of human cells that are responsible for inhibition of homologous membrane attack complex formation. Similar rabbit proteins may or may not inhibit formation of the human membrane attack complex efficiently, or at all. The interaction of human complement with rabbit cells may be facilitated because of a relative lack on rabbit cells of a reactive decay accelerating factor. The latter is a known regulator of complement activation and formation of C5b-9.\(^5\),\(^6\) Thus, the lack of these homologous inhibitors may maximize the effectiveness of human complement activation in the model in which rabbit tissue is exposed to human plasma (complement). However, the appearance of the membrane attack complex on myocytes as a result of in vivo ischemia and reperfusion\(^5\),\(^15\),\(^16\),\(^6\) demonstrates that complement activation occurs even in the presence of homologous inhibitors of membrane attack complex formation. The extent of complement activation in the rabbit heart model, as compared with an in vivo state, is unknown. Complement activation in vivo occurs in the presence of a full titer of plasma complement proteins, whereas only 6% plasma was used in these experiments, possibly limiting the extent of complement-mediated damage. Differences in the mechanisms and sites of complement activation may exist in the experimental model as compared with in vivo activation of the system. The events responsible for complement activation in either setting have not been fully elucidated.

Activation of the complement system, in response to tissue injury, results in proinflammatory and cytolytic effects. The inflammatory response is mediated by several peptide fragments, C3a, C4a, and C5a (anaphylatoxins), whereas the cytolytic consequences of complement activation result from the assembly and insertion into the cell membrane of the membrane attack complex composed of C5b-9. The inflammatory reaction to tissue injury is a natural response, whereas unregulated complement activation and formation of the terminal complex lead to irreversible tissue injury. The ability to interrupt the complement cascade may provide an innovative approach to suppressing tissue damage. CR1 is one of several membrane bound proteins that regulate the activation of complement at the cell surface, thereby protecting the host cell from injury. CR1 has the most inhibitory potential. In both the classical and alternative pathways, CR1 inactivates C3 and C5 convertases by dissociating the protein subunits of the catalytic complex and enhancing their proteolytic degradation by factor I.\(^5\),\(^6\) The in vivo efficacy of recombinant soluble CR1 in preventing complement-mediated tissue injury has been demonstrated.\(^5\),\(^6\) The soluble CR1 had complement inhibitory and anti-inflammatory activities in a rat model of reperfusion injury of ischemic myocardium, reducing myocardial infarction size and reducing the cytolytic action of the membrane attack complex. These observations provide strong support for the cytotoxic role of complement and the membrane attack complex as mediators of tissue injury.

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