Mechanism of Complement Activation After Coronary Artery Occlusion: Evidence That Myocardial Ischemia in Dogs Causes Release of Constituents of Myocardial Subcellular Origin That Complex With Human C1q In Vivo


To evaluate whether ischemic myocardium releases molecules that react with the first component of complement, we studied cardiac lymph from eight dogs before and at intervals after coronary artery occlusion and reperfusion. Before occlusion, the dogs were injected intravenously with radiolabeled human C1q. Labeled C1q could be detected in the cardiac lymph within minutes following injection. Rabbit antisera, prepared against substances precipitated from postreperfusion cardiac lymph by anti-human C1q, also reacted with specific constituents of isolated cardiac sarcoplasmic reticulum and mitochondria. To evaluate whether mitochondria are the source of these C1q-binding proteins, we isolated intramyofibrillar and subsarcolemmal mitochondria from canine heart and incubated sonicates of these with purified C1q, immobilized on nitrocellulose. Molecules bound to the immobilized C1q were removed with 0.1% sodium dodecyl sulfate, fractionated under reducing conditions by polyacrylamide gel electrophoresis, and transferred electrophoretically to nitrocellulose paper. Antisera prepared against postreperfusion lymph reacted with a 31,000-32,000-dalton protein in these nitrocellulose paper replicas. Since this protein originates from mitochondria, binds to C1q, and is recognized by antibodies made against postreperfusion lymph, this protein is likely to be one of the subcellular constituents that, upon release from ischemic cells, activates the complement cascade. To evaluate the clinical relevance of these observations, we tested sera from 53 patients obtained 48–72 hours after hospitalization for suspected myocardial infarction. Sera from 12 of 15 patients (80%) with documented myocardial infarctions contained abnormally large quantities of substances that formed macromolecular complexes with C1q; C1q-binding substances were found in sera from only 5 of 38 patients (13%) who did not fulfill the criteria for myocardial infarction (p < 0.001). Serial tests of sera from 19 patients showed that high levels of C1q-binding activity were temporally associated with significantly depressed C4 and/or C3 levels in 4 of 6 patients with and only 1 of 13 patients without myocardial infarctions. These observations are consistent with the hypothesis that myocardial ischemia results in the release of subcellular constituents of cardiac muscle that bind C1q. These C1q-binding substances may activate the complement cascade, trigger release of leukotactic anaphylatoxins, and stimulate infiltration of inflammatory cells that may extend the myocardial tissue injury associated with ischemia. (Circulation Research 1988;62:572–584)

Infiltrates of inflammatory cells are frequently evident in myocardium that has experienced prolonged ischemia. Several lines of evidence suggest that these inflammatory-cell infiltrates may contribute to ischemic myocardial injury. Reduction in myocardial necrosis can be achieved experimentally by inducing neutropenia before coronary artery occlusion. Reduction in myocardial damage can also be achieved by treating with superoxide dismutase and catalase as free-radical scavenging systems, or by inactivation of the complement cascade. Activation of complement within ischemic tissue may be necessary for the development of intracardiac inflammatory-cell infiltrates. For example, previous work from this laboratory showed a significant correlation between the quantity of radiolabeled C1q that localizes in ischemic myocardium and the numbers of leukocytes that accumulate after coronary occlusion and reperfusion.

These studies support the concept that complement fixed by ischemic myocardium generates a chemotactic substance, presumably C5a, that induces leukocyte infiltration and inflammation. Lysozomal enzymes and oxygen radicals released into the microenvironment of ischemic tissues by these leukocytes may augment the...
tissue damage that results from anoxia. It is not clear, however, how inflammatory responses that follow ischemic injury spread beyond severely ischemic zones to injure myocardium that might otherwise escape injury.

One possible explanation is that damaged myocardial cells release subcellular constituents that activate the complement cascade in extracellular fluids bathing these cells. Complement activation products may then disseminate through the vasculature or lymphatics. If this activation hypothesis is correct, one might find macromolecular complexes incorporating components of complement and subcellular constituents of myocardial cells in the lymphatic fluids of the heart after a myocardial occlusion. We present evidence in this report that cardiac lymph, collected after reperfusion of ischemic tissue, contains molecules of cardiac subcellular origin, bound to Clq, one of the constituents of the first component of complement. We postulate that formation of macromolecular complexes of this type may be among the stimuli that cause the spread of inflammation in ischemic myocardium.

Materials and Methods

Experimental Subjects

Eight dogs, weighing 18–20 kg, were anesthetized and prepared for open thoracotomy. Polyvinyl catheters were placed in the femoral artery and the inferior vena cava. The cardiac lymph duct was located after injection of Evans blue dye and was cannulated. Doppler flow probes were placed around the circumflex coronary artery to verify reduction in flow after injection of 1.0-1.1 x 10^6 cpm/µg.

After 1 hour, the occlusion was verified by measuring coronary artery flow; myocardial ischemia was verified by standard limb lead electrocardiograms. Sample collections continued at 30-minute intervals. After 1 hour, the occluder was released and resumption of distal flow in the occluded segment was demonstrated. In four dogs, the chest was closed 4 hours after the occlusion; sampling catheters for cardiac lymph and venous blood were led through the skin so that additional blood and lymph samples could be collected. The animals were resuscitated; sample collection and monitoring were then continued for up to 20 hours.

Blood was collected into Vacutainer tubes (Beckton-Dickinson, Rutherford, New Jersey) containing 3.76 x 10^-3 M disodium-EDTA as anticoagulant. Lymph was collected on ice in the presence of 0.02 M EGTA, 1 mM phenylmethylsulfonfluoride (PMSF), and 1% aprotonin (Trasylol). Cells were removed by centrifugation at 2,500g for 10 minutes at 4°C, and the supernatants were stored at -70°C, except when tested immediately.

Preparation and Evaluation of Clq

Clq was isolated from pooled human serum and if not used immediately, it was stored at -70°C. Purity was evaluated by immunoelectrophoresis in 1% agarose containing (M) sodium acetate 0.1, sodium barbital 0.1, and EDTA 0.001, pH 8.5, and by polyacrylamide gel electrophoresis (PAGE). When heated at 100°C for 5 minutes in 0.1% sodium dodecyl sulfate (SDS) and 2% mercaptoethanol (2-ME) before application to the stacking gel, Clq produced three bands with estimated molecular weights of 21,400, 26,800, and 28,000. These molecular weights closely approximate those of 22,000, 27,000, and 29,000 reported for the II-3, I-2, and I-1 subunits of reduced and alkylated Clq.

Clq was labeled with 125I by the lactoperoxidase method. Most of the radioactivity was associated with the lowest molecular-weight fraction. Unbound radiiodine was removed by dialysis at 4°C.

The ability of Clq to react with aggregated IgG was evaluated by the Clq-binding test using freshly heat-aggregated (63°C, 20 minutes) human IgG (HAGG) in concentrations between 0.125 and 3.0 mg/ml. The ability of Clq to react with aggregated IgG was evaluated by the Clq-binding test using freshly heat-aggregated (63°C, 20 minutes) human IgG (HAGG) in concentrations between 0.125 and 3.0 mg/ml as substrate. Before testing, the labeled Clq was centrifuged at 26,700g for 30 minutes at 0°C to remove aggregates.

To evaluate the ability of the radiolabeled Clq, circulating in blood and lymph, to react with natural substrates, 50 µl of plasma or lymph, containing at least 10,000 cpm of Clq-associated radioactivity, was added to a range of concentrations of HAGG diluted in heat-inactivated (56°C, 30 minutes) normal human serum containing 0.01 M EDTA. HAGG-associated [125I]Clq was separated from [125I]Clq by precipitation with polyethylene glycol at a final concentration of 2.7%. This same Clq-binding test was also used to detect Clq-binding substances in human sera. The percent Clq bound was a function of the concentration of HAGG or immune complex-like materials in the sera and was calculated as previously described: Percent Clq bound = (cpm[125I]Clq, experimental) – (background)/(cpm[125I]Clq, 10% TCA) – (background) x 100, where cpm indicates counts per minute, and TCA indicates trichloracetic acid.

Isolated human Clq, rather than dog Clq, was used in these investigations because in a previous study it had not been possible to isolate canine Clq with a high

Table of Abbreviations

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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<td>DAB</td>
<td>diaminobenzidine</td>
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<td>GARP</td>
<td>goat anti-rabbit peroxidase</td>
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<td>HAGG</td>
<td>heat-aggregated IgG</td>
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<td>kDa, kilodaltons</td>
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<td>2-ME, 2-mercaptoethanol</td>
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<td>NCP</td>
<td>nitrocellulose paper</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
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<tr>
<td>PBS</td>
<td>0.15 M NaCl buffered at pH 7.4 with .01 M sodium phosphate</td>
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<td>PMSF</td>
<td>phenylmethylsulfonfluoride</td>
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<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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biological activity. However, we formally demonstrated in that report that purified human Clq effectively combines with canine Clr and Cls to form an active C1 complex that, in the presence of appropriate substrate, activates the classic complement pathway in dog plasma.

**Preparation of Clq Solid Phase Immunoadsorbents**

Nitrocellulose paper strips (NCP), 1 x 5 cm, were incubated with purified Clq diluted in 0.15 M NaCl buffered at pH 7.1 with 0.01 M sodium phosphate (PBS) to a concentration of 25 μg/ml in slowly rotating sealed tubes for 5 hours at 6°C. Thereafter, Clq-coated NCP strips were washed four times for 30 minutes each with 1% bovine serum albumin (BSA) in PBS at 6°C. These washes provided a coating with BSA to prevent subsequent nonspecific uptake of irrelevant macromolecules and removed 96% of poorly adherent Clq as shown by monitoring the release of [125I]Clq into the wash solution. Clq-coated strips were subsequently stored in 1% BSA-PBS at 6°C until used.

Control (nonspecific) adsorbents were prepared by coating NCP strips of the same size with 1% BSA-PBS under the same conditions. The ability of Clq as compared with BSA-coated adsorbents to bind preferentially to substrates known to have an affinity for Clq was tested by incubating Clq- and BSA-coated NCP strips for 5 hours or more with [125I]Fab' fragments of IgG, diluted in 0.1% BSA-PBS containing 0.05% Tween 20. Thereafter, the NCP strips were washed four times in 0.1% BSA-PBS with 0.05% Tween 20, and the remaining bound radioactivity was measured by counting the strips. To determine how much of the bound [125I]Fab' could be removed, each NCP strip was incubated in 200 μl of a buffer containing 3% SDS, 0.0625 M Tris, 10% glycerol at pH 6.85 for 30 minutes at 56°C. These analyses showed that the Clq-NCP adsorbents bound 11.6-16.4 times as much labeled IgG as did BSA-NCP adsorbents of the same dimensions (range of four experiments). However, the Clq-NCP adsorbents had no selective ability to bind [125I]Fab'.

**Preparation of Subcellular Fractions of Cardiac Cells**

Cardiac sarcoplasmic reticulum was isolated from canine hearts using previously described methods. Briefly, cardiac muscle, stripped of fat and coronary vessels, was homogenized in 10 mM bicarbonate, pH 6.8, containing 5 mM sodium azide, 1% aprotinin, and 1 mM PMSF. The homogenate was centrifuged at 500g for 10 minutes; the resulting pellet was homogenized a second time under the same conditions. The supernatants from these two homogenizations were combined and centrifuged at 3,000g for 10 minutes. The pellet was washed twice in the same mannitol and sucrose buffer and stored at -70°C until used.

Subsarcolemmal and intramyofibrillar mitochondria were isolated from approximately 20 g of left ventricular myocardium, homogenized in buffer containing 220 mM mannitol, 70 mM sucrose, 5 mM morpholinopropanesulfonic acid, 2 mM EGTA, and 0.2% BSA at pH 7.4. Two centrifugations at 500g produced supernatants that were combined and centrifuged at 3,000g. This final pellet was suspended in buffer solution (without BSA) to give the fraction representing SSL mitochondria.

The pellet from the 500g spin was suspended in a buffer solution of 100 mM KCl, 50 mM morpholinopropanesulfonic acid, 2 mM EGTA, and 0.2% BSA at pH 7.4. Nagarse (type VII bacterial proteinase) was then added at 5 mg/g wet wt, and the suspension was immediately homogenized and diluted twofold with buffer solution. Centrifugation at 5,000g for 5 minutes yielded a pellet that was then resuspended in the same buffer solution and spun at 500g for 10 minutes. The 500g wash was repeated, and the two supernatants were combined. Final centrifugation at 3,000g for 10 minutes produced a pellet representing intramyofibrillar mitochondria. The total protein concentration of the mitochondrial preparations was measured and the material was stored at -70°C until used.

**Identification of Clq-Binding Proteins in Cardiac Lymph**

To harvest antigens that may have bound to the human Clq in the canine cardiac lymph, we added sufficient rabbit anti-human Clq (Lot 041-1, Boehringer-Mannheim Biochemicals, Indianapolis, Indiana) to precipitate more than 95% of the [125I]human Clq present in the sample. In preliminary experiments, we showed that addition of dog serum to mixtures of [125I]human Clq and anti-Clq at equivalence did not interfere with precipitation of the [125I]human Clq, whereas addition of the same quantities of human serum to this mixture of antibody and antigen inhibited precipitation of human Clq by 81%. Thus, we concluded that canine Clq was unlikely to compete for the human Clq used in these experiments and inhibit its precipitation by anti-Clq. To minimize nonspecific precipitation, both the lymph and rabbit anti-Clq were centrifuged at 6,500g for 10 minutes immediately before mixing. To reduce the chance that we would harvest canine Clr and Cls along with the human Clq, the reaction mixture was made 0.13 M in EDTA. Proteolysis and bacterial growth during subsequent incubations were inhibited by adding, respec-
tively, 100 kIU aprotinin, 1 mM PMSF, and 0.1% sodium azide. The reaction mixture was incubated for 30 minutes at 37°C and then at 4°C for 70 hours.

**Polyacrylamide Gel Electrophoresis; Transfer of Fractionated Proteins to Nitrocellulose Paper**

Samples were incubated at 100°C in the presence of 0.1% SDS and 2% 2-ME for 10 minutes, layered on 5% polyacrylamide stacking gels and fractionated in 5%, 7.5%, or 10% polyacrylamide in the presence of 0.1% SDS. Electrophoresis was performed at 22°C using 30 mA/slab gel. Proteins resolved by this procedure were stained with 0.1% Coomassie blue after fixation with 10% trichloracetic acid. Immunological characterization of the proteins in these slab gels was accomplished after the fractions were transferred by electrophoresis at 150 V for 4 hours at 22°C to NCP. To identify proteins specifically transferred by this procedure, NCP replicas were stained with amido black. To identify specific antigens in these NCP replicas, they were incubated in calf serum for 30 minutes to block nonspecific protein binding and then exposed to specific rabbit antisera for 30 minutes more. After three washes in 0.01 M Tris, 0.015 M NaCl, pH 7.5, containing 0.1% Tween 20 (vol/vol), the strips were incubated with horseradish peroxidase-conjugated goat anti-rabbit serum (GARP) for 30 minutes. After three more washes in the same buffer, the color reaction was developed with ortho-dianisidine and H2O2. Molecular-weight estimates, based on the distance of migration of standard proteins of known molecular weight in these gels, were calculated from a linear plot, determined by the method of least squares, that related the distance of migration to the logarithm of the molecular weight.

**Preparation of Antisera to the Clq-Binding Proteins in Cardiac Lymph**

The components of cardiac lymph that had reacted with Clq and therefore could be precipitated by adding anti-Clq were harvested by centrifugation at 6,500g for 5 minutes and washed four times with veronal-buffered saline containing the same concentrations of sodium azide, PMSF, and aprotinin. These washed precipitates were used to immunize 2-kg adult rabbits intracutaneously at multiple sites on the dorsal skin. A preimmunization blood sample was collected from each rabbit. The initial antigen preparation was homogenized with complete Freund's adjuvant. Booster injections containing the same quantity of immunoprecipitate, homogenized in incomplete Freund's adjuvant, were given 2 and 4 weeks after the first injection. Antisera were harvested 2 weeks after the last immunization and pooled after verification that the preimmunization sera contained no reactivity against these antigens.

The resulting antisera were adsorbed with the proteins from whole dog serum, conjugated to CNBr-activated Sepharose, at a ratio of 1 ml antiserum to 2 mg whole dog serum protein. Sodium azide (0.1%), as well as 100 kIU/ml aprotinin, and 1 mM PMSF were added to the rabbit sera before these adsorptions. Also Na+ was added to monitor dilution and nonspecific adsorption caused by sequential additions of dog serum Sepharose. Generally, five serial adsorptions were required to remove all reactivity to dog serum protein, as demonstrated by double diffusion in agar gels.

These adsorbed antisera, hereafter called antisera to postreperfusion lymph, were used to identify specific antigenic constituents of cardiac lymph. To concentrate molecules that bind to Clq in the lymph, sufficient anti-Clq was added to precipitate greater than 95% of the Clq-labeled Clq in the lymph. The packed precipitate, approximately 0.03–0.05 ml, was resuspended by vortexing, washed three times at 7,000g with 1 ml of ice-cold PBS at pH 7.4, dissolved in 0.1% SDS with 2% 2-ME, and was fractionated by SDS-PAGE. The resulting fractions were transferred electrophoretically to NCP. The NCP replicas of these gels were then incubated with appropriately diluted antisera to postreperfusion lymph. Bound rabbit antibodies were detected with GARP and DAB.

To differentiate those molecules that precipitate nonspecifically under the conditions of these experiments from those that selectively precipitate because they were bound to Clq, we used equivalent quantities of IgG and Fab fragments of IgG that had no anti-Clq activity as substitutes for anti-Clq in sham immunoprecipitation experiments. These sham immunoprecipitates were washed, fractionated, and transferred to NCP alongside the immunoprecipitates made with anti-Clq and were developed with the same antisera to postreperfusion lymph, followed by GARP and DAB.

We also investigated whether these antisera to postreperfusion lymph reacted with molecules found in subcellular fractions of cardiac muscle under the hypothesis that subcellular components of heart muscle cells may be released into the lymph as an early consequence of ischemic injury.

To determine whether these antisera reacted with mitochondria, we fractionated mitochondrial proteins soluble at 100°C in 0.1% SDS and 2% 2-ME by PAGE. The fractions were transferred to NCP replicas that were then incubated with the adsorbed rabbit antiserum. Binding of rabbit IgG to specific proteins in these NCP replicas was identified with GARP and DAB.

In the case of cardiac sarcoplasmic reticulum, this direct method failed to detect specific binding of antibody to any protein (see "Results"). Therefore, cardiac sarcoplasmic reticulum was centrifuged at 6,500g for 10 minutes to separate insoluble from soluble proteins. The pellet was taken up in 0.5 ml PBS and sonicated at full gain in an ice bath for 15 seconds in a Branson sonicator (Heat Systems-Ultrasonics, Inc., Plainview, New York). This material was centrifuged again at 6,500g for 10 minutes. Resulting supernatants were pooled and combined at a 1:6 (vol/vol) ratio with the adsorbed rabbit antiserum and incubated for 30 minutes at 37°C and then for 44 hours at 4°C. Precipitated proteins were removed by centrifugation at 2,000g for 30 minutes at 4°C and washed.
three times with PBS containing aprotinin, PMSF, and sodium azide. Soluble complexes remaining in the supernatant were also harvested by adsorption to staphylococcal protein A conjugated to Sepharose. Sufficient staphylococcal protein A to bind all the remaining IgG was added to these supernatants and gently swirled for 60 minutes in an ice bath. The staphylococcal protein A carrying these soluble complexes was then washed three times with 0.01 M NaCl buffered with 0.015 M Tris at pH 7.4, containing 0.05% Tween 20. Staphylococcal protein A–bound proteins were eluted with 0.1% SDS and 2% 2-ME at 63°C for 30 minutes and added to the washed immunoprecipitates, likewise dissolved in SDS containing 2-ME. These proteins were fractionated by PAGE, transferred electrophoretically to NCP, and tested for reactivity to antibodies in the adsorbed rabbit antisera using GARP and DAB in the presence of H₂O₂.

Further Characterization of C1q-Binding Components of Canine Cardiac Mitochondria

The subsarcolemmal and intramyofibrillar mitochondria at concentrations between 14 and 25 mg/ml were sonicated on a wet ice slurry at a power setting of 6, using a Kontes ultrasonic cell disrupter with a 2-mm tip (Kontes Glass Co., Vineland, New Jersey). Sonication was carried out for a total of 4 minutes, using a 30-second initial pulse, followed by a 30-second cooling period, then a series of 15-second pulses, each followed by a 15-second cooling period. Immediately after thawing the mitochondria, 1 mM PMSF and 1% aprotinin were added to protect against proteolysis. The sonicates were diluted in 0.1% BSA-PBS with 0.05% Tween 20 containing the same concentrations of PMSF and aprotinin to a final concentration of 4.6 mg/ml in the case of the subsarcolemmal mitochondria and 1.9 mg/ml in the case of the intramyofibrillar mitochondria. BSA and Tween 20 were added to inhibit nonspecific binding of mitochondrial proteins to the NCP. NCP (1 × 5 cm) strips coated either with C1q or BSA were then incubated for 14 hours with these mitochondrial sonicates in slowly rotating sealed tubes at 4–6°C. Afterwards, the NCP strips were washed with four changes of 0.1% BSA-PBS containing 0.05% Tween 20 over a period of 24 hours to remove nonspecifically bound mitochondrial constituents. Each NCP strip was then cut up into fragments 2 mm² and placed in the barrel of a 3-ml syringe containing 1 ml of 0.1% SDS and 2% 2-ME. These were incubated for 1 hour at 56°C and then overnight at 22°C. The next morning the fluid was expressed from the NCP fragments, using the plunger of the syringe, heated at 100°C for 10 minutes, and then fractionated by SDS-PAGE followed, in some experiments, by electrophoretic transfer of the fractions to NCP. These transblots were then incubated with the adsorbed antisera to the postreperfusion cardiac lymph.

Clinical Studies

Two groups of patients were studied. In the first group of 53 patients admitted with a diagnosis of possible myocardial infarction, we tested serum from a single sample of clotted blood that was obtained within 48 hours of admission to the coronary care unit. In a second subgroup of 19 patients with the same admission diagnosis, samples of blood were obtained 4 hours after admission, every 12 hours for 72 hours, and subsequently at 48-hour intervals for the remainder of the hospital stay. The blood was allowed to clot at 22°C for 1 hour; it was centrifuged, and the serum was harvested and frozen at −70°C until studied. The diagnosis of myocardial infarction was established by the evolution of characteristic electrocardiographic changes and/or serial changes in serum creatine phosphokinase and other serum enzymes.31

Sera from these patients were tested for constituents that can form macromolecular complexes with C1q, which are precipitable upon addition of 2.7% polyethylene glycol.32 These methods measure the percentage of added [¹²⁵I]C1q that precipitates in association with macromolecular C1q-binding complexes present in the sample. A C1q-binding activity of less than 8.7% was in the 95th percentile for serum samples from 24 healthy age-matched donors who were tested six or more times concurrently as controls.

Serum levels of C3 and C4 were measured by the microtiter method,33 using EAC1,4, EAC1 and functionally purified complement components obtained from Cordis Laboratories, Miami, Florida. Pooled normal donor sera, run as controls in each microtiter tray, had C4 titers greater than 1:2,000 and C3 titers greater than 1:6,000. Levels of C4 and C3 were considered significantly depressed if their respective titers were more than fourfold less than the lower limit of these measurements in the normal controls.

The C reactive protein was identified qualitatively in sera by double immunodiffusion in agarose gels using rabbit anti-human C reactive protein (Lot 28715, Behring Diagnostics, American Hoechst Corp., Woodbury, New Jersey). Precipitin reactions were graded as negative, 1+, or 2+ in gels that had been allowed to diffuse for 48 hours before washing and staining with amido black. Specificity of the anti-C reactive protein was verified by double diffusion in agar against purified C reactive protein, isolated from human sera by Sepharose C-polysaccharide adsorption–elution.34 The C-polysaccharide was the generous gift of Dr. Emil Gottschlich, Rockefeller University, New York.

Heart-reactive antibody was measured by testing twofold serial dilutions of patient sera by indirect immunofluorescence against tissue sections of normal human heart.34 Only the serial samples were studied for heart-reactive antibodies. Specificity controls included tissue from human skeletal muscle, rat skeletal muscle, and myocardium. Sera from 16 healthy donors, tested concurrently, had titers in this assay of less than or equal to 1:16 against human heart and less than or equal to 1:20 against human skeletal muscle. Titers of normal donor sera against the rat tissue were twofold lower.
Results

Survival of Functionally Active Radiolabeled Human C1q in the Dog Circulation and Its Appearance in Cardiac Lymph

125I-Labeled human C1q equilibrated in the dog circulation within the first 30 minutes following intravenous injection. Thereafter, the levels declined to half-maximal values within 20–24 hours as shown in Figure 1. In both plasma and lymph, 97% of the 125I was protein bound. Between 84% and 86% of the [125I]C1q in the circulation and lymph remained biologically active for the duration of these experiments, as shown by its ability to react with HAGG (Figure 1). Maximum lymph concentrations of [125I]C1q were achieved by the third hour following injection, even in dogs that had coronary artery occlusion followed by reperfusion. The level of [125I]C1q attained in the lymph was 40–50% of that found in the plasma.

Composition of Canine Cardiac Lymph After Coronary Artery Occlusion and Reperfusion, as Demonstrated by Polyacrylamide Gel Electrophoresis

Novel protein constituents, not evident in cardiac lymph collected before coronary artery occlusion, were demonstrated by PAGE in lymph collected within the first 30 minutes after reperfusion in seven of the eight experimental animals (Figure 2). The PAGE profiles shown in Figure 2 are representative of those seen in samples from these seven dogs. Arrows indicate the three most prominent components of the postreperfusion lymph. These proteins had estimated molecular weights of 38,500, 40,000, and 51,500 daltons. The last lane of Figure 2 shows the PAGE profile of the [125I]human C1q used in these experiments. Radioau-
To evaluate whether any of the proteins in the cardiac lymph had formed complexes with the injected human Clq, sufficient rabbit anti-human Clq was added to the lymph to precipitate more than 95% of the [125I]Clq contained therein. The immunoprecipitates were washed and analyzed by PAGE; results representative of all five dogs studied in this manner are shown in Figure 3. Components of cardiac lymph precipitated nonspecifically by anti-Clq were determined by adding anti-Clq to lymph obtained before injection of [125I]Clq (Lane B, Figure 3). There was no qualitative change in the protein composition of these precipitates following injection of Clq nor after coronary artery occlusion, as shown by the PAGE profiles in Lanes C and D. However, after reperfusion, the lymph samples contained several new components (Lanes E through I). Radioactivity remained associated only with the three intrinsic constituents of Clq, as shown by the radioautographs of the sample obtained 20 hours after reperfusion (Lane J).

To differentiate between lymph proteins that bound specifically to Clq and proteins that were inadvertently trapped within the precipitate, we prepared sham immunoprecipitates with lymph and nonspecific IgG that lacked antibody activity to human Clq. Similar controls were prepared with lymph and the Fab' fragments of that nonspecific IgG. Comparison of the proteins precipitated by specific anti-Clq versus nonspecific IgG (Figure 4) suggested that most proteins having molecular weights larger than 40,000 were precipitated nonspecifically. Four components, however, appeared to be distinctively precipitated by anti-Clq (Figure 4). Their estimated molecular weights were 32,100, 30,400, 29,500, and 26,800 in gels stained with Coomassie blue (Lane B). They were not evident in the sham immunoprecipitates prepared by adding nonspecific IgG to postreperfusion lymph samples (Lanes C and D, respectively).

To investigate the source of the Clq-binding proteins in postreperfusion lymph, we prepared rabbit antisera against the immunoprecipitates obtained with anti-human Clq. The resulting antisera were adsorbed repeatedly to remove antibodies against canine plasma proteins. The antisera were then tested for reactivity against immunoprecipitates made by adding anti-Clq to lymph (Figure 4), fractionated components of dog heart mitochondria (Figure 5), and sarcoplasmic reticulum (Figure 6).

The immunoprecipitates from postreperfusion lymph were fractionated by SDS-PAGE, and transblots were prepared. The antisera reacted with four molecules with estimated molecular weights of 32,100, 30,400, 29,500, and 26,800 in gels stained with Coomassie blue (Lane B). They were not evident in the sham immunoprecipitates prepared by adding nonspecific IgG to postreperfusion lymph samples (Lanes C and D, respectively).

Identification of Proteins in Canine Cardiac Lymph That Can Form Complexes With Clq

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Demonstration of Proteins of Cardiac Subcellular Origin In Postreperfusion Lymph

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The immunoprecipitates from postreperfusion lymph were fractionated by SDS-PAGE, and transblots were prepared. The antisera reacted with four molecules with estimated molecular weights of 32,100, 30,400, 29,500, and 26,800 in gels stained with Coomassie blue (Lane B). They were not evident in the sham immunoprecipitates prepared by adding nonspecific IgG to postreperfusion lymph samples (Lanes C and D, respectively).
Identification of Clq-Binding Substances in Mitochondria That React With Antibodies to Postreperfusion Lymph

An approach was then devised to concentrate the Clq-binding proteins in mitochondria since previous studies had suggested that molecules that can activate the classic pathway in myocardium may be localized in mitochondria. Sonicates of subsarcolemmal or intramyofibrillar mitochondria were incubated with human Clq, which was previously immobilized on nitrocellulose. To control for nonspecific binding to nitrocellulose, we incubated aliquots of the same mitochondria with BSA, similarly immobilized on nitrocellulose. After repeated washings, molecules bound to these adsorbents were removed with 0.1% SDS and 2% 2-ME and analyzed by PAGE under reducing conditions.

No qualitative differences in the proteins eluted from Clq- and BSA-coated adsorbents were seen when the

found among molecules precipitated by adding nonspecific IgG or Fab' fragments of IgG to the lymph (Lanes G and H).

To investigate whether any of the antigens were subcellular constituents of myocardial cells, we fractionated isolated mitochondria and sarcoplasmic reticulum by SDS-PAGE under reducing conditions and probed transblots of these fractionations with antisera to postreperfusion lymph. These antisera reacted with a single component of mitochondria (estimated molecular weight of less than 10,000 daltons) that was smaller than our lowest molecular weight standard (Figure 5).

The antisera failed to react with any component of unconcentrated extracts of sarcoplasmic reticulum. However, we were able to concentrate the antigens in sarcoplasmic reticulum that react with the antisera by routine immunoprecipitation techniques, and we were able to analyze transblots of these after fractionation by SDS-PAGE. These transblots showed that sarcoplasmic reticulum contains a single 20,200-dalton antigen that is recognized by antibodies to postreperfusion lymph (Figure 6). But there was no evidence to suggest that sarcoplasmic reticulum was the source of any of the four higher molecular-weight antigens previously identified in postreperfusion lymph.

**FIGURE 5.** Identification of a low molecular-weight component of cardiac mitochondria by antisera to postreperfusion cardiac lymph. Soluble proteins of isolated cardiac mitochondrial proteins, fractionated by SDS-PAGE, and transferred electrophoretically to NCP strips were incubated with (Lane A) rabbit antibodies to postreperfusion cardiac lymph or (Lane B) calf serum. These immunoblots were developed with peroxidase conjugated goat anti-rabbit IgG. Lane C shows molecular-weight markers. Note that the rabbit antibodies reacted with a protein of less than 10,000 daltons in the transblots of the cardiac mitochondria (arrows).

**FIGURE 6.** Immunologic recognition of a 20,200-dalton protein in cardiac sarcoplasmic reticulum by antibodies made against postreperfusion cardiac lymph. Lane A, precipitate that resulted from mixing soluble cardiac sarcoplasmic reticulum antigens with antibodies to postreperfusion lymph after fractionation in SDS-PAGE under reducing conditions in 10% polyacrylamide gel, electrophoretic transfer to NCP strips, and staining with amido black. Lanes B–E, same transblot, incubated with preimmunization rabbit serum (Lane B); antiserum to postreperfusion lymph containing antibodies to canine serum proteins (Lane C); antiserum to postreperfusion lymph containing antibodies to canine serum proteins removed (Lane D); and calf serum, a nonspecific control (Lane E). Each of the transblots in Lanes B–E were subsequently incubated with peroxidase conjugated goat anti-rabbit IgG and then with substrate to localize the site of deposition of the peroxidase-conjugated antibodies. Lane F, molecular-weight markers.
PAGE gels were stained with Coomassie blue (Lanes A and B, Figure 7). However, development of transblots from these gels with antisera to postreperfusion lymph demonstrated a 31,600-dalton component in eluates from Clq adsorbents exposed either to subsarcolemmal or to intramyofibrillar mitochondria (Lanes E and F). This molecule was not evident in eluates from BSA adsorbents that were incubated with these mitochondria (Lanes C and D). This 31,600-dalton molecule corresponded closely in molecular weight to the 32,100-dalton Clq-binding protein previously identified in postreperfusion lymph by the same antisera (Figure 4).

Subunits of reduced Clq were also present and demonstrable in the material that was eluted from these Clq-adsorbents (Lane G). However, the largest subunit was smaller than 31,600 daltons. Moreover, subunits of Clq were not recognized by these antisera under the conditions used in these experiments (Lane H).

To confirm that Clq-binding molecules of mitochondrial origin react with antibodies to postreperfusion lymph and to verify that these were undetectable until they had been selectively concentrated by adsorption to immobilized Clq, we repeated the experiment with another preparation of mitochondria. Figure 8 compares the antigens recognized by antibodies to postreperfusion lymph in unconcentrated mitochondria (Lane E) with those recognized in the Clq-binding fraction of mitochondria (Lane G) when 200 μm of each were fractionated and transblotted under identical conditions. The antibodies to postreperfusion lymph react with a single low molecular weight component of unconcentrated mitochondria faintly visible at the bottom of Lane E. They react with 5 antigens in mitochondrial extracts enriched for molecules that bind Clq. Prominent among these is a 31,100-dalton antigen (Lane G), which is likely the same as the 31,600-dalton antigen identified in Lanes E and F. These antibodies to postreperfusion lymph also react weakly with four other mitochondrial antigens having estimated molecular weights of 63,300, 48,400, 29,600, and 24,800 (Lane G).

Adsorption/elution from immobilized Clq isolates many proteins from mitochondria of which few are recognized by the antisera to postreperfusion lymph (Lane H). However, this step significantly enriches for the 31,000-dalton molecule that is recognized by the antisera. This molecule is undetectable in the crude lysate but prominent among the proteins eluted from Clq adsorbents.

Included in the nitrocellulose eluates were BSA, which was positively identified in Lane A of Figure 8 by staining with anti-BSA, and Clq, which was identified in Lane F by staining with anti-Clq. However, antigens associated with BSA were not recognized either by anti-Clq or by antisera to postreperfusion lymph (Lanes B and C).

Demonstration of Clq-Binding Macromolecules in Sera of Patients Within the First 48–72 Hours Following Myocardial Infarction

To evaluate whether Clq-binding subcellular fragments are released in humans suffering significant cardiac ischemia, we obtained sera from patients with suspected myocardial infarction shortly after their hospitalization. These sera contained significantly increased quantities of material that will form macromolecular complexes with added [125I]Clq. The Clq-
postreperfusion lymph (Lane B); and anti-Clq (Lane C).

Although BSA is prominent in eluates from the Clq immunoadsorbents (Lane H), neither anti-Clq nor antisera to postreperfusion lymph (Lane E). Control experiments, BSA, fractionated by SDS-PAGE, was incubated with anti-BSA (Lane A); antisera to postreperfusion lymph and Lane F with anti-Clq. Lane G shows a prominent 31,100-dalton antigen, possibly the same as that assigned a molecular weight of 31,600 daltons in Lanes E and F of Figure 7. This component is not evident in transblots of unconcentrated mitochondria when probed with antisera to postreperfusion lymph (Lane E). Control experiments, BSA, fractionated by SDS-PAGE, was incubated with anti-BSA (Lane A); antisera to postreperfusion lymph (Lane B); and anti-Clq (Lane C). Although BSA is prominent in eluates from the Clq immunoadsorbents (Lane H), neither anti-Clq nor antisera to postreperfusion lymph reacts with this antigen.

binding substances were identified in sera from 12 of 15 patients with documented myocardial infarctions but in only 5 of 38 patients without objective evidence of myocardial injury ($\chi^2 = 22.1, p < 0.001$, Table 1).

The temporal relations between high levels of serum Clq-binding activity and creatine phosphokinase measurements are shown for two representative patients with and for two without documented myocardial infarction (Figure 9).

Coincidental with the high-serum Clq-binding activity, C4 levels were below the lower limit for normal donors in sera of 4 of 6 patients with and in 1 of 13 without myocardial infarction. C3 levels were also significantly depressed in sera from 3 of the 6 with and from 1 of 13 patients without a myocardial infarction.

All patients, regardless of their hospital course, had titers of IgG and IgM heart-reactive antibody less than 1:8 at the time of admission to the hospital. Normal limits are titers less than 1:16 as determined by concurrent testing of healthy volunteers. Serial sera were collected for a minimum of 5 days and a maximum of 12 days from 6 patients with and from 12 patients without subsequently documented myocardial infarctions. There were no increases in the titers of anti-heart antibodies throughout this time period. Measurement of antibody to human skeletal muscle and rat heart and skeletal muscle likewise failed to demonstrate abnormal levels of or a change in titer of antibody against striated muscle in either patient group. Thus, it is unlikely that the increased levels of serum Clq-binding activity, found in sera of patients with myocardial infarction, indicated the presence of immune complexes, caused by reaction of anti-heart antibodies with antigens released from ischemic heart tissue.

Similarly, it is unlikely that the Clq-binding substances in these patients’ sera were caused by C reactive protein. All patients with and all but one of those without a subsequently documented myocardial infarction had readily detectable levels of CRP.

**Discussion**

Although previous studies have suggested that subcellular fractions rich in heart muscle mitochondria can activate both the classic and alternative pathways of the complement cascade in vitro and although immunohistologic studies have shown that complement is deposited in ischemic myocardium 4 hours or more after coronary occlusions, there has been little information until recently concerning the molecular interactions likely to trigger complement activation after coronary artery occlusion in vivo.

Our previous studies suggest that Clq begins to accumulate in the ischemic tissues within 15 minutes following coronary artery occlusion. In those studies, as in the present ones, we used highly purified radioiodinated human Clq to identify Clq-reactive tissue components. The choice of this reagent was dictated in part by our inability to isolate functionally active canine Clq in an immunochemically pure form. However, since human Clq effectively interacts with canine Clr and Cls to form a highly functional C1 complex and since studies of isolated Clq from diverse species show that these molecules are similar both in structure and function, the use of human Clq should not cause reservations about the interpretation of these experiments. Indeed, there were specific advantages, associated with the use of human Clq, especially in the
experiments in which lymph proteins were allowed to bind to human C1q and then were precipitated with anti-human C1q. We found that canine serum proteins did not inhibit precipitation of [125I]human C1q by the anti-C1q we used. Thus, the precise quantity of anti-C1q needed to precipitate 95% or more of the human C1q present in the lymph could be determined and added without fear of competition from the unknown quantities of canine C1q in the sample. Not infrequently, antisera prepared against C1q of one species fails to react or reacts poorly with C1q from another species. Moreover, since the purpose of these immunoprecipitation experiments was to trap and identify molecules that bound to C1q during or after the ischemic period, there was a distinct advantage to adding an exogenous C1q that does not cross-react immunologically with canine C1q. Conceivably some isologous C1q in dogs may normally be associated with circulating macromolecules that are irrelevant to the events that follow coronary occlusion. These would not be precipitated by the anti-human C1q reagent.

If, indeed, leukocyte-mediated injury in ischemic myocardium in vivo is initiated by molecules, released from ischemic cardiac cells, that activate the classic complement pathway, one should be able to demonstrate that 1) C1q, a relatively large molecule, that normally circulates in a Ca2+-dependent 774,000-dalton complex with C1r and C1s, is present in appreciable quantities in extracellular fluids of the heart both before and after coronary artery occlusion; 2) C1q can indeed react with one or more molecules of cardiac subcellular origin in vivo; and 3) novel complexes incorporating C1q and subcellular molecules of myocardial origin are found in extracellular fluids of the heart after an ischemic insult. This report provides evidence for each of these postulates.

Comparison of the concentrations of [125I]-labeled C1q in the circulation and cardiac lymph indicates that detectable quantities of biologically active C1q are present in the extracellular fluids of the heart, even before coronary artery occlusion. Analysis of cardiac lymph by PAGE also demonstrates C1q in this fluid. After coronary artery occlusion and reperfusion, the concentration of C1q in the lymph continues to rise. The composition of cardiac lymph increases in complexity, as does the number of its components that coprecipitate with C1q.

To demonstrate that C1q-binding molecules of subcellular origin are found in cardiac lymph after reperfusion of ischemic myocardium, we prepared antisera against those molecules precipitated by anti-C1q. Antibodies to dog serum proteins were removed from these antisera by adsorption. We first sought to discover which molecules in cardiac lymph react with these antibodies. Therefore, we used these antibodies to analyze the composition of immunoprecipitates prepared by adding anti-C1q to postreperfusion lymph. The antisera recognized four molecules in these immunoprecipitates that were not found in sham precipitates of cardiac lymph prepared by using nonspecific IgG in place of the anti-C1q.

The principal C1q-binding protein recognized by these antisera among subcellular constituents of myocardium was 31,100 daltons. An antigen of similar
molecular weight was precipitated by anti-C1q from cardiac lymph. This antigen has characteristics expected of a cardiac subcellular molecule that can activate the classic pathway: it is a constituent of cardiac mitochondria; it binds C1q; and it is recognized by antibodies prepared against molecules that are complexed to C1q in postreperfusion lymph. It is clearly not a major component of mitochondria. Indeed, it was demonstrated only in mitochondrial extracts that had been enriched for molecules that bind to and can be eluted from immobilized C1q.

These results are in keeping with previous in vitro studies13,36 that suggested that mitochondria may be a source of molecules that can activate complement. Our previous studies demonstrated that the principal ligand of the classic pathway, C1q, indeed localizes in ischemic myocardium in vivo.4 In the present study, we have identified the subcellular C1q-binding molecule that may trigger complement activation. Isolation of this molecule will permit study of its ability to activate complement both when cell-associated and when present in cell-free fluids. Such investigations will further define its role as a molecule that links complement activation and leukocyte-mediated injury.4,7-11 in ischemic myocardium.

Our studies do not exclude the possibility that there are other cardiac subcellular molecules that may activate complement when released by myocardial cells. Transblots from fractionated mitochondria and sarcoplasmic reticulum contain additional antigens that react with antibodies developed against postreperfusion lymph. This is evidence that other subcellular constituents are released into extracellular fluids during an ischemic episode. Previous studies suggest that cardiac cells become permeable and release intracytoplasmic enzymes as early as 10 minutes after onset of ischemia in dogs,14 and 15 minutes after onset of ischemia in baboons.43

Our investigations also provide evidence that C1q-binding molecules are released following myocardial infarction in humans. Patients with documented myocardial infarctions have increased levels of circulating C1q-binding complexes; these complexes are associated, temporally, with acute depression of serum C3 and C4 activities. Thus, in the clinical setting of myocardial infarction, it is likely that damaged myocardial cells also release C1q-binding molecules that activate the classic complement pathway.11 The C1q-binding substance cannot be C reactive protein33 since the presence of this acute phase reactant did not correlate with the presence of elevated serum C1q-binding activity.

It is also unlikely that the increased serum C1q-binding activity represented an autoimmune response to myocardial cell constituents, resulting in the formation of C1q-binding, circulating immune complexes.44,45 since none of the patients had or later developed significant titers of anti-heart antibody. Moreover, this increased serum C1q-binding activity was demonstrated invariably within the first 72 hours, whereas previous studies have shown that circulating anti-heart antibodies and immune complexes appear 7 days or more after the ischemic event.44-46

References


Mechanism of complement activation after coronary artery occlusion: evidence that myocardial ischemia in dogs causes release of constituents of myocardial subcellular origin that complex with human C1q in vivo.

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