Cardiolipin-Protein Complexes and Initiation of Complement Activation After Coronary Artery Occlusion


Abstract Specific rabbit anti-cardiolipin (anti-CL) antibodies were used to investigate the hypothesis that cardiolipin, associated with mitochondrial membrane proteins, binds C1 and facilitates activation of the complement cascade following reperfusion of ischemic myocardium. By immunoelectron microscopy, anti-CL localized to subsarcolemmal mitochondria, emerging through breaks in membranes of damaged cardiac myocytes. Anti-CL reacted with >15 mitochondrial constituents, most of which comigrated with the proteins that bind C1q in transblots of subsarcolemmal mitochondria, fractionated by polyacrylamide gel electrophoresis under reducing conditions in the presence of sodium dodecyl sulfate. A subset of the C1q-binding proteins >24 to 37 kD served as stable sites for assembly of C3, C5, and C9. Cardiac lymph, collected during the first hour after reperfusion of ischemic myocardium, contained proteins of diverse size that reacted with both anti-CL and C1q. Cardiac lymph, collected before occlusion and 4 to 5 hours after reperfusion, in comparison, had few if any C1q or anti-CL reactive proteins. Treatment with phospholipase suppressed the C1q-binding activity and anti-CL reactivity of the proteins in reperfusion lymph and those with similar properties in mitochondrial extracts. Our data suggest that during ischemia, mitochondria, extruded through breaks in the sarcolemma, unfold and release membrane fragments in which cardiolipin and protein are intimately associated. By binding C1 and supplying sites for the assembly of later-acting complement components, these fragments provide the means to disseminate the complement-mediated inflammatory response to ischemic injury. (Circ Res. 1994;75:546-555.)

Key Words • cardiolipin • ischemia • coronary artery occlusion

Antibody-independent activation of the complement cascade has long been recognized as a consequence of myocardial ischemia. Clinical and laboratory investigations have demonstrated that activation of the complement cascade under these circumstances proceeds principally by the classic pathway. In experimental models of myocardial ischemia/reperfusion injury, one can show that cardiac extracellular fluids contain leukocyte chemotactic activity that is completely inhibited by antisera to C5a. Release of this complement-dependent anaphylatoxin, after reperfusion of ischemic myocardium, activates circulating neutrophils and causes these to localize in regions of myocardium with significantly decreased blood flow. Complement-mediated inflammation accounts for a significant portion of ischemic injury, as shown by the myocardial salvage that results from treatments that suppress complement activation during ischemia and reperfusion.

Although a number of studies have suggested that the proinflammatory stimulus can come from products of damaged cardiac myocytes, the initial trigger for this proinflammatory event remains unknown. Membranous protein components of cardiac mitochondrial origin that bind C1q and provide sites for the assembly of later-activating complement components have particularly been implicated as possible activating stimuli in vitro. Although it is not clear how these may be released by ischemic myocardial cells, the likelihood that cardiac subcellular constituents bind and activate C1 has been increased by the recognition that cardiac lymph from experimental animals after myocardial ischemic injury contains C1q bound to molecules of cardiac subcellular origin. The idea that proteins of mitochondrial origin provide stimuli for this inflammatory response has been challenged by studies reporting that the cardiac mitochondrial proteins are comparatively weak C1 activators in vitro when rigorously separated from membrane phospholipids. Among mitochondrial constituents, it has been suggested that cardiolipin has the greatest ability to bind and activate C1.

To identify the initiating stimulus for complement activation in ischemia/reperfusion injury, we tracked the relation between cardiolipin release and complement activation in vivo after coronary artery occlusion and reperfusion in dogs. Our studies suggest that the initiating stimulus for complement activation may be cardiolipin-containing proteins, displayed by mitochondria as they are released through the torn subsarcolemmal membranes of damaged myocytes. Relatively large fragments of mitochondrial membrane that contain both protein and cardiolipin appear to be required to bind C1 and sustain the activation of the complement cascade through the membrane attack complex.
Materials and Methods

Immunologic Recognition of Antigens Immobilized in Nitrocellulose or Nylon Membranes

Cardiolipin, phosphatidylinositol, phosphatidyl-L-serine, phosphatidylethanolamine, lecithin, and cholesterol (all obtained from Sigma Chemical Co) were dissolved in 100% ethanol, dotted onto nitrocellulose membranes by means of a micropipette, and allowed to dry thoroughly before exposure to antisera or Clq. Subsarcolemmal mitochondria, isolated and solubilized in Nonidet P-40 (NP-40) as previously described, were diluted serially in veronal-buffered saline containing 0.2% sodium dodecyl sulfate (SDS) and applied to nitrocellulose or nylon membranes in a similar manner. Nitrocellulose strips were initially incubated in calf serum to suppress nonspecific protein binding before exposure to specific antisera or Clq. Membrane-bound primary antibody was demonstrated with a second antibody conjugated to horseradish peroxidase. This enzyme was visualized by conversion of the substrate 3,3′-diaminobenzidine in the presence of hydrogen peroxide. In later experiments, especially those designed to identify fragments that contain cardiolipin and/or remove human Clq, nonspecific binding was blocked by incubation for 1 hour at room temperature (23°C) or overnight at 6°C in veronal-buffered saline containing 1.5 × 10⁻⁵ mol/L Ca²⁺, 5 × 10⁻⁴ mol/L Mg²⁺, and 4 g/100 mL 4% human (HSA) or bovine serum albumin (BSA), as determined by preliminary experiments. Bound immunoglobulin was detected with affinity-purified alkaline phosphatase–conjugated goat anti-rabbit IgG (Pierce Chemical Co). Second antibodies were adsorbed with canine IgG and canine cardiac mitochondrial proteins, each immobilized on cyanogen bromide (CNBr)-activated Sepharose (Pharmacia) to remove cross-reactive antibodies. Localization of alkaline phosphatase–conjugated antibodies was identified by conversion of 5-bromo-4-chloro-3-indolyl phosphate in the presence of p-nitro blue tetrazolium chloride (Bio-Rad Laboratories). Clq-reactive targets were detected by means of 125I-human Clq, labeled as previously described. Clq was purchased from Quidel. To measure membrane-bound radiolabeled Clq, we used a two-dimensional radialonclide counter equipped with a collimator and computer software sufficient to resolve images created by focal deposits of 125I (Ambus 1000 Radioanalytic Imaging System, Ambus Systems). Increased sensitivity of detection of bound Clq, as required in some experiments, was achieved by using purified monospecific goat antibodies to Clq. These, in turn, were detected by means of biotin-conjugated affinity-purified goat anti-rabbit IgG. This anti-rabbit IgG had been adsorbed both with canine IgG and cardiac mitochondrial proteins conjugated to CNBr-activated Sepharose. Biotinylated second antibody was detected by use of streptavidin, followed by alkaline phosphatase–conjugated biotin (Pierce Chemical Co). Localization of the alkaline phosphatase conjugate was detected by conversion of phosphate-containing substrates as described above. It should be emphasized that all nitrocellulose or nylon replicas containing tissue fragments or specific lipids were copiously washed with buffers containing 8% albumin between exposure to each reagent used to detect bound Clq or anti-cardiolipin antibodies. Moreover, each component of the detection system, including second antibodies, streptavidin, and alkaline phosphatase–conjugated biotin, was tested and adsorbed, as necessary, to ensure that at the concentrations required for these studies, none reacted with human Clq or fragments of sonicated canine mitochondria or proteins in cardiac lymph.

Preparation of Rabbit Anti-Cardiolipin Antibodies

Cardiolipin, diluted in absolute ethanol to a final concentration of 2 mg/mL, was mixed volume for volume with isolated keyhole limpet hemocyanin (KLH, Calbiochem) also at a concentration of 2 mg/mL in 0.15 mol/L NaCl and buffered with 0.01 mol/L sodium-potassium phosphate (PBS) at pH 7.4. Twenty-five microliters of the mixture was blotted onto 0.22-µm nitrocellulose paper and allowed to dry. The nitrocellulose containing the dried mixture was cut into small strips and stuffed into the bore of 14-gauge needles. These needles were used as trocars to insert the dried nitrocellulose strips subcutaneously at multiple sites into the shaved backs of five rabbits. The animals were boosted with 1 mg cardiolipin mixture with 1 mg KLH + 0.05 mg cardiolipin deposited on nitrocellulose membranes in the same manner at weekly intervals for 4 weeks before blood was collected for analysis. Sera from two of the five rabbits reacted with cardiolipin deposited on nitrocellulose membranes at a concentration of 0.2 µg/cm²; serum from one was reactive when diluted at >1:500. This antiserum, when tested at concentrations 2-fold higher than needed to detect cardiolipin, was unreactive with phosphatidylinositol, phosphatidyl-L-serine, phosphatidylethanolamine, or lecithin blotted in concentrations up to 10-fold higher on nitrocellulose membranes. Pretreatment of the blotted cardiolipin with phospholipase C (PLC) or phospholipase D (PLD), using conditions described below, abolished reactivity with this antiserum.

Affinity Purification of Anti-Cardiolipin Antibodies

Cardiolipin-containing liposomes were prepared by mixing cardiolipin, cholesterol, and dicetylphosphate at molar ratios of 2:1.5:0.22, respectively, by use of modifications of the methods of Alving and Richards. These lipids were placed in a 15-mL Corex tube containing 1 mL ethanol and 1 mL chloroform and dissolved by vortexing vigorously. The lipid mixture was dried on the glass walls of the tube under a stream of N₂, with continued vortexing and stored under vacuum until needed. Liposomes in which phosphatidylethanolamine was substituted for cardiolipin and liposomes that contained only cholesterol were also prepared. To hydrate the dried lipids and prepare liposomes, three glass beads were added to the tubes along with 1 mL of 0.15 mol/L NaCl; the tube was vigorously vortexed, producing an opalescent aqueous suspension of the lipids. One milliliter of anti-cardiolipin antiserum was then added; this mixture was incubated for 1 hour at 23°C. The liposome-antiserum suspension was then centrifuged at 27 000g for 5 minutes, the supernatant was removed, and the pelleted liposomes were washed once with 5 mL ice-cold saline. The pellet was resuspended in 1 mL fresh ice-cold saline, and 1 mL chloroform was added to extract the lipids. The mixture was then vortexed and allowed to stand without manipulation for 10 minutes, after which it was centrifuged at 12 000g for 5 minutes. Isolated antibodies were recovered in the aqueous fraction.

Canine Subsarcolemmal Mitochondria: Isolation and Identification of Complement-Reactive Components

Mitochonrida were isolated as previously described and stored at a concentration of 5 mg/mL in 200-µL aliquots at -80°C. Aliquots were thawed as needed and diluted in veronal-buffered saline. Mitochondria were sonicated on wet ice by use of a Kontes ultrasonic cell disrupter (Kontes Glass Co) with a 2-mm tip at a power setting of 6, tuned between 3 and 4 as previously described. To isolate constituents that bind Clq, sonicated mitochondria were incubated for 14 to 16 hours at 4°C or 6°C with isolated human Clq immobilized on nitrocellulose membranes. After washing these adsorbents, bound proteins were eluted with 1.5% SDS containing 2.5% 2-mercaptoethanol and fractionated by SDS–polyacrylamide gel electrophoresis (PAGE) under reducing conditions. Parallel strips of the Western-blotted replicas of these gels were stained either with amido black or probed with highly purified Clq to identify Clq-binding proteins. Additional transblots from these polyacrylamide gels were incubated with fresh human serum to identify those mitochondrial components that provide sites for partial or complete assembly of the comple-
ment cascade. After the strips were washed thoroughly, fractions binding C1q, C3, C5, and C9 were identified by means of specific antibodies (Quidel, Inc) followed by horseradish peroxidase–conjugated second antibody and substrate (Bio-Rad Laboratories) as previously described.4 Appropriate control experiments were performed to establish that the antisera to C1q, C3, C5, and C9 and the second antibodies used in these experiments did not react with any components in the transblots of the fractionated mitochondria unless these replicas had first been incubated with fresh human serum to provide a source of complement.5 To further demonstrate that specific components of these fractionated mitochondrial extracts could activate the complement cascade, other replicas of the fractionated mitochondria were incubated in guinea pig complement supplemented with isolated human C1q for 1 hour at 22°C.6 They were then washed and overlaid with 1% agarose containing 0.5% sheep red blood cells in veronal-buffered saline containing 15×10⁻³ mol/L Ca²⁺ and 5×10⁻⁴ mol/L Mg²⁺ for 4 hours at 37°C. As reported previously, these methods resulted in uniform lysis of the red blood cell overlay except over sites where complement had been activated and consumed by specific mitochondrial proteins immobilized at that site in the transblot.2

Electrophoretic Separation of Components of Mitochondria and Cardiac Lymph

We used modifications of previously described methods14 to fractionate canine cardiac mitochondria and lymphatic fluids by PAGE and Western blotting of the fractionated proteins to nitrocellulose or nylon membranes. Briefly, two gel electrophoresis formats were used: Large format gels were used principally to fractionate sonicated mitochondria; these were run in an SE 600 vertical slab gel apparatus (Hoefer Scientific). Small polyacrylamide gels, used principally to fractionate canine cardiac lymph, were run in a Bio-Rad Mini-Protein II system (Bio-Rad Laboratories). Samples were taken up in loading buffer (0.0625 mol/L Tris-HCl, containing 4% SDS, 26% glycerol, and 10% 2-mercaptoethanol), placed in a boiling water bath for 1.5 minutes, and then cooled over wet ice just before electrophoresis. They were fractionated at 30 mA per gel at 22°C in 1.5-mm running gels, 10% polyacrylamide for mitochondria and 15% polyacrylamide for canine cardiac lymph. A 4% stacking gel was used; the tank buffer was composed of 0.025 mol/L Tris-HCl, pH 8.7, with 0.192 mol/L glycine and 0.1% SDS. Resolved proteins were transferred to nitrocellulose or nylon membranes at 100 V for 1 hour at 22°C in 0.025 mol/L Tris, pH 8.3, containing 0.192 mol/L glycine and 20% methanol. Molecular weights were estimated with reference to a set of molecular weight standards, incorporated in each gel, ranging in size from 14 200 (lysozyme) to 200 000 (myosin heavy chain).

Treatment With Phospholipases

Nitrocellulose or nylon membranes on which test substances had been immobilized were immersed in phospholipases, typically for 2 hours at 37°C, in tightly sealed polypropylene tubes, which were rotated end over end at three to five revolutions per minute to ensure intimate contact between the enzymes and the membrane surface. PLD (type VI, P 8023 from S chromofuscus, Sigma Chemical Co) was dissolved in 0.1 mol/L Tris-HCl, pH 8.0, containing 5% HSA, 0.05 mol/L CaCl₂, and 3 μmol/L SDS. PLD (type I, P 7758, Sigma) was diluted in 0.2 mol/L sodium acetate, pH 5.8, containing 0.039 mol/L CaCl₂, 5% BSA, and 3 μmol/L SDS. PLC (type XII, P 5527 from B cereus, Sigma, later replaced by Sigma type XI, P 7147) was diluted in Tris-saline, pH 7.3, containing 1% HSA. Just before use, CaCl₂ was added to bring its final concentration to 2.2×10⁻² mol/L. The phospholipases were used at concentrations of 10 and 100 U/mL.

Experimental Animal Studies

Eight mongrel dogs weighing between 18 to 25 kg were anesthetized and prepared for thoracotomy as previously described.14,20 Cardiac lymphatic vessels were identified by injection of Evans blue dye in the posterolateral wall of the left ventricle. The largest lymphatic vessel was cannulated =2 to 5 cm from the base of the heart; noncardiac lymphatic vessels and tracheobronchial lymphatic connections in the same anatomical region were ligated. Polyvinyl catheters were placed in the left and right atria for blood sampling. A hydraulic occluder was placed around the circumflex coronary artery distal or just proximal to the first diagonal branch and a Doppler flow probe downstream from the occluder to verify reduction in blood flow after occlusion. The catheters and the occluder were led through the skin before closure of the thoracic cavity. The animal was allowed to recover for 3 days to reduce the levels of tissue debris and proinflammatory substances in the cardiac lymph. To collect cardiac lymph, the animal was placed in a nylon mesh sling. After administration of 0.1 to 0.2 mg/kg IV or SC pentazocine lactate for analgesia, the coronary artery occluder was inflated until flow distal to the occluder ceased, as determined by the Doppler flow probe. After 1 hour, during which time flow through the distal segment remained at zero, the occluder was slowly deflated, allowing blood flow through the formerly ischemic segment. Ischemia was documented by persistent ST-segment elevation during occlusion. Cardiac lymphatic fluids were collected for 1 hour before the occlusion, during occlusion, and at 30-minute intervals after release of the occluder for 5 hours. Cardiac lymph was collected on ice into 1.5-mL polypropylene tubes in the presence of 0.02 mol/L disodium EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, and 100 kallikrein inhibiting units of aprotinin. Individual lymph samples were centrifuged to remove cellular elements, divided into 100- to 200-μL aliquots, and frozen at −70°C until tested. Twenty-four hours after the occlusion, the dogs were killed by intravenous injection of sodium thiamaal and potassium chloride, the heart was promptly removed, and the left ventricle was isolated, sectioned transversely, and stained with 2,3,5-triphenyltetrazolium chloride to identify zones of infarcted tissue, which were then measured planimetrically.20

Cardiac lymph samples, diluted 1:30, 1:60, 1:90, and 1:120 in loading buffer, were fractionated by SDS-PAGE and blotted to nylon membranes, which were then cut in the direction of migration in parallel strips to facilitate immunologic identification and comparison of embedded tissue fragments reactive with C1q, and anti-cardiolipin antibodies. Care was taken to fractionate sequential samples from a single dog under identical conditions to facilitate accurate comparisons of the relative mobilities of reactive species found at each time point. Paired transblots of prereperfusion and postreperfusion lymph from each dog, diluted to the same end point and fractionated by SDS-PAGE under identical conditions, were incubated for 2 hours at 37°C in 0.2 mol/L sodium acetate, pH 5.8, with 0.039 mol/L CaCl₂, 5% BSA, and 3 μmol/L SDS with or without 3.75 μg/mL type I PLD to study the effect of this enzyme on the reactivity of specific proteins in the lymph with both C1q and the anti-cardiolipin antibodies.

Electron Microscopic Studies of Isolated Myocardial Cells Exposed to Anti-Cardiolipin Antibodies

Canine cardiac myocytes were isolated as previously described.21,22 Briefly, nonischemic hearts were delivered through a left lateral thoracotomy under sterile conditions into ice-cold saline. By means of an aortic cannula, the vasculature was perfused initially with Jokik’s modified minimum essential medium equilibrated with 95% O₂/5% CO₂, containing 2 g/L sodium bicarbonate and 0.1% fatty acid–free BSA, until the vessels were cleared of blood. Thereafter, 120 U/mL collage-
nase type III (Worthington Biochemical) was added to the perfusate, and perfusion was continued for 10 additional minutes. Ten grams of left ventricular myocardium was then minced and incubated on a shaker platform at 35°C in the presence of 95% O₂/5% CO₂ in Joklik’s medium containing collagenase and bicarbonate for an additional 20 minutes. The supernatant was filtered repeatedly through cheesecloth and then incubated again in the presence of fresh medium with collagenase, until a sufficient number of single myocytes was obtained with >80% viability by trypan blue dye exclusion. Canine cardiac myocytes harvested in this manner were stored on wet ice and used up to 48 hours after isolation. To identify sites reactive with anti-cardiolipin antibodies, myocytes were allowed to adhere to polylysine-coated coverslips and then incubated either with affinity-purified IgG anti-cardiolipin or isolated normal rabbit IgG. Thereafter, they were washed, exposed to gold colloid–labeled anti-rabbit IgG, washed again, fixed in phosphate-buffered 1% glutaraldehyde for 1 hour, and then prepared for scanning and transmission electron microscopy as previously described.23

### Results

#### Identification of Mitochondrial Proteins That Show Reduced C1q-Binding Activity After Treatment With Phospholipase

Transblots of sonicated mitochondria that had been fractionated by SDS-PAGE under reducing conditions and transferred to nylon membranes were cut into strips parallel to the direction of migration and probed with 125I-labeled C1q (Fig 1). Before exposure to C1q, one of these strips was treated with PLD type VI (S. chromofuscus) to identify the phospholipase-sensitive C1q-binding constituents of mitochondria. C1q reacted strongly with six and weakly with five additional constituents of the fractionated mitochondria (inset, Fig 1). PLD treatment significantly reduced the quantity of 125I C1q bound by all but one of the six proteins resolved by two-dimensional counting of the transblot and abolished visual recognition of all but the 32.3-, 29.9-, 28.1-, and 17.8-kD C1q-reactive proteins in Western blots, where bound C1q was visualized with a stack of reagents that included anti-C1q, biotinylated second antibody, streptavidin, alkaline phosphatase biotin, and substrate (inset, Fig 1). Although inspection of the transblot suggests that phospholipase treatment may have reduced the C1q-binding activity of the 32.3-kD band, radionuclide counting of 125I C1q indicated that there was no significant difference in the quantities of C1q bound by the 32.3-kD protein before and after exposure to this enzyme.

### Association of Cardiolipin With the C1q-Binding Proteins in Cardiac Mitochondria

To investigate the relation between cardiolipin content and the C1q-binding activity of these mitochondrial proteins, we prepared a rabbit antiserum to cardiolipin. This reacts with 15 or more constituents of mitochondria, fractionated under reducing conditions on SDS-PAGE and blotted to nylon membranes (Fig 2). Affinity-purified anti-cardiolipin antibodies reacted with the same mitochondrial constituents as were recognized by antibodies in whole serum (compare lanes D and E; Fig 2), whereas antibodies isolated from the same antiserum, using phosphatidylethanolamine as the immunoadsorbent, reacted with a subset of these mitochondrial proteins (lane C, Fig 2). In contrast, immunoglobulins isolated from this antisem under the same conditions using dicetyl phosphate–cholesterol liposomes as the immunoadsorbent did not recognize any mitochondrial constituent (lane B, Fig 2). The numbers of proteins recognized by the anti-cardiolipin reagent were similarly decreased when fractionated mitochondria lysates, transblotted to nylon membranes, were incubated with PLC (Fig 3).

To ascertain the relative abilities of the C1q-binding mitochondrial proteins to activate complement and provide sites for assembly of the membrane attack complex, we isolated the proteins in NP-40 lysates of canine cardiac mitochondria, which react with C1q by using immobilized C1q as an affinity-binding reagent.9 The C1q-binding mitochondrial proteins eluted from this adsorbent were fractionated by SDS-PAGE and transferred to nitrocellulose membranes (Fig 4). The transblots were then incubated in fresh serum, washed, and evaluated for their ability to bind anti-C3, anti-C5, and anti-C9. Twenty-one or more C1q-binding proteins, ranging in size from >67 kD to <14 kD, were isolated

![Figure 1](http://circres.ahajournals.org/)

**Figure 1.** Bar graph shows the effect of phospholipase D on the C1q-binding activity of mitochondrial proteins. The major C1q-binding constituents of mitochondria were detected by binding 125I-C1q (counts=SD) before (horizontally striped bars) and after (diagonally hatched bars) incubation with phospholipase D (type VI). Inset shows a photograph of the transblotted proteins after incubation with anti-C1q and appropriate developing reagents needed to visualize the bound antibody before (A) and after (B) incubation with phospholipase D.
Fig 2. Anti-cardiolipin antibodies react with specific proteins of canine cardiac mitochondria after these have been fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions. Lanes are as follows: A, amido black stain for protein; B, negative control (blot probed with IgG, isolated from rabbit anti-cardiolipin antiserum by using dicetyl phosphate–cholesterol liposomes as an immunoadsorbent); C, proteins reactive with antibodies affinity-purified from the anticaldilipin antiserum by using phosphatidylethanolamine-cholesterol liposomes as the immunoadsorbent; D, proteins reactive with antibodies affinity-purified from this antiserum by using cardiolipin liposomes; and E, proteins reactive with unfractiated anti-cardiolipin antiserum. MW indicates molecular weight.

in this manner from NP-40 lysates of cardiac mitochondria (Fig 4, lane A). Only C1q-binding proteins >24 kD appeared to provide sites for the attachment of C3 and C5, as shown by their reactivity with anti-C3 (lane B) and anti-C5 (lane C, Fig 4). Only the \( \geq 37 \)-kD proteins reacted with anti-C9 under the conditions of these experiments, suggesting that mitochondrial fragments this size or larger may be required to provide a stable platform for the assembly of the membrane attack complex (Fig 4, lane D). Analysis of the complement-activating proteins in whole NP-40 lysates of cardiac mitochondria (Fig 4, lane E) similarly demonstrated that fixation and consumption of complement sufficient to prevent lysis of sensitized sheep erythrocytes was also mainly a property of the \( \geq 24 \)-kD mitochondrial proteins (Fig 4, lane F).

**Effect of Phospholipase on the Ability of Proteins in Canine Cardiac Lymph to Bind C1q and React With Anti-Cardiolipin Antibodies**

To evaluate whether the C1q-binding proteins, known to be present in cardiac extracellular fluids after ischemia/reperfusion injury,14 contain immunoreactive cardiolipin and to determine whether their ability to bind C1q and react with anti-cardiolipin antibodies is phospholipase sensitive, we analyzed the composition of cardiac lymph samples collected at intervals up to 5...
hours after circumflex coronary artery occlusion for 1 hour (Fig 5). Before occlusion, three major proteins were detectable in amido black-stained replicas of lymph, fractionated by SDS-PAGE (lane A, Fig 5). No reaction was seen with anti-cardiolipin (lane B); one of the proteins, possibly a plasma contaminant or a cardiac cellular protein left over from the inflammatory response to cannulation of the cardiac lymph ducts 3 days before the experiment, reacted faintly with C1q (lane C). One hour after reperfusion, the lymph contained numerous proteins ranging from >68 to <14 kD, identifiable in amido black-stained replicas of the fractionated lymph (lane D). Thirteen or more reacted with anti-cardiolipin antibodies (lane E). All of those reactive with anti-cardiolipin and at least four others bound C1q (lane F). Treatment with type I PLD abolished the C1q-binding activity of all but eight of these proteins (lane G). After 5 hours of reperfusion (lane H), a number of discrete proteins were visible in the amido black-stained replicas of the fractionated cardiac lymph, but the pattern and distribution of the proteins in the 5-hour sample were distinctly different from those found in the 1-hour reperfusion sample. All but one were nonreactive both with anti-cardiolipin (lane I) and with C1q (lane J). Note the similarity in the patterns and distribution of the C1q-binding proteins in the postischemic cardiac lymph (Fig 5, lane F) and in the mitochondrial lysates (Fig 4, lane A), especially those that consume complement (Fig 4, lane F).

Analysis of cardiac lymph from all eight dogs in the present study (summarized in Fig 6) demonstrated that samples collected within 1 hour after reperfusion contained numerous proteins, ranging in size from >200 to <15 kD, that reacted both with C1q and with anti-cardiolipin antibodies.

Source of Immunoreactive Cardiolipin: Binding of Anti-Cardiolipin Antibodies to Membranes of Mitochondria Released From Damaged Cardiac Myocytes

To identify the source of C1q-binding proteins reactive with anti-cardiolipin antibodies in postreperfusion lymph and in fractionated mitochondria, we incubated isolated cardiac myocytes, maintained in tissue culture, with affinity-purified anti-cardiolipin antibodies, followed by gold colloid-conjugated second antibody. Whereas >80% of these cells were viable by trypan blue dye exclusion, damaged cells and subcellular organelles, including mitochondria, were identifiable in all preparations. Binding of the gold colloid-conjugated second antibody was not observed, except in samples previously treated with anti-cardiolipin–specific antibodies (as shown by comparing the experiments shown in Fig 7A and 7C with control experiments), which were treated first with normal rabbit IgG (Fig 7B and 7D). In samples treated first with rabbit anti-cardiolipin, gold beads were localized on the unfolding membranes of mitochondria that were released from damaged cardiac myocytes (Fig 7A). By scanning electron microscopy, subsarcolemmal mitochondria, recognized as round protrusions or symmetrical swellings in the sarcolemma membrane, were occasionally labeled with gold beads (Fig 7C). Transmission electron micrographs (Fig 8A)
demonstrated the reaction of some of the protruding mitochondria with the anti-cardiolipin antibodies in the scanning electronmicrographs. Wherever mitochondria protruded through breaks in the sarcolemma membrane, mitochondrial membrane epitopes reactive with the anti-cardiolipin became available (see arrow; left side of Fig 8A). Binding of the anti-cardiolipin antibodies was not seen wherever these were covered by an intact sarcolemma, as shown in the right side of Fig 8A and in the lower part of Fig 8B. Mitochondria, fully liberated from the confines of the cardiac myocyte (Fig 8B), were manifestly swollen and displayed numerous membrane sites reactive with the anti-cardiolipin antibodies. We wish to emphasize that healthy myocytes with intact cell membranes were unreactive with these antibodies and that even in damaged myocytes, the anti-cardiolipin antibodies did not react with membranes of mitochondria protected by an intact sarcolemma (Fig 8A and 8B).

Discussion

After ischemic and other types of myocardial injury, plasma proteins extravasate into the injured cardiac muscle; albumin, immunoglobulin, complement, and fibrinogen are readily identifiable in extracellular de-
direct evidence in vivo that focal deposits of activated complement can provide stimuli that cause leukocytes to localize preferentially in ischemic myocardium.\(^8\)

Complement activation by cardiac mitochondrial membranes, in vitro, proceeds principally via the classic pathway, although some subcellular elements of myocardium may activate the alternative pathway as well.\(^5,9,15,18,32,33\) Prior in vitro studies from this laboratory suggested that certain myocardial subcellular proteins, principally those associated with mitochondrial membranes, bind Clq, activate C1, and propagate the complement cascade.\(^9,14\) Kovacsovics, Peitsch, and their colleagues,\(^15-18\) on the other hand, suggested that when cardiac mitochondrial proteins are rigorously separated in vitro from membrane phospholipids, the proteins are weak C1 activators and that the membrane phospholipid, cardiolipin, appeared to be the most avid ligand and activator of C1.

Although these studies suggested that cardiolipin could activate C1, there were no data, until the present studies were undertaken, to suggest that this phospholipid is released in vivo from ischemic myocardium or that it could play a role in the complement-mediated response to injury that follows (sometimes within 15 minutes) the reperfusion of ischemic myocardium.\(^5,10\) The present studies were designed to evaluate these possibilities. Before we initiated these investigations, we recognized the futility of seeking answers from histological sections of ischemic myocardial tissues, since histological sectioning was likely to expose antigens associated with cardiolipin wherever mitochondria are transected, eg, throughout the myocardium. Therefore, we chose to study freshly isolated myocytes from healthy hearts to see if the surfaces of any of these cells displayed cardiolipin. Although most of the isolated myocytes were viable, we recognized that a few of these cells would be damaged in the course of isolation. We postulated that the damaged but still intact myocytes would allow us to test the hypothesis that cell surface display of mitochondrial membrane cardiolipin is an early feature of myocyte injury, regardless of cause. By immunogold electron microscopy, using highly specific polyclonal antibodies to cardiolipin, we found that cardiolipin is abundantly expressed on mitochondria, emerging through breaks in the sarcolemma. We also found cardiolipin to be abundant in membranes of swollen and unfolded mitochondria that had escaped from injured cardiac myocytes. Most important, cardiolipin was not detected on the surfaces of myocytes with intact sarcolemmal membranes.

Although cardiolipin is generally considered to be a constituent of the inner mitochondrial membrane,\(^15,34\) there are some reports suggesting that small quantities of this phospholipid may also be demonstrated in outer mitochondrial membranes.\(^35\) The identification of this phospholipid in membranes of mitochondria as they are being released from damaged cardiac myocytes and in the unfolding membranes of mitochondria that have separated from cardiac cells is consistent with the hypothesis that injured myocytes provide a direct and proximate source of substances that can activate C1.

Thus, in this model of injury, cardiolipin, expressed on membranes of mitochondria protruding through the sarcolemmal membranes of injured myocytes, provides a potential trigger to activate the complement cascade. Such protrusions of mitochondria through the membranes of damaged cardiac myocytes, as discussed below, have previously been recognized as an ultrastructural feature of early ischemic myocardial injury.\(^36-39\)

To evaluate whether cardiolipin might contribute to the Clq-binding abilities of the complement-activating proteins previously identified in extracts of cardiac mitochondria and cardiac lymph collected after reperfusion of ischemic myocardium,\(^9,14\) we analyzed these materials for cardiolipin. Our results show that within the limits of the methods used in these and previous studies to resolve the Clq binding proteins in mitochondrial extracts and reperfusion cardiac lymph, the relative molecular weights of those that bind Clq and those that react with anti-cardiolipin antibodies are closely homologous. Since the conformation adopted by proteins cannot be closely controlled as they transfer to nylon or nitrocellulose membranes from polyacrylamide gels after fractionation by SDS-PAGE, differences in the ability of specific proteins to bind Clq or rabbit anti-cardiolipin antibodies once they have been immobilized in these transblots does not necessarily mean that there are Clq-binding proteins in mitochondria that lack cardiolipin or, conversely, cardiolipin-containing elements that cannot react with Clq. To investigate whether cardiolipin contributed to their ability to bind Clq, we treated these isolated mitochondrial proteins with phospholipase. Treatment with PLC or PLD reduced, and in some cases abolished, reactivity both with Clq and anti-cardiolipin antibodies. We concluded from these experiments (1) that phospholipids, especially cardiolipin, are an integral constituent of these proteins and (2) that these phospholipids are not easily dissociated by exposure to detergents such as SDS, heating to 100°C, and reducing agents such as 2-mercaptoethanol that are normally effective in separating integral membrane phospholipids and proteins during fractionation by SDS-PAGE.

Recognizing that binding of Clq does not ensure binding and activation of the later-acting complement components,\(^40,41\) we evaluated the complement-activating abilities of the Clq-binding proteins in cardiac mitochondria. We found that only a subset of these provide sites for assembly and activation of the later-acting complement components (Fig 4). Specifically, when tested for their ability to bind C1 and activate the later complement components, only proteins >24 kD provided sites for attachment of C3 and C5 (Fig 4). Assembly of the membrane attack complex, as demonstrated by binding of anti-C9, was evident only with proteins >37 kD. We resolved at least eight additional mitochondrial elements <24 kD by SDS-PAGE that contain cardiolipin (Fig 2) and react with Clq (Fig 4). These did not activate the complement cascade under the conditions used in these experiments. Therefore, we conclude that only the higher-molecular-weight mitochondrial proteins appear to provide both cardiolipin, with its high affinity for C1, together with proteins that provide favorable sites for binding and activating C3\(^42,43\) and a microenvironment that protects these from complement-regulatory proteins that might otherwise interfere with the assembly of the later-acting elements of the complement cascade.\(^44\)

Previously, we observed that antisera prepared against the Clq-binding proteins in reperfusion lymph...
react with specific mitochondrial proteins. Figs 5 and 6 in the present report show that the great majority of Clq-binding molecules in cardiac lymph, collected 1 hour after onset of reperfusion, contain cardiolipin. This provides additional evidence that most of the molecules that activate complement in the course of ischemic myocardial injury in vivo come from mitochondria. Reactivity with both Clq and anti-cardiolipin antibody was significantly suppressed and in some cases abolished by treatment with phospholipases, further emphasizing the role of cardiolipin in the Clq-binding activity of these substances. Notably, Clq-binding anti-cardiolipin–reactive molecules were rare to absent in cardiac lymph collected before coronary artery occlusions; they were maximally evident 1 hour after reperfusion and virtually gone from lymph samples collected between 4 and 5 hours after reperfusion. This timing coincides with the time of appearance and disappearance of C5a-like activity in canine cardiac lymph after ischemia/reperfusion injury and suggests that the appearance of these Clq-binding proteins in the cardiac extracellular fluids may be responsible for the generation of this anaphylatoxin during reperfusion.

Previous histopathologic studies of myocardial cells undergoing ischemia/reperfusion injury have demonstrated that blebs or microblister form between the sarcolemma and the myofilaments, possibly as the result of the accumulation of osmotically active metabolites during the first 10 to 30 minutes after the onset of ischemia. These blebs often contain subsarcolemmal mitochondria. In cats, these blebs or microblisters can break after only 10 minutes of ischemia, releasing subsarcolemmal mitochondria into the extracellular environment. In our studies of freshly isolated cardiac myocytes, we have observed that breaks in the cell membrane overlying subsarcolemmal mitochondria (Figs 7 and 8) allow anti-cardiolipin antibodies to react with cardiolipin in the membranes of these mitochondria. We suggest that the subcellular fragments of mitochondrial origin that bind and activate Cl after ischemia/reperfusion injury are released from ruptured subsarcolemma in ischemic myocytes. Once released from the confines of the cardiac cell, the mitochondria swell, their inner membranes unfold, and they release fragments rich in cardiolipin and protein that can bind Clq and activate the complement cascade, at least to the point where the leukotactic anaphylatoxin C5a is generated and promotes infiltration of circulating leukocytes into the injured myocardium, thereby initiating a classic acute reaction to injury.

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Cardiolipin-protein complexes and initiation of complement activation after coronary artery occlusion.

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