Molecular Basis of Complement Activation in Ischemic Myocardium: Identification of Specific Molecules of Mitochondrial Origin That Bind Human Clq and Fix Complement

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Mitochondria may be a source of molecules that activate complement during ischemic injury to myocardium, providing therewith a stimulus for infiltration of polymorphonuclear leukocytes. To identify specific molecules that activate the classical complement pathway, detergent lysates of canine cardiac mitochondria were fractionated by polyacrylamide gel electrophoresis and transferred electrophoretically to nitrocellulose paper (NCP). The NCP replicas of the gels were incubated with isolated Clq and fresh sera as a source of complement, washed briefly, and overlaid with sensitized sheep erythrocytes (RBC) in agarose. A cluster of four to six molecules between 45 and 53 kDa as well as four others, 34, 30, 26, and 23 kDa, consumed complement thereby preventing complement-mediated lysis of sensitized sheep RBC in the agarose overlay. Additional molecules reactive with Cl were identified by their ability to bind isolated human Clq and to serve as assembly sites for later acting complement components. Sites of localization of complement were demonstrated by incubating NCP replicas of fractionated mitochondria with antisera specific for Clq, C3, C5, and C9, followed by peroxidase-conjugated anti-immunoglobulin and substrate. A total of 12 Clq binding molecules ranging in size from 67 kDa to 23 kDa, which can fix later acting complement components, were identified. At least two of these reacted with antisera prepared against canine cardiac lymph collected in the first 3–4 hours after a 45-minute coronary artery occlusion. These studies present direct evidence that specific molecules, released from subcellular fractions of myocardial cells rich in mitochondria, can activate the complement cascade.

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Several lines of evidence suggest that activation of the complement cascade is required for the recruitment of the intracardiac inflammatory cell infiltrates in hypoxemic myocardium. Treatment with agents that interfere with complement activation or the function and products of infiltrating neutrophils significantly reduce the myocardial cell injury that follows episodes of cardiac ischemia. Recent studies suggest that myocardial injury, associated with ischemic episodes of short duration, may also be mediated by infiltrating granulocytes attracted by complement-derived chemotactic stimuli.

In vitro studies of possible mechanisms that might initiate complement activation in ischemic myocardium have repeatedly implicated mitochondria. Only recently, however, has it been possible to show in vivo that extracellular fluids in ischemic myocardium contain molecules of cardiac mitochondrial origin, some of which react with Clq, the molecule that triggers activation of the classical complement pathway. The present studies were designed to identify and characterize specific molecules in cardiac mitochondria that can bind Clq and serve as sites for assembly and activation of the later-acting complement components.

Materials and Methods

Reagents

Human, guinea pig or canine serum, absorbed repeatedly with packed sheep red blood cells were used
as sources of hemolytic complement. Polyclonal goat or rabbit antisera to human C1q, C5, and C9 as well as canine C3 were purchased from Cytotech, San Diego, California. C1q was isolated from human serum and analyzed for purity as previously described.7 It was tested for biological activity by the C1q binding test, using heat aggregated immunoglobulin G (IgG) as substrate.7-8 Serum from a child with severe combined immunodeficiency, obtained before an attempted bone marrow transplantation,19 provided a source of complement devoid of natural antibodies reactive with mitochondrial proteins.

Isolation of Cardiac Mitochondria

Subsarcolemmal mitochondria were obtained from 20 g of left ventricular myocardium. The tissue was homogenized in buffer containing 220 mM mannitol, 70 mM sucrose, 5 mM morpholino-propanesulfonic acid, 2 mM ethyleneglycol-bis-(β-aminoethylether)-N,N,N',N' -tetraacetic acid (EGTA), and 0.2% bovine serum albumin (BSA) at pH 7.4.20 Two centrifugations of the homogenate at 500g produced supernatants that were combined and centrifuged again at 3,000g. The pellet was suspended in buffer solution, without BSA or EGTA to produce the fraction rich in mitochondria. This material was stored at ~80°C in small aliquots and was thawed immediately before use.

Preparation of Solid Phase Adsorbents for C1q Binding Proteins

Nitrocellulose paper (NCP) strips 1x5 cm were incubated with 25 μg/ml isolated C1q diluted in 0.15 M NaCl buffered at pH 7.1 with 0.01 M sodium phosphate (PBS) in slowly rotating sealed polystyrene tubes for 12 hours at 6°C. The C1q-coated NCP strips were then washed three times for 30 minutes each in tubes containing 12 ml 1% BSA in PBS at 6°C. These washes removed excess C1q and provided a coating of unrelated protein to reduce subsequent nonspecific binding of substrate to the C1q-coated strips. Preliminary experiments demonstrated that this number of washings was sufficient to remove >96% of radiolabeled C1q, which was loosely adherent to the NCP. Control adsorbents were prepared by incubating NCP strips of the same dimensions in 1% BSA in PBS under the same conditions. The C1q-NCP adsorbents bound 11.6 to 16.4 times as much 125I-IgG as the BSA-NCP strips when incubated for 1 or more hours with this reagent. However, 125I-Fab' did not bind preferentially to the C1q-NCP strips.8

Adsorption/Elution of Mitochondrial Molecules to C1q, Immobilized on NCP

The mitochondria were disrupted in the presence of 1 mM phenylmethylsulfonyl fluoride (PMSF) and 100 Kallikrein Inactivator Units of aprotinin in glass tubes, chilled by a wet ice slurry, at protein concentrations of 14–25 mg/ml at a power setting of 6, using a Kontes ultrasonic cell disrupter (Kontes Glass Co, Vineland, New Jersey) with a 2-mm tip for a total of 4 minutes as previously described.8 The sonicated mitochondria were subsequently diluted to a protein concentration of 4.6 mg/ml in 0.1% BSA-PBS containing 0.05% Tween 20 and the same concentrations of PMSF and aprotinin. The sonicates were incubated with NCP strips coated either with C1q or BSA for 14 hours in slowly rotating tubes at 4–6°C. The optimal incubation time and temperature and the concentrations of BSA and Tween 20 that facilitated specific and minimized nonspecific binding of proteins to coated NCP strips was established by studying the conditions that optimized binding of 125I-labeled C1q to IgG-coated NCP as compared with NCP coated with Fab' fragments of IgG, as described above. After the incubation period, the strips were removed from the mitochondrial sonicate and washed four times in 12 ml PBS containing 0.1% BSA and 0.05% Tween 20.

Protein bound to the NCP strips was then eluted with 1.5% sodium dodecyl sulfate (SDS) containing 2.5% 2-mercaptoethanol and fractionated by SDS polyacrylamide gel electrophoresis (PAGE), followed, in some cases, by electrophoretic transfer of the fractions to NCP.

Polyacrylamide Gel Electrophoresis; Transfer of Fractionated Proteins to NCP

NP-40 extracts of mitochondria were incubated at 100° C in the presence of 1.5% SDS and 2.5% 2-mercaptoethanol for 10 minutes, layered onto 5% polyacrylamide stacking gels, and fractionated in 10% polyacrylamide gels with 0.1% SDS.21 Unless stated otherwise, we loaded 250 μg of mitochondrial protein to each centimeter of gel. Electrophoresis was performed at 22° C using 30 mA per slab gel. Immunological characterization of the fractions was accomplished after transfer by electrophoresis at 150 mA for 2½ hours at 22°C to NCP.22 Protein bands in NCP replicas were identified by staining with amido black. Antigens in these transblots were identified with specific antisera, diluted 1:50 to 1:250 in calf serum. Before exposure to these antisera, the NCP strips were incubated in calf serum to block nonspecific protein binding sites23 except as noted below. Subsequently, NCP strips that had been exposed to these antisera were washed four times in 0.05% Tween 20, Tris-buffered saline (TWETS). Next, they were incubated in horseradish peroxidase–conjugated goat anti-rabbit IgG. After three additional washes, localization of the peroxidase enzyme was visualized with 3,3'-diaminobenzidine in the presence of appropriate concentrations of hydrogen peroxide. Molecular weight estimates based on the distance of migration of standard proteins of known molecular weight were calculated from a linear regression plot, determined by the method of least squares, which related distance of migration to the logarithm of the molecular weight.
Preparation of Antisera to the Clq Binding Proteins in Cardiac Lymph

Components of cardiac lymph, which had bound to Clq and could therefore be harvested by adding rabbit anti-Clq, were collected in immunoprecipitates prepared by adding anti-human Clq, at equivalence, to canine cardiac lymph collected from dogs previously injected with 125I-labeled human Clq. The lymph in question was collected from dogs that had undergone complete occlusion of the left circumflex artery for 45 minutes, after which the occluder was released and the ischemic segment reperfused. The lymph, used as a potential source of Clq binding proteins, was collected during the first 3 to 4 hours after reperfusion.

In preliminary experiments, we estimated the quantity of antibody needed to precipitate >95% of the 125I associated with Clq. These immunoprecipitates were washed four times with 10 volumes of ice-cold PBS, homogenized with complete Freund’s adjuvant, and injected subcutaneously into the dorsal skin of 3-kg adult rabbits. Booster injections, homogenized in incomplete Freund’s adjuvant, were administered 2 and 4 weeks after the first injection. Antibodies, harvested 1 week after the final booster injection, were adsorbed with whole dog serum protein conjugated to CNBr-activated Sepharose, as previously described. These adsorbed antisera, hereafter called antisera to postreperfusion lymph, were used to probe fractions of canine cardiac mitochondria to investigate whether antigens recognized by these antibodies are associated with specific molecular components of cardiac mitochondria.

Identification of Complement Binding Macromolecules in Cardiac Subcellular Fractions

NCP replicas of PAGE gels were incubated in a solution containing 6 CH₄O units of guinea pig complement [guinea pig serum diluted 1:10 in veronal buffered saline (VBS) with 15×10⁻⁵ M Ca²⁺ and 5×10⁻⁴ M Mg²⁺] supplemented with 20 μg/ml purified human Clq for 1 hour at 22°C. The NCP strips were washed once for 2 minutes at 4°C in VBS with Ca²⁺ and Mg²⁺ and then overlaid with 1% agarose in VBS with Ca²⁺ and Mg²⁺ containing 0.5% sheep red blood cells sensitized with rabbit amboceptor. This NCP strip with its immobilized red blood cell overlay was then placed in a humidified incubator at 37°C for 4 hours. This incubation time was sufficient to cause uniform lysis of the red cells in the overlay except at those sites where complement had been previously activated and consumed by molecules adsorbed to the NCP strips, before the application of the sensitized sheep cells. We established 1) the optimum times and temperature of incubation, 2) the dilutions of sera used as source of hemolytic complement, and 3) concentration of sensitized red blood cells required in the agarose overlay in preliminary experiments in which serial dilutions of heat aggregated IgG, applied in 5-μl aliquots to the NCP, supplied the substrate for activating the complement cascade. These “dots” of IgG provided easily identifiable circular zones in which complement-mediated lysis of the red cell overlay was inhibited when the appropriate concentration of complement was applied to NCP.

In other experiments, NCP replicas were incubated with purified Clq, washed repeatedly with TWETS, and then incubated with antisera specific for Clq. Alternatively, the NCP strips were incubated with fresh human or dog serum, diluted 1:10 in VBS with Ca²⁺ and Mg²⁺; localization of human C3, C5, or C9, or canine C3 to specific zones in these strips was demonstrated with antisera reactive with these complement components. To identify molecules that might cause nonspecific binding of these antibodies, we incubated control NCP replicas with human or canine serum that had been heat inactivated at 56°C for 30 minutes and made 0.01 M in EDTA.

In experiments in which specific antibody reagents were used to identify complement in the NCP strips, 4% bovine or human serum albumin diluted in VBS was used in place of calf serum to block nonspecific binding of extraneous proteins to the transblots before incubation with canine or human sera and antibodies to C3, C5, or C9. The localization of antibodies to specific complement components in the transblots was identified with horseradish peroxidase-conjugated goat anti-rabbit IgG and substrate as described above.

Results

Identification of Cardiac Mitochondrial Proteins That Activate Complement

Previous studies by Pinckard and his colleagues have shown that canine mitochondria bind human Clq and can activate the classical pathway in human and guinea pig serum, even when these sera lack specific anti-mitochondrial antibodies. To identify specific molecules that exhibit this function, we fractionated detergent lysates of mitochondria by SDS-PAGE and transferred the fractions to NCP. The nitrocellulose replicas were then incubated with human Clq and a source of hemolytic complement, previously adsorbed with sheep red blood cells. After a brief wash, these replicas were overlaid with agarose containing sheep red blood cells sensitized with rabbit anti-sheep red blood cell antibodies. We reasoned that the complement, adsorbed to the NCP, would lyse the sheep red blood cells in the agarose overlay except at sites where Clq binding mitochondrial molecules had activated and consumed the available complement within their immediate microenvironment, in the interval before the addition of the sensitized red cell overlay. Indeed, as shown in Figure 1, lanes A and C, all the sheep red blood cells in the overlay were lysed except at sites occupied by molecules between 45 kDa to 53 kDa, and at locations that correspond to molecules having molecular weights of 34, 30, 26, and 23 kDa. If human Clq was omitted from the serum used
FIGURE 1. Identification of complement fixing molecules in NP-40 lysates of canine cardiac mitochondria: A nitrocellulose paper replica of subsarcolemmal mitochondria, fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, under reducing conditions, was incubated in 10% guinea pig serum supplemented with 20 μg/ml purified human Clq, washed briefly, and then overlaid with sheep red cells sensitized with rabbit antibody. Lane A shows that after incubation the red cells were lysed except at sites occupied by molecules between 45 and 53 kDa and 34, 30, 26, and 23 kDa. Lane C is a transilluminated photograph of the agarose overlay from lane A after it was removed from the nitrocellulose to illustrate that the agarose overlay transmits light poorly, except where activated complement on the nitrocellulose paper had lysed the red blood cells. Lane B is a trans blot from the same gel, which was incubated with fresh human serum, washed, and then incubated with antisera specific for C5, followed by horseradish peroxidase-conjugated anti-immunoglobulin and substrate. The anti-C5 binds to the same molecules identified by inhibition of complement-mediated red cell lysis, shown in lanes A and C, plus additional molecules >94 kDa to 55 kDa.

as a source of complement in these experiments, no inhibition of lysis was seen.

To validate the hypothesis that the complement fixing sites (identified in Figure 1, lanes A and C) were the locations of molecules that can serve as assembly points for the complement cascade, we incubated additional NCP replicas with fresh serum, washed them, and then incubated them with antibodies to individual complement components. Figure 1, lane B shows a nitrocellulose replica, identical to the ones used in lanes A and C, which was incubated with fresh human serum, washed, and then incubated with antibody reagents that identify sites of localization of C5. We see that C5 was also bound by the cluster of molecules 45 to 53 kDa and by the 34, 30, 26, and 23 kDa molecules, previously identified by inhibition of complement fixation in lanes A and C. Several additional molecules >94 to 55 kDa also provided sites for localization of C5 (Figure 1, lane B). This experiment (Figure 1, lane B), also reveals that the 30 and 23 kDa complement fixing sites identified in lanes A and C, each contain two complement binding molecules, closely similar in molecular weight. One contains molecules 28 and 30 kDa the other contains molecules 22 and 23 kDa.

Proteins Between 45 and 53 kDa Are the Most Abundant Complement Fixing Molecules in Mitochondria

To identify the most abundant complement fixing molecules in these mitochondrial lysates, we performed the experiment shown in Figure 2. Fractionation of 200 μg of the mitochondrial lysate by SDS-PAGE allowed us to identify 22 molecules that provide C5 fixing sites after transfer of the fractionated proteins to NCP (Figure 2, lane B). These molecules ranged from 14 to 83 kDa. Application of fourfold less mitochondrial lysate to the gel (Figure 2, lanes C and D) significantly decreased the numbers of C5-reactive molecules detected. When only 12.5 μg mitochondrial protein was applied (Figure 2, lanes E and F) we saw no C5-reactive molecules. In lane G we see only one band of C5-reactive molecules, and we interpret this as a single band of C5 fixing molecules, since these were the conditions used in the nitrocellulose overlay experiments (Figure 1, lane C). The lane H shows that the only C5 fixing sites were identified in fractions between 45 and 53 kDa.
Complement binding molecules in fractionated subsarcolemmal mitochondria may be identified by detecting those that bind purified C1q as well as those that serve as sites for localization of human C5 and C9 or canine C3. Lanes A and A' are controls: Nitrocellulose replicas of fractionated mitochondria incubated with anti-C1q(A) and then with peroxidase conjugated anti-immunoglobulin and substrate(A'). In B and B', the transblots were incubated with C1q before exposure to anti-C1q. Lanes C and E were incubated with fresh human serum and developed with anti-C5 and anti-C9, respectively. Lanes D and F were incubated with heat-inactivated serum containing 0.01 M EDTA before exposure to anti-C5 (lane D) or anti-C9 (lane F). Lane G was incubated with fresh canine serum and lane H, with heat inactivated and EDTA-treated dog serum. Both lanes G and H were incubated with anti-dog C3 followed by horseradish peroxidase-conjugated anti-immunoglobulin and substrate.

2, lanes E and F) anti-C5 detected only six molecules in well-washed transblots previously incubated with fresh serum. These corresponded closely in molecular weight to the cluster of molecules between 45 kDa, demonstrated by the modification of the traditional complement fixation test shown in Figure 1, lanes A and C.

Canine Mitochondrial Molecules That Bind Human C1q Serve as Sites for Localization of C5 and C9 and React With Canine Complement

To investigate whether specific mitochondrial molecules bind C1q, NCP replicas of fractionated canine mitochondria were incubated with isolated human C1q. After this incubation, the NCP replicas were washed and then incubated with anti-C1q to identify molecules that had bound C1q. Control experiments (Figure 3, lanes A and A') show that none of the mitochondrial molecules react with anti-C1q alone, nor do they react with the horseradish peroxidase-conjugated goat anti-rabbit IgG used to identify molecules that had bound the anti-C1q.

Figure 3, lanes B and B' demonstrate that 20 or more constituents of mitochondria provide sites for attachment of C1q. Incubation of these same nitrocellulose replicas with human serum, followed by antibodies to human C5 (Figure 3, lane C) or C9 (Figure 3, lane E), or dog serum followed by antibodies to canine C3 (Figure 3, lane G) illuminate the same array of molecules that react with human C1q, suggesting that mitochondrial molecules that bind C1q provide sites for assembly of the entire complement cascade.

Five additional mitochondrial molecules, one >94 kDa, the others 50.5, 42.8, and 22.7 kDa, were identified by anti-dog C3, horseradish peroxidase-conjugated goat anti-rabbit IgG in transblots initially incubated with fresh dog serum as a source of complement (Figure 3, lane G). These molecules were not evident in transblots incubated with C1q (lanes B and B') nor in those exposed to fresh human serum followed by anti-C5 (lane C) or anti-C9 (lane E). Therefore, the molecules uniquely react with anti-C3 (lane G) may be mitochondrial constituents that activate the alternative complement pathway or molecules that provide complement binding sites because they express antigens that bind naturally occurring anti-mitochondrial antibodies, present in canine serum.

Localization of human C5 and C9 or canine C3 was greatly reduced by adding EDTA and heat
Isolation of Clq Binding Mitochondrial Molecules by Solid Phase Immunoabsorbents Coated With Clq

To concentrate the Clq binding molecules in mitochondria, we incubated NP 40 mitochondrial lysates with Clq, adsorbed to NCP strips. After 14 hours of incubation at 6°C in the presence of 0.05% Tween 20 and 4% BSA, the strips were extensively washed and the bound proteins removed with 1.5% SDS. Figure 4 demonstrates that these eluates contained 22 distinct molecular species ranging in size from 67 kDa to much less than 14 kDa. Two of these reacted prominently with antibodies prepared against Clq binding proteins that are found in cardiac lymph of dogs that have had a recent coronary artery occlusion (Figure 4, lane A). Twelve of the Clq binding molecules in the mitochondrial lysate provide sites in vitro for the assembly of C3 and C5 (Figure 4, lanes B and E, respectively), including the 64-kDa molecule identified in lane A by antibodies to the Clq binding proteins in postreperfusion cardiac lymph. The most prominent of these are 51- and 53-kDa molecules, which are among the four mitochondrial proteins that provide sites for localization of all complement proteins through C9 (Figure 4, lane G). These proteins are closely similar in molecular weight to the 45-53 kDa cluster of complement fixing mitochondrial molecules previously described in Figure 1. As in previous experiments, heat-inactivated, EDTA-treated sera were used as controls to demonstrate that the antibodies to human C3, C5, and C9 used in this experiment had little if any intrinsic ability to react with the molecules in these mitochondria. Compare lanes B and C, D and E, and F and G in Figure 4.

Formation of an Antigen Antibody Complex Is Not Necessary for Mitochondrial Proteins to Fix Complement

It was conceivable that the sera used as a source of complement in these investigations might contain antibodies that could bind to specific mitochondrial molecules and create antigen-antibody complexes that would provide sites for localization of Clq and later-acting complement components. To investigate this possibility, as opposed to the alternative hypothesis that mitochondrial molecules, identified after incubation with fresh serum and antibodies specific for C3, C5, or C9, are molecules that directly activate the classical complement pathway, we compared the reactions in NCP replicas of fractionated mitochondria caused by fresh normal donor serum and serum from a patient with severe combined immunodeficiency syndrome, used as a source of complement.

The serum from the patient with severe combined immunodeficiency syndrome had no detectable IgG or IgM antibody to mitochondrial proteins as shown by the experiments in lanes C and D of Figure 5. In contrast, serum from a normal adult donor, shown in lanes E and F of Figure 5 contained IgG antibodies to 17 mitochondrial components and IgM antibodies to at least three. Nevertheless, when that normal donor serum was used as a source of complement and the reaction was developed with anti-C5, horseradish peroxidase-conjugated goat anti-rabbit IgG, and substrate (Figure 5, lane I) the pattern of staining by the peroxidase substrate was very different from that caused by localization of IgM or IgG antibodies. Molecule for molecule, the bands identified with anti-C5 matched those attributable to the localization of isolated Clq, as shown in lane H, Figure 5. Addition of EDTA and heating at 56°C for 30 minutes significantly suppressed the localization of anti-C5 when this serum was used as a complement source (lane J).
isolated Clq, as shown in lane H, Figure 5. Addition of EDTA and heating at 56°C for 30 minutes significantly suppressed the localization of anti-C5 when this serum was used as a complement source (lane J).

Notably, when the serum from the patient with severe combined immunodeficiency was used as a source of complement and developed with anti-C5, horseradish peroxidase-conjugated goat anti-rabbit IgG and substrate (lane K), the reactions closely matched those seen in the strip incubated with isolated Clq, followed by anti-Clq. There was also a strong homology between the reactions developed by normal donor serum (lane L) and the serum from the patient with severe combined immunodeficiency syndrome (lane M) when these were used as a source of complement and developed with anti-C9, horseradish peroxidase-conjugated goat anti-rabbit IgG, and substrate. Considering the congenital inability of this donor to make antibodies and the demonstrated lack of IgG or IgM antibodies to molecules of mitochondrial origin in the serum from this immunodeficient patient, it seems likely that the reactions demonstrated when this serum is used as a complement source truly indicate molecules that directly activate complement. Considering also the homology between reactions caused by isolated Clq and the reactions caused by the immunodeficient patient's serum, it seems likely that these mitochondrial molecules activate the classical pathway.

Discussion

Since the seminal investigations of Hill and Ward, it has been evident that muscle injury, particularly ischemic injury to cardiac muscle, can activate the complement cascade. A series of investigations by Pinckard and his colleagues suggested that molecules associated with mitochondria might supply the stimuli that activate complement following ischemic injury independent of the presence of antibodies reactive with subcellular membranes or organelles. The earliest investigations indicated that an interaction of C1 and mitochondria is sufficient for complement activation. Although subsequent studies by Giclas et al suggested that activation of complement by heart subcellular membranes may involve both classical and alternative pathways, the very high affinity of Clq for mitochondrial and other subcellular membranes (Ks, 108 to 1010 M−1) and
complement activation following tissue damage sufficient to bring complement in contact with subcellular organelles. These studies also suggested that the Cl binding sites were integral membrane components since they could not be removed by washing the membranes in high salt or EDTA. Kovacsovics et al. in a study of the ability of phospholipids to mediate antibody-independent activation of Cl, suggested that mitochondria and other subcellular membranes may trigger Cl activation because they incorporate integral phospholipids that contain regions with high density negative charges. These are attributes of some, but no means all, biological materials that can cause "nonimmune" activation of Cl.

The present attempt to identify and enumerate specific molecular constituents of mitochondria that can bind Cl and activate the complement cascade was stimulated by earlier work from this laboratory, which demonstrated that radioiodinated Clq, injected into animals undergoing experimental coronary artery ischemia, localizes preferentially in regions of the myocardium that have the lowest perfusion. Even after reperfusion, the deposits of labeled Clq persist and serve as markers for sites of accumulation of radiolabeled autologous leukocytes, suggesting that the deposits of Clq indicate sites where complement activation has caused the release of the leukotactic anaphylatoxin, C5a. Studies of the lymphatic fluids that drain from formerly ischemic myocardium, after reperfusion, suggested that they contain a number of molecules that are not present in normal canine cardiac lymph; some appear to originate from mitochondria or sarcoplasmic reticulum. But evidence that these molecules activate the complement cascade was indirect, since it was based on the observation that antisera prepared against molecules precipitated from postreperfusion cardiac lymph by anti-Clq react with molecules of mitochondrial origin, which can form complexes with Clq in vitro.

The present studies were designed to directly identify any canine cardiac mitochondrial molecules that not only bind Clq but also activate the complement cascade. Most abundant among the many molecules that bind Clq is a cluster of four to six molecules between 45 and 53 kDa, which retain the ability to serve as assembly sites for complement when only 12.5 μg of mitochondrial protein was fractionated and transferred to NCP. These molecules, as well as four others having estimated molecular weights of 34, 30, 26, and 23 kDa, bound human Clq and activated guinea pig complement, causing localized inhibition of complement-mediated lysis of sensitized sheep red blood cells, in a modified complement fixation test.

Using antibodies specific for Clq, C3, C5, and C9, we were able to demonstrate additional molecules that appeared to provide sites for binding Clq and for assembly of later-acting complement proteins. Considering that fresh human or canine sera were used as a source of complement in these experiments, we were concerned that these methods would as easily reflect complement localization caused by antigen-antibody complexes as antibody-independent complement localization. However, comparison of the results obtained with fresh frozen serum from a patient with severe combined immunodeficiency syndrome who had no antibodies to any of the components in mitochondria (Figure 5) with those obtained with normal human serum suggest that the first explanation is unlikely. We could demonstrate that normal human sera contains IgG and IgM antibodies that react with specific molecular constituents of mitochondria. But under the conditions employed in these studies, the patterns of localization of C5 and C9 achieved with severe combined immunodeficiency syndrome were identical. They did not correspond to the localization of IgG or IgM antibodies from normal donor sera in the NCP replicas of fractionated mitochondria. Thus, it is likely that the mitochondrial constituents we identified with antibodies to C3, C5, and C9 are molecules that cause antibody-independent complement activation.

Antibodies prepared against the Clq-binding proteins in postreperfusion lymph react prominently with only two of these mitochondrial molecules; one of these provides a site of assembly for complement proteins through C9. It is not clear why these antibodies recognize so few of the complement fixing molecules in mitochondrial lysates since it is likely that others are released into the extracellular fluids after ischemic injury. It is conceivable that lysosomal proteases, released by activated leukocytes that were attracted to the ischemic myocardium after complement activation, may degrade many of the Clq binding molecules released from mitochondria with the result that antisera prepared against those recoverable in postreperfusion lymph only recognize the few which are relatively resistant to such degradative enzymes.

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