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Animal Model

Time Course of Complement Activation and Inhibitor Expression after Ischemic Injury of Rat Myocardium

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Activation of the complement (C) system has been documented in both experimental and clinical studies of myocardial infarction, but the exact time course and mechanisms leading to C activation have remained unclear. Our earlier postmortem study on human beings showed that formation of the membrane attack complex (MAC) of C was associated with loss of CD59 (protectin), an important sarcolemmal regulator of MAC, from the infarcted area. The recent discovery of a rat analogue of CD59 has now allowed the first experimental evaluation of the temporal and spatial relationship between C component deposition and loss of CD59 in acute myocardial infarction (AMI). After ligating the left coronary artery in rats the earliest sign of C activation, focal deposition of C3, was observed at 2 hours. Deposition of the early (C1, C3) and late pathway (C8, C9) components in the AMI lesions occurred at 3 hours. Glycophosphoinositol-anchored rat CD59 was expressed in the sarcolemmal membranes of normal cardiomyocytes. In Western blot analysis extracts of normal rat heart CD59 appeared as a band of 21 kd of molecular weight under nonreducing conditions. Loss of CD59 in the AMI lesions was observed in association with deposits of MAC from day one onward. Our results show that C activation universally accompanies AMI in vivo. It is initiated within 2 hours after coronary artery obstruction via deposition of C3, which may be due to generation of the alternative pathway C3 convertase in the ischemic area. Deposition of C1 and late C components also starts during the early hours (2 to 4 hours) after ischemia. Subsequent loss of the protective CD59 antigen may initiate postinjury clearance of the irreversibly damaged tissue. (Am J Pathol 1994, 144:1357–1368)

Although activation of the complement (C) system in acute myocardial infarction (AMI) has been demonstrated in several studies,[1–6] the precise time course and the initiating events in C activation are not well known. The role of C in the pathogenesis of AMI has been established in experimental models, where depletion of C with cobra venom factor[3,7] or its inhibition by the soluble recombinant human C receptor type 1, sCR1,[8] have reduced the size of AMI lesions. Although it is clear that tissue injury in the infarcted myocardium is primarily due to the ischemia itself, activation of the C system during the reperfusion stage will increase its extent. The C anaphylatoxins, C3a and C5a, are capable of attracting and activating leukocytes in the infarcted area.[9–13] The membrane attack complex of C (MAC)[14] may cause direct cell lysis or lead to changes in cell metabolism by increasing calcium influx into the cells.[15–17]

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Table 1. *Immunofluorescence Analysis of Complement Deposits and Expression of Rat CD59 in the Hearts of Variously Treated Rats*

Group*	Age of Infarction†	C1	C3	C8	C9	Rat CD59
I	1 hour	–	–	–	–	+
II	2 hours	–	+ (2/4)	–	–	+
III	3 hours	+	+	+	+	+
IV	6 hours	+	+	+	+	±
V	24 hours	+	+	+	+	±/–
VI	72 hours	+	+	+	+	–
VII	Sham-operated rats	–	–	–	–	+
VIIIa‡	No infarction	–	–	–	–	+
VIIIb‡	6 hours	+	+	+	+	±

* n = 4 for each group.
† Time between ligation of left coronary artery and death of the animal.
‡ Autolysis control rats.

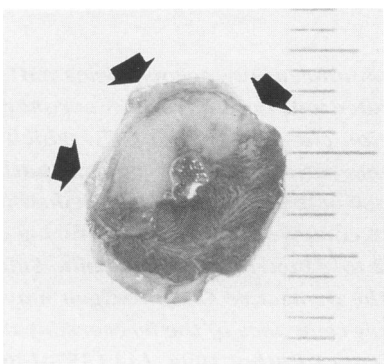


Figure 1. *Cross-section of infarcted rat myocardium. Tissue section is stained with NBT for detection of myocardial infarction* (**arrows**). *The age of infarction is 3 hours* (×2.7).

Activation of the human C system is strictly controlled by soluble and membrane-associated C regulatory proteins.[18] At the C3 and C5 steps C activation is inhibited by both membrane-associated (CR1, decay accelerating factor [DAF] and membrane cofactor protein [MCP]) and soluble regulators (C4 binding protein [C4bp] and factor H). The terminal pathway of C is controlled by C8 binding protein (C8bp) and CD59 on cell membranes and by vitronectin (S protein) and clusterin (apo J, SP40,40) in the fluid phase. In human myocardium the main membrane regulator of C is CD59,[6,19] which is a low molecular weight glycophosphoinositol (GPI-)-anchored inhibitor of homologous C.[20–23] CD59 inhibits MAC formation by preventing the incorporation and polymerization of C9 on cell membranes.[24,25]

Recently, the first membrane-associated inhibitor of MAC in rat, initially called rat inhibitory protein (RIP), was purified from rat erythrocytes.[26] RIP is a 21-kd glycoprotein linked to cell membranes by a GPI anchor. The functional properties, NH_2-terminal amino acid sequence, and ability of RIP to inhibit MAC of rat demonstrate that RIP is the rat homologue of human CD59[26] and is hence here referred to as rat CD59.

In our previous study we observed that human CD59 was strongly expressed in normal myocardium and selectively lost from AMI lesions.[6] In postmortem specimens the loss of CD59 was associated with deposits of MAC in the AMI lesions aged 8 hours or more. This study left the following questions unanswered: 1) what is the time course of C activation in the infarcted lesions? and 2) what is the relationship between loss of CD59 and formation of MAC in the AMI lesions? The primary aim of this study was to seek an answer to these questions by an analysis under more defined conditions in a rat model for AMI. Identification of the rat analogue of CD59 has now for the first time allowed examination of its pathophysiological role in an experimental animal model.

Materials and Methods

Animals

Male Wistar rats (Unit of Clinical Physiology, Minerva Institute for Medical Research, Helsinki, Finland) used in this study were 4 to 6 months old and weighed 400 to 600 g. Rats ate standard food and had standard laboratory animal care. The study protocol was approved of by the ethical committee of our institute and by the local health authority (STO 2534).

Animal Preparation and Experimental Protocol

Myocardial infarctions were produced in rats according to methods previously described.[27,28] Rats were anesthetized with ketamine (Ketalar, Parke-Davis, Barcelona, Spain), 150 mg/kg intraperitoneally, and xylazine (Rombun, Bayer Corp., Leverkusen, Germany), 6 mg/kg intramuscularly, tracheostomized and ventilated with a custom-made positive pressure respirator (Medith Corp., Helsinki, Finland). The animals underwent left thoracotomy and the left coronary

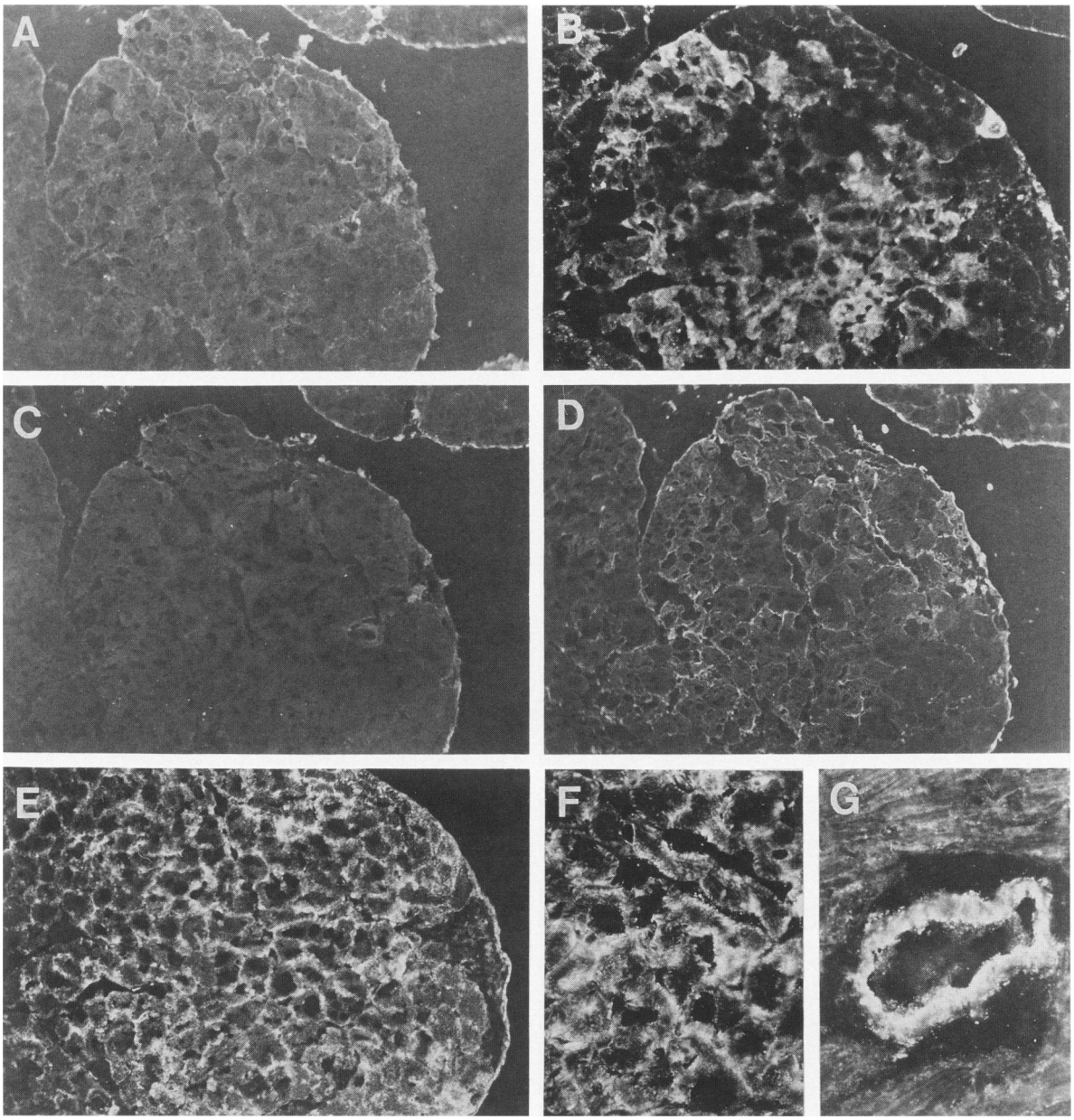
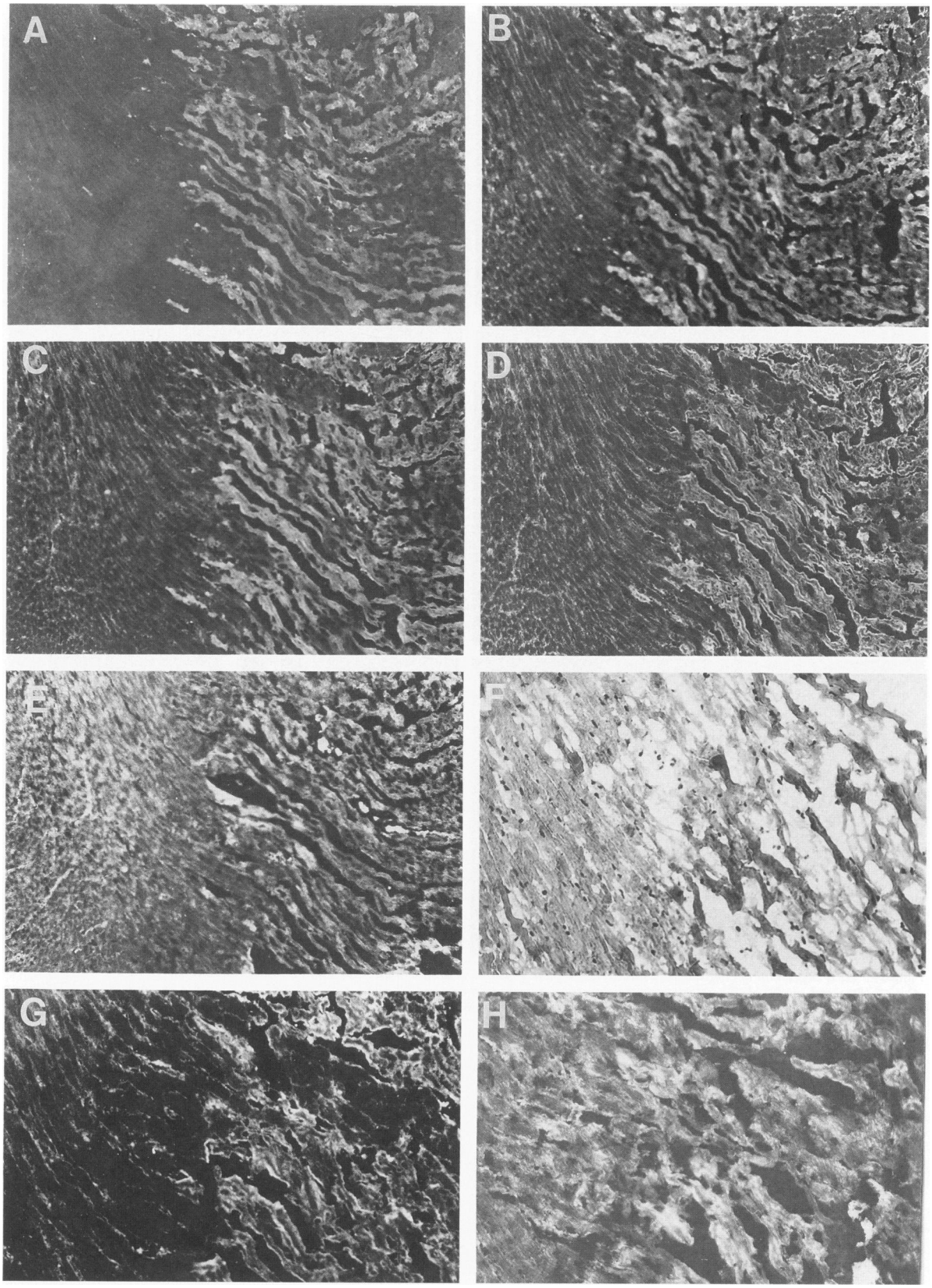


Figure 2. *Early deposition of complement in infarcted papillary muscle. A 2-hour infarction lesion was immunostained for rat C1* (**A**), *C3* (**B**), *C8* (**C**), *C9* (**D**), *and rat CD59* (**E**). *C3 is deposited in a focal pattern in the lesion. No similar deposits of C1, C8, or C9 are present. Rat CD59 is expressed on sarcolemmal membranes* (**E**). *The controls show CD59 expression on normal cardiomyocytes* (**F**) *and on the endothelium of a coronary artery* (**G**). **A–E**, *×100;* **F** *and* **G**, *×400.*

artery was ligated between the pulmonary artery outflow tract and the left atrium. The thoracotomy and tracheostomy wounds were closed and the rats were allowed to recover. Sham-operated rats underwent the same procedure without coronary artery ligation. The rats were sacrificed by carbon dioxide inhalation and decapitation.

Animals were divided into eight groups (see Table 1), each of which consisted of four animals. In groups I to VI rats were killed 1, 2, 3, 6, 24, or 72 hours after the permanent coronary artery ligation. Animals without diagnostic signs of AMI in nitroblue tetrazolium (NBT) and hematoxylin and eosin stainings (n = 5) were excluded from groups III to VI. In groups I and II the NBT and hematoxylin and eosin staining methods were too insensitive for the detection of AMI lesions aged less than 3 hours. Group VII consisted of sham-operated rats that were killed 72 hours after the thoracotomy. In group VIII two rats were and two rats were not subjected to coronary artery ligation. Rats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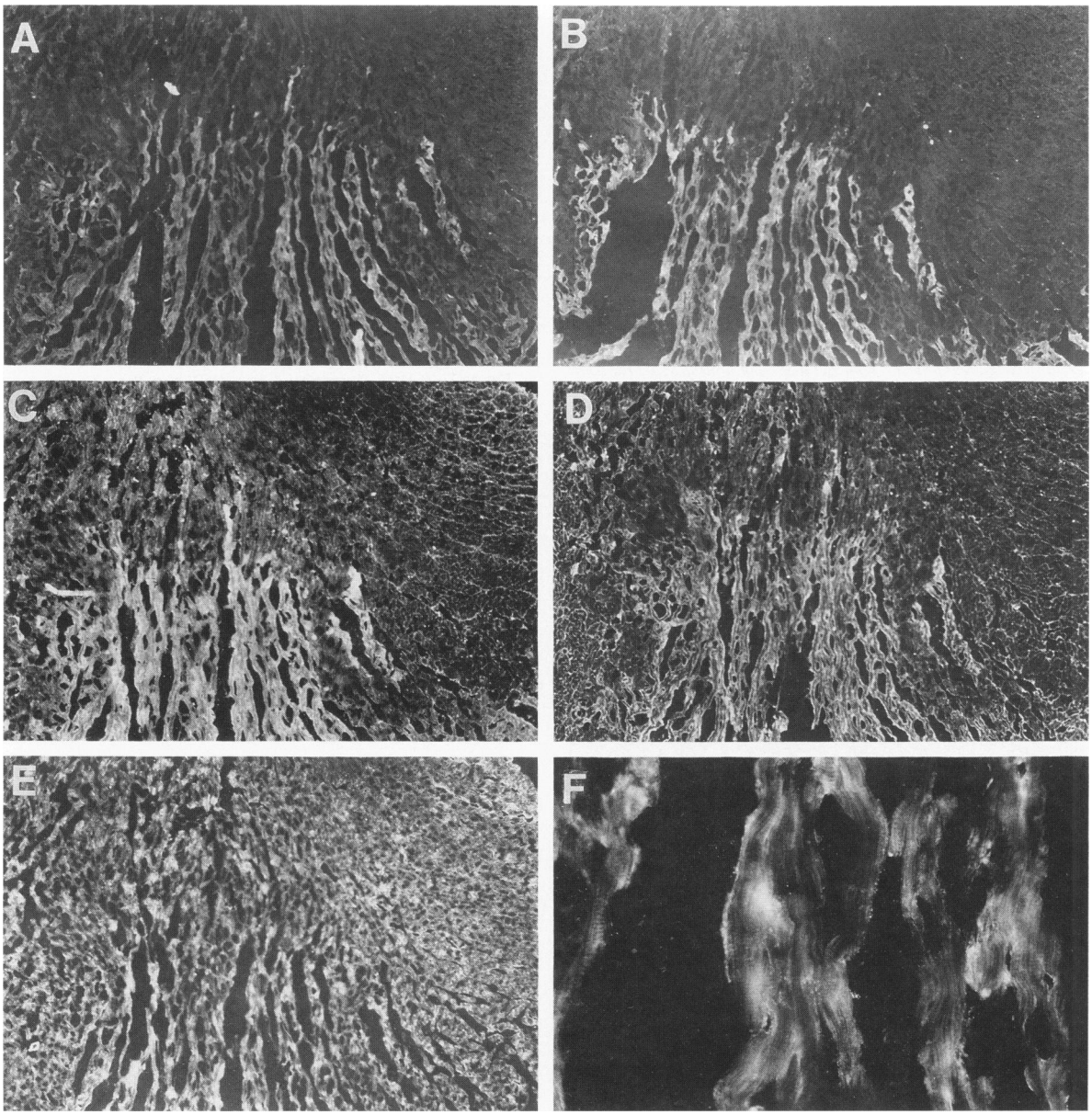

Figure 4. *Deposition of C1* (A), *C3* (B), *C8* (C), *and C9* (D) *in a border area of a 6-hour ischemic lesion (bottom). The expression of rat CD59 in AMI lesions was slightly diminished 6 hours after coronary artery ligation* (E, F). A–E, ×100; F, ×350.

were killed at 6 hours, whereafter they were kept at 4 C for 3 days. This group was used for the examination of the effects of postmortem autolysis on the results of immunofluorescence (IFL) stainings. In addition, hearts (n = 4) of nonoperated normal rats were examined as controls for IFL stainings.

Diagnosis of Myocardial Infarction

For macroscopic detection of AMI, NBT vital staining[29] was used. Immediately after killing of the rat horizontal sections of the heart, approximately 3-mm thick, were incubated in the NBT staining solution (15

Figure 3. *Deposition of early, C1* (A), *C3* (B), *and late, C8* (C), *C9* (D, G), *components of C and expression of rat CD59* (E, H) *in a border area of a 3-hour AMI lesion. The infarcted area is on the right. The localization of C1, C3, and C8 is both sarcolemmal and intracellular, whereas C9 is found mostly on cell membranes. No loss of CD59 is apparent at this stage. Hematoxylin and eosin staining of a border area between infarction and normal tissue* (F). A–E, ×100; F–H, ×300.

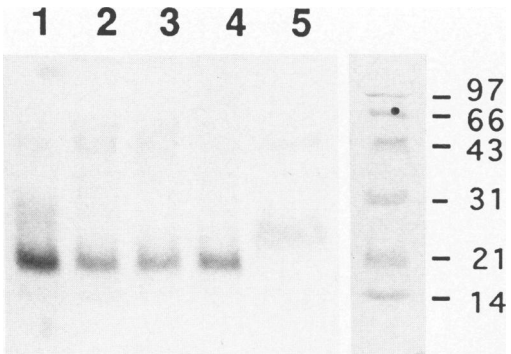

Figure 5. *Western blot analysis of rat CD59. A solubilizate of rat erythrocyte ghosts (lane 1), homogenates from noninfarcted rat heart tissue (lanes 2 and 3), aorta (lane 4), and urinary proteins (lane 5) were subjected to 12% SDS-PAGE under nonreducing conditions. After transfer to a nitrocellulose membrane rat CD59 was detected with a monoclonal antibody (TH9) and alkaline phosphatase-conjugated anti-mouse IgG secondary antibody. The apparent molecular weights of membrane-associated (lanes 1 to 4) and soluble (lane 5) rat CD59s are 21 and 24 kd, respectively.*

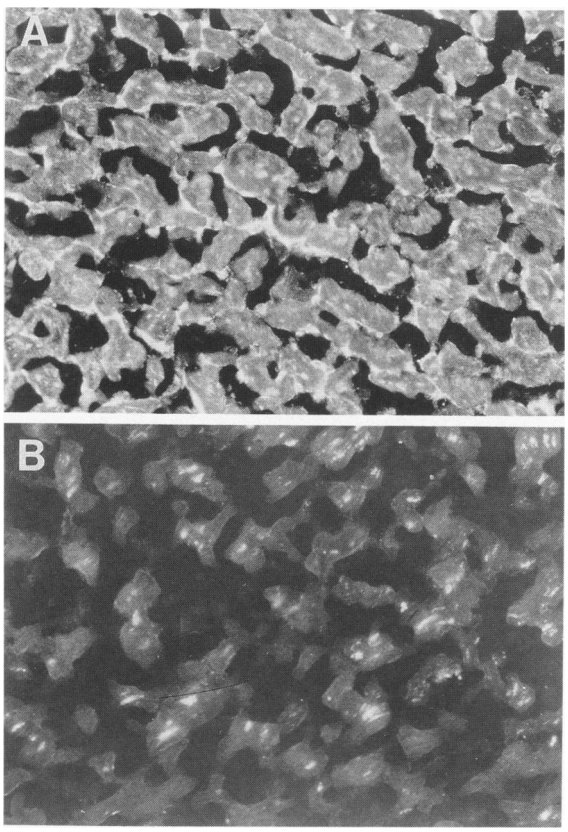

Figure 6. *Sensitivity of rat CD59 in myocardium to PIPLC (1 IU/ml, 30 minutes at 37 C). IFL staining of frozen sections of normal rat heart for CD59 (TH9 mAb) before* (A) *and after* (B) *treatment with PIPLC (×300).*

minutes, 37 C). Frozen sections (5 μ, see below) of the heart were fixed with cold acetone (10 minutes, −20 C) and stained with hematoxylin and eosin. His-

Figure 7. *Loss of expression of rat CD59 in a necrotic AMI lesion aged 24 hours. A border area between an infarcted lesion (bottom, right) and adjacent normal tissue (top, left) is shown (×300).*

topathological diagnosis of AMI was assessed by the presence of neutrophil infiltration, eosinophilia, cellular edema, nuclear changes, hyperchromasia, loss of crossstriation, or complete cellular necrosis.

Immunohistochemistry

Hearts were removed immediately after killing of the animals. Tissue blocks were prepared for suitable horizontal tissue sections. One section was used for NBT staining and other parallel sections were placed in Tissue-Tek embedding medium (Ames Co., Elkhardt, IN), frozen using dry ice and stored at −70 C. Frozen sections (5 μ) were fixed with cold acetone (10 minutes, −20 C) for indirect IFL microscopy. Serial sections were incubated for 30 minutes at 22 C with the following antibodies: mouse anti-rat CD59 mAb (TH9)[26] or rabbit antibodies against rat C1, C8, or C9.[30] After washing three times with phosphate-buffered saline (PBS), pH 7.4, the sections were treated with fluorescein isothiocyanate (FITC-)-conjugated antibodies against rabbit or mouse (Dakopatts, Glostrup, Denmark) immunoglobulins, respectively. For detection of C3 FITC-conjugated antibodies against rat C3 (Cappel Laboratories, Malvern, PA) were used. IFL staining analyses were controlled by omitting the primary antibody, by using nonimmune sera, or by using mouse antibodies against rat albumin or rat IgG (Dakopatts). The IFL slides were mounted with Mowiol[31] and examined on a Zeiss Standard microscope equipped with a filter specific for FITC fluorescence. For photography, Kodak Tri-X Pan films were used.

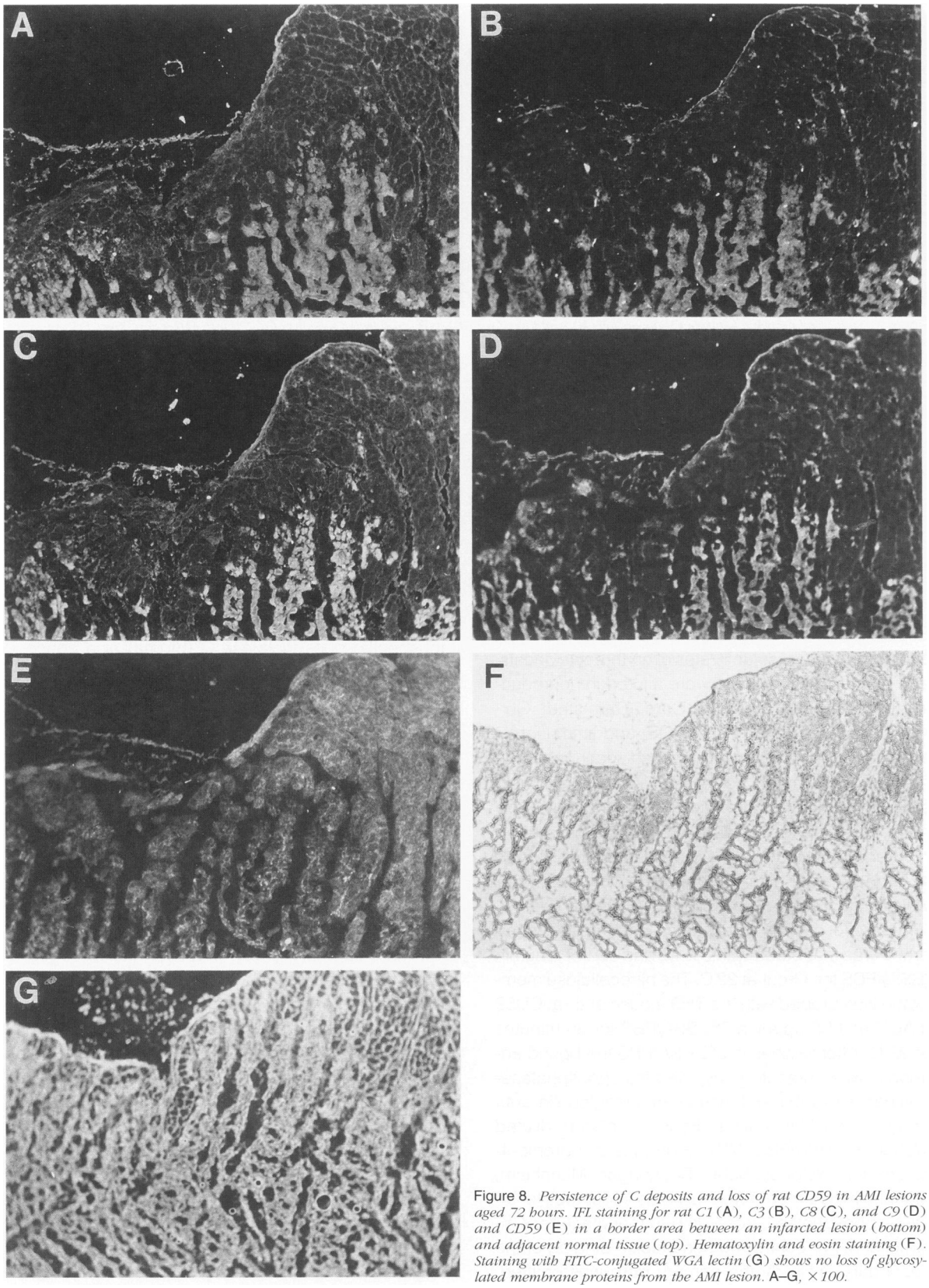

Figure 8. *Persistence of C deposits and loss of rat CD59 in AMI lesions aged 72 hours. IFL staining for rat C1* (A), *C3* (B), *C8* (C), *and C9* (D) *and CD59* (E) *in a border area between an infarcted lesion* (*bottom*) *and adjacent normal tissue* (*top*). *Hematoxylin and eosin staining* (F). *Staining with FITC-conjugated WGA lectin* (G) *shows no loss of glycosylated membrane proteins from the AMI lesion.* A–G, ×100.

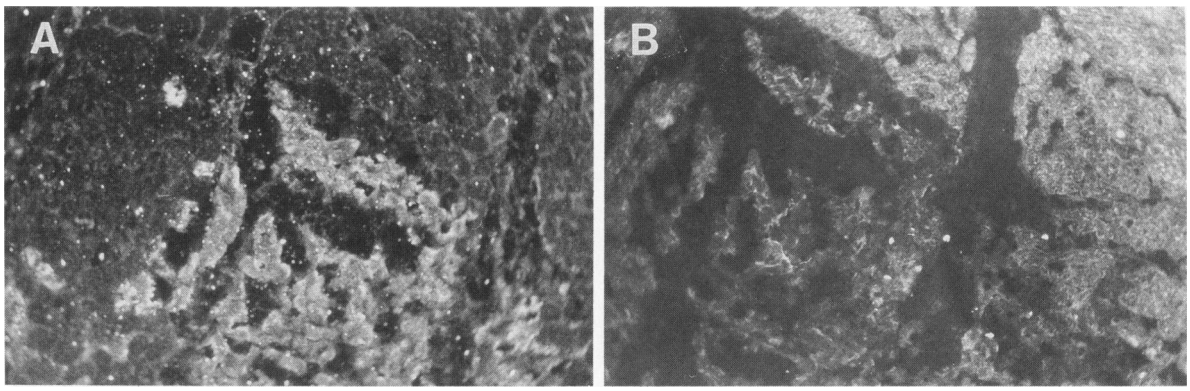

Figure 9. *Spatial relationship between C9 deposition* (**A**) *and loss of rat CD59* (**B**) *in parallel sections of a 72-hour AMI lesion. The CD59-negative area is slightly wider than the area of C9 deposition* (×*300*).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting

Detergent extracts of rat and human tissues (heart and aorta) were obtained after first rinsing the tissues with ice-cold PBS and then homogenizing and solubilizing them in 60 mmol/L octylglucoside (Sigma Chemicals, St. Louis, MO) in PBS for 1 hour at 0 C. Supernatants of the detergent extracts of the heart and aortic tissues, solubilizates of erythrocyte ghosts, and concentrates of urine were diluted in nonreducing SDS-PAGE buffer (Merck Corp., Darmstadt, Germany) and used for SDS-PAGE and immunoblot analyses. SDS-PAGE was performed according to the method of Laemmli[32] using 15% gels and a minigel system (Bio-Rad Laboratories, Richmond, CA). Low molecular weight standards were from Bio-Rad.

After electrophoresis the proteins were transferred to nitrocellulose filters (Bio-Rad). The molecular weight standards were stained with Ponceau S (Sigma). To prevent nonspecific binding the membrane was incubated with 3% bovine serum albumin (BSA)/PBS for 1 hour at 22 C. The nitrocellulose membrane was probed with the TH9 mouse anti-rat CD59 mAb[26] at 11.5 μg/ml in 3% BSA/PBS for 30 minutes at 22 C. After washes in 3% BSA/PBS the bound antibody was detected by alkaline phosphatase-conjugated rabbit anti-mouse immunoglobulin antibody (Orion-Diagnostica, Espoo, Finland) diluted 1/20 in 3% BSA/PBS. NBT (Sigma) and 5-bromo-4-chloro-3-indolylphosphate (Boehringer Mannheim, Mannheim, Germany) were used as substrates for alkaline phosphatase, according to manufacturer's instructions. For controlling nonspecific binding of the secondary antibody the primary mAb was omitted. BRIC 229, a mouse anti-human CD59 mAb (Bio-Products Laboratory, Elstree, UK), was also used for Western blot analysis of rat and human heart homogenates and urine concentrates.

Treatment of Rat Myocardium with Phosphatidylinositol-Specific Phospholipase C

Cryostat sections of normal nonfixed rat myocardium were treated with *Bacillus cereus* phosphatidylinositol-specific phospholipase C (Sigma) at a concentration of 1 IU/ml in PBS or with PBS alone for 30 minutes at 37 C in a moist chamber. After washing three times with PBS, the sections were immunostained for rat CD59 as described above.

Results

C Deposition in Myocardial Infarction Lesions and Normal Myocardium of Rat

AMI to rats was caused by permanent ligation of the left main coronary artery. Infarction lesions aged 3 hours or more were macroscopically detectable by NBT staining (Figure 1). Cross-sectional areas of the AMI lesions ranged from 16 to 36% of the total horizontal area. Microscopically, by hematoxylin and eosin staining, the earliest signs of AMI could be detected 3 to 6 hours after coronary artery ligation.

No apparent cardiomyocyte-associated C deposits were observed in normal hearts or in normal areas of infarcted hearts. Occasionally, the basement membranes of blood vessels stained positive for C3, C8, and C9. Initial signs of C activation could be demonstrated in AMI lesions by IFL staining 2 hours after ligation of the coronary artery. In the 2-hour infarction group two of four rats had foci of C3 deposition in the myocardial areas supplied by the ligated coronary

artery but no corresponding deposition of C1, C8, or C9 (Figure 2). By 3 hours both early (C1 and C3) and late components (C8 and C9) of C were deposited in the histologically diagnosed AMI lesions (Figure 3). In the 6-hour or older myocardial infarction lesions clear-cut deposits of C were detected (Figures 4, 8, and 9). C components were deposited exclusively in the infarcted lesions. Control stainings by omitting the primary antibody, by using nonimmune rabbit serum, or antibodies against rat albumin or rat IgG appeared negative in both normal and infarcted myocardial areas.

Expression of CD59 in Normal Rat Myocardium and Its Sensitivity to Phospholipase C Treatment

Frozen sections of each of the infarcted and noninfarcted rat heart specimens were stained with the TH9 mouse anti-rat CD59 mAb. Rat CD59 was expressed in the sarcolemmal membranes of normal cardiomyocytes throughout the myocardium, in endocardium, and in endothelial cells of blood vessels (Figure 2, E-G). Western blot analysis of homogenates of normal rat myocardium showed a major band of 21 kd and faint minor bands of higher molecular weight reacting with the TH9 mAb (Figure 5, lanes 2 and 3). A similar major band was observed in rat erythrocyte ghost preparations (Figure 5, lane 1) and in a homogenate of rat aorta (Figure 5, lane 4). In human urine the CD59 antigen exists in a soluble form without a lipid anchor and has a slightly higher apparent molecular weight than the lipid-anchored form in SDS-PAGE.[6] In Western blot analysis of a rat urine concentrate the TH9 mAb reacted with a band of approximately 24 kd of molecular weight (Figure 5, lane 5).

Homogenates of normal human heart and a concentrate of human urine showed no reaction with the TH9 mAb in the Western blots. When the primary antibody was omitted to control for nonspecific binding no reaction of the secondary antibody with the nitrocellulose-bound proteins was detected. Thus, the minor bands in Figure 5 probably represent nonspecific reactivity of the primary mAb. Analysis of the rat heart homogenate and urine showed no reaction with the BRIC 229 mAb against human CD59. In human heart homogenate and urine concentrate the BRIC 229 antibody detected bands with apparent molecular weights of 19 to 24 and 21 kd, respectively.

When the frozen sections of rat heart were treated with phosphatidylinositol-specific phospholipase C (PIPLC; 1 IU/ml for 30 minutes) the expression of rat CD59 in the sarcolemmal membranes, endothelial layers of cardiac blood vessels, and endocardium virtually disappeared indicating GPI-type anchorage (Figure 6).

Temporal and Spatial Relationship Between Loss of Rat CD59 and Deposition of C8 and C9 in Infarcted Myocardium

In our earlier study[6] we observed that human CD59 was lost from infarcted areas of myocardium in conjunction with deposition of components of MAC. A similar analysis of rat CD59 in our experimental model of AMI showed that the expression of rat CD59 gradually diminished from the 6- to 72-hour AMI lesions (Table 1). In most AMI lesions aged 24 hours or more the expression of rat CD59 was nearly absent (Figures 7, 8, and 9). In earlier AMI lesions (Figures 3 and 4) CD59 was still present but often appeared in condensed patches. Regularly, deposits of C (C1, C3, C8, and C9) were observed within the CD59-negative infarction lesions (Figures 8 and 9). No loss of rat CD59 was detected in the hearts of sham-operated rats or rats that had undergone a 3-day postmortem autolysis period (Table 1). Control stainings with sialic acid reactive FITC-conjugated wheat germ agglutinin (WGA) lectin showed that terminal sialic acid residues were present throughout the normal cardiac muscle and no major changes in their distribution were observed in the AMI lesions (Figure 8G).

Discussion

In this study we observed that the immunopathological features of experimental AMI in rats were similar to those earlier seen in humans. In the rat model it could be demonstrated that C activation is initiated approximately 2 hours after coronary artery ligation. The rat analogue of the human MAC inhibitory protein, protectin, or CD59 was abundantly expressed in normal rat myocardium. The expression of rat CD59 diminished with increasing time and development of the AMI lesions between 6 and 72 hours, whereas deposits of C8 and C9 were already evident at 3 hours.

The observation that C3 but not C1 was deposited in two of four 2-hour myocardial infarction hearts suggests that C activation is initiated by spontaneous activation of C3 and of the alternative pathway. Deposition of C1 in older lesions indicates that the classical pathway is recruited 2 to 3 hours after the beginning

of AMI. C becomes fully activated in the infarcted myocardium by the end of the third hour after permanent coronary artery ligation. This is in accordance with earlier studies showing C3 deposition in a 4-hour baboon AMI[1] and human autopsy studies showing complement MAC deposition in 6- to 8-hour AMI lesions.[2,6,19] It has been shown that heart mitochondrial membranes activate C via the classical pathway via an antibody-independent mechanism.[33] On the basis of our study it seems that C activation is initiated through the alternative pathway and continues later via the classical pathway after exposure of intracellular components like mitochondrial membranes and intermediate filaments to plasma.

Our previous study suggested that human heart seems to be protected better against the late than the early C cascade because regulators of the C3/C5 step, CR1, DAF, and MCP, were absent or expressed at low level in cardiomyocytes, whereas regulators of MAC, CD59, and C8bp were strongly expressed.[19] This implies that during AMI the contact of plasma with insufficiently protected cardiomyocytes may lead to generation of the alternative and classical pathway C3/C5 convertases. Complement activation during the primary phase of AMI will lead to the production of chemotactic and vasoactive components of C, primarily C3a and C5a, that can act as triggers of inflammation in the ischemic lesions.[9–13] Although activation of C appears to have progressed to the level of C9 within 3 hours from the beginning of AMI, it is not clear whether actual MACs have become formed by this time. Expression of CD59 may allow the deposition of some C9 molecules but would still prevent their insertion and polymerization into cell membranes.[24]

In our earlier study of human CD59 we observed that GPI-anchored CD59 was strongly expressed in sarcolemmal membranes of normal human cardiomyocytes.[6] Similarly, in this study rat CD59 was clearly detected in rat myocardium by both IFL and immunoblot analysis. Rat CD59 was found in normal myocardial sarcolemmal membranes and in the endothelia of blood vessels. Sensitivity of the sarcolemmal rat CD59 to the PIPLC enzyme indicates that it is tethered to the membranes via a PIPLC-sensitive GPI anchor. Similarities in the distribution, type of anchor, and apparent molecular weight between rat CD59 and human CD59 in myocardium further strengthen the conclusion that they represent species analogues of the same molecule.[26]

In the study of human autopsy material CD59 was lost from the AMI lesions in patients whose infarctions were aged 8 hours or more.[6] In this study the loss of expression of rat CD59 was observed to occur gradually between 6 hours and 3 days. The selectivity of the loss of rat CD59 was suggested by WGA lectin stainings, which showed no apparent loss of WGA-reactive sialylated glycoproteins in the rat CD59-negative AMI lesions. In control studies it was found that a 3-day postmortem interval did not lead to further loss of CD59 in noninfarcted or infarcted rat hearts. This indicates that the loss of CD59 is not caused by postmortem artefacts like autolysis but is a consequence of the pathophysiological process that follows myocardial ischemia. CD59 is not lost from the infarcted area before activation of the C system. Despite the presence of CD59, C activation can proceed until the C8/C9 stage. It is possible that CD59 is lost from myocardial cells in small membrane microparticles or vesicles.[6,34] Alternatively, CD59 may be "used up" in trying to prevent MAC-mediated damage. The diminished expression of CD59 after the initial C activation period may then gradually allow full assembly of the cytolytic MAC in the AMI lesions.

Although it is evident that activation of the C system may cause additional damage to the myocardium, during the late phase of AMI the role of C in the infarcted myocardial areas could also be related to the clearance of injured cardiomyocytes. The loss of CD59 from the infarcted myocardium during the late phase of AMI might sensitize nonviable cells to the membranolytic activity of MAC. Clearance of the membrane fragments and MAC complexes could be aided by the soluble multifunctional proteins, clusterin and vitronectin, as suggested by previous studies of human AMI.[19,35,36]

In conclusion, this experimental animal model study has verified and extended previous observations on C activation during AMI in humans. The availability of antibodies against rat C components and the identification of the rat analogue of CD59 (protectin) now allow more detailed experimental investigations of the role of C-mediated injury in various ischemic conditions and possible intervention studies using recombinant rat CD59.

Acknowledgments

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