Myocardial Infarction and Apoptosis After Myocardial Ischemia and Reperfusion
Role of the Terminal Complement Components and Inhibition by Anti-C5 Therapy

Antti P. Vakeva, MD; Azin Agah, PhD; Scott A. Rollins, PhD; Louis A. Matis, MD; Lan Li, PhD; Gregory L. Stahl, PhD

Background—Myocardial ischemia and reperfusion (MI/R)–induced tissue injury involves necrosis and apoptosis. However, the precise contribution of apoptosis to cell death, as well as the mechanism of apoptosis induction, has not been delineated. In this study, we sought to define the contribution of the activated terminal complement components to apoptosis and necrosis in a rat model of MI/R injury.

Methods and Results—Monoclonal antibodies (mAbs; 18A and 16C) raised against the rat C5 complement component bound to purified rat C5 (ELISA). 18A effectively blocked C5b-9–mediated cell lysis and C5a-induced chemotaxis of rat polymorphonuclear leukocytes (PMNs), whereas 16C had no complement inhibitor activity. A single dose (20 mg/kg IV) of 18A blocked >80% of serum hemolytic activity for >4 hours. Administration of 18A before myocardial ischemia (30 minutes) and reperfusion (4 hours) significantly reduced (91%) left ventricular free wall PMN infiltration compared with 16C treatment. Treatment with 18A 1 hour before ischemia or 5 minutes before reperfusion significantly reduced infarct size compared with 16C treatment. A significant reduction in infarct size (42%) was also observed in 18A-treated rats after 30 minutes of ischemia and 7 days of reperfusion. DNA ladders and DNA labeling (eg, TUNEL assay) demonstrated a dramatic reduction in MI/R-induced apoptosis in 18A-treated compared with 16C-treated rats.

Conclusions—Anti-C5 therapy in the setting of MI/R significantly inhibits cell apoptosis, necrosis, and PMN infiltration in the rat despite C3 deposition. We conclude that the terminal complement components C5a and C5b-9 are key mediators of tissue injury in MI/R. (Circulation. 1998;97:2259-2267.)

Key Words: infarction complement C3b reperfusion complement C5

Tissue injury and cell death occur after periods of prolonged myocardial ischemia. Although early reperfusion can salvage tissue, reperfusion also increases cell mortality by increasing the inflammatory response. Thus, understanding the mechanisms of cell mortality and the mechanisms involved in the inflammatory process will lead to the development of novel therapeutic interventions.

Several lines of investigation support a role for complement in the pathogenesis of MI/R injury. First, experimental studies have demonstrated localization of complement components (ie, C1q, C3, C4, and C5) in infarcted myocardium.1–5 Second, depletion (ie, cobra venom factor) or inhibition (ie, C1 esterase inhibitor or sCR1) of complement attenuates MI/R injury.6–8 These data collectively suggest that complement plays an important role in MI/R injury. However, the importance of specific complement components, particularly the role of early (ie, C3b, C3a, and iC3b) versus late (ie, C5a and C5b-9) complement components and the mechanisms of complement-mediated injury in MI/R are poorly characterized.

C3b, iC3b, C3a, C5a, and C5b-9 have proinflammatory activity that could be involved in MI/R injury. C3b is important in the immune response, particularly for opsonization and phagocytosis, whereas iC3b is an important ligand for CD11b-mediated neutrophil (PMN) adherence.9 C3a and C5a mediate PMN activation, whereas only C5a is chemo- tactic.10 C5a and C5b-9 have been shown to induce CD62P upregulation, resulting in increased PMN adherence.11–12 C5b-9 is also cytolytic and can directly induce myocardial injury.13,14 Furthermore, C5b-9 directly attenuates endothelium-dependent relaxation of vascular smooth muscle.15–17 Thus, each of these complement components, either alone or in concert with other factors, could induce MI/R injury.

Recent experimental evidence suggests that in addition to necrosis, apoptosis may also contribute to cell loss after...
MI/R.18–22 Hypoxia-induced apoptosis of cultured neonatal rat cardiac myocytes involves increased p53 activity.23 However, myocyte apoptosis during acute myocardial infarction appears to occur independently of p53 in vivo.24 Hypoxia-induced apoptosis of cultured myocytes also involves increased expression of p21 (Ras).25 Because sublytic amounts of C5b-9 induce Ras26 and C5a receptor activation also involves Ras,26 complement may play an important role in MI/R-induced apoptosis. Thus, inhibition of complement activation, particularly inhibition of C5a and C5b-9 production, may inhibit MI/R-induced apoptosis in addition to attenuating cell necrosis.

We have previously shown that treatment of swine with a functionally inhibitory mAb to porcine C5a in a model of MI/R reduced infarct size.27 Inhibition of porcine C5a in that study, unlike inhibition of complement with sCR1 or C1 esterase inhibitor, did not attenuate PMN accumulation in the infarct area, suggesting that additional mechanisms (ie, iC3b or C5b-9) may be required for PMN recruitment or adhesion to the ischemic area.4 Because the anti–porcine C5a antibody did not inhibit porcine C5b-9 or iC3b formation, the role of these complement components in MI/R injury could not be evaluated. In the present study, we developed a panel of mAbs to purified rat C5 to (1) evaluate the importance of early (ie, C3a, C3b, and iC3b) versus late (ie, C5a and C5b-9) complement components in MI/R injury, (2) investigate the contribution of the early versus late complement components in PMN infiltration, and (3) investigate the effects of anti-C5 therapy on MI/R-induced apoptosis.

### Methods

**Purification of Rat C5**

Rat C5 was purified by use of a modification of the methods of Van den Berg et al.25 Briefly, 250 mL of rat serum containing 0.5 mmol/L PMSF and 5 mmol/L EDTA was precipitated with 4% PEG (Sigma Chemical Co), and the pellet was discarded. The remaining supernatant was again precipitated with 4% PEG and dialyzed overnight against Buffer A. The supernatant was then loaded onto a Q Sepharose Fast Flow anion exchange column (Pharmacia) and eluted with a linear (0 to 1.0 mol/L) NaCl gradient. The resulting fractions containing rat C5 activity and pure C5 protein were pooled and loaded onto two Superose 12 gel filtration columns (Pharmacia) connected in series. The fractions were then analyzed by a hemolytic assay and SDS-PAGE followed by silver staining, and fractions containing active pure (>90%) rat C5 were pooled and used as the immunogen for the production of anti-rat C5 mAbs.

**Rat Serum Hemolytic Assays**

The purified anti-rat C5 mAbs (18A and 16C) were tested in vitro for complement inhibitory activity. mAb 18A (n = 3) effectively blocked rat serum complement activity in a dose-dependent manner in 20% rat serum hemolytic assays. mAb 16C (n = 3) did not block complement activity in these assays. Values shown are mean ± SEM of triplicate determinations from a single experiment and are representative of 5 similar assays. B. Inhibition of PMN chemotaxis by anti-C5 mAb. mAb 18A10.6 (n = 5) dose-dependently inhibited ZAS-induced rat PMN chemotaxis under agarose vs control mAb 16C–treated (n = 4) or vehicle-treated (HBSS; n = 5) ZAS. Daily chemotactic index of nonactivated rat serum was subtracted from chemotactic index of ZAS in each treatment group to normalize data. Values are mean ± SEM. *P < 0.05 vs PBS- and 16C–treated sera.

**Production and Characterization of Anti-Rat C5 mAbs**

mAbs directed against rat C5 were produced with standard hybridoma technology. Briefly, C5-deficient mice (B10.D2-OSNJ, Jackson Laboratories) were immunized with an intraperitoneal injection of rat C5 (50 μg in Titermax, Vaxcel) followed by 3 weekly intraperitoneal injections of rat C5 in PBS. Fusions were performed with the SP-20-Ag4 cell line (ATCC), and cells were cloned by limiting dilution. Clones were screened for C5 blocking activity in hemolytic assays with 20% rat serum, and binding to rat C5 was determined by ELISA. One clone, 18A10.62 (IgG2b, referred to as 18A) bound to the α-chain of rat C5 and functionally blocked both C5b-9–dependent serum hemolytic activity and C5a-dependent neutrophil migration (Figure 1). A second clone, 16C9.10 (IgG2b, referred to as 16C) bound to rat C5 but did not block C5b-9–mediated hemolysis or C5a-dependent neutrophil migration and was therefore used as an isotype-matched control mAb in these studies (Figure 1).

**Rat Serum Hemolytic Assays**

The purified anti-rat C5 mAbs (18A and 16C) were serially diluted 1:2 (50 to 0 μg/mL) in GVB27 buffer (gelatin veronal–buffered...
saline: 0.1% gelatin, 141 mmol/L NaCl, 0.5 mmol/L MgCl₂, 0.15 mmol/L CaCl₂, 1.8 mmol/L sodium barbital) and added in triplicate (50 μL/well) to a 96-well plate. Rat serum was diluted to 40% vol/vol with GVB⁺ buffer and added (50 μL/well) to the rows of the same 96-well plate such that the final concentration of rat serum in each well was 20%. The plate was then incubated at room temperature for ~30 minutes. Chicken erythrocytes (5×10⁶/mL in 4 mL of GVB⁺) were sensitized with anti-chicken erythrocyte polyclonal antibody (InterCELL Technologies, 0.1% vol/vol) and incubated at 4°C for 15 minutes. The cells were washed two times with GVB⁺ and resuspended to a final volume of 2.4 mL in GVB⁺. The erythrocytes (30 μL/well, 2.5×10⁶ cells) were added to the plate containing serum and anti-C5 mAb as described above, mixed well, and incubated at 37°C for 30 minutes. The plate was then centrifuged at 1000g for 2.0 minutes, and 85 μL of the supernatant of the supernatant was transferred to a new 96-well microtiter plate. The plate was read at 415 nm with a microplate reader, and the percent serum complement hemolytic activity was determined by the following formula (where OD is optical density):

\[
\text{% Hemolysis} = \frac{(\text{OD}_{\text{sample}} - \text{OD}_{\text{GVB}^+ \text{ control}})}{(\text{OD}_{\text{100% lysed control}} - \text{OD}_{\text{GVB}^+ \text{ control}})} \times 100
\]

where 100% lysed control is 100% control obtained by addition of 100 μL GVB⁺ containing 0.1% NP-40 to the 30 μL of chicken erythrocytes as prepared above.

**Pharmacodynamics of Anti-Rat C5 mAbs**

The pharmacodynamic profile of complement inhibition by the anti-rat C5 mAbs was determined after a single intravenous injection in rats. Briefly, the mAbs 18A and 16C were injected intravenously (20 mg/kg dose in PBS) into adult male Lewis rats. The rats were then bled at various times (0, 0.5, 1, 2, 4, 8, 12, and 24 hours) after mAb injection, and the serum complement hemolytic activity was determined with the assay described above.

**Zymosan-Activated Serum**

ZAS, a source of rat C5a/C5a des Arg, was made as described previously. Briefly, rat serum (Sigma) was diluted 1:2 with HBSS (Sigma). The serum was activated with zymosan (10 mg/mL; Sigma) for 60 minutes at 37°C. Rat serum (50%) was also activated by zymosan in the presence of 18A (50 to 100 μg/mL) or 16C (50 to 100 μg/mL) mAb and used for rat PMN chemotaxis experiments.

**Neutrophil Chemotaxis**

Rat neutrophils were isolated from rat blood with a commercially custom-made hole punch in each plate. The chemoattractant (ZAS, a source of rat C5a/C5a des Arg, was made as described previously. Briefly, rat serum (Sigma) was diluted 1:2 with HBSS (Sigma). The serum was activated with zymosan (10 mg/mL; Sigma) for 60 minutes at 37°C. Rat serum (50%) was also activated by zymosan in the presence of 18A (50 to 100 μg/mL) or 16C (50 to 100 μg/mL) mAb and used for rat PMN chemotaxis experiments.

**MIR/R Studies: Animal Preparation and Protocols**

Adult male Lewis rats (220 to 260 g) were anesthetized with sodium pentobarbital (50 mg/kg IP). Rat hearts were tragacanthostomized and ventilated with a SAR-830 small-animal ventilator (CWE Inc). Expired CO₂ was monitored continuously with a microcapnometer and was maintained at 4% to 5% by adjustment of the respiratory rate and/or tidal volume. Polyethylene catheters were placed in the internal carotid artery and the external jugular vein for measurement of mean arterial blood pressure and infusion of antibody or PBS (ie, vehicle), respectively.

MIR/R was produced as described previously, with some modifications. Briefly, the LAD was ligated 3 to 4 mm from its point of origin with 6–0 silk after a left thoracotomy. Ischemia was produced by tightening the previously placed reversible ligature around the LAD to completely occlude the vessel. Sham-operated animals underwent the same surgical procedures but without ligation of the LAD suture. The ligature was untied after 30 minutes, and the ischemic myocardium was reperfused for 4 hours. Animals were randomly divided into the following groups: (1) 18A (20 mg/kg) given 60 minutes before ischemia (n = 4), (2) 18A (20 mg/kg) given 5 minutes before reperfusion (n = 6), (3) 16C (20 mg/kg) given 60 minutes before ischemia (n = 3), and (4) PBS given 60 minutes before ischemia (n = 5). The following groups of rats underwent 4 hours of ischemia and no reperfusion: (1) 18A given 5 minutes before ischemia (n = 5) and (2) PBS given 5 minutes before ischemia (n = 6). All the rat hearts listed above were used for calculation of myocardial infarct size (see below).

Additional rats underwent 30 minutes of ischemia and 7 days of reperfusion. These rats did not undergo catheter placement, and the chest wall was closed in layers after the initiation of reperfusion. The rats were randomly placed into the following groups: (1) PBS (n = 4), (2) 18A (20 mg/kg 5 minutes before reperfusion; n = 3), or (3) 16C (20 mg/kg 5 minutes before reperfusion; n = 3). Infarct size (ie, ratio of necrosis to total LV volume) was analyzed after 7 days of reperfusion in these animals as described below (“Infarct Size Analysis”).

Myocardial tissue CK and MPO activities were analyzed in additional groups of rats undergoing 30 minutes of ischemia and 4 hours of reperfusion as follows: (1) mAb 18A (20 mg/kg) given 60 minutes before ischemia (n = 4), (2) mAb 16C (20 mg/kg) given 60 minutes before ischemia (n = 5), (3) PBS given 60 minutes before ischemia (n = 5), and (4) sham-operated rats (n = 8).

Ischemia was confirmed in all rats by the presence of ventricular ectopy, discoloration of the ischemic area, and LV dyskinesia. Reperfusion was confirmed by ventricular ectopy and the return of color to the ischemic area. The injection of mAb or PBS and all analyses were performed in a blinded manner. Blood pressure and heart rate were measured before and after antibody or PBS administration, before ischemia, before reperfusion, and at 1, 2, 3, and 4 hours of reperfusion in all rats.

**Infarct Size Analysis**

Infarct size was measured as previously described with the following modifications. The ligature around the left coronary artery was tightened, and 1 mL of 5% patent blue violet (Sigma) was given intravenously to stain the RA negatively after 4 hours of reperfusion. The heart was rapidly removed and placed in ice-cold 0.9% saline, intravenously to stain the RA negatively after 4 hours of reperfusion. The heart was rapidly removed and placed in ice-cold 0.9% saline, and the atria, right ventricle, and great vessels were removed. The LV was sliced transversely into sections 2 mm thick. The unstained portion (ie, ischemic but nonnecrotic area) with the aid of a
surgical microscope (Zeiss). All three portions of the LV myocardium (ie, nonischemic, ischemic nonnecrotic, and ischemic necrotic) were weighed individually. The results are expressed as the percentage of the infarct area to the RA or the total LV.

**CK Activity Assay**
CK activity of the rat heart was assessed as described previously. The LVFW and the septum were separated, weighed, and homogenized in 4 mL of ice-cold 0.25-mol/L sucrose buffer containing 0.1 mmol/L EDTA and 10 mmol/L 2-mercaptoethanol. The homogenates were centrifuged at 30 000g at 4°C for 30 minutes. The supernatants were then assayed for CK activity and protein analysis as described previously. The pellets from the CK homogenates were frozen on dry ice and stored (−80°C) for analysis of MPO activity. The extent of myocardial injury was calculated as described previously. Loss of CK activity from the LVFW was calculated by subtracting the LVFW CK activity from the septal CK activity. CK activity was expressed as IU/mg protein.

**Tissue MPO Activity**
The myocardial MPO activity, an index of neutrophil infiltration, was determined as described previously, with the following modifications. The CK homogenate pellets were resuspended in 20 mL of 5 mmol/L phosphate buffer (pH 6) to remove 2-mercaptoethanol as previously described. The samples were centrifuged at 30 000g at 4°C for 30 minutes. After freezing, thawing, and sonication (3 times), the ice-cold myocardium was homogenized in 0.5% hexadecyltrimethyl ammonium bromide (Sigma) dissolved in 500 mmol/L potassium phosphate buffer (pH 6.0). The change in absorbance was measured spectrophotometrically at 460 nm for several minutes, and the linear portion of the tracing was used for analysis. One unit of MPO activity was defined as that quantity of enzyme that hydrolyzed 1 μmol H2O2/min at 25°C. MPO activity was expressed as units per gram wet tissue weight.

**Immunohistochemistry**
Additional hearts treated with 18A (n=3) or 16C (n=3) underwent 30 minutes of ischemia and 4 hours of reperfusion. The great vessels, atria, and right ventricle were removed after occlusion of the coronary artery and injection of blue dye at the end of the experimental protocol. The LV cavity was embedded in OCT compound (Miles, Inc) and frozen in methylbutanol (−80°C). The myocardium was then sectioned (4 μm) and stained for rat C5 (Cappel).

**Analysis of Cell Apoptosis in Hearts Undergoing Ischemia and Reperfusion**
Rats were subjected to myocardial ischemia (30 minutes) followed by reperfusion (4 hours). Apoptosis was determined by both ex vivo end labeling with a commercially available kit (Boehringer Mannheim) and DNA laddering as described previously. For 3′ end labeling experiments, the RA was immediately excised, embedded in OCT compound (Miles Pharmaceuticals), and frozen in methylbutanol at −80°C. For DNA laddering experiments, rat hearts were sectioned into RA versus nonischemic myocardium after injection of blue dye.

**Statistical Analysis**
All values in the text and figures are presented as mean±SEM of n independent experiments. All data were subjected to one-way ANOVA followed by the Student-Newman-Keuls post hoc test. Differences were considered significant at P<0.05. SigmaStat software (Jandel Scientific) was used for statistical analysis.

**Results**
**Functional Characterization of Anti-Rat C5 mAbs**
mAbs were screened by ELISA for binding to the rat C5 protein, and functional inhibition of C5 activation was determined by serum complement hemolytic and C5a-dependent neutrophil migration assays. Two isotype-matched mAbs (18A and 16C) were found by ELISA to bind rat C5. Functional characterization of these antibodies revealed that clone 18A but not clone 16C effectively blocked the lysis of chicken erythrocytes by rat complement in a dose-dependent manner (Figure 1A). In these assays, 18A completely blocked complement-mediated lysis at a concentration of 25 μg/mL in 20% rat serum. Concomitant with these observations, 16C failed to inhibit ZAS-induced rat PMN chemotaxis compared with vehicle controls, whereas 18A inhibited ZAS-induced rat PMN chemotaxis in a dose-dependent manner (Figure 1B). These data revealed that the 18A mAb bound to rat C5 in a manner that effectively inhibited the generation of both C5a and C5b-9.

**Pharmacodynamic Profile of 18A in Rats**
To determine the duration of the complement inhibitory effect of 18A in vivo, rats were injected with the mAb, and hemolytic assays were performed on serum samples drawn at 0, 0.5, 1, 2, 4, 8, 12, and 24 hours after 18A injection. As shown in Figure 2, 18A effectively blocked complement activity (>80%) for more than 4 hours after administration. In these experiments, significant complement inhibition was also observed (>70%) at 8 hours after 18A injection, and complement levels returned to normal by 12 hours after mAb administration. Similar experiments performed with the 16C control mAb demonstrated that this antibody did not functionally block complement activity in vivo.

**Hemodynamic Data**
Administration of 18A or 16C (20 mg/kg IV) had no significant effect on the double product (ie, index of myocardial oxygen demand) compared with PBS-treated rats (data not presented). Thus, any in vivo protective effects of these antibodies cannot be attributed to a reduction in myocardial oxygen demand.
Assessment of Myocardial Injury

**MPO Analysis**

Inhibition of neutrophil infiltration into tissues has been shown to protect the tissue and vasculature from reperfusion-induced injury and endothelial dysfunction. We observed a significant increase in LV MPO (ie, index of neutrophil infiltration) activity after 30 minutes of ischemia and 4 hours of reperfusion in vehicle-treated rats compared with sham-operated rats (P < 0.05; Figure 3A). We observed no significant difference in LV MPO activity between PBS- and control mAb (16C)-treated animals after MI/R. Rats treated with 18A mAb had a significantly lower LV MPO activity than 16C- or PBS-treated rats (P < 0.05). There was no significant difference in LV MPO activity between 18A-treated and sham-operated rats. These data demonstrate that the increase in LV MPO activity after MI/R can be significantly attenuated by inhibiting rat C5.

**CK Analysis**

One biochemical assessment of myocardial injury is the loss of CK from the myocardium. PBS-treated rats undergoing 30 minutes of myocardial ischemia and 4 hours of reperfusion demonstrated a significant loss of LV CK compared with sham-operated rats (Figure 3B). Pretreatment (20 mg/kg IV 60 minutes before ischemia) with a nonblocking mAb to rat C5 (16C, 20 mg/kg) failed to significantly reduce LV CK loss. In contrast, pretreatment with 18A (20 mg/kg) significantly reduced LV CK loss compared with PBS- or 16C-treated rats. The LV CK loss in 18A-treated rats was not significantly different from sham-operated rats. These data demonstrate a protective effect of C5 inhibition against CK loss from the ischemic/reperfused myocardium.

**Myocardial Infarct Size**

Another index of myocardial injury is the size of the infarct area after MI/R. The RA was 28 ± 5%, 30 ± 2%, 35 ± 7%, and 30 ± 8% for the PBS-treated, 16C-treated, and 18A-treated (preischemia and prereperfusion) rats, respectively. These values were not significantly different from each other. Infarct size (eg, infarct area to RA) did not significantly differ in rats treated with PBS or control mAb 16C (Figure 4). Treatment with mAb 18A (20 mg/kg IV) 60 minutes before coronary artery ligation significantly reduced infarct size by 66% and 67% (P < 0.05) compared with mAb 16C and PBS-treated rats, respectively. Infarct size was reduced by almost 50% (P < 0.05) compared with control mAb 16C- or PBS-treated rats when mAb 18A (20 mg/kg IV) was given 5 minutes before reperfusion (ie, treatment 25 minutes after induction of ischemia). Similar findings were also observed if the infarct size was evaluated as a ratio of necrotic area to total LV area. Thus, inhibition of rat C5 decreases infarct size after myocardial ischemia and 4 hours of reperfusion.

We also investigated the influence of anti-C5 therapy on infarct size in nonreperfused ischemic hearts. The RAs of 18A- or PBS-treated rats undergoing 4 hours of ischemia and no reperfusion were not significantly different (30 ± 3% and 41 ± 4%, respectively; P > 0.05). Treatment of rats with 18A (20 mg/kg, 5 minutes before ischemia) significantly reduced the infarct size (ie, IA/RA × 100) compared with PBS-treated rats (57 ± 8% and 94 ± 2%, respectively; P < 0.05). These data demonstrate that anti-C5 treatment also reduces infarct size induced by ischemia alone.

Because anti-C5 therapy may have only delayed the onset of necrosis, additional rats were subjected to 30 minutes of ischemia and reperfused for 7 days. Rats were treated with a single dose of 16C (20 mg/kg IV; n = 3) or 18A (20 mg/kg IV; n = 3) or given a bolus (=300 μL) of PBS 5 minutes before ischemia (Isch.) or 5 minutes before reperfusion (Rep.) significantly (P < 0.05) reduced infarct size vs vehicle- or control mAb (16C9.10)-treated rats. IA/RA*100 = infarcted area as percent of risk area. Values are mean ± SEM. PBS (n = 5); 18A (18A10.6 mAb; Isch. n = 4; Rep. n = 6), and 16C (16C9.10 control mAb; n = 3). *P < 0.05 vs PBS- and 16C-treated rats. C5 (16C, 20 mg/kg) failed to significantly reduce LV CK loss. In contrast, pretreatment with 18A (20 mg/kg) significantly reduced LV CK loss compared with PBS- or 16C-treated rats. The LV CK loss in 18A-treated rats was not significantly different from sham-operated rats. These data demonstrate a protective effect of C5 inhibition against CK loss from the ischemic/reperfused myocardium.
reperfusion. We observed a significant reduction (42%) in infarct size in 18A-treated rats compared with PBS- or 16C-treated rats (Figure 5). Thus, a single bolus of anti-C5 antibody significantly attenuates infarct size in rats after 30 minutes of myocardial ischemia and 7 days of reperfusion.

**Analysis of Complement Activation/Apoptosis**

C3 deposition in 18A- and 16C-treated hearts after 30 minutes of ischemia and 4 hours of reperfusion was observed by immunohistochemistry (Figure 6). Equivalent C3 deposition was observed in 18A-treated and 16C-treated hearts undergoing MI/R. No C3 deposition was observed in sham-operated rats or the nonischemic LV regions of rats undergoing MI/R. These data demonstrate that anti-C5 treatment does not inhibit C3 deposition in the ischemic/reperfused myocardium. These data further suggest that the mechanism of PMN adherence in the ischemic/reperfused myocardium is not mediated by iC3b.

Previous studies have also demonstrated that apoptosis is observed in the ischemic/reperfused myocardium. Because a portion of MI/R injury may also involve apoptosis and the known biological effects of C5a and C5b-9 are conducive to apoptosis initiation, we investigated the effects of anti-C5 therapy on MI/R-induced apoptosis. Gel electrophoresis of LV DNA was performed to evaluate the extent of apoptosis in 16C- and 18A-treated rats. DNA ladders were not observed in the nonischemic LV of either 16C- or 18A-treated rats (Figure 7, lanes A and C, respectively). We observed DNA ladders in the RA of 16C-treated rats but not in 18A-treated rats (Figure 7, lanes B and D, respectively).

To confirm the DNA laddering experiments, we used TUNEL staining to locate apoptotic nuclei. We observed a significant number of apoptotic cell nuclei in ischemic/reperfused rat hearts treated with 16C (Figure 8, top). In sharp contrast, very few apoptotic cell nuclei were observed in ischemic/reperfused rat hearts after treatment with 18A (Figure 8, bottom). These data demonstrate that in addition to inhibiting MI/R-induced necrosis, anti-C5 therapy attenuates MI/R-induced apoptosis. Thus, two separate techniques demonstrate that MI/R-induced apoptosis can be attenuated by anti-C5 therapy.

**Discussion**

This study demonstrates that inhibition of the complement system with an antibody against C5 significantly reduces MI/R-induced necrosis, apoptosis, and PMN infiltration in the rat. We also demonstrate a central role of C5a/C5b-9–mediated PMN accumulation in the infarcted myocardium in the presence of C3 deposition. Thus, these data establish the importance of the activated terminal complement components in MI/R-induced apoptosis and necrosis.

**Reduction of MI/R-Induced Necrosis**

It is generally accepted that complement plays an important role in MI/R injury. However, the specific complement components involved in MI/R injury are poorly characterized. Previous studies have shown that complement inhibition at the level of C3 (ie, sCR1) or C1 (ie, C1 esterase inhibitor) reduced myocardial infarct size. The present study allowed evaluation of the specific role of the terminal complement components (ie, C5, C6, C7, C8, and C9) without attenuating the activation of the earlier complement components or C3 deposition. Biochemical (ie, MPO and CK assays) and histochemical (ie, infarct size) data presented in the present study indicate a significant reduction of MI/R injury in mAb 18A10.6–treated rats compared with 16C-treated rats. Inhibition of C5 did not simply delay the onset of necrosis, because a significant 40% reduction in infarct size was observed in the anti-C5–treated rats after 7 days of reperfusion. Further, no C5b-9 deposition (evaluated by

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**Figure 5.** Infarct size in rats after 7 days of reperfusion. Treatment with mAb 18A10.6 (20 mg/kg IV) 5 minutes before reperfusion significantly (P<0.05) reduced infarct size vs vehicle- or control mAb (16C9.10)–treated rats after 7 days of reperfusion. IA/TLV (%)=infarcted area as percent of total LV. Values are mean±SEM.

PBS (n=4), 18A (18A10.6 mAb; n=3), and 16C (16C9.10 control mAb; n=3). *P<0.05 vs PBS or 16C-treated rats.

**Figure 6.** Immunohistochemical staining for rat C3. LVFWs of rats undergoing 30 minutes of ischemia and 4 hours of reperfusion were processed for immunohistochemical staining for rat C3 as described in “Methods.” Equivalent C3 staining was observed in 16C- (top) and 18A- (bottom) treated rats. Figures are representative of at least 3 separate experiments.
antibody to rat C9) was observed in the infarct area of rats treated with 18A, whereas 16C rats did not demonstrate C9 staining (data not presented). These data are consistent with the conclusion that the terminal complement components (ie, C5a and C5b-9) are the key complement components in MI/R-induced necrosis.

Reduction of PMN Infiltration

PMNs play an important role in MI/R injury.34 There are several mechanisms by which the complement system may play a critical role in PMN-induced myocardial injury. First, C5a and C5b-9 can induce CD62P upregulation on endothelial cells, resulting in increased PMN adherence.12,38 Second, deposition of iC3b on endothelial cells can mediate PMN adhesion via CD11b/CD18 interactions.9,39 Third, sublytic amounts of C5b-9 have been shown to induce MCP-1 and interleukin-8 in human endothelial cells.40 Finally, C5a, a potent PMN chemotactic factor, is present at elevated levels in cardiac lymph after myocardial ischemia.41 However, we have demonstrated previously that a functionally inhibitory antibody to porcine C5a reduced porcine MI/R injury without significantly reducing PMN accumulation, suggesting that additional mechanisms are involved in PMN recruitment to the infarct area.27

Anti-C5 treatment, which inhibits C5a and C5b-9 but not C3 deposition, significantly reduced MPO accumulation (ie, PMN infiltration) in the LV in the present study. These data demonstrate that the terminal complement pathway plays a central role in the recruitment of PMNs into the ischemic area. Our data suggest that C5b-9 and not iC3b deposition or

C5a production is an important complement component for PMN accumulation in the ischemic/reperfused heart.27 In vitro data from others would support a central role of C5b-9 in PMN recruitment to the ischemic myocardium.38,40 Thus, complement has the potential to initiate and/or augment PMN adherence, recruitment, transmigration, and activation during MI/R.

Reduction in MI/R-Induced Apoptosis

Several studies have demonstrated that myocardial ischemia induces apoptosis in animal models and humans.18,21,22 However, the mechanism(s) of MI/R-induced apoptosis are not well elucidated. Prolonged (ie, 72 hours) periods of hypoxia induce apoptosis in cultured rat cardiac myocytes by increasing p53 activity and p21 (Ras) expression.23 Recent evidence, however, suggests that p53 is not involved in acute myocardial infarction–induced apoptosis in vivo.24

C5a receptor activation or sublytic amounts of C5b-9 activate Ras.25,26 Furthermore, C5b-9 can induce significant changes in intracellular calcium fluxes and production of oxygen-derived free radicals.42–45 Because the mechanisms of apoptosis induction during MI/R may also involve several of these complement signaling events, the terminal complement components (ie, C5-C9) are well suited to provide a molecular switch for MI/R-induced apoptosis. We have demonstrated that inhibition of rat C5 in vivo dramatically
attenuated MI/R-induced apoptosis. These data suggest that the terminal complement components play an important role not only in MI/R-induced necrosis but in apoptosis as well. Furthermore, these data suggest that anti-C5 therapy in humans may attenuate complement-dependent apoptosis. Future studies on the mechanism of complement-induced apoptosis are warranted.

From the data presented in this study, it is clear that anti-C5 therapy significantly reduces myocardial necrosis. The mechanism of inhibition of MI/R-induced apoptosis is less clear. Because anti-C5 therapy also inhibited PMN infiltration, it is possible that inhibition of the release of toxic PMN products (reactive oxygen species, azurophilic granule contents, etc) may play an important role in this process. In addition, we have also demonstrated that C5b-9 directly induces apoptosis in human vascular endothelial cells.83 Regardless of the mechanism of myocardial ischemia-induced apoptosis in the present study, inhibition of the terminal complement component C5 attenuated apoptosis in the rat. Elucidating the apoptotic mechanism in vivo will require additional studies and the development of additional antibodies against either C6 or C7 that functionally inhibit C5b-9 formation yet allow C5a generation.

Conclusions
Inhibition of complement activation at C5 significantly reduces MI/R-induced necrosis and apoptosis. The decrease in myocardial injury was associated with a reduction in PMN accumulation, despite significant C3 deposition. Inhibition of the complement system at C5 represents a novel and effective mechanism of inhibition of MI/R-induced necrosis and apoptosis. The decrease in myocardial injury was associated with a reduction in PMN infiltration, despite significant C3 deposition. Inhibition of the complement system at C5 represents a novel and effective therapeutic intervention for MI/R injury while preserving the immunoprotective effects of C3b.

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Antti P. Vakeva, Azin Agah, Scott A. Rollins, Louis A. Matis, Lan Li and Gregory L. Stahl

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