Cardioprotective Actions of a Monoclonal Antibody Against CD-18 in Myocardial Ischemia-Reperfusion Injury

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Background. Previous studies have demonstrated that polymorphonuclear leukocytes (PMNs) are locally activated in reperfused myocardium and contribute to the myocardial cell injury associated with reperfusion. It has been suggested that the adhesion of activated PMNs in reperfused myocardium is mediated by the PMN adhesion molecule CD-18. In the present study, we performed experiments to determine if the specific anti-CD-18 monoclonal antibody (MAb) R15.7 can prevent PMN adhesion and PMN-mediated reperfusion injury in the heart.

Methods and Results. Studies were performed with isolated, Langendorff-perfused rat hearts (nine per group) in which the hearts were subjected to 20 minutes of global ischemia followed by 45 minutes of reperfusion. Human PMNs (50 million) and rat plasma (HNRPs) were infused directly into the coronary circulation of nonischemic and postischemic hearts. When HNRPs was administered to nonischemic hearts, no significant alterations in coronary flow, left ventricular developed pressure, or left ventricular end-diastolic pressure were observed. When hearts were reperfused in the presence of HNRPs, however, marked impairment of contractile function was observed with more than 90% reduction in coronary flow throughout the reperfusion period (P<0.001 versus baseline). In addition, left ventricular developed pressure was significantly depressed (P<0.001 versus baseline) throughout the reperfusion period in the HNRPs group and recovered to only 13.0±3.0% at 45 minutes of reperfusion. Moreover, left ventricular end-diastolic pressure was significantly elevated (P<0.001) in the HNRPs group throughout the reperfusion period. Treatment with the anti-CD-18 monoclonal antibody MAb R15.7 (20 μg/mL) at the time of reperfusion resulted in a 92.9±4.9% recovery of coronary flow (P<0.001 versus HNRPs) as well as a 71.0±10.1% recovery of left ventricular developed pressure (P<0.001 versus HNRPs). Administration of MAb R15.7 also very significantly attenuated the elevation in left ventricular end-diastolic pressure that was observed in the untreated HNRPs (30.2±7.8 versus 110.3±10.3 mm Hg, P<0.001) at 45 minutes of reperfusion. Cardiac myeloperoxidase activity, an index of PMN accumulation, was markedly reduced in the MAb R15.7 group at 45 minutes of reperfusion compared with the HNRPs group (0.3±0.05, P<0.001). To determine that the protective effect of MAb R15.7 was based on functional blocking of CD-18, additional experiments were performed with identical concentrations of MAb 3.1, which binds to the α-subunit of LFA-1. This PMN-binding but non-CD-18–blocking antibody had little effect on the recovery of postischemic function or coronary flow and did not reduce tissue myeloperoxidase activity.

Conclusions. The administration of a specific anti-CD-18 monoclonal antibody, MAB R15.7, attenuates much of the PMN-mediated contractile dysfunction associated with this in vitro model of myocardial ischemia-reperfusion injury by limiting PMN accumulation. We conclude that CD-18–mediated adhesion may play a critical role in the pathogenesis of PMN-induced myocardial injury. (Circulation. 1993;88[part 1]:1779-1787.)

Key Words • antibodies • myocardium • ischemia • reperfusion • neutrophils

When ischemic myocardial tissue is reperfused with oxygenated blood, a series of inflammatory events rapidly ensue. These events include the production of oxygen-derived free radicals, activation of the complement system, coronary endothelial dysfunction, the adherence of neutrophils (polymorphonuclear leukocytes [PMNs]) to the coronary en-
The complement system plays a pivotal role in the recruitment and activation of PMNs within the ischemic-reperfused myocardium. Maroko et al. originally observed significant reductions in myocardial damage following coronary occlusion if the complement system had been inactivated with cobra venom factor. More recently, Rosen and coworkers have determined that neutrophils selectively accumulate in regions of ischemic myocardium that are rich in Clq, a subunit of the first component of complement. Moreover, Rosen et al. have also demonstrated that myocardial ischemia induces the release of subcellular components of cardiac muscle that bind to Clq, and they proposed that these Clq-binding substances may activate the complement cascade. Taken together, these studies suggest that activation of the complement system following myocardial ischemia-reperfusion can trigger the release of leukotactic mediators that in turn stimulate the activation and infiltration of PMNs into ischemic myocardium, thereby extending the myocardial injury associated with ischemia.

Furthermore, it appears that adherence of PMNs to the coronary circulation is a prerequisite for PMN infiltration and PMN-induced myocardial cell injury. Several studies have demonstrated that monoclonal antibodies directed against adhesion proteins located on PMNs have substantially reduced myocardial cell necrosis following ischemia and reperfusion. Studies in canine models of myocardial ischemia-reperfusion have determined that the anti-CD-11b monoclonal antibody (Mab) 904 significantly reduced infarct size, and in addition, studies, Mab R15.7 has been shown to diminish PMN accumulation and reduce myocardial injury. In more recent studies, it has been demonstrated that an anti-ICAM-1 antibody can reduce the adhesion of PMNs to cardiac myocytes. In addition, Ma and coworkers demonstrated marked coronary endothelial and cardiac protective effects of a Mab directed against ICAM-1 in a feline model of myocardial ischemia-reperfusion.

Recently, we developed and validated an in vitro model of myocardial ischemia-reperfusion in which isolated rat hearts are perfused with human PMNs in combination with rat plasma. Our initial studies have demonstrated that after ischemia, plasma factors are required for the activation of PMNs, which in turn generate oxygen free radicals resulting in a marked degree of contractile dysfunction. We have also observed that much of the PMN-mediated contractile dysfunction associated with this model of myocardial ischemia-reperfusion can be attenuated with soluble complement receptor 1, human sCr1. However, it is presently unknown what role, if any, CD-18-mediated PMN adhesion plays in the profound impairment of cardiac contractile function that occurs after reperfusion of ischemic myocardium. Therefore, the purpose of the present study was to determine if a potent and specific anti-CD-18 Mab, Mab R15.7, can reduce the extent of PMN-mediated contractile dysfunction observed following myocardial ischemia-reperfusion.

Methods

Isolated Heart Perfusion

Female Sprague-Dawley rats (250 to 500 g) were heparinized with 1000 units Na heparin (Elkins-Sinn, Inc, Cherry Hill, NJ) and anesthetized with intraperitoneal sodium pentobarbital (Steris Laboratories, Inc, Phoenix, Ariz) at a dose of 35 mg/kg. The hearts were rapidly excised, the ascending aorta was cannulated, and retrograde reperfusion was initiated. The hearts were then perfused with Krebs bicarbonate perfusate of (in mmol/L) glucose 17, sodium chloride 120, sodium bicarbonate 25, calcium chloride 2.5, EDTA 0.5, potassium chloride 5.9, and magnesium chloride 1.2 at 37°C at a constant pressure of 80 mm Hg. The perfusate was bubbled with 95% O2-5% CO2. Two sidearms in the perfusion line located just proximal to the heart cannula allowed infusion of PMNs and plasma directly into the heart (Fig 1). To assess contractile function, a latex balloon was inserted into the left ventricular cavity and connected to a pressure transducer as described previously. The balloon was initially inflated with the volume of distilled water required to produce an end-diastolic pressure between 8 and 14 mm Hg. Subsequent measurements of developed pressures were calculated as the difference between the peak systolic and end-diastolic pressures. Left ventricular pressure was recorded with a Gould RS3400 four-channel recorder. Coronary flow and heart rates were measured periodically every 5 minutes before a 20-minute period of zero-flow global ischemia and after ischemia for a 45-minute period of reperfusion.

Leukocyte Preparation

Human neutrophils were prepared by the method of Kensler and Trush, which yields PMNs with a purity of more than 95%. Freshly sampled blood (50 mL) was drawn from volunteer donors and collected in plastic syringes containing 100 units/mL sodium heparin. The whole blood was then transferred to 50-mL plastic centrifuge tubes (Sarstedt, Newton, NC) and centrifuged at 500g at 4°C for 15 minutes in a Beckman GS-6R refrigerated centrifuge (Beckman Instruments, Palo Alto, Calif). The plasma and buffy coat layers were aspirated and discarded, leaving the red cell layer along with the neutrophils. The cells were then mixed with an equal volume of 6% dextran. The 6% dextran solution was prepared by adding 1.0 g of 500 K dextran (Pharmacia LKB Biotechnology, Piscataway, NJ) and 5.7 g of 70 K dextran (Pharmacia LKB Biotechnology) to 100 mL of 0.9% normal saline (Abbott Labs, North Chicago, III). The dextran-cell mixture was then transferred to 30-mL plastic syringes (Becton Dickinson, Rutherford, NJ).
The supernatant was centrifuged and resuspended in ice-cold ACK lysing buffer (0.155 mol/L NH₄Cl, 0.01 mol/L KHCO₃, and 0.1 mmol/L EDTA at pH 7.4) and respun for 15 minutes at 500g. The supernatant was aspirated, and the cell pellet containing purified PMNs was resuspended and washed twice in Dulbecco’s phosphate-buffered saline (PBS) containing (in mmol/L) CaCl₂ 0.9, KCl 2.7, KH₂PO₄ 1.1, MgCl₂ 0.5, NaCl 138.0, Na₂HPO₄, and 1.0% glucose ( Gibco Laboratories, Grand Island, NY). The PMNs were then counted using a hemocytometer, and viability was determined by assessing exclusion of 0.8% Trypan blue (Sigma Chemical, St Louis, Mo).

**Rat Plasma**

Whole rat blood was obtained by performing a closed-chest intracardiac puncture with a 10.0-mL plastic syringe (Becton Dickinson) containing 2000 units Na heparin (Elkkins-Sinn). To obtain platelet-poor plasma, the whole blood was immediately spun in a Beckman GS-6R refrigerated centrifuge (Beckman Instruments) at 1500g and 4°C for 20 minutes. The plasma layer was collected and stored at 4°C until it was used in the isolated perfused heart or electron paramagnetic resonance (EPR) experiments.

**Fluorescence Flow Cytometry**

Measurements of antibody binding to PMNs were performed by fluorescence flow cytometry, and these measurements were used to determine the antibody dose required to saturate CD-18. PMNs were suspended in PBS with 0.2% bovine serum albumin (BSA) and incubated with the desired concentration of the primary antibody Mab R15.7 at 0°C on ice for 30 minutes. The PMNs were then centrifuged at 8000 rpm in an Eppendorf model 5414 centrifuge for 20 seconds, the supernatant was removed, and the cells were resuspended in PBS with 0.2% BSA. The cells were then washed twice. The cell pellet was resuspended, and the secondary antibody, phycoerythrin-conjugated, goat anti-mouse immunoglobulin G (TAGO, Inc, Burlingame, Calif) was added 1:100 vol/vol and incubated with the cells in the dark on ice. The cells were then washed two times and resuspended in PBS with 0.2% BSA. Flow cytometry measurements were performed immediately using a Coulter EPICS Flow Cytometer with 250,000 cells per measurement.

**Electron Paramagnetic Resonance Measurements**

In additional experiments, the effect of Mab R15.7 on the production of oxygen-derived free radicals by human PMNs was determined using EPR spectroscopy. Human PMNs were isolated as described previously and stored at 4°C until use. Aliquots of the PMNs (1.0 × 10⁶) were activated with either activated zymosan, leukotriene B₄ (100 nmol/L), or phorbol myristate acetate (PMA, 10 ng/mL) in the presence or absence of Mab R15.7 (20 μg/mL) and placed in a quartz EPR flat cell (Wilmad Glass Co). EPR spectra were recorded at room temperature with a Bruker-IBM ER 300 spectrometer operating at X-band using a TM₃₁₀ cavity, a modulation frequency of 100 kHz, a modulation amplitude of 0.5 G, a microwave power of 20 mW, and a microwave frequency of 9.77 GHz. Each spectral acquisition file was the sum of 10 1-minute scans. The digital Bruker spectral data were transferred to an Intel 486-based personal computer for analysis. Software capable of isotropic spectral simulation, developed in this laboratory, was used for component analysis of experimental spectra as described previously. Spectral simulations consisting of isotropic combinations of the component signals were performed to match the observed spectra. From the weighted intensities of each component in these simulations, the relative amount of each component signal was determined. The total radical concentration was then determined from the ratio of the double integral of the observed spectra to the known concentration of 2,2,6,6-tetramethylpiperidinoxy free radical in aqueous solution as previously described.

**Cardiac Myeloperoxidase Determination**

Myeloperoxidase activity, an enzyme virtually exclusive for PMNs, was determined in the rat hearts according to the method of Bradley et al and modified by Mullane et al and used as an index of PMN accumulation in the heart. After the 45-minute reperfusion period, the hearts were removed from the perfusion apparatus and rapidly frozen in liquid nitrogen. The hearts were stored at −80°C until homogenization. The myocardial tissue was homogenized in 0.5% hexadecyltrimethyl ammonium bromide (Sigma Chemical) and dissolved in 50 mmol/L K phosphate buffer at pH 6.0 using a Polytron (PCU-2) homogenizer (Kinematika GmbH, Lucerne, Switzerland) for 15 seconds twice at 7000 rpm. Homogenates were centrifuged for 20 minutes at 12 000g and 2°C. The supernatants were decanted and added to 0.167 mg/mL O-dianisodine hydrochloride (Sigma Chemical) and 0.005% H₂O₂ in 50 mmol/L phosphate buffer at pH 6.0. The change in absorbance was measured spectrophotometrically at 460 nm. One unit of myeloperoxidase activity is defined as the quantity of enzyme degrading 1 μmol peroxide/min at 25°C. The measured myeloperoxidase activity of 10⁶ PMNs was 0.60 units.

**Experimental Protocol**

The experimental protocol for the isolated heart perfusion studies is depicted in Fig 2. After a 10- to 15-minute equilibration period, baseline left ventricular developed pressure, left ventricular end-diastolic pressure, and coronary flow were measured. At the onset of ischemia, the balloon was deflated. The intraventricular balloon volume was then reinflated with the previous volume immediately after the onset of reflow. The hearts were reperfused for the first 5 minutes with human PMNs (50 million) and rat plasma (5%) along with standard Krebs buffer, with or without Mab R15.7 (20 μg/mL). After this, perfusion was continued with Krebs buffer alone for an additional 40 minutes, during which serial measurements of coronary flow and developed pressure were performed every 5 minutes. One or more sidearm ports were placed just above the aortic cannula, allowing administration of cells, plasma, or both (Fig 1). As reported previously, similar infusion of PMNs and plasma in normally perfused hearts not
subjected to ischemia does not result in significant alterations in contractile function or coronary flow; however, in postischemic hearts, marked changes are seen.24 This postischemic injury was observed only in presence of both PMNs and plasma. In a series of hearts reperfused with plasma alone or PMNs alone, the recovery of contractile function and coronary flow was identical to that of hearts reperfused in the absence of PMNs and plasma. When hearts were reperfused in the presence of PMNs and plasma, marked injury was seen.24 It was shown that complement was required for PMN activation with the generation of oxygen free radicals and that C5a was the required plasma factor.

Validation of Antibody Concentration and Function

The monoclonal antibody to CD-18, MAb R15.7, was obtained from Dr Robert Rothlein of Boehringer Ingelheim. This antibody is a potent functional adhesion-blocking antibody.20-22 The binding nonblocking antibody used, MAb R3.1, was also obtained from Dr Rothlein. It is an antibody to the α-subunit of LFA-1, CD-11a. Because the MAC-1, CD-18–CD-11b heterodimer is largely responsible for PMN adhesion, not LFA-1, R3.1 has been previously used as a control binding nonblocking antibody.21 The concentration of MAb R15.7 used was selected from measurements of antibody binding to PMNs by fluorescence flow cytometry, with the concentration of 20 μg/mL determined to be sufficient to achieve more than 90% saturation. This concentration was demonstrated to be highly effective at blocking PMN adhesion to cultured endothelial cells, and this concentration was sufficient to totally block complement-mediated free radical generation (see “Results”).

Statistical Analysis

Data are presented as the mean±SEM. Comparisons between the groups during ischemic control conditions as well as after ischemia were made by a one-way ANOVA designed for repeated measures. Because multiple comparisons were made, a conventional value of P≤.05 was accepted as statistically significant.

Results

In Vitro Measurement of Antibody Binding and Function

Antibody binding to isolated PMNs. To determine the concentration of MAb R15.7 required to saturate PMN CD-18, flow cytometry studies were performed to measure the dose response of antibody binding. One million PMNs were suspended in 1 mL of PBS with 0.2% BSA and activated by incubation with 100 nmol/L LTB4 for 30 minutes. The cells were then washed with PBS and incubated with MAb R15.7 concentrations ranging from 0.5 to 40 μg/mL. The cells were processed for fluorescence flow cytometry as described above. From these measurements, it was observed that MAb R15.7 concentrations of 20 μg/mL were sufficient to provide more than 90% saturation of neutrophil CD-18 (Fig 3). Further experiments measuring the effect of the antibody on PMN adhesion to human umbilical vein endothelial monolayers demonstrated that 20 μg/mL of MAb R15.7 blocked PMN adhesion by more than 90%, while the non–CD-18–blocking antibody had no significant effect on PMN adhesion.

PMN free radical generation. To determine if treatment with 20 μg/mL of MAb R15.7 was sufficient to completely inhibit complement-mediated PMN free radical generation, we conducted additional experiments measuring free radical generation using electron paramagnetic resonance spectroscopy. The effects of R15.7 on activated PMNs were studied in aliquots of freshly isolated human PMNs. Human PMNs were initially stimulated with zymosan activated plasma, 5% volume activated with 200 μg/mL zymosan, in the presence of a 50-nmol/L concentration of the spin-trap agent DMPO. A profound burst of oxygen free radical generation was observed with a prominent DMPO-OH 1:2:2:1 quartet signal whose magnitude peaked at 60 to 90 minutes after activation (Fig 4A). In the presence of 100 units/mL of recombinant human superoxide dismutase (HSOD), this signal was completely quenched, demonstrating that the observed DMPO-OH adduct was derived from superoxide. Pretreatment with R15.7 (20 μg/mL) almost completely abolished free radical generation by PMNs stimulated with activated zymosan (Fig 4B). In contrast, R15.7 had little or no effect on PMN free radical generation by PMNs activated with leukotriene B4, (100 nmol/L) or phorbol ester (10 ng/mL). The CD-18 PMN adhesion molecule also functions as complement receptor 3, CR3. Therefore, the MAb R15.7, which binds to CD-18, apparently prevents complement-mediated PMN activation and free radical generation by blocking this cellular complement receptor.
Hemodynamic Measurements and PMN Adhesion in the Postischemic Heart

Left ventricular developed pressure. Preischemic baseline values of left ventricular developed pressure did not differ significantly in the two groups studied; mean pressures of 124±7 and 114±4 mm Hg were observed in the human neutrophils and rat plasma (HNRP) only and HNRP+MAb R15.7 groups, respectively (Fig 5). After 20 minutes of global ischemia and 5 minutes of reperfusion with human neutrophils and rat plasma, a marked decline in left ventricular developed pressure to 12±5 mm Hg was observed in the HNRP group. Treatment with MAb R15.7 for the first 5 minutes of reperfusion resulted in a much higher recovery of developed pressure to 44±10 mm Hg (P<.05 versus HNRP). Moreover, administration of MAb R15.7 resulted in recovery of left ventricular developed pressure to 60±9% of baseline at 10 minutes of reperfusion with further recovery to 90±9% of baseline at 45 minutes of reperfusion (Fig 5). In contrast, recovery of hearts subjected to HNRP infusion alone was consistently less than 15% of baseline from 10 minutes of reperfusion to 45 minutes of reperfusion (P<.01 compared with HNRP+MAb R15.7). Thus, MAb R15.7 administration markedly improved the recovery of left ventricular developed pressure throughout the time course of reperfusion.

Rate-pressure product. The product of heart rate and left ventricular developed pressure, the rate-pressure product, was used as an additional index of cardiac performance in the present study. The HNRP hearts exhibited a markedly lower (P<.001) rate-pressure product than did the MAb R15.7–treated hearts throughout the reflow period as shown in Fig 6. Hearts receiving MAb R15.7 exhibited substantially higher recovery of the rate-pressure product through the reperfusion period to a final value of 65±6% of the baseline value at 45 minutes of reperfusion (P<.001 versus HNRP). Recovery of rate-pressure product in the HNRP group without antibody was limited to less than 12% of baseline throughout the reflow period.

Left ventricular end-diastolic pressure. Baseline left ventricular end-diastolic pressure (Fig 7) was very similar in the two groups of hearts. After ischemia and reperfusion with HNRP for 5 minutes, left ventricular end-diastolic pressure was elevated significantly to 100.2±15.8 mm Hg compared with an elevation to 51.8±9.4 mm Hg in the hearts receiving MAb R15.7 in addition to HNRP at reperfusion (P<.01). Left ventricular end-diastolic pressure remained significantly elevated to levels above 100 mm Hg in the HNRP hearts throughout the 45-minute reflow period. In contrast, the left ventricular end-diastolic pressure steadily declined during the reflow period in the hearts treated with MAb R15.7. At 45 minutes of reperfusion, the left ventricular end-diastolic pressure was 110.3±10.3

![Graph showing hemodynamic measurements and PMN adhesion in the postischemic heart.](image)

**Fig 4.** Representative electron paramagnetic resonance (EPR) spectra of free radical generation by human neutrophils stimulated with 5% volume of zymosan activated rat plasma (ZAP) in the absence (A) and in the presence (B) of 20 μg/mL of monoclonal antibody (MAb) R15.7. EPR spectra were recorded 60 minutes after ZAP activation of the neutrophils. The 20-μg/mL concentration of MAb R15.7 blocked complemeni-mediated free radical generation by more than 90%.

![Graph showing plots depicting the percent recovery of rate-pressure product.](image)

**Fig 5.** Plot showing left ventricular developed pressure (LVDP) during the 15-minute control period and during the 45-minute reperfusion period for hearts receiving saline vehicle or monoclonal antibody (MAb) R15.7. HNRP indicates human neutrophils+rat plasma. Treatment with MAb R15.7 significantly enhanced the recovery of LVDP throughout reperfusion compared with hearts receiving vehicle.

**Fig 6.** Plot depicting the percent recovery of rate-pressure product (heart rate multiplied by left ventricular developed pressure) in the vehicle- and monoclonal antibody (MAb) R15.7–treated hearts during the 45-minute reflow observation period. The recovery of rate-pressure product was significantly enhanced (P<.001) in the MAb R15.7–treated hearts compared with human neutrophils+rat plasma (HNRP) alone.
FIG 7. Plot of left ventricular end-diastolic pressure (LVEDP) during the control period and after global ischemia. Monoclonal antibody (MAb) R15.7 treatment largely attenuated (P<.001) the profound increase in LVEDP observed immediately after the infusion of HNRP.

mm Hg in the HNRP hearts compared with 30.2±7.8 mm Hg in the hearts given MAb R15.7 in addition to HNRP (P<.001).

Coronary flow. During baseline conditions, coronary flow (Fig 8) was identical in both groups (18.8±1.1 and 19.0±1.8 mL/min for the HNRP and HNRP+MAb R15.7 groups, respectively). After global ischemia and reperfusion with HNRP, coronary flow was depressed to 1.1±0.9 mL/min at 5 minutes of reperfusion. Coronary flow remained similarly reduced in the HNRP group throughout reflow and recovered to only 37% of control (5.0±1.1 mL/min) at 45 minutes of reperfusion. Coronary flow remained at values very similar to baseline at all of the reflow time points studied in the hearts receiving MAb R15.7 in addition to HNRP. At 5 minutes of reflow, coronary flow had returned to 84% (16.3±2.6, P<.001 versus HNRP alone) of the baseline value. Coronary flow continued to recover during reperfusion to more than 90% (17.3±2.2 mL/min) of baseline at 20 minutes of reperfusion and to 95% (18.1±2.3 mL/min) of baseline at 45 minutes of reflow. Coronary flow was significantly greater (P<.001) at all time points studied in the hearts treated with MAb R15.7 compared with the hearts receiving HNRP alone.

Cardiac myeloperoxidase activity. At the end of reperfusion (45 minutes), cardiac myeloperoxidase activity was 0.3±0.05 units/100 mg of tissue in the HNRP group compared with 0.02±0.01 units/100 mg tissue in the HNRP+MAb R15.7 group (P<.001) (Fig 9). This finding is consistent with markedly attenuated PMN accumulation following global ischemia and reperfusion in hearts receiving MAb R15.7. The average total myeloperoxidase activity per heart was 4.5 units in the HNRP group, indicating that approximately 7.5×10^6 PMNs, which corresponds to 15% of the infused PMNs, remained in the heart after 45 minutes of reflow. In the HNRP+MAb R15.7 group, the average total myeloperoxidase activity per heart was 0.3 units, corresponding to 0.5×10^6 PMNs, or 1% of those infused.

Experiments With a PMN-Binding Nonblocking Antibody

To prove that the effect of MAb R15.7 was specifically due to the blocking of CD-18, additional experiments were performed in which hearts were reperfused with a PMN-binding antibody that has been previously shown to be ineffective in blocking adhesion via CD-18. For these experiments, MAb R3.1 was used, which is a specific antibody to the α-subunit of LFA-1, CD-11a. Hearts were subjected to the same protocol of 20 minutes of ischemia and 45 minutes of reperfusion that was used in the MAb R15.7 experiments with identical antibody concentration and infusion duration. Despite this antibody administration, marked injury was observed with severe impairment of contractile function and coronary flow (Table). For all physiological parameters studied, the recovery with MAb R15.7 was significantly higher than that with MAb R3.1 with P<.01. The differences were striking and almost as marked as those seen with simple vehicle infusion. Tissue myeloperoxidase activity was measured after 45 minutes of reflow and was found to be much higher than in MAb R15.7–treated hearts, with a value similar to that of the HNRP group (Table).

Discussion

Although early reperfusion of ischemic myocardium may limit ischemic injury, experimental evidence sug-
suggests that reperfusion itself may contribute to additional myocardial cell injury. In particular, PMNs have been implicated as a primary mediator of myocardial cell injury, myocardial contractile dysfunction, and, ultimately, myocardial cell necrosis. For PMNs to induce myocardial cell injury, a number of steps in the PMN activation cascade must occur. The essential initial step involved with PMN-mediated myocardial cell injury is the interaction of the PMN with the coronary vascular endothelium, resulting in PMN adhesion. Firm adhesion of PMNs to the endothelium is followed by further activation of the PMNs, diapedesis, and extravascular migration of the PMNs into the surrounding myocardium. Accordingly, agents that interfere with the adhesion of PMNs to ischemic-reperfused coronary endothelial cells may be of tremendous therapeutic value in the setting of myocardial reperfusion injury.

Adhesion of PMNs to endothelial cells occurs after interaction between adhesion molecules, which are expressed on the surface of PMNs, and counter receptor adhesion molecules, which are expressed on the endothelium. These adherence molecules are currently grouped into three major families—the integrin family (eg, LFA-1, Mac-1, and p150,95), the immunoglobulin superfamily (eg, intercellular adhesion molecule–1, vascular cell adhesion molecule–1), and the selectin family (eg, L-selectin, P-selectin). Following activation of the circulating PMNs and the endothelium by inflammatory stimuli, PMNs begin to roll along the endothelium and adhere. These initial PMN–endothelial cell interactions are mediated by adhesion molecules belonging to the selectin family. In particular, it is currently believed that rapid redistribution of P-selectin from the Weibel–Palade bodies to the surface of endothelial cells following stimulation may play a crucial role in initial PMN adhesion following ischemia–reperfusion. Recent studies by Weyrich et al and Winn et al have clearly demonstrated that MAbs against P-selectin are quite effective in limiting the extent of tissue injury following ischemia–reperfusion. In a feline model of myocardial ischemia–reperfusion, the P-selectin MAb, PB1.3, very significantly reduced the degree of myocardial necrosis following 1.5 hours of ischemia and 4.5 hours of reperfusion.

PMN rolling is also mediated by L-selectin, which is expressed on the surface of the PMN. Upon activation, the binding affinity of L-selectin is rapidly enhanced, followed by PMN adhesion and L-selectin shedding. Monoclonal antibodies against L-selectin inhibit up to 80% of intravascular PMN rolling and thereby inhibit PMN adhesion to the endothelium. It is not known, however, if L-selectin is involved in PMN adhesion to the coronary endothelium following myocardial ischemia–reperfusion. Although the selectin family of adhesion molecules is clearly involved in the initial tethering of PMNs to the endothelium, more firm adhesion is a result of interactions between the integrins and the immunoglobulin superfamily of proteins.

Previous studies have indicated that the common β-chain of neutrophil adhesion glycoproteins (ie, CD-18) as well as the endothelial ligand for the CD-18 complex (ie, ICAM-1) is involved in the pathogenesis of PMN-mediated myocardial reperfusion injury. Studies by Winquist et al and Ma et al using the anti–CD-18 MAb, R15.7, clearly demonstrated salvage of ischemic-reperfused myocardium by administration of MAb R15.7. In addition, the study conducted by Ma et al determined that MAB R15.7 treatment preserved endothelium-dependent reactivity of the coronary vasculature, suggesting a possible mechanism of cardioprotection. Ma and coworkers have also demonstrated endothelial and myocardial protective effects following the administration of the anti–ICAM-1 MAb, RR1/1, in a feline model of myocardial reperfusion injury. The results of these studies support the concept of PMN adhesion as critical step in the evolution of myocardial injury following ischemia and demonstrate that agents that interfere with PMN adhesion can very significantly reduce the extent of myocardial reperfusion injury.

In the present study, we demonstrated that administration of the specific CD-18 MAb, R15.7, during the initial 5 minutes of reflow significantly reduces the extent of PMN-mediated myocardial contractile dysfunction following myocardial ischemia–reperfusion in the isolated heart. Treatment with MAb R15.7 very significantly preserved coronary flow while also markedly enhancing left ventricular developed pressure and attenuating the marked increase in left ventricular end-diastolic pressure that was observed in untreated hearts. The cardiac myeloperoxidase data very strongly suggest that treatment with MAb R15.7 significantly limited the degree of PMN accumulation following ischemia–reperfusion. In additional studies, we also determined that MAb R15.7 very significantly inhibited complement-dependent generation of oxygen free radicals, apparently by blocking CD-18, which also functions as complement receptor 3. While the specific CD-18 antibody R15.7 exhibited these marked protective effects, equal concentrations of the control PMN-binding but non–CD-18–blocking antibody R3.1 had little if any protective effect on contractile function or

### Recovery of Physiological Parameters at 45 Minutes of Reperfusion

<table>
<thead>
<tr>
<th></th>
<th>Vehicle (n=9)</th>
<th>MAb R3.1 (n=5)</th>
<th>MAb R15.7 (n=9)</th>
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<tr>
<td>LVDP, %</td>
<td>12.8±2.8</td>
<td>29.0±8.0</td>
<td>70.7±10.4*</td>
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<tr>
<td>RPP, %</td>
<td>11.3±2.5</td>
<td>25.8±7.9</td>
<td>65.4±10.4*</td>
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<td>Coronary flow, %</td>
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<td>36.0±8.0</td>
<td>93.1±4.8*</td>
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<td>MPO, units/100 mg</td>
<td>0.30±0.05</td>
<td>0.31±0.09</td>
<td>0.02±0.01*</td>
</tr>
</tbody>
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MAb indicates monoclonal antibody; LVDP, left ventricular developed pressure; RPP, rate-pressure product; and MPO, myeloperoxidase activity.

*P<.01 vs vehicle or MAb R3.1.
coronary flow. MAb R3.1 also did not decrease PMN adhesion in the postischemic heart, as determined from tissue myeloperoxidase measurements. This antibody binds to the α-subunit of LFA-1, CD-11a, and can only block LFA-1–mediated adhesion while not affecting PMN adhesion, which occurs via the MAC-1, CD-11a–CD-18 heterodimer. As such, these studies suggest that MAC-1, not LFA-1, is responsible for PMN-mediated adhesion in the postischemic heart. 

Previous studies with isolated rabbit papillary muscles\(^4,5\) have demonstrated that activated PMNs produce a significant concentration-dependent decrease in contractile function via the release of H\(_2\)O\(_2\). Furthermore, the studies by Kraemer et al\(^4\) also reveal that PMN-mediated reduction in myocardial contractility could be reversed by a monoclonal antibody directed against the neutrophil CD-11b–CD-18 glycoprotein adhesion complex. Entman et al\(^6\) have clearly demonstrated a profound burst of H\(_2\)O\(_2\) following the adhesion of PAF-stimulated canine PMNs to canine cardiac myocytes and subsequent myocyte injury that was largely attenuated with MAb R15.7 treatment. Ambrosio et al\(^6\) as well as Bolli et al\(^6\) have proposed that oxygen-derived free radicals are responsible for much of the myocardial stunning observed following brief coronary occlusions as contractile dysfunction is ameliorated by antioxidant therapy. We found that MAb R15.7 almost completely inhibited the complement-mediated generation of superoxide radicals by human PMNs, whereas free radical generation induced by stimulation with phorbol ester or leukotriene B\(_4\) was unaffected by MAb R15.7. A potential mechanism for the observed recovery of contractile function in the present study is the reduction of oxygen free radical production by activated PMNs.

It has previously been demonstrated that mechanical removal of PMNs at the time of reflow significantly enhances regional coronary perfusion as well as contractile function following 15 minutes of coronary artery occlusion.\(^6,7\) These studies suggest that myocardial stunning is related to injury induced by activated PMNs resulting in coronary endothelial cell injury and myocardial cell injury. Endothelial dysfunction, as evidenced by decreased vasodilator response to acetylcholine, has been demonstrated by Tsao et al\(^7\) in isolated, buffer-perfused cat hearts. Interestingly, in the study by Tsao et al\(^7\), the addition of activated cat PMNs accentuated the degree of coronary endothelial injury, and this injury was inhibited by MAb R15.7. In the present study, hearts receiving MAb R15.7 exhibited a 95% recovery of coronary flow following ischemia with a concomitant and similar recovery of left ventricular contractile function. It is likely in the present study that a major mechanism of the observed cardioprotection with MAb R15.7 is the preservation of coronary endothelial function. This suggests that the alleviation of myocardial stunning resulting from neutralization of CD-18 may also be related to the maintenance of coronary vasodilator reserve via preservation of endothelium-derived relaxing factors.

In summary, the profound protective effects of MAb R15.7 may be due to inhibition of complement-mediated free radical generation by PMNs as well as marked attenuation of PMN adhesion. Treatment with MAb R15.7 resulted in salutary effects on coronary flow and contractile function throughout the duration of reflow.

Taken together, the results of this study provide strong evidence that anti–CD-18 therapy can prevent PMN-mediated reperfusion injury and can markedly enhance the recovery of cardiac function following myocardial ischemia–reperfusion.

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