Limitation of reperfusion injury by a monoclonal antibody to C5a during myocardial infarction in pigs

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Amsterdam, Ezra A., Gregory L. Stahl, Hui-Lin Pan, Stephen V. Rendig, Mark P. Fletcher, and John C. Longhurst. Limitation of reperfusion injury by a monoclonal antibody to C5a during myocardial infarction in pigs. Am. J. Physiol. 268 (Heart Circ. Physiol. 37): H448–H457, 1995.—The complement system has been implicated in reperfusion injury during acute myocardial infarction. We therefore attempted to reduce reperfusion injury with a monoclonal antibody (MAb) to the complement component, C5a. In 13 control pigs and 9 pigs pretreated with this MAb, ischemia was induced by a 50-min occlusion of the left anterior descending coronary artery, followed by 3 h of reperfusion. Infarct area (as percent of risk area) was reduced from 58 ± 5% in controls to 38 ± 7% (P < 0.05) in MAb-treated animals. Heart rate-systolic blood pressure product, left ventricular (LV) first derivative of pressure, LV end-diastolic pressure, and coronary blood flow were similar (P > 0.05) in the two groups. At 15 min of reperfusion, immunoreactive factor Bb began to increase significantly (P < 0.05) in regional coronary venous plasma, consistent with activation of the alternative complement pathway. The anti-C5a MAb did not attenuate formation of the membrane attack complex (C5b-9) as assessed by a hemolytic complement assay. Myocardial myeloperoxidase activity, a marker of tissue neutrophil concentration, was similar in the risk regions of the two groups, suggesting that neutrophil infiltration was unaltered by the MAb. However, in vitro the MAb (15 and 30 μg/ml) reduced C5a-stimulated neutrophil aggregation (67.4 and 70.9%), chemotaxis (52.5 and 81.4%), degranulation (66.7 and 75.8%), and superoxide generation (26.7 and 100%). In conclusion, myocardial infarction-associated with neutrophil extraction from the coronary circulation (26, 27) or by pharmacological inhibition of neutrophil function (1, 26, 27).

We have demonstrated that neutrophils are involved in the release of thromboxane, reduction of coronary blood flow, and impairment of contractile function produced by intracoronary infusion of both purified porcine C5a (12, 25, 37, 38) and zymosan-activated serum (ZAS; 38) in pigs. By contrast, Ito and co-workers (8, 20–22) have reported that resident cardiac mast cells rather than circulating neutrophils release leukotrienes, as well as thromboxane, both of which mediate these C5a-induced cardiac actions. Our evidence supporting the role of neutrophils is derived from several lines of investigation. First, the cardiac effects of C5a are associated with neutrophil extrusion from the coronary circulation (2, 25, 37, 38). Second, a monoclonal antibody (MAb; IB4) directed against CD18, the common β-chain of the white cell integrins, attenuates these C5a-induced cardiac effects (38). In addition, a MAb to C5a inhibits its cardiac actions while preventing myocardial extrac tion of neutrophils (38). These findings support the concept that cardiac derangements induced by exogenous C5a are partially dependent on neutrophil trapping by a CD18-mediated aggregation-adherence mechanism. Furthermore, these results suggest that inhibition of C5a activity has the potential to limit cardiac damage during myocardial infarction-reperfusion by attenuating C5a-induced neutrophil activation. Therefore, we evaluated the following hypotheses: 1) a MAb to porcine C5a reduces the extent of experimental myocardial infarction in pigs; and 2) the decrease in infarct size is associated with attenuation of C5a-induced neutrophil...
activity as indicated by a reduction of neutrophil infiltration into ischemic tissue and in vitro inhibition of neutrophil cytotoxic activity.

**METHODS**

**Instrumentation**

Adult domestic pigs of either sex, weighing 16-35 kg, were preanesthetized with ketamine (20 mg/kg im), atropine (0.05 mg/kg im), and thiamylal sodium (10 mg/kg iv). Animals were intubated and ventilated with positive pressure. After intubation, anesthesia was maintained with thiamylal sodium (10-20 mg·kg⁻¹·h⁻¹).

The chest was opened by a midline sternotomy. Recording electrodes were placed subcutaneously for constant monitoring of the electrocardiogram (ECG). Both internal mammary veins were cannulated for administration of anesthesia and drugs. An internal mammary artery was cannulated for measurement of arterial blood pressure and for blood sampling. Arterial blood gases were measured every 30 min (ABL3, Radiometer, Copenhagen, Denmark) and were maintained within physiological limits by adjusting the respirator and administering bicarbonate and supplemental oxygen. The right carotid artery was cannulated with a 7-Fr catheter-tipped pressure transducer (Millar Instruments, Houston, TX), which was passed retrogradely into the left ventricle (LV). In seven animals, catheters were introduced into the left atrium and femoral artery for measurement of regional myocardial blood flow by the radioactive microsphere technique (17). The pericardium was opened, and the length of the left anterior descending coronary artery (LAD) was measured from the origin to the LV apex. The midpoint of the LAD was dissected free from surrounding tissue. Topical lidocaine was applied to the LAD during its dissection to reduce spasm. A nonocclusive catheter was placed in the anterior cardiac vein adjacent to the LAD midpoint.

**Purified Porcine C5a and ZAS**

Porcine C5a was prepared from porcine ZAS by the method of Gerard and Hugli (16). The purity of the polypeptide was established by amino acid analysis after acid hydrolysis. ZAS was prepared as we have previously described (37). Briefly, fresh pig blood was allowed to clot, centrifuged (2,000 g, 4°C, 20 min), and the serum was frozen at −45°C. On the day before the experiment, the serum was thawed at room temperature and was incubated with zymosan (10 mg/ml; Sigma, St. Louis, MO) for 1 h at 37°C. Particulate zymosan was removed by centrifugation at 5,000 g for 30 min. ZAS was then dialyzed at pH 7.4 for 16-18 h at 4°C against Ringer buffer.

**MAbs to C5a.** A murine antiporcine C5a MAb (288-26F7), immunoglobulin (Ig) G₁ (38), was obtained from Cetus (Emeryville, CA) and was stored at −45°C in phosphate-buffered saline (PBS) at concentrations of 0.6-1.2 mg/ml. This MAb has an affinity for porcine C5a of 8.8×10⁶ M⁻¹ and does not immunoprecipitate human C5a or Pseudomonas endotoxin, and blocks porcine C5a-induced neutrophil chemotaxis, polarization, and chemiluminescence. The MAb was warmed at 37°C for 30 min, centrifuged at room temperature (40,000 g), and decanted before administration. In preliminary experiments, flow cytometric assessment of direct binding of the MAb to porcine neutrophils indicated no binding at concentrations of 15-60 μg/ml, whereas the anti CD18 MAb, IB4, readily stained the neutrophils, as previously indicated (12, 38).

**Control MAb.** An irrelevant murine MAb, IgG₁K, to a membrane protein of human intestinal mucosa was prepared and stored at −70°C in PBS at a concentration of 1.0 mg/ml. The antibody was warmed, centrifuged, and decanted as described in MAb to C5a before administration.

**Experimental protocol.** The ECG, aortic blood pressure, LV pressure, and LV first derivative of pressure (dP/dt) were monitored continuously and were recorded at each measurement period. The heart rate-blood pressure product was calculated by multiplying systolic arterial pressure and heart rate. After a 20-min period of stabilization following instrumentation, control data were recorded. MAb (0.5 mg/kg in 7 animals, 1.0 mg/kg in 2 animals) was administered by intravenous infusion over 10 min. Blood samples were obtained for measurement of circulating MAb levels at control, 45 min of ischemia, and 1, 2, and 3 h of reperfusion. One group of control animals (n = 8) received neither intervention (n = 6) or intravenous infusion of MAb vehicle (PBS, n = 2). A second group of controls (n = 5) received the control MAb (0.5 mg/kg) by intravenous infusion over 10 min.

Ten minutes after completion of infusion of MAb, vehicle, or the control MAb, hemodynamic variables and ECG were recorded and baseline regional myocardial blood flow was assessed in a subgroup of five control and three treated animals. The LAD was then occluded at its midpoint for 50 min by a nontraumatic occluder. Epicardial cyanosis was apparent in the area distal to the LAD occlusion in all animals. Hemodynamic data and ECG were recorded every 15 min during the period of occlusion. Radioactive microsphere blood flow measurements were repeated at 5 and 45 min during the period of occlusion. After 50 min, the occluder was removed, and the LAD was reperfused for 3 h while hemodynamic data were recorded every 30 min. During reperfusion, the color of the epicardium distal to the previously occluded site returned toward normal. Blood flow measurements by microsphere injection were obtained at 45 and 180 min of reperfusion.

No parenteral pharmacological agents other than those indicated above were administered. Direct current counter-shock (30 J) was applied to the epicardium (remote from the risk area [RA]) for sustained ventricular tachyarrhythmias associated with severe hypotension. If hemodynamic stability was not restored within 3 min or if more than two shocks were required, the animal was excluded from the study.

In six of the eight control pigs and four additional pigs, coronary venous plasma samples (2.0 ml) were collected in iced test tubes containing EDTA and heparin before coronary ischemia, at 15 and 45 min after onset of occlusion, and 15, 30, 60, 120, and 180 min after reperfusion for measurement of factor Bb by intravenous infusion over 10 min.

**Measurement of Infarct Size**

Myocardial infarct size was estimated by histochemical staining to identify normal, infarcted, and viable myocardium. After 3 h of reperfusion, the LAD was briefly reoccluded at the original site of occlusion, and 60 ml of 0.5% patent blue dye (Sigma) was injected into the left atrium to identify the region of myocardium at risk, indicated by absence of blue stain. Animals were then killed by ventricular fibrillation induced with electrical current (9 V) applied directly to the myocardium remote from the RA. Hearts were rapidly removed and rinsed, and the LV was sectioned into four to six transverse sections, ~1 cm thick, from apex to base. The slices were weighed and traced onto clear acetate sheets to demarcate the
normal area (blue stain) and the RA (unstained). The slices were then incubated at 37°C in a 1% solution of triphenyltetrazolium chloride (TTC) in 10 mM potassium phosphate buffer (pH 7.4). After 20 min, the slices were removed from the TTC and retracted on the same acetate sheets to demarcate the area of myocardial salvage, indicated by red staining (TTC positive), within the RA. The tracings were magnified and scanned by computer. Several myocardial tissue samples in each pig were excised from the control, salvage, and infarct areas, frozen in liquid nitrogen, and stored at -45°C until assayed. MPO activity was determined in a blinded fashion by counting 25 random fields (0.01 mm² each), which were averaged to give the number of neutrophils per millimeter squared. The ratio of neutrophils in vessels to interstitium was then calculated.

**Neutrophil Degranulation**

Polymorphonuclear leukocytes (PMN; 10 x 10⁶ cells/ml) were incubated in the presence and absence of C5a MAb (15 and 30 μg/ml, respectively) or vehicle for 15 min at 37°C. The cells were then incubated for 20 min at 37°C with C5a (final concentration 100 ng/ml) and cytochalasin B (5 μg/ml). After being cooled to 4°C and being centrifuged (2,500 g, 15 min), the cell-free supernatant was mixed (1:1) with 1% HTAB in 50 mM potassium phosphate buffer at pH 6.0 before sonication in an ice bath for 15 s. Suspensions were centrifuged, and the peroxidase activity of the resulting supernatants was measured as described above. A comparable aliquot of neutrophil (10 x 10⁶/ml) served as the nonstimulated (100%) control.

**Neutrophil Aggregation**

C5a-induced neutrophil aggregation was studied with a modified platelet aggregometer (Chrono-Log, Haverton, PA) as we have previously described (38). Briefly, after a preincubation period of 10 min at 37°C with either MAb or its vehicle (mHBSS), neutrophils (10 x 10⁶/ml mHBSS) were placed in cuvettes (0.45 ml). After a 1-min preincubation period, Ca²⁺ and Mg²⁺ (1.0 and 0.5 mM final concentration, respectively) were added. Finally, C5a [100 ng/ml final concentration, prepared in 0.25% bovine serum albumin (BSA) in HBSS] was added to the neutrophil suspension, and the aggregation response was recorded as an increase in light transmission.
suspension (10 × 10^6 cells/ml) were preincubated with ferricytochrome C (1.4 mg/ml, Sigma) in the presence of cytochalasin B (20 μM) for 5 min at 37°C, then stimulated with C5a (100 ng/ml) for 20 min. Separate aliquots were preincubated with C5a MAb concentrations of 15 and 30 μg/ml. A duplicate set of tubes contained SOD (30 U/ml). Supernoxide generation was spectrophotometrically determined at 550 nm by the difference in absorbance between the samples with and without SOD, utilizing an extinction coefficient of 21.1 × 10^-3 M^-1 cm^-1. Results are expressed as nanomoles O2^- per 10^6 cells per 20 min.

Neutrophil Chemotaxis

Porcine neutrophil chemotaxis was assessed utilizing a 48-well microchemotaxis chamber and 3.0-μm pore polycarbonate filter sheets (Costar, Cambridge, MA). PMN (0.5 × 10^6/ml) previously incubated for 10 min with MAb (15 and 30 μg/ml) or its vehicle (HBSS) were placed in the upper compartment of this chamber. The cells were separated from the lower compartment containing 5% ZAS or vehicle (HBSS) by the filter and were incubated for 30 min (6% CO2, 100% humidity, 37°C). After incubation, the cells that were adherent to the filters were fixed and stained. The number of cells migrating to the lower surface were then counted in 10 high-power (×400) fields, and the results were averaged.

Immunoreactive Factor Bb Assay

In pilot studies, a murine MAb against human factor Bb (Quidel, San Diego, CA) was found to cross react with porcine factor Bb in an immunoblot assay. The MAb did not react with any peptides in deproteinized [trichloroacetic acid (TCA)] pig serum. In contrast, the MAb did react with deproteinized ZAS. This MAb was used to develop an antibody-capture immunoassay. Coronary venous blood samples (2.0 ml) in 10 pigs collected during occlusion-reperfusion were deproteinized with 50 μl of 1.0 M TCA, and samples of the supernatant (100 μl) were incubated in duplicate at 4°C for 18 h in 96-well microtiter plates (Corning, Corning, NY). A standard curve was constructed in duplicate with a range of 20 ng/ml to 200 μg/ml purified human factor B (Quidel). After incubation, the plate was washed three times and then blocked with 350 μl of 5% BSA for 1.5 h at room temperature. The plate was thoroughly emptied, and 50 μl of a MAb (1:500) against the human complement fragment Bb was incubated at room temperature for 1.5 h. The plate was washed three times, and 50 μl of a goat anti-mouse antibody (1:10,000) labeled with peroxidase (Sigma) was incubated at room temperature for 30 min. The plate was washed three times and developed with 3,3',5,5'-tetramethyl benzidine dihydrochloride (Sigma). The plate was read at 650 nm with a microplate reader (Molecular Devices, THERMOmax, Menlo Park, CA). Sample values (ng/ml) were calculated from the standard curve with a log-logit curve using the SOFTmax software system (Molecular Devices).

Measurement of Mouse IgG (Anti-C5a MAb) in Pig Plasma

Venous blood samples (2 ml) were collected in heparinized syringes, centrifuged at 2,000 g for 15 min at 4°C, and the plasma was stored at −45°C until analyzed. The level of circulating MAb to C5a during the course of the ischemia-reperfusion protocol was assessed by a standard enzyme-linked immunosorbent assay, utilizing purified MAb as a standard. Briefly, 100-μl aliquots of known concentrations of the MAb were used to coat the wells of a 96-well microtiter plate. Plasma to be analyzed for the presence of mouse MAb to C5a was also coated overnight at 4°C at 1:10, 1:100, and 1:1,000 dilutions in carbonate coating buffer. Thereafter, the plates were washed three times with PBS containing 0.05% Tween 20 (Sigma) and were then blocked with 1% BSA in PBS. Optimally diluted peroxidase-conjugated goat anti-mouse (100 μl) antibody (Tago, Burlingame, CA) was then added to the well and incubated at room temperature for 1 h to detect the presence of mouse antibodies. The plates were then washed three times with PBS containing 0.05% Tween 20 and were developed with 100 μl of 0.55 mg/ml 2,2'-azino-bis(ethylenbenz-thiazoline-6-sulfonic acid) (Sigma) and 0.06% hydrogen peroxide. Color development was arrested by the addition of 100 μl of 0.5% sodium dodecyl sulfate after 10 min. All results were read at 405 nm. A standard curve was established in each plate, and the unknown concentrations were deduced from the linear portion of the standard curve.

Hemolytic Complement Assay (CH50)

The ability of anti-C5a MAb to inhibit formation of the membrane attack complex was assessed by measurement of the effect of this MAb on the hemolytic activity of porcine serum (5). Briefly, porcine serum from four healthy domestic pigs was stored at −20°C until use. In each row of a 96-well round-bottom microtiter plate, serum was serially diluted in PBS to give a series of dilutions: 1:12, 1:17, 1:24, 1:34, 1:48, 1:69, 1:98, 1:138, and 1:196 (final volume 50 μl). A separate aliquot of each serum was incubated with anti-C5a MAb (final concentration 30 μg/ml) for 1 h at 37°C and was diluted identically. In two experiments, an aliquot of serum was incubated at 56°C for 1 h to denature the complement, and the serum was then serially diluted. With the plate on ice to retard activation, 50-μl aliquots of antibody-sensitized sheep erythrocytes (Sigma) in gelatin-veronal buffer (1 × 10^6 cells/ml) were added to each well and mixed. A negative control (cells + PBS) and a positive control (cells + distilled H2O) were included in each row. The plate was incubated at 37°C for 30 min and was centrifuged for 10 min. Supernatants (50 μl) were transferred to a flat-bottom plate and read at 405 nm on a microtiter plate reader. The percent hemolysis was calculated for each serum dilution and was converted to the term, log(y/1-y), where y = percent lysis, and was plotted against log(1/dilution) to yield a straight line from which the 50% hemolysis intercept was read (15). In this method, the CH50 is defined as the serum dilution giving 50% hemolysis of an equal volume of red blood cells.

Data Analysis

Data are presented as means ± SE. Student’s t test for unpaired data was used for single comparisons between control and C5a MAb groups. Multiple comparisons of hemodynamic values were analyzed by two-way repeated-measures analysis of variance (ANOVA), and a Bonferroni test was applied if differences were significant. A one-way repeated-measures ANOVA was used to analyze the factor Bb data. The Williams post hoc test was applied to paired comparisons between control and each subsequent measurement period. Categorical variables were analyzed by $\chi^2$. Differences were considered to be significant if $P < 0.05$.

RESULTS

Study Group

Exclusions. All animals surviving the full coronary arterial occlusion-reperfusion protocol were included in
the data analysis. Two control pigs were excluded because of arrhythmic deaths during occlusion-reperfusion. There were no deaths during the experimental protocol in the MAb-treated pigs.

Arrhythmias. Ventricular tachyarrhythmias occurred during occlusion-reperfusion in all animals. Direct current countershock was required in three of the eight control animals (two during occlusion, one during reperfusion), including the two nonsurvivors. Countershock was required in five pigs in the MAb group (two during occlusion and three during reperfusion) and was successful in restoring sinus rhythm in all cases.

Myocardial Infarct Size

Infarct size (IA/RA) was similar in the two control groups (no therapy 60 ± 9%, neutral antibody 56 ± 5%). Therefore, they were combined to comprise a single control group (IA/RA 58 ± 5%). IA/RA was less (P < 0.05) in the group treated with the anti-C5a MAb (38 ± 7%) than in the controls. RA as a percentage of total LV mass was similar in the controls and the animals treated with the anti-C5a MAb (Fig. 1).

Hemodynamic Function

There were no significant differences in hemodynamic data between the control and MAb groups at any of the measurement points during the control, occlusion, and reperfusion periods (Fig. 2).

Regional Myocardial Blood Flow

Hemodynamic variables measured immediately before and after each microsphere injection confirmed steady-state conditions during myocardial blood flow determinations. Regional myocardial blood flow fell markedly during occlusion and rose at 45 min of reperfusion (Table 1). There were no significant differences in regional myocardial blood flow in the RAs (salvage + infarcted regions) of the control and MAb-treated groups at the points measured during the control, occlusion, and reperfusion periods.

Myocardial MPO Activity and Neutrophil Distribution

MPO activity was greater (P < 0.05) in the RA (salvage + infarcted) than in the normal region of LV in both the control and MAb groups (Fig. 3). However, there were no significant differences in MPO activity between the two groups in the respective normal and risk areas.

Tissue neutrophils in the myocardial risk region were preponderantly distributed in the interstitium, rather than within vessels, in both the control and treated animals. The ratio of neutrophils in vessels to intersti-
DECREASED INFARCT SIZE BY ANTI-C5a ANTIBODY

Table 1. LV transmural blood flow

<table>
<thead>
<tr>
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<th>LV Blood Flow, ml g⁻¹ min⁻¹</th>
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<tbody>
<tr>
<td></td>
<td>Pre-Occ 5-min Occ 45-min Occ 45-min Reperf 180-min Reperf</td>
</tr>
<tr>
<td>Control</td>
<td></td>
</tr>
<tr>
<td>Norm</td>
<td>1.02 ± 0.10</td>
</tr>
<tr>
<td>RA</td>
<td>0.84 ± 0.09</td>
</tr>
<tr>
<td>Antibody</td>
<td>0.77 ± 0.16</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of pigs. Occ, occlusion of left anterior descending coronary artery; Reperf, reperfusion; Norm, normal left ventricular (LV) myocardium; RA, LV risk region (infarcted and salvaged left ventricular myocardium).

Neutrophil Function

The effects of MAb on neutrophil function in vitro are shown in Table 2. At a concentration of 15 µg/ml, the MAb produced >50% inhibition of aggregation, chemotaxis, and degranulation. All neutrophil functions were reduced by >70% at a MAb concentration of 30 µg/ml. Superoxide generation, which was least affected at the lower MAb concentration, was completely inhibited at the higher antibody concentration.

Factor Bb Assay

Factor Bb was unchanged from control (221 ± 58 ng/ml) during the ischemic period but was increased significantly at 15 min of reperfusion (P < 0.05) and remained elevated during the entire reperfusion period (Fig. 4).

Anti-C5a MAb Plasma Levels

Plasma concentrations of MAb were between 18.2 ± 1.4 and 20.4 ± 0.7 µg/ml at the points measured (45 min of occlusion and 60, 120, and 180 min of reperfusion).

These levels were comparable to those that inhibited C5a-induced responses of neutrophils in vitro (Table 2).

Hemolytic Complement Assay (CH₅₀)

There were no significant differences in hemolysis of antibody-sensitized sheep erythrocytes by control serum and serum preincubated with the anti-C5a MAb (Fig. 5). By contrast, heat-inactivated serum demonstrated only minimal hemolytic activity. The CH₅₀ for serum alone was 72 ± 7, and for serum plus anti-C5a MAb it was 65 ± 10 (NS).

DISCUSSION

This study demonstrates that a MAb to the anaphylatoxin, C5a, limits myocardial infarct size in pigs. The cardioprotective action of this MAb was not associated with decreased infiltration of neutrophils into injured myocardium but was accompanied by in vitro attenuation of C5a-induced neutrophil aggregation, degranulation, and superoxide production, functions with proinflammatory and cytotoxic potential. In addition, our findings provide evidence for activation of the complement system by the alternative pathway during reperfusion, as reflected by the rise in factor Bb in coronary venous blood during this period. The complement hemolytic assay indicated that the anti-C5a MAb did not prevent generation of the C5b-9 membrane attack complex, which supports inhibition of C5a activity as the mechanism of cardioprotection. The absence of any effects of this MAb on hemodynamic function or coronary blood flow compared with controls indicates that its beneficial effects were not related to alteration of the major determinants of myocardial oxygen supply and demand. This study, therefore, is the first to provide evidence that experimental myocardial infarction-reperfusion injury 1) is associated with activation of the complement system by the alternative pathway and 2) can be limited by selective inhibition of the anaphylatoxin, C5a, by a MAb, most likely through attenuation of C5a-induced neutrophil activation.

Complement-induced reperfusion injury associated with myocardial infarction has been attributed to the direct effects of the C5b-9 membrane attack complex and indirect actions mediated by the leukotactic properties of C5a. Recent experimental studies have extended prior evidence (7, 29, 32) for the role of both mechanisms in reperfusion injury. Thus Weisman and coworkers (40) reduced postischemic cardiac inflamma-

![Fig. 3. Myocardial myeloperoxidase (MPO) activity in control and treated (MAb) animals. MPO activity was markedly increased in salvage and infarct regions of hearts in both groups (P < 0.05), but there were no differences in MPO activity between groups in any of the myocardial regions (normal, salvage, or infarct).](image-url)
Table 2. Effect of anti-C5a antibody (MAb) on neutrophil functions activated by C5a

<table>
<thead>
<tr>
<th>Neutrophil Functions</th>
<th>n</th>
<th>Control (HBSS)</th>
<th>Control (C5a, 10^-8 M)</th>
<th>MAb (15 μg/ml)</th>
<th>MAb (30 μg/ml)</th>
<th>%Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aggregation, AU</td>
<td>4</td>
<td>17.3 ± 1.1</td>
<td>33.4 ± 2.7</td>
<td>29.5 ± 1.8</td>
<td>22.0 ± 2.1</td>
<td>67.4</td>
</tr>
<tr>
<td>Chemotaxis, no. cells/10 HPF</td>
<td>3</td>
<td>8.7 ± 1.5</td>
<td>97.7 ± 7.4</td>
<td>51.0 ± 4.6</td>
<td>25.3 ± 3.1</td>
<td>52.5</td>
</tr>
<tr>
<td>Degranulation, U MPO</td>
<td>4</td>
<td>0.00</td>
<td>0.33 ± 0.02</td>
<td>0.11 ± 0.03</td>
<td>0.08 ± 0.03</td>
<td>66.7</td>
</tr>
<tr>
<td>Superoxide generation, nmol O2-· 10^6 cells·l^(-1)·20 min^(-1)</td>
<td>3</td>
<td>6.46 ± 2.00</td>
<td>24.64 ± 7.70</td>
<td>19.78 ± 6.42</td>
<td>5.90 ± 2.20</td>
<td>26.7</td>
</tr>
</tbody>
</table>

Values (except for %inhibition) are means ± SE; n, no. of experiments. The negative control data [Hanks’ balanced salt solution (HBSS)] were subtracted from the positive control data (C5a) and from the results with monoclonal antibody (MAb) for each function to calculate %inhibition. AU, arbitrary units; HPF, high-power fields; MPO, myeloperoxidase.

The protective effect of the inhibitor was associated with reduced deposition of C5b-9 on capillary endothelium and decreased myocardial accumulation of leukocytes, suggesting that both direct damage by the membrane attack complex and indirect injury related to anaphylatoxin-directed neutrophil activity were inhibited by sCR1.

In considering the mechanisms of the protective effect of the anti-C5a MAb, it was essential to assess the influence of the MAb, which can cross-react with C5, on formation of the membrane attack complex. C5b-9 has been shown to produce direct myocardial injury in an isolated heart preparation (19). The CHSO assay measures in vitro hemolytic capacity of serum, which is related to generation of the membrane attack complex. The anti-C5a MAb did not significantly inhibit formation of the membrane attack complex, as indicated by lack of an effect on hemolysis. These data suggest that reduction of infarct size with the anti-C5a MAb was not related to an action on C5b-9. Thus, while there is evidence that the membrane attack complex may play a role in myocardial reperfusion injury (29, 40), our findings suggest that C5a also significantly contributes to this process.

Although the vasoconstrictive actions (30) and PMN-related effects (12, 25, 37, 38) of C5a may contribute to its role in reperfusion injury, its direct effects on the myocardium appear to be limited. We have shown that purified porcine C5a has a biphasic, predominantly positive, but modest contractile action on isolated pig myocardium (2). Furthermore, Homeister et al. (19) were unable to reproduce the deleterious myocardial actions attributed to C5b-9 with C5a. However, the isolated rabbit heart preparation utilized by these investigators is limited by absence of ischemia and lack of neutrophils, two factors that are essential for anaphylatoxin-induced myocardial reperfusion injury. Prior studies support an interaction between C5a and neutrophils in reperfusion injury. Under the chemotactic and activating influence of C5a, neutrophils engage in a series of proinflammatory processes (1, 26, 27) that include aggregation, trapping in the microcirculation, migration...
to injured tissue, and release of cytotoxic mediators. Furthermore, depletion of complement (29) or prevention of its activation (40) reduces infarct size in association with reduced myocardial infiltration by neutrophils. Finally, infarct size can be limited by depletion of neutrophils (26, 27) or inhibition of their function by several methods (1, 26, 27). Ito and co-workers (8, 20–22) reported that resident cardiac mast cells rather than circulating neutrophils are the source of eicosanoids that mediate the deleterious cardiac effects of C5a. However, their studies relate only to intracoronary injection of exogenous C5a in nonschematic animals and thus may not be relevant to experimental myocardial infarction.

Our method of complement system inhibition to achieve a cardioprotective effect during myocardial ischemia-reperfusion differed from previous approaches (29, 40). Our study involved selective inhibition of a single complement component and its des-Arg derivative (38). By contrast, prior studies have been nonselective in regard to suppression (40) or depletion (29) of complement, and the precise mechanism of therapeutic efficacy was therefore not clarified. Our data demonstrate that inhibition of C5a and its des-Arg derivative is associated with diminished reperfusion injury. This beneficial effect was not related to reduced neutrophil accumulation in injured myocardium, despite in vitro evidence of inhibition by the MAb of C5a-induced neutrophil proinflammatory functions such as chemotaxis. Because this MAb did not bind directly to neutrophils, as indicated by our preliminary experiments, it could not have exerted its beneficial effects by a nonspecific interaction with these leukocytes. In addition, infarct size in control animals treated with a control IgG MAb did not differ from that of the vehicle-treated group, indicating that the protective action of the anti-C5a MAb was not a nonspecific effect of this antibody class.

Assessment of myocardial MPO activity demonstrated that treatment with anti-C5a MAb did not alter neutrophil infiltration into injured myocardium despite the inhibitory effect of the MAb on chemotaxis in vitro. Persistence of myocardial neutrophil accumulation in vivo, despite attenuation of C5a-induced chemotaxis in vitro, may be attributable to multiple chemotactic factors other than C5a that promote this process in the intact animal, including oxidized plasma lipids, 5-lipoxygenase products, and platelet-derived factors (4, 26, 39). Furthermore, several lines of investigation provide evidence of intact neutrophil accumulation with diminished or absent neutrophil-induced tissue injury. A recent study utilizing an isolated ischemic heart preparation is relevant to our observations in showing that in the absence of a source of complement, neutrophils could still accumulate in the myocardium during reperfusion without causing injury (35). By contrast, if a source of complement were present or if C5a was provided, there was marked neutrophil-mediated damage. Furthermore, lower stimulation thresholds have been demonstrated for chemotaxis than for other neutrophil functions such as superoxide anion generation (13) and lysosome release (14). Thus concentrations of C5a that induce neutrophil chemotaxis may not be sufficient to initiate release of cytotoxic mediators. In addition, it has been shown in noncardiac tissue that dual lipoxygenase-cyclooxygenase antagonists can attenuate neutrophil release of cytotoxic factors, such as leukotrienes and toxic oxygen species, without decreasing accumulation of neutrophils at sites of inflammation (18, 34). In this regard, we have demonstrated that the dual lipoxygenase-cyclooxygenase inhibitor, BW755C, reduces infarct size in the pig in association with diminished production of cytotoxic mediators without reducing neutrophil infiltration of injured myocardium (1).

We assessed myocardial neutrophil content by measurement of tissue MPO activity. This method is based on the specificity of MPO as a marker for neutrophils (36). The validity of tissue MPO activity as a measure of neutrophil content is supported by prior findings that demonstrate concordance between tissue MPO activity and histological determination of neutrophils in acutely infarcted myocardium (36). Although neutrophils labeled with radioisotopes may provide more direct quantitative data, this method has the potential to significantly alter cellular integrity (31).

It is possible that the protective effect of the anti-C5a MAb was related to prevention of neutrophil migration from the circulation into myocardial tissue, an effect that would not be detected by measurement of tissue MPO activity. However, histological analysis of myocardium from controls and animals treated with the anti-C5a MAb revealed no difference in the distribution of intravascular and interstitial neutrophils in the two experimental groups.

Prior studies have provided evidence that complement is involved in the cardiac damage produced during myocardial infarction. The complement components, C1q, C3, C4, and C5, have been identified in infarcted tissue (29, 33), and inhibition of complement activity reduces infarct size (29, 40). Our data provide the first direct evidence that the alternative pathway of the complement cascade is activated during myocardial infarction-reperfusion, as indicated by the rise of plasma factor Bb during the reperfusion phase. Activation of the alternate pathway results in cleavage of factor B, a serum protein specific to the alternative pathway, to factors Ba and Bb (23). Elevation of plasma Bb concentration in the coronary venous effluent therefore indicates activation of the alternative pathway. Although we measured factor Bb in the coronary venous effluent, our study allows no conclusions as to whether activation of the alternative pathway was primarily intra- or extravascular in origin. Rosen et al. (32) have reported that subcellular fragments released from ischemic, reperfused myocardium may activate complement in the interstitium, while intravascular activation of complement in infarction-reperfusion has been recently demonstrated (40).

Several potential limitations of this study require consideration. Our conclusions regarding the mechanism of the cardioprotective effect of the anti-C5a MAb
are based partially on indirect evidence obtained from in vitro studies of neutrophil function and hemolytic activity of serum. Caution must be exercised in extrapolating the inhibitory actions of this MAb on isolated neutrophils to an in vivo action, since the pathophysiological milieu in the intact animal may alter these effects. However, attenuation of certain important C5a-induced cytotoxic actions of neutrophils by the MAb in vitro provides a possible mechanism for a cardioprotective effect during infarction-reperfusion in the intact animal. Another potential limitation of this study is the determination of myocardial neutrophil accumulation indirectly by assessing tissue peroxidase activity as a measure of MPO content. However, although indirect, this approach has certain advantages over other methods to quantitate myocardial neutrophil content, as previously noted. Finally, the cardioprotective effects demonstrated in our study were achieved by administration of the MAb before coronary occlusion. Before this approach can be considered for clinical application, its utility must be demonstrated with MAb administered after the onset of coronary occlusion.

In summary, our findings in experimental myocardial infarction in pigs indicate for the first time that 1) reperfusion during infarction activates the alternative pathway of the complement system, and 2) a MAb to C5a that inhibits C5a-mediated cytotoxic functions of neutrophils in vitro, and has no effect on the membrane attack complex, decreases reperfusion injury and reduces infarct size. These data support an important role for C5a in the porcine model of cardiac reperfusion injury.

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