Inhibition of Complement C5 Reduces Local and Remote Organ Injury After Intestinal Ischemia/Reperfusion in the Rat

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Background & Aims: Complement activation plays an important role in the local pathogenesis of ischemia/reperfusion (I/R) injury. We investigated the action of anti-C5 monoclonal antibody (mAb) on local and remote organ injuries after intestinal I/R in the rat. Methods: Under anesthesia, functional anti-rat C5 mAb (18A), an isotype-matched control anti-C5 mAb (16C), or vehicle (phosphate-buffered saline) was administered 60 minutes before the superior mesenteric artery was occluded for 90 minutes and reperfused for 60 minutes. Tissue injury was assessed by lactate dehydrogenase release, myeloperoxidase activity, and microvessel relaxation. Tumor necrosis factor (TNF)-α, interleukin (IL)-1α, and intercellular adhesion molecule (ICAM)-1 expression was assessed by reverse-transcription polymerase chain reaction and immunohistochemistry. Results: The loss of endothelium-dependent relaxation of microvessels from the superior mesenteric artery after I/R was significantly attenuated by 18A but not by 16C. Intestinal lactate dehydrogenase release after I/R was significantly reversed by 18A treatment. Anti-C5 treatment significantly inhibited the increased myeloperoxidase activity in the lung and intestine after intestinal I/R. Furthermore, increased intestinal TNF-α, IL-1α, and vascular ICAM-1 expression after I/R were significantly inhibited by anti-C5 mAb. Conclusions: Anti-C5 therapy significantly improved intestinal I/R tissue injury as well as lung injury.

It is well known that gastrointestinal ischemia/reperfusion (I/R) induces organ dysfunction and tissue injury after surgery for bowel obstruction or abdominal aortic aneurysm, hemorrhagic shock, or necrotic enterocolitis or after cardiopulmonary bypass.1-5 Recently, the mortality rate for supravisceral aortic cross-clamping (above the superior mesenteric or celiac artery) in humans was correlated to postoperative visceral ischemia/infarction,6 and mortality from acute mesenteric ischemia ranges from 60% to 90%.7

Local gastrointestinal I/R induces the systemic release/synthesis of various inflammatory mediators (i.e., tumor necrosis factor [TNF]-α, interleukin [IL]-1α6,8) and activates leukocytes, which may also induce remote (second) organ injury.1,2,8 Intestinal I/R is an important effector of multisystem organ failure, with which pulmonary injury and development of adult respiratory distress syndrome is highly associated.3,5,9 The mortality associated with multisystem organ failure is high,4,7,9 and the design of specific interventions to prevent local and remote organ injury after mesenteric I/R is clearly needed.

Several reports have indicated that complement activation mediates I/R injury of the myocardial, skeletal, and intestinal circulations.10-12 Inhibition of complement by soluble complement receptor type 1 (sCR1) can protect the intestine from I/R injury.13-16 Complement activation results in the formation of the proinflammatory molecules C5a and C5b-9. Therapeutic inhibition of the complement cascade at C5 preserves the immunoprotective function of the early complement components (i.e., C3b, C3b) while blocking the formation of the C5-derived inflammatory mediators, C5a and C5b-9.17 This may be particularly important in gastrointestinal diseases, in which local flora may enter the cardiovascular system, resulting in sepsis and adult respiratory distress syndrome. Along these lines, C5a- or C5a-receptor knockout mice are more susceptible to bacterial infections than controls.18,19 In contrast, C5a inhibition protects rats from the lethal effects of sepsis.20 In previous studies, we demonstrated that anti-C5 monoclonal antibodies (mAbs) inhibit leukocyte infiltration and decrease tissue injury after I/R of the kidney or heart.17,21 In this study, we report the actions of anti-C5 mAb on local intestinal and remote organ injury after intestinal I/R in rats.

Abbreviations used in this paper: BK, bradykinin; GADPH, reduced glyceraldehyde-phosphate dehydrogenase; ICAM-1, intercellular adhesion molecule 1; IL, interleukin; I/R, ischemia/reperfusion; LDH, lactate dehydrogenase; mAb, monoclonal antibody; MPO, myeloperoxidase; PMN, polymorphonuclear leukocyte; sCR1, soluble complement receptor type 1; TNF, tumor necrosis factor.

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Materials and Methods

Anti-rat mAbs and Reagents

Anti-rat C5 mAbs have been described previously. One mAb, 18A10.62 (18A; immunoglobulin [Ig] G2b isotype), functionally blocks C5b–9 and C5a formation, whereas 16C9.10 (16C; IgG2b isotype) binds rat C5 but does not block C5b–9 or C5a formation. All antibodies were raised in tissue culture and purified by affinity chromatography as previously described. All treatments were given 60 minutes before ischemia (20 mg/kg intravenously [IV]) as described previously. U-46619 was a gift from Upjohn (Kalamazoo, MI). Bradykinin (BK) was purchased from Sigma (St. Louis, MO). Monospecific rabbit anti-rat C9 polyclonal antibody was a gift from Dr. B. Paul Morgan (Cardiff, Wales). Anti-rat intercellular adhesion molecule 1 (ICAM-1) mAb (clone 1A29; Pharmingen, San Diego, CA) and TNF-α polyclonal antibody (Research & Diagnostic Systems, Minneapolis, MN) were used for immunohistochemical staining and Western blotting, respectively.

I/R-Induced Intestinal Injury

Adult male Sprague–Dawley rats weighing 220–250 g were fasted for 16–18 hours before the experiments and allowed free access to water. Intestinal I/R was produced by occlusion of the superior mesenteric artery as previously described, with some modifications. Briefly, the rats were anesthetized with sodium pentobarbital (50 mg/kg intraperitoneally), and the superior mesenteric artery was occluded with a small clamp for 90 minutes. This degree of ischemia is similar to that observed by patients undergoing supraceliac aortic cross-clamping during thoracoabdominal aortic aneurysm repair. The rats were killed after 60 minutes of reperfusion. Sham-operated animals underwent the same surgical procedures without clamping.

Intestinal Tissue Homogenates: Measurements of Lactate Dehydrogenase Activity in Perfusates or Tissue Homogenates

Intestinal lactate dehydrogenase (LDH; Sigma) activity from tissue homogenates was measured as an index of cell injury. TNF-α was also evaluated by Western blot analysis. Brieﬂy, tissue samples (10–30 cm above the cecum) were homogenized either in 5 mmol/L phosphate buffer (LDH activity) or in phosphate-buffered saline containing 1% Nonidet P-40 and 2 mmol/L phenylmethysulfonyl fluoride (TNF-α) and centrifuged, and the supernatant was assayed. Cell lysates (9 μg of protein per lane) for Western analysis were separated by 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis under nonreducing conditions, transferred to nitrocellulose, and blocked. A polyclonal anti-rat TNF-α antibody (Research & Diagnostic Systems; 1:500) and horseradish peroxidase–conjugated goat anti-rabbit IgG were used to develop the Western blot. LDH activity was normalized to total protein concentration (Bio-Rad, Hercules, CA).

Measurement of Myeloperoxidase Activity in Intestine or Lung

Tissue samples of intestine and lung were collected, and myeloperoxidase (MPO) activities (U/mg protein) were measured as described previously.

Rat Mesenteric Microvessels

Rat superior mesenteric microvessels (100–150 μm in diameter, 1–2 mm in length) were isolated after the I/R procedure, placed into a vessel chamber (Living System Instrumentation, Burlington, VT), superfused with Krebs buffer (37°C), and pressurized to 40 mm Hg as previously described. The microvessels were precontracted to 30%–50% of the resting baseline diameter with U-46619 (0.1–1 mmol/L). Increasing concentrations of BK were added to induce endothelial cell–dependent vasorelaxation, which was measured as previously described.

H&E Staining

Tissue samples for histologic staining were taken and fixed in 3.7% formaldehyde–phosphate-buffered saline at 4°C overnight. The samples were dehydrated and embedded in paraffin. Sections (7 μm) were cut and stained with H&E.

Immunohistochemistry

The Vectastain ABC kit (Vector Laboratories, Burlingame, CA) was used with the 3,3′-diaminobenzidine substrate kit (Vector Laboratories) according to the manufacturer’s instructions after removal of paraffin.

Semiquantitative Reverse-Transcription Polymerase Chain Reaction

Total RNA was extracted from frozen intestinal tissue using acid guanidinium thiocyanate as described previously. RNA (DNase treated; Gibco Life Technologies Inc., Gaithersburg, MD) was extracted by phenol chloroform and precipitated with ethanol. Complementary DNA (cDNA) was synthesized using the RT system (Promega, Madison, WI) according to the manufacturer’s instructions. ICAM-1, reduced glyceraldehyde-phosphate dehydrogenase (GAPDH), and IL-1α were amplified using the following primers: ICAM-1: 5′-atggcttcaacccgtgccaggc-3′ and 5′-tcagggagggggcttgac-3′ and GAPDH: 5′-tgaccaccaactgcta-3′ and 5′-ggatggaggggcttgac-3′. TNF-α and IL-1α were amplified using commercially available primers (Clontech, Palo Alto, CA). Amplification products were resolved by electrophoresis, digitized, and analyzed (Kodak Digital Science). Net band intensity was normalized to GAPDH. To ensure that amplification was in the linear range, the number of polymerase chain reaction cycles for ICAM-1, TNF-α, IL-1α, and GAPDH was titrated, and the optimal cycle numbers were used. Water samples or RNA samples containing no reverse transcripase were amplified in parallel to ensure that no contaminating DNA was present during polymerase chain reaction.
**Figure 1.** Gastrointestinal injury after I/R. (A–C) Microscopic images of intestinal tissue sections from (A) sham, (B) I/R + vehicle, and (C) I/R + 18A (original magnification 100×). (D) Tissue LDH activity from sham, I/R + vehicle, and I/R + 18A groups. Each bar and bracket represents the mean ± SEM from 6–9 different animals. I/R induced intestinal injury that was significantly attenuated by anti-C5 mAb. *P < 0.05 vs. sham group or 18A.

**Figure 2.** Effect of anti-C5 mAb on vascular complement deposition and function. C9 deposition was significantly increased on the vascular endothelium of (B) I/R animals compared with (A) sham-operated controls. (C) Anti-C5 inhibited vascular C9 deposition on the vasculature. (D) Summarized results from BK-induced relaxation of mesenteric microvessels from sham, I/R control, and I/R + 18A groups. I/R attenuated BK-induced relaxation of microvessels, and anti-C5 mAb reversed this vascular dysfunction. Each symbol and bracket represents the mean ± SEM of 3–5 independent experiments.

**Figure 3.** Effect of anti-C5 mAb on leukocyte infiltration into intestinal tissue after I/R. (A–C) Microscopic images of intestinal tissue sections from (A) sham, (B) I/R + vehicle, and (C) I/R + 18A groups (original magnification 1000×). Leukocytes were observed in the vasculature after I/R and were attenuated by anti-C5 mAb. (D) MPO activity (U/mg protein) of intestinal tissues from sham, I/R plus vehicle, I/R + 18A, and I/R + 16C groups. Each bar and bracket represents the mean ± SEM of 5–8 experiments. *P < 0.05 compared with sham- or 18A-treated animals.

**Figure 4.** Effect of anti-C5 mAb on leukocyte infiltration into lung tissue after intestinal I/R. (A–C) Microscopic images (H&E) of lung sections from (A) sham, (B) I/R + vehicle, and (C) I/R + 18A (original magnification 200×). Leukocytes were observed in the lung after I/R and were attenuated by anti-C5 mAb. (D) MPO activity of lung tissues from sham, I/R plus vehicle, I/R + 18A, and I/R + 16C was measured and expressed as U/mg protein. Each column represents the mean ± SEM of 5–8 experiments. *P < 0.05 compared with sham- and 18A-treated animals.

**Figure 5.** Intestinal ICAM-1 expression after I/R. (A–C) Microscopic images of intestinal microvessels staining for ICAM-1 from (A) sham, (B) I/R + vehicle, and (C) I/R + 18A. ICAM-1 was localized to the vascular endothelium after intestinal I/R. Treatment with 18A inhibited ICAM-1 protein expression (original magnification 1000×). (D) Semiquantitative reverse-transcription polymerase chain reaction (RT-PCR) for ICAM-1 mRNA from sham, I/R, and I/R + 18A–treated intestine. ICAM-1 RT-PCR products were digitized, and the net band intensity was normalized to GAPDH for each sample. Bars and brackets represent means ± SEM of 3 independent experiments. A representative experiment is shown below the histogram. *P < 0.05 compared with sham- or 18A-treated animals.
Statistical Analysis

All results are expressed as means ± SEM. Data analysis was performed using SigmaSTAT software (Jandel Scientific, San Rafael, CA). Statistical comparisons were made using Student–Newman–Keul’s test after analysis of variance (ANOVA). The results were considered significantly different at P < 0.05.

Results

Effects of Anti-C5 Antibody on I/R-Induced Intestinal Injury

Mucosal destruction, loss of villi and epithelial cells, hemorrhage, and infiltration of inflammatory cells were observed microscopically in the rat intestine after I/R (Figure 1B) compared with sham-operated controls (Figure 1A).

Treatment with anti-C5 mAb (18A) dramatically improved these microscopic alterations (Figure 1C). Intestinal tissue LDH levels significantly decreased in vehicle-treated animals subjected to I/R compared with sham-operated animals (Figure 1D). Treatment with 18A, but not 16C, significantly inhibited the loss of LDH from intestinal tissues. These results show that anti-C5 mAb treatment attenuates I/R-induced intestinal injury in vivo.

Anti-C5 mAb Improves Microvessel Dysfunction

Vascular deposition of C5b-9 attenuates endothelium-dependent relaxation of vascular smooth muscle and up-regulates endothelial cell adhesion molecules in vitro. C9 deposition on the vascular endothelium was observed on the small vessels of the submucosa (Figure 2B) compared with the sham-operated group (Figure 2A) and decreased after treatment with 18A (Figure 2C). The median effective concentration for BK-induced relaxation increased significantly from 27 ± 5 nmol/L in sham-operated animals to 624 ± 42 nmol/L (P < 0.05) after I/R (Figure 2D). Treatment with 18A significantly attenuated the loss of BK-dependent relaxation (median effective concentration, 90 ± 5 nmol/L) compared with untreated animals (P < 0.05). Relaxation in response to sodium nitroprusside (i.e., endothelial cell-independent relaxation) was not significantly different between any of the groups (data not presented). These results indicate that the terminal complement pathway plays an important role in microvascular dysfunction after I/R in vivo.

Effect of Anti-C5 mAb on Leukocyte Infiltration After Intestinal I/R

Inhibition of neutrophil infiltration after intestinal I/R results in local tissue protection. I/R (Figure 3B) induced a significant influx of leukocytes, particularly polymorphonuclear leukocytes (PMNs), into the intestine compared with sham-operated animals (Figure 3A).

I/R induced a significant increase in intestinal MPO activity in vehicle-treated rats compared with sham-treated animals (Figure 3D). Treatment with 18A (Figure 3C and D), but not 16C (Figure 3D), significantly inhibited the increase in PMN infiltration and MPO activity compared with vehicle-treated animals. These results show that inhibition of C5 attenuates the influx of PMNs into the intestine after I/R.

Remote tissue injury is often observed after reperfusion of the ischemic gut. Intestinal I/R induced a remarkable increase in pulmonary congestion and an influx of inflammatory cells into the lung (Figure 4B) compared with sham-treated animals (Figure 4A).

MPO activity in the lung was significantly increased in vehicle-treated rats after intestinal I/R compared with sham-operated animals (Figure 4D). Treatment with 18A (Figure 4C and D), but not 16C (Figure 4D), significantly inhibited the increase in lung MPO activity. Thus, blocking the complement component C5 significantly attenuates PMN infiltration and lung injury after intestinal I/R in the rat.

Effect of Anti-C5 mAb on ICAM-1 Expression in the Intestine

Gastrointestinal inflammatory diseases are associated with increased ICAM-1 expression and may play an important role in PMN recruitment from the vasculature. ICAM-1 protein expression within the gastrointestinal tissue, particularly within the vascular endothelium, was significantly increased in vehicle-treated animals after I/R (Figure 5B) compared with sham-operated animals (Figure 5A).

Anti-C5 treatment inhibited ICAM-1 protein expression within the vasculature after I/R (Figure 5C). Further, intestinal ICAM-1 messenger RNA (mRNA) expression was significantly increased in vehicle-treated rats after I/R compared with sham-operated animals (Figure 5D). Anti-C5 mAb treatment significantly attenuated ICAM-1 mRNA expression. These data show that inhibition of the late complement component C5 inhibits ICAM-1 mRNA and protein expression in the gastrointestinal tract after I/R.

TNF-α and IL-1α Expression After Gastrointestinal I/R

Inflammatory cytokines (e.g., TNF-α and IL-1α) are thought to play a major role in the inflammatory process in multiple human diseases, including gastrointestinal I/R. TNF-α protein levels were evaluated in
intestinal tissues in vehicle-treated animals after I/R (Figure 6A, lane 2) compared with sham-operated animals (Figure 6A, lane 1).

Anti-C5 treatment inhibited TNF-α protein expression within gastrointestinal tissues after I/R (Figure 6A, lane 3) and are consistent with our mRNA findings. Further, intestinal TNF-α and IL-1α mRNA were significantly increased in vehicle-treated animals after I/R compared with sham-operated animals (Figure 6B and C). Anti-C5 mAb treatment significantly attenuated TNF-α and IL-1α mRNA expression. These data show that inhibition of the late complement component C5 inhibits expression of the proinflammatory mediators IL-1α and TNF-α in the gastrointestinal tract after I/R.

**Discussion**

Complement activation is considered an important effector in the pathologic alterations induced by intestinal I/R in animals. Whereas C3- or C5α-receptor knockout mice are more susceptible to bacterial infections, C5α inhibition protects rats from the lethal effects of sepsis. Thus, inhibition of complement at the level of C3 (e.g., sCR1) may not be a clinically viable therapeutic option for conditions associated with acute mesenteric I/R because translocation of bacterial flora is a potential contributing factor to the high mortality rate observed in patients after acute mesenteric ischemia. In this study, inhibition of C5α and C5b-9 with an anti-C5 mAb dramatically improved I/R-induced intestinal tissue injury. The leakage of LDH from intestinal tissue and accumulation of neutrophils was attenuated by anti-C5 mAb after I/R. A strikingly significant increase in TNF-α and IL-1α expression by I/R was abolished by anti-C5 treatment. This is the first report to show the protective actions of anti-C5 mAb therapy on intestinal I/R injury in vivo. These results indicate that cleavage of C5 is a critical step in intestinal I/R injury and that anti-C5 therapy can provide tissue protection.
Leukocyte infiltration is a major mechanism of I/R-induced gastrointestinal tissue injury. Although the molecular mechanisms involved in leukocyte recruitment from the vasculature during intestinal I/R are not completely elucidated, up-regulation of vascular leukocyte adherence molecules plays an important role in the process. Inflammatory cytokines, like TNF-α and IL-1α, are presumed to play an important role in the inflammatory process and multiorgan failure in humans after gastrointestinal I/R. In the present study, we show that inhibition of C5 attenuates the up-regulation of the vascular leukocyte adherence molecule ICAM-1 and the potent inflammatory cytokines, TNF-α and IL-1α. It is possible that anti-C5 treatment attenuates ICAM-1 expression by decreasing inflammatory cytokine expression. Previously, Kilgore et al. showed that C5b-9 augmented TNF-α-induced up-regulation of ICAM-1 on human endothelial cells in vitro, an observation consistent with our in vivo findings. Although complement has been shown to induce adherence molecule expression in vitro, this report shows that inhibition of the terminal complement components (i.e., C5a and C5b-9) inhibits the up-regulation not only of ICAM-1, but also of inflammatory cytokines in vivo. Because the activated leukocytes, TNF-α and IL-1α are presumed to play an important role in human gastrointestinal I/R, anti-human C5 mAbs (i.e., 5G1.1 and 5G1.1SC) may be useful in clinical situations of gastrointestinal I/R or other inflammatory bowel diseases.

I/R of tissues induces endothelial dysfunction and complement activation during the early phases of reperfusion in vivo. Endothelial dysfunction is mediated in part by leukocytes and complement activation. We demonstrated a loss of BK-induced relaxation of microvessels after intestinal I/R and a protective effect elicited by anti-C5 mAb. Although we cannot ascertain whether the protective actions of anti-C5 on vascular integrity are caused solely by anticomplement or anti-leukocyte actions or by a combination of the 2 events, it is clear that inhibition of anti-C5 provides multiple anti-inflammatory actions in this model.

One of the serious consequences of gastrointestinal I/R is multiorgan failure, also referred to as remote (second) organ injury. We show that anti-C5 mAb attenuates the accumulation of leukocytes into lung after intestinal I/R. The molecular mechanisms regulating pulmonary injury after intestinal I/R are not fully elucidated but probably involve complement activation and cytokine/chemokine generation. Further studies investigating the molecular mechanisms of pulmonary injury after gastrointestinal I/R in this model are warranted.

Based on the findings in this study, the terminal complement components appear to be a keystone to the inflammatory process after gastrointestinal I/R. But how can so many protective aspects be observed by inhibition of only a single plasma protein? We speculate that complement activation is an initial and early event that takes place after reperfusion at the level of the ischemic gastrointestinal vascular endothelium. Complement activation releases a number of proinflammatory and biologically active components, including the anaphylatoxins and C5b-9. Complement can mediate endothelial dysfunction, loss of endothelium-dependent relaxation, up-regulation of adherence molecules, and adherence of leukocytes to the endothelium and leukocyte activation. Activation and transmigration of PMNs across the vasculature and ultimately the epithelial cells may then allow translocation of bacteria to the cardiovascular system, leading to sepsis and increased proinflammatory gene expression. Because inhibition of C5a protects against the lethal effects of sepsis and endotoxemia is thought to play an important role in multiorgan failure to acute mesenteric ischemia, inhibition of the terminal complement components may be an important clinical therapeutic for disease management.

In conclusion, anti-C5 mAb therapy protects the rat intestinal and pulmonary tissues from acute intestinal I/R injury. Complement inhibition of the terminal complement component, C5, decreases the loss of BK-induced microvascular relaxation and up-regulation of the endothelial-leukocyte adhesion molecule, ICAM-1. I/R-induced expression of TNF-α and IL-1α was significantly inhibited by anti-rat C5 mAb. These data suggest that the protective actions of anti-C5 are multifaceted and that the terminal complement components are a primary inducer of tissue injury and inflammation after intestinal I/R. Anti-C5 mAb therapy may be useful for the clinical treatment of intestinal injury associated with bowel obstruction, necrotic enterocolitis, abdominal aortic aneurysm repair, and surgery for acute mesenteric ischemia.

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