BASIC RESEARCH STUDIES

From the Society for Vascular Surgery

Complement C5a receptor antagonist attenuates multiple organ injury in a model of ruptured abdominal aortic aneurysm

Denis W. Harkin, MD,[a,b] Alex Romaschin, PhD,[a] Stephen M. Taylor, PhD,[c] Barry B. Rubin, MD, PhD,[a] and **Thomas F. Lindsay, PhD,[a]** *Toronto, Ontario, Canada; Belfast, Ireland; and Queensland, Australia*

Objective: **Abdominal aortic aneurysm (AAA) rupture is associated with a systemic inflammatory response syndrome, characterized by increased microvascular permeability and neutrophil sequestration, leading to multiorgan dysfunction. We examined the role of a novel complement factor 5a (C5aR) receptor antagonist, the cyclic peptide AcF-(OpdChaWR), in attenuation of pathologic complement activation and tissue injury in a model of AAA rupture.**

Methods: **Anesthetized rats were randomized to sham (control) or shock and clamp (s+c) groups. Animals in the s+c group underwent 1 hour of hemorrhagic shock (mean arterial blood pressure ≤50 mm Hg), followed by 45 minutes of supramesenteric aortic clamping, then 2 hours of resuscitated reperfusion. Animals in the s+c group were randomized to receive an intravenous bolus of C5aR antagonist at 1 mg/kg or saline solution control at the end of hemorrhagic shock. Intestinal and pulmonary permeability to iodine 125–labeled albumin was measured as an indicator of microvascular permeability. Tissue myeloperoxidase activity, proinflammatory cytokine tissue necrosis factor-α (TNF-α) protein and mRNA, and C5aR mRNA levels were measured as indicators of neutrophil sequestration and inflammatory signaling, respectively.**

Results: **Lung permeability index was significantly increased in the s+c group compared with the sham group (4.43 ± 0.96 vs 1.30 ± 0.17; $P < .01$), and prevented with treatment with C5aR antagonist (1.74 ± 0.50; $P < .03$). Lung myeloperoxidase activity was significantly increased in the the s+c group compared with the sham group (2.41 ± 0.34 U/mg vs 1.03 ± 0.29 U/mg; $P < .009$), and significantly attenuated with treatment with C5aR antagonist (1.11 ± 0.09 U/mg; $P < .006$). Lung TNF-α protein levels were significantly elevated in both s+c groups, whereas lung TNF-α mRNA expression was significantly downregulated in both s+c groups compared with the sham group. Intestinal permeability index was significantly increased in animals in the s+c groups during reperfusion, compared with sham ($P < .001$), which was attenuated in early reperfusion with treatment with C5a receptor antagonist. Data represent mean ± SEM, group comparisons with analysis of variance and post hoc Scheffé test.**

Conclusions: **These results indicate that a potent antagonist of C5a receptor protects the rat intestine and lung from neutrophil-associated injury in a model of AAA rupture. These data suggest that complement-mediated inflammation can be modulated at the C5a receptor level, independent of proinflammatory TNF-α production, and prevent acute local and remote organ injury. (J Vasc Surg 2004;39:196-206.)**

Abdominal aortic aneurysm (AAA) rupture continues to confer a 40% to 75% in-hospital mortality rate, despite improvements in surgical techniques and perioperative care.[1] The combined effects of hemorrhagic shock and subsequent clamp-related lower torso ischemia are believed to represent a "two hit" ischemic injury,[2] which on reperfusion initiates a systemic inflammatory response syndrome, characterized by increased microvascular permeability and neutrophil sequestration, leading to multiple organ dysfunction syndrome.[3] Multiple organ dysfunction syndrome remains the primary cause of death in 70% of patients who survive the initial surgery and a contributory cause in the remainder.[4,5] Reperfusion of the ischemic lower torso after prolonged ischemia initiates a systemic inflammatory response syndrome, characterized by proinflammatory cytokine[3,6] and increased circulating polymorphonuclear (PMN) leukocyte activation.[7] Pulmonary sequestration of activated neutrophils is associated with development of acute pulmonary microvascular injury[8,9] and acute respiratory distress syndrome,[10] and high subsequent mortality.

Complement system activation occurs in ruptured AAA repair[7] and in experimental models of ischemia-reperfusion

From Division of Vascular Surgery, Department of Surgery, The Toronto Hospital (General Division), Faculty of Medicine, University of Toronto,[a] Regional Vascular Surgery Unit, The Royal Victoria Hospital, Belfast,[b] and Department of Physiology and Pharmacology, University of Queensland.[c]

Supported by the Physicians of Ontario through Physician's Services Incorporated Foundation Grant No. 98-049.

Recipient of the 2003 Lifeline Foundation Resident Research Prize award on behalf of The American Association for Vascular Surgery/The Society for Vascular Surgery.

Competition of interest: none.

Presented at the Fifty-sixth Annual Meeting of the Society for Vascular Surgery, Chicago, Ill, Jun 8-11, 2003.

Reprint requests: Denis W. Harkin, MD, MB, FRCSI, FRCS(Gen)Ed, Division of Vascular Surgery, The Toronto Hospital (General Division), Eaton Building 5-306, 200 Elizabeth St, Toronto, Ont, Canada M5G 2C4 (e-mail: D.W.Harkin@qub.ac.uk).

0741-5214/2004/$30.00 + 0

doi:10.1016/j.jvs.2003.07.001

injury to the lower limb[11] and intestine.[12] Activated products of the complement pathway, C3a and C5a, are potent inflammatory mediators with myriad effects, including alteration of blood vessel permeability and tone,[13] leukocyte chemotaxis,[14] and activation of multiple inflammatory cell types.[15] Support for the role of complement in inflammatory tissue injury is evidenced by the attenuation of such injury with anti-C5 antibody[16] and C5a receptor (C5aR) antagonist.[17] However, the role of complement in inflammatory tissue injury after ruptured AAA remains largely unknown.

The purpose of this study was to determine for the first time the effects of a novel small molecule C5aR antagonist, AcF-(OpdChaWR) (Phe[Orn-Pro-D-cyclohexylalanine-Trp-Arg]), in a model of ruptured AAA. We hypothesized that complement-mediated inflammation could be attenuated at the C5a receptor level, reducing local intestinal and remote acute lung injury.

MATERIAL AND METHODS

Animal care guidelines. All animals used in this study were maintained in an accredited facility and cared for in accordance with the recommendations of the Canadian Council on Animal Care, the requirements of the Animals for Research Act of the Province of Ontario, and the regulations of the Animal Care Committee, The Toronto Hospital, and the Guide for the Care and Use of Laboratory Animals [DHSS Publication No. (NIH)86-23, revised 1985].

Experimental design. Male Sprague-Dawley rats (350-500 g) were used throughout the experiment. All animals were anesthetized with pentobarbital sodium (50 mg/kg intraperitoneally). A tail vein and the right carotid artery were cannulated with 22-gauge angiocatheters for intravenous access and continuous monitoring of mean arterial pressure (MAP), respectively.

Animals were randomized into two groups, sham (n = 6) and shock + clamp (s+c; n = 19). Animals in the s+c group were further randomized into C5aR antagonist–treated (n = 9) and control (n = 10) groups. In the treated group, small-molecule C5aR anatgonist, AcF-(OpdChaWR) (Promics Pty Ltd, Queensland, Australia) was administered intravenously over 2 minutes at the end of hemorrhagic shock at a dose 2 mg/kg; the control group received saline solution infusion. In all cases the operator was blinded to the treatment given.

After a midline laparotomy, the abdominal aorta was isolated at the superior mesenteric artery and just proximal to the iliac bifurcation. Intestinal permeability was used as an index of intestinal injury, and was measured as described. In short, a 5-cm segment of jejunum was isolated and perfused continuously with percutaneous inflow and outflow cannulas with 37°C Ringer lactate solution at a rate of 0.3 mL/min with an infusion pump (model AVI 480; 3M, St Paul, Minn). For determination of intestinal and pulmonary permeability, animals then received iodine 125–labeled albumin (~1 μCi) via the tail vein catheter, and were allowed to stabilize for 30 minutes to establish postoperative equilibrium. Intestinal perfusate was collected every 10 minutes throughout the experiment, and samples of blood (0.3 mL) were withdrawn at 1-hour intervals to measure total albumin concentration and the specific activity of ^{125}I-albumin for calculation of intestinal albumin loss.

In appropriate groups, shock was induced with blood withdrawal from the carotid artery cannula into a plastic heparinized syringe (500 U), which was maintained at room temperature on a tube rocker, to reduce and maintain MAP at 50 mm Hg for 1 hour. After 60 minutes of shock or an equivalent control period, clamps were applied to the abdominal aorta just proximal to the superior mesenteric artery and at the iliac bifurcation. At this time, half of the shed blood was infused into the tail vein. The clamps remained in place for 45 minutes. Just before clamp removal the remainder of the shed blood was reinfused. Additional Ringer lactate solution was also administered, as required, to resuscitate and maintain MAP at 100 mm Hg. Reperfusion was continued for 120 minutes, at which time the animals were killed with an overdose of pentobarbital sodium. Tissue samples were excised, washed in ice-cold saline solution, and rapidly frozen in liquid nitrogen and stored at −70°C until analyzed for reverse transcriptase polymerase chain reaction (RT-PCR), myeloperoxidase (MPO), and cytokine levels.

Determination of pulmonary permeability. The left lung was lavaged three times with 3.5 mL of Ringer lactate solution, and the effluent bronchoalveolar lavage (BAL) was collected. Blood and BAL fluid were weighed and counted for ^{125}I activity, and lung permeability index (LPI) was calculated with the formula LPI = BAL-^{125}I(cpm/g)/blood-^{125}I (cpm/g).

Determination of intestinal permeability. To calculate intraluminal intestinal albumin loss, all 10-minute effluent collections from the intestinal perfusion were weighed, and a 1-mL sample of each was assayed for ^{125}I-albumin activity with a gamma counter. Each blood sample drawn during the experimental procedure was centrifuged at 100,000 rpm, and 100 μL of plasma was removed for determination of albumin content and ^{125}I-albumin activity. The level of ^{125}I in the blood samples was regressed against time, and the slope of the curve was used to determine the activity of this isotope in whole blood. This was used to determine the specific activity of ^{125}I per microgram of total albumin to calculate intestinal albumin loss in milligrams per gram dry weight of the perfused intestinal segment.

Measurement of circulating complement activity. To assess complement activation, total serum hemolytic activity was measured with the total hemolytic complement (CH_{50}) technique, as described.[11] Total hemolytic complement activity (CH_{50}) was determined in serum with Compquik CH_{50} (Sigma, St Louis, Mo) with antibody-coated sheep erythrocytes and compared with a reference standard. Frozen serum samples (−80°C) were assayed as a single batch; intra-assay variability was less than 5%.

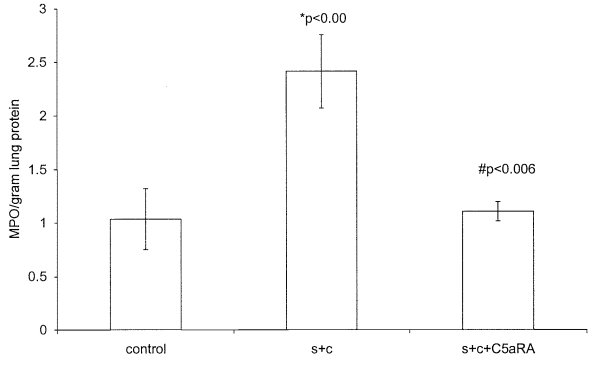

Fig 1. Lung permeability index to iodine 125–labeled albumin was significantly increased in the shock and clamp group *(s+c)* compared with the sham group (*$P < .01$), and was significantly prevented with treatment with C5a receptor antagonist (*C5aRA*; #$P < .03$). *MPO,* Myeloperoxidase.

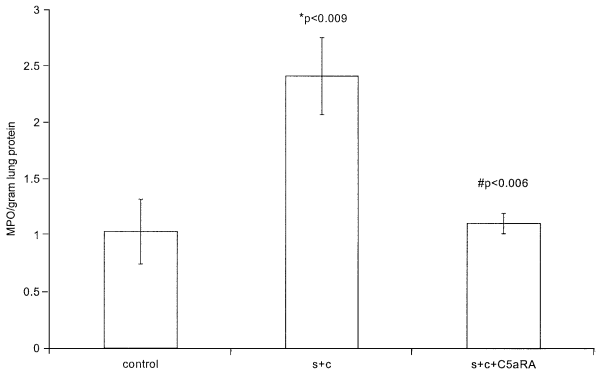

Fig 2. Lung tissue myeloperoxidase *(MPO)* activity was significantly increased in the shock and clamp group *(s+c)* compared with the sham group (*$P < .009$), and was significantly attenuated with treatment with C5a receptor antagonist (*C5aRA*; #$P < .006$).

Determination of MPO activity. Tissues were assayed for MPO, an index of neutrophil sequestration, as described.[18] In short, MPO activity was assessed at 37°C by monitoring the change in absorbance at 655 nm over 3 minutes in a Cobas FARA II centrifugal analyzer (Roche Diagnostic Systems, Montclair, NJ). The reaction mixture contained 16 mmol/L of 3,3′,5,5′-tetramethylbenzidine dissolved in *N,N*-dimethylformamide in 0.22 mol/L of phosphate-buffered saline solution containing 0.11 mol/L of sodium chloride (NaCl) at pH 5.4. The reaction was initiated with addition of 3 mmol/L of hydrogen peroxide. One unit of activity was defined as a one-unit change in absorbance per minute at 37°C. Protein content of pulmonary and intestinal samples was determined with the bicinchoninic acid protein assay system (Pierce, Rockford, Ill). MPO activity was expressed as units per milligram of protein.

Measurement of TNF-α in lung. One hundred milligrams of tissue was homogenized in 1 mL of phosphate-buffered saline solution (0.4 mol/L of NaCl and 10 mmol/L of $NaPO_4$) containing antiproteases (0.1 mmol/L of phenylmethyl sulfonyl fluoride, 0.1 mmol/L of benzethonium chloride, 10 mmol/L of ethylenediaminetetraacetic acid, and 20 KI [potassium chloride] of aprotinin A) and 0.05% Tween 20. The samples were then centrifuged for 10 minutes at 3000*g*, and the supernatant was immediately used for commercial enzyme-linked immunosorbent assay at a 1:2 dilution in assay dilution buffer, according to the manufacturer's instructions (R&D Systems, Minneapolis, Minn). Protein contents of samples were determined with the bicinchoninic acid protein assay system (Pierce). Cytokine concentrations were expressed as picograms per milligram of protein.

RNA isolation and semiquantitative RT-PCR. Lung tissue from rats was obtained at the end of the experiment and prepared as described above. Total RNA was isolated with the TRTzol method (Life Technologies, Rockville, Md) according to the manufacturer's instructions. Digestion of any contaminating DNA was achieved with treatment of samples with RQ1 Rnase-free Dnase (Promega, Madison, Wis).

Semiquantitative RT-PCR was performed with 1 μg of RNA with Superscript II RNase H Reverse Transcriptase (Life Technologies, Grand Island, NY) according to the manufacturer's instructions and as described.[19] The primers for the "housekeeping" gene GAPDH were 5′ primer 5′-GCC TCG TCT CAT AGA CAA GAT G-3′ and 3′ primer 5′-CAG TAG ACT CCA CGA CAT AC-3 ′. PCR was performed with primers for C5aR—5′ primer 5′-TAT AGT CCT GCC CTC GCT CAT-3′ and 3′ primer 5′-TCA CCA CTT TGA GCG TCT TGG-3′—and TNF-α—5′ primer 5′-GGC AGG TCT ACT TTG GAG TCA TTG C-3′ and 3′ primer 5′ ACA TTC GAG GCT CCA GTG AAT TCG G-3′. For each primer couple the following PCR conditions were used: C5aR, after a "hot start" for 5 minutes at 94°C, 30 cycles were used for amplification, with a melting temperature of 94°C and an annealing temperature of 60°C, and an extending temperature of 72°C, each for 1 minute, followed by a final extension at 72°C for 8 minutes; TNF-α, after a "hot start" for 3 minutes at 94°C, 32 cycles were used for amplification, with a melting temperature of 94°C and an annealing temperature of 60°C, and an extending temperature of 72°C, each for 1 minute, followed by a final extension at 72°C for 10 minutes. The RT-PCR product was confirmed with electrophoresis of samples in a 1.2% agarose gel. Experiments were conducted in which total RNA from sample was amplified with different cycle numbers for GAPDH and C5aR primers to ensure that RNA bands after 30 cycles were detected within the linear part of the amplifying curves. To rule out contaminating DNA as responsible for results, controls for the samples were performed in which RT-PCR was performed similarly, except for absence of reverse transcriptase. These controls showed no detectable bands for C5aR mRNA or TNF-α, respectively (data not shown). The amount of amplified product was esti-

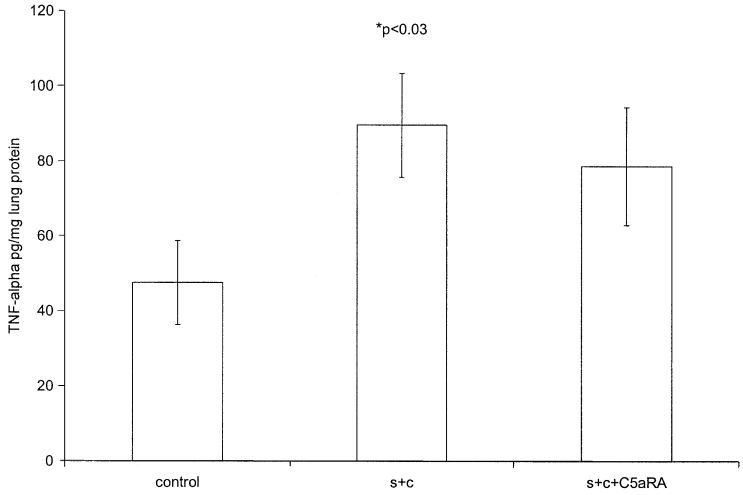

Fig 3. Lung tissue necrosis factor-α *(TNF-α)* levels were significantly elevated in the shock and clamp group *(s+c)* compared with the sham group (*$P < .03$), but were not prevented with treatment with C5a receptor antagonist (*C5aRA*; P = NS).

mated with densitometry of ethidium bromide–stained 1.2% agarose gels with a CCD camera and Imagemaster VDS software (Pharmacia, Uppsala, Sweden). Results are presented semiquantitatively, referring to equal loading of the relative amount of transcribed mRNA.

Statistical analysis. Summary values are presented as mean ± SEM, and differences between group mean were considered significant at $P < .05$. End point data were analyzed with one-way ANOVA, and with the post hoc Scheffé test for individual between-group comparison if significance was determined with ANOVA. Repeated measures, such as blood pressure and intestinal permeability index (IPI), were analyzed with repeated measures two-way ANOVA, with group and time as independent variables.

RESULTS

Pulmonary permeability. LPI to ^{125}I-labeled albumin was significantly increased in the s+c group compared with the sham group (4.43 ± 0.96 vs 1.30 ± 0.17; $P < .01$), and was significantly prevented with treatment with C5a receptor antagonist (1.74 ± 0.50; $P < .03$; Fig 1).

Pulmonary MPO activity. Lung tissue MPO activity was significantly increased in the s+c group compared with the sham group (2.41 ± 0.34 U/mg vs 1.03 ± 0.29 U/mg; $P < .009$), and significantly attenuated with treatment with C5a receptor antagonist (1.11 ± 0.09 U/mg; $P < .006$; Fig 2).

Pulmonary TNF-α concentration. Lung TNF-α levels were significantly elevated in the s+c group compared with the sham group (89.70 ± 13.83 pg/mg protein vs 47.57 ± 11.22 pg/mg protein; $P < .03$), but was not prevented with treatment with C5a receptor antagonist (78.71 ± 15.78 pg/mg protein; P = NS; Fig 3).

Intestinal permeability. The rate of intraluminal intestinal albumin loss, IPI, remained stable throughout the entire experimental period in sham animals. In s+c animals IPI remained stable during stabilization, hemorrhage, and clamp periods; however, at reperfusion there was a statistically significant increase in IPI (Fig 4). After 30 minutes of reperfusion IPI was significantly increased in s+c animals, compared with pre-shock levels ($8.05 \times 10^{-2} \pm 3.59 \times 10^{-2}$ vs $0.72 \times 10^{-2} \pm 0.51 \times 10^{-2}$; $P < .0001$) and control levels ($8.05 \times 10^{-2} \pm 3.59 \times 10^{-2}$ vs $1.75 \times 10^{-2} \pm 0.33 \times 10^{-2}$; $P < .0001$), and remained at similar levels throughout the 120-minute reperfusion. Treatment with C5a receptor antagonist significantly reduced the increased IPI in early reperfusion; after 30 minutes of reperfusion IPI was significantly reduced in C5aR antagonist-treated animals compared with untreated s+c animals ($2.82 \times 10^{-2} \pm 0.91 \times 10^{-2}$ vs $8.05 \times 10^{-2} \pm 3.59 \times 10^{-2}$; $P < .01$). However, as reperfusion progressed IPI increased, even in the treated group, to mirror untreated s+c levels (Fig 4).

Intestinal MPO activity. Intestinal tissue MPO activity was not significantly increased in the s+c group compared with the sham group (3.93 ± 0.66 U/mg vs 3.34 ± 0.53 U/mg; P = NS). Of interest, intestinal MPO activity was significantly reduced in C5a receptor antagonist–treated animals compared with untreated s+c animals (1.86 ± 0.26 U/mg vs 3.93 ± 0.66 U/mg; $P < .01$) and sham animals (3.34 ± 0.53 U/mg; $P < .017$; Fig 5).

Intestinal TNF-α concentration. Intestinal TNF-α levels were significantly elevated in s+c animals compared with sham animals (73.02 ± 10.12 pg/mg protein vs 45.42 ± 6.23 pg/mg protein; $P < .038$), but not prevented with treatment with C5a receptor antagonist (72.00 ± 13.95 pg/mg protein; P = NS; Fig 6).

Blood pressure and resuscitation requirements. MAP remained stable throughout the entire experimental period in sham animals (Fig 7, *A*), requiring minimal intravenous fluid resuscitation with Ringer lactate solution

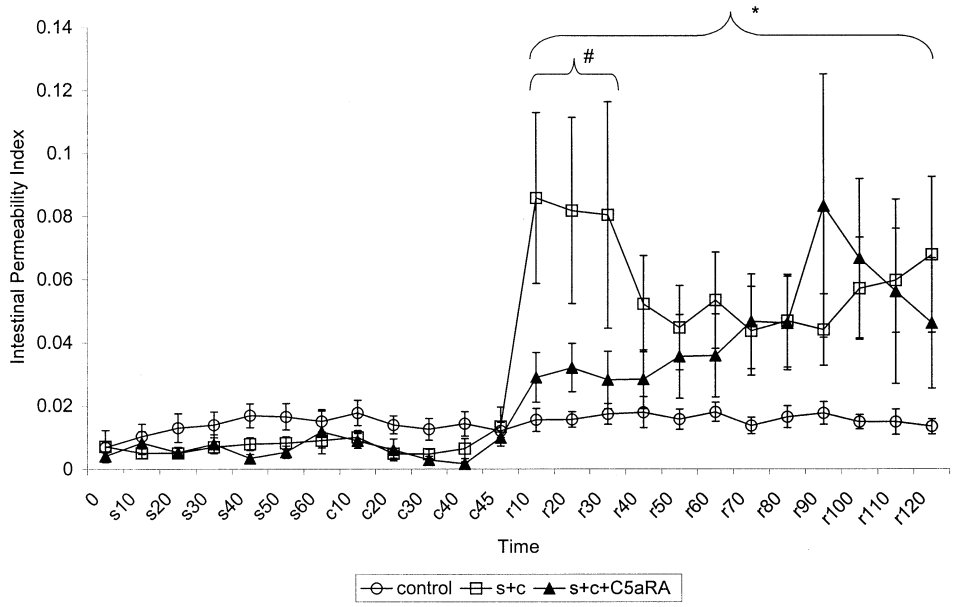

Fig 4. Rate of intraluminal intestinal albumin loss, intestinal permeability index (IPI), remained stable throughout the entire experimental period in sham animals. In shock and clamp animals *(s+c)*, IPI remained stable during stabilization, hemorrhage, and clamp periods. During reperfusion IPI was significantly increased in s+c animals, compared with pre-shock levels (*$P < .0001$) and with control levels (*$P < .0001$), and remained at similar levels throughout the 120-minute reperfusion. Treatment with C5a receptor antagonist *(C5aRA)* significantly reduced the increased IPI in the first 60 minutes of (early) reperfusion, compared with untreated s+c animals (#$P < .01$). However, as reperfusion progressed IPI increased, even in the treated group, to mirror untreated s+c levels. *s,* Shock; *c,* clamping; *r,* reperfusion.

(Fig 7, *B*). In s+c animals MAP was reduced during hemorrhagic shock to 50 mm Hg or less for 1 hour, as defined by the protocol; with application of a supramesenteric aortic clamp, MAP increased significantly compared with pre-shock levels (158 ± 9.0 mm Hg vs 117 ± 3.0 mm Hg; $P < .001$). In s+c animals after removal of the aortic clamp MAP decreased progressively during reperfusion to a nadir after 120 minutes of reperfusion (68 ± 6.0 vs pre-shock 117 ± 3.0; $P < .001$), despite vigorous fluid resuscitation with intravenous infusion of Ringer lactate solution (69.3 ± 8.5 mL). Animals treated with C5a receptor antagonist maintained significantly better MAP during reperfusion compared with untreated s+c animals (95 ± 5.3 mm Hg vs 68 ± 6.0 mm Hg; $P < .01$; Fig 7, *A*), and required less intravenous fluid resuscitation (60.0 ± 7.0 mL vs 69.3 ± 8.5 mL; $P < .1$, NS; Fig 7, *B*).

Complement activation (CH_{50}) in model of ruptured AAA. Compared with control animals, CH_{50} levels dropped significantly in our model of ruptured AAA (s+c), and this was not altered with treatment with C5aR antagonist (Fig 8).

Expression of C5aR and TNF-α in lung tissue. Expression of C5aR mRNA in lung tissue was significantly reduced in our model of ruptured AAA (s+c) compared with sham animals (0.44 ± 0.04 vs 2.03 ± 0.63; $P < .012$). This was only partially prevented with treatment with C5aR antagonist (Fig 9, *A, B*).

Expression of TNF-α mRNA in lung tissue was significantly reduced in our model of ruptured AAA (s+c) compared with sham animals (0.57 ± 0.22 vs 0.08 ± 0.04; $P < .019$). This was unaltered with treatment with C5aR antagonist (Fig 10, *A, B*).

DISCUSSION

Ruptured AAA initiates a systemic inflammatory response syndrome, characterized by increased microvascular permeability and activated neutrophil sequestration,[3] leading to multiple organ dysfunction syndrome,[6] which is a major factor in most of the 35% to 70% in-hospital mortality rate reported by most specialist units.[1] The development of a variety of novel anti-complement compounds has renewed interest in complement as a therapeutic target in the critically ill. In this ruptured AAA model, hemorrhagic shock for 1 hour at MAP 50 mm Hg, followed by 45 minutes of supramesenteric aortic clamping and 120 minutes of reperfusion, resulted in significant intestinal and pulmonary injury, and refractive shock, despite vigorous fluid resuscitation. Significant reduction in total serum hemolytic activity (CH_{50}) suggests a major role for complement activation in the early inflammatory response in this model of ruptured AAA. The CH_{50} assay measures both the classic activation and the terminal complement components, and is frequently used in rat models as an indicator of total complement activity in blood.[20] Complement activation occurs in the early stages of inflammation, releasing activated complement components C3a, C4a, C5a, potent anaphylatoxins that alter vascular tone and permeability. They also chemotactically recruit and activate

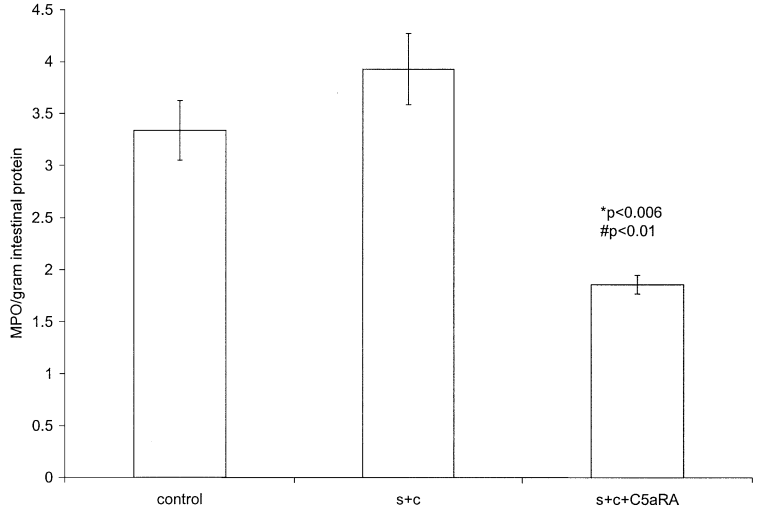

Fig 5. Intestinal tissue myeloperoxidase *(MPO)* activity was not significantly increased in the shock and clamp group *(s+c)* compared with the sham group (P = NS). Of interest, intestinal MPO activity was significantly reduced in animals treated with C5a receptor antagonist *(C5aRA)* compared with untreated s+c animals ($*P < .01$) and the sham group ($\#P < .017$).

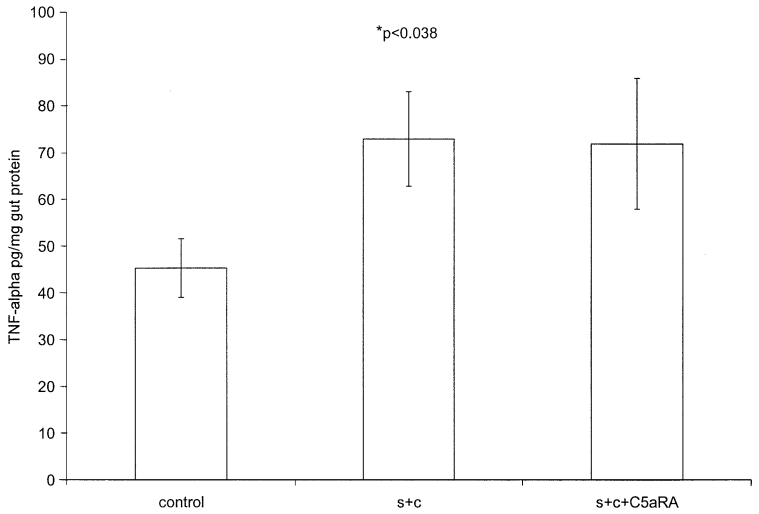

Fig 6. Intestinal tissue necrosis factor-α *(TNF-α)* levels were significantly elevated in the shock and clamp group *(s+c)* compared with the sham group ($*P < .038$), but were not prevented with treatment with C5a receptor antagonist (*C5aRA;* P = NS).

inflammatory cells, in particular C5a, and lead to release of cytokines, such as TNF-α. Subsequent formation of the membrane attack complex C5b-C9 is directly lytic to cells. In this study we have demonstrated for the first time that the local and systemic injury associated with ruptured AAA can be attenuated with a novel complement component C5a receptor antagonist, the cyclic peptide AcF-(OpdChaWR), in a rat model.

The additive effects of hemorrhagic shock and lower torso ischemia-reperfusion injury comprise the major etiopathologic basis of organ injury in ruptured AAA.[2] Our previously reported rat model of ruptured AAA reproduces the sequential and synergistic harmful effects of hemorrhagic shock followed by aortic clamp–induced lower torso ischemia-reperfusion injury.[18] Although infrarenal aortic clamping may often be sufficient to gain control of ruptured AAA, suprarenal or even supraceliac clamping is not uncommon, and is essential in unstable patients and those with juxtarenal aneurysms. In our model a direct clamp-related ischemic injury involved the renal and intestinal circulation, and although this is not the most common clinical scenario, it is associated with a severe inflammatory

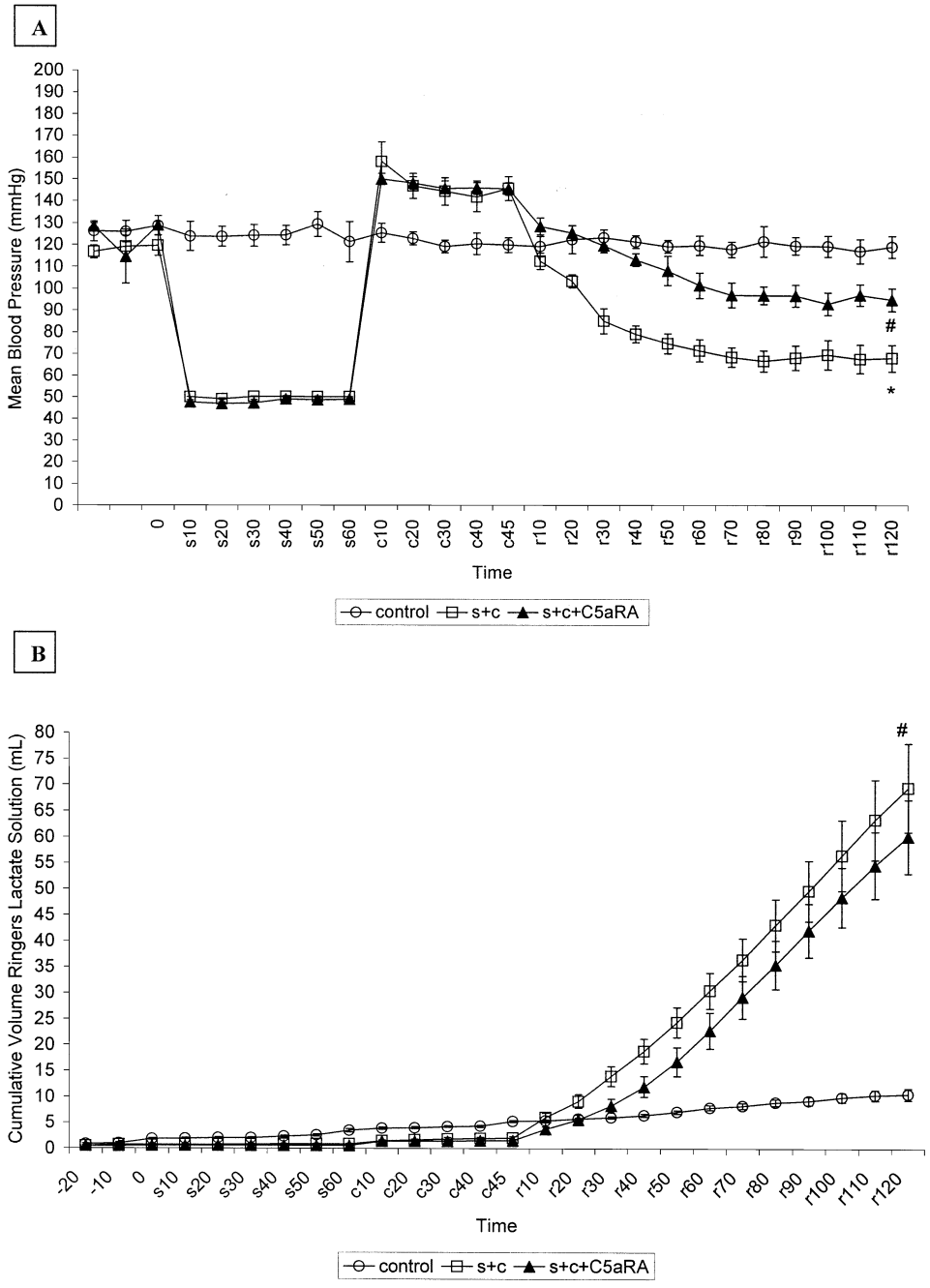

Fig 7. Mean arterial blood pressure (MAP) remained stable throughout the entire experimental period in sham animals **(A)**, requiring minimal intravenous fluid resuscitation with Ringer lactate solution **(B)**. In shock and clamp animals *(s+c)*, MAP was reduced during hemorrhagic shock to 50 mm Hg or less for 1 hour, as defined by the protocol. On application of the supramesenteric aortic clamp, MAP increased significantly compared with pre-shock levels ($P <$ .001). In s+c animals after removal of the aortic clamp, MAP decreased progressively during reperfusion to a nadir after 120 minutes of reperfusion (#$P <$ .001), despite vigorous fluid resuscitation with intravenous infusion of Ringer lactate solution (69.3 ± 8.5 mL). Animals treated with C5a receptor antagonist *(C5aRA)* maintained significantly better MAP during reperfusion compared with untreated s+c animals (#$P <$ .01; **A**), and required less intravenous fluid resuscitation ($P <$ .1, NS; **B**). *s,* Shock; *c,* clamping; *r,* reperfusion.

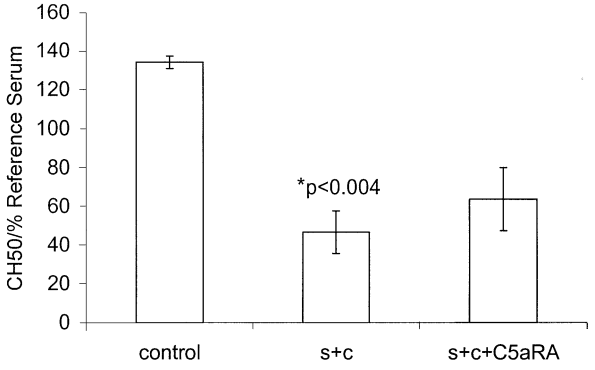

Fig 8. Total serum hemolytic activity *(CH_{50})* measured at the end of the experimental period is expressed as a percentage of reference serum value. Significant reduction in serum hemolytic activity is noted in the shock and clamp group *(s+c)*, and this was not significantly attenuated with treatment with C5a receptor antagonist (*C5aRA*; $P < .004$, analysis of variance).

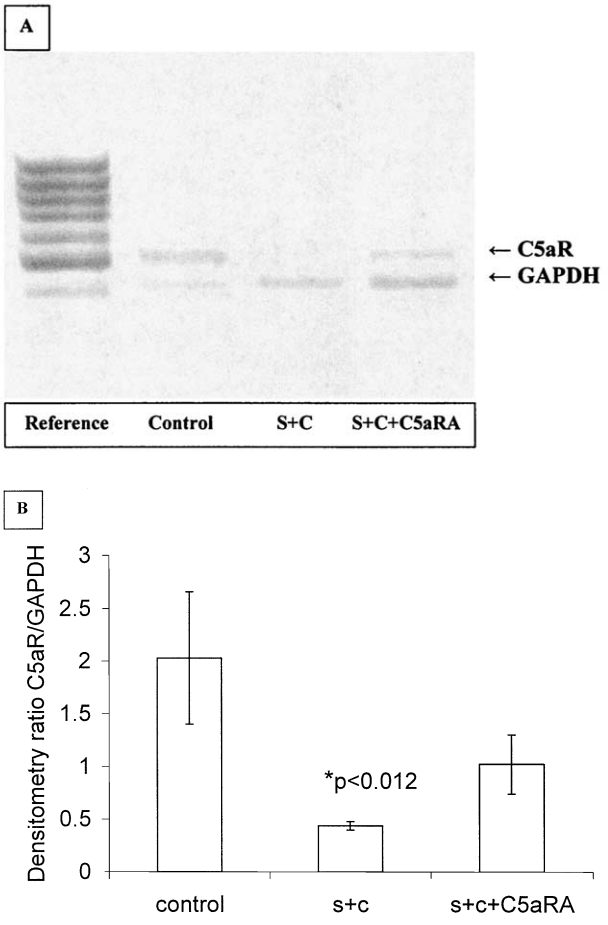

Fig 9. A, Expression of C5a receptor antagonist *(C5aRA)* in lung tissue is downregulated from control levels by shock and clamp injury *(s+c)*, and this is only partially attenuated in the C5aRA-treated group. **B,** Densitometry analysis demonstrates significant downregulation of C5aRA in the s+c group, which is prevented in the C5aRA-treated group ($P < .012$, analysis of variance).

response, making our successful modulation of injury with C5aR antagonism even more impressive. Intestinal injury in this model was associated with a significant increase in intestinal capillary permeability to ^{125}I-albumin immediately after release of the supramesenteric aortic clamp, an increase that persisted throughout the reperfusion period. Treatment with the C5aR antagonist significantly prevented increased intestinal permeability in early reperfusion; however, in late reperfusion intestinal permeability increased to that in nontreated s+c animals. Increased intestinal permeability has been reported after ruptured AAA and elective abdominal[21] and thoracoabdominal[22] aneurysm repair in human beings, and is associated with increased morbidity and mortality. Injury to the intestine is twofold in that the initial global hypoxia during hemorrhagic shock is compounded by the direct ischemia-reperfusion injury during and after release of the supramesenteric aortic clamp. Intestinal ischemia-reperfusion injury is associated with neutrophil sequestration and increased microvascular permeability, and can be modulated with neutrophil depletion or antibodies directed against neutrophil adhesion molecules.[23] Activated complement components alter vascular tone and permeability, chemotactically attract and activate neutrophils, and are integral to intestinal reperfusion injury.[12] By inhibiting early interaction between the activated complement component C5a and its target cells on the intestinal vascular endothelium, and circulating immune cells, we have reduced the severity of the initial gut injury in this model. Furthermore, limb ischemia-reperfusion injury, when associated with systemic inflammation, induces intestinal injury associated with translocation of bacterial fragments across a damaged intestinal capillary barrier,[24] with the resultant endotoxemia[25] producing an exaggerated inflammatory response. This has led many to suggest that the gut drives the inflammatory response to a variety of critical illnesses.[26,27] Complement is important in neutrophil activation in response to endotoxin[28]; therefore complement receptor blockade could decrease the proinflammatory effects of endotoxin in early reperfusion. However, as reperfusion continues intestinal injury increases, most likely as a result of the cellular effects of ischemia-reperfusion injury and parallel activation of cytokine and proinflammatory mediator cascades. Our findings are in agreement with those of previous studies, which show that direct intestinal ischemia-reperfusion injury can be attenuated by blocking the complement cascade at various points.[17,29] The reduction in intestinal MPO concentration compared in both untreated s+c groups is interesting, and may suggest that local complement-induced neutrophil chemotaxis and activation are crucial to neutrophil sequestration and intestinal injury in this model. Complement activation in vitro stimulates rapid adhesion of neutrophils to endothelial target cells.[30] In this study s+c animals had significantly increased intestinal levels of proinflammatory cytokine TNF-α compared with sham animals,

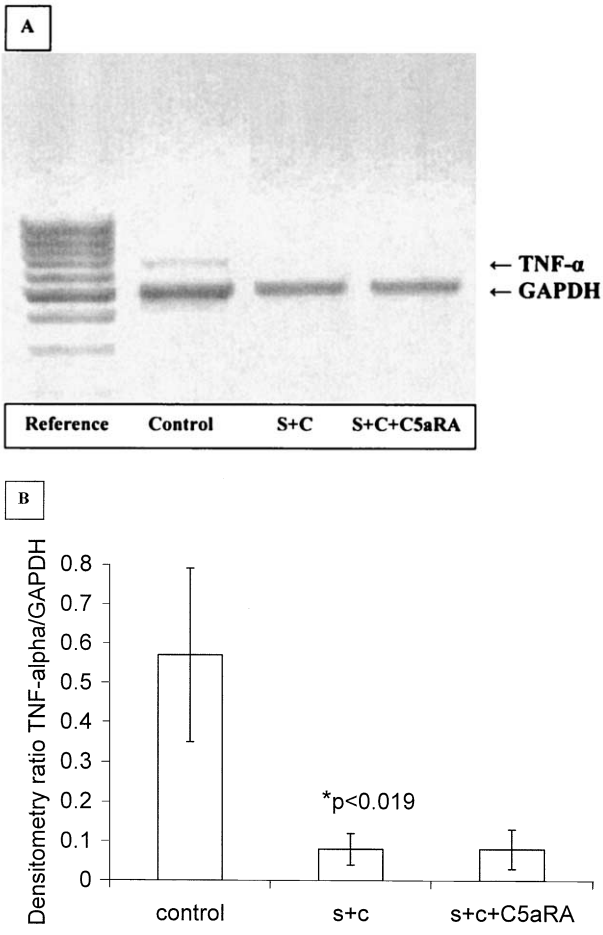

Fig 10. A, Expression of tissue necrosis factor-α *(TNF-α)* in lung tissue is downregulated from control levels by shock and clamp injury *(s+c)*, and this is only partially attenuated in the group treated with C5a receptor antagonist *(C5aRA)*. **B,** Densitometry analysis demonstrates significant downregulation of TNF-α in the s+c group, which is not altered with treatment with C5aRA ($P <$ .019, analysis of variance).

and this was not altered with treatment with C5aR antagonist. Although activated complement causes release of TNF-α from a variety of cell types, including immune cells, by a receptor-mediated effect, a variety of other mediators created after ischemia-reperfusion injury, such as arachidonic acid metabolites, also stimulate cytokine release.

Ruptured AAA is associated with noncardiac acute interstitial pulmonary edema and associated hypoxemia, analogous to acute respiratory distress syndrome, which has a grave prognosis. In this model of ruptured AAA the s+c group had significantly increased pulmonary permeability to ^{125}I-albumin and lung MPO levels. Treatment with C5aR antagonist significantly prevented increased pulmonary permeability and neutrophil sequestration. The mechanism whereby lung injury in this model is primarily due to neutrophil adherence, sequestration, and subsequent respiratory burst-induced oxidative injury is supported by the observation that lung injury can be attenuated with anti-CD18 monoclonal antibodies.[18] In this model, antagonism of the C5a receptor on target cells reduces neutrophil sequestration and subsequent microvascular hyperpermeability. In this model the combination of the systemic hemorrhagic shock injury, compounded by lower torso ischemia-reperfusion, produces a severe acute lung injury. Abrogation of C5a-induced neutrophil chemotaxis and activation in the pulmonary circulation may in part explain the attenuation of injury in this study. Significant downregulation of C5aR mRNA expression in the lung is noted after s+c injury in this model, which is attenuated in the C5aR antagonist–treated group. C5aR activation is associated with downregulation of C5aR expression, and we have shown this. However, it is not possible to define the exact mechanism by which this downregulation occurs from these data. C5aR activation has been reported to cause upregulation of β_2-integrin on neutrophils[30] and endothelial P-selectin and intercellular adhesion molecule–1,[31] and thus would promote neutrophil adherence and transmigration into the lung parenchyma, where it would induce oxidative injury. Therefore prevention of C5aR activation may explain the diminution of lung injury in our model. C5a receptor blockade does not affect formation of the cytolytic membrane attack complex C5b-C9, which has been implicated in acute lung injury[32]; however, its effects may be more important in the lower torso and intestine, where direct ischemic injury occurred, than in the remotely injured lung. In this acute model modulation of this early complement-dependent injury may be sufficient to convert lethal acute lung failure to recoverable acute lung dysfunction. As with the intestinal injury, TNF-α levels in the lung significantly increase after s+c, and this is not affected with treatment with C5aR antagonist. TNF-α is produced after a variety of stresses, and induces direct lung injury.[33] Conversely, expression of TNF-α mRNA is reduced significantly in both injured groups of animals. Taken together, inasmuch as both C5aR and TNF-α mRNA expression are reduced, there may be a common signal involved. Alternatively, reduced expression at this early stage of reperfusion may reflect the severity of hypoxic cellular injury in this model. The phenomenon of "cell stunning" suggests that an injured cell may have reduced nonessential metabolic processes, and therefore nonessential transcription or translation processes are reduced.[34]

Throughout the experimental procedure sham animals maintained stable blood pressure with minimal requirement for fluid resuscitation. Animals in the s+c group required significant fluid resuscitation from the start of the reperfusion period, after aortic clamp release, to maintain MAP. After initial response to fluid resuscitation in the first hour of reperfusion, shock refractory to fluid resuscitation developed in the second hour of reperfusion, requiring large volumes of intravenous fluid to maintain blood pressure. Treatment with C5aR antagonist significantly prevented the severe hypotension observed in the untreated group, and required less fluid resuscitation. Aortic clamp

release is associated with a variety of vasoactive effects, including hypovolemia from peripheral vasodilation and increased vascular permeability, reperfusion of ischemic tissues with circulation of vasoactive mediators and metabolites, and myocardial depressant factors.[35] Hemorrhagic shock itself initiates a cascade of proinflammatory mediator induction,[36] and oxidative injury is associated with the degree of complement activation.[37] Reducing microvascular permeability and immune cell activation via complement receptor–specific pathways reduces the degree and duration of hypotension in this model. This is probably a reflection of the reduced third space fluid loss from prevention of complement and complement-dependent increases in microvascular permeability. Complement activation also affects vascular tone and histamine release, prevention of which may also help maintain vascular resistance.[38] Reduced organ injury, and perhaps reduced myocardial depression, may also allow the animal to better handle the fluid load required to maintain target blood pressure.

In conclusion, we have shown for the first time that a novel C5a receptor antagonist can reduce local intestinal and remote lung injury in a model of ruptured AAA. This treatment appears to mediate its effects by reducing the inflammatory stimulus to tissue neutrophil sequestration, by reducing activated complement–immune cell interaction. Antagonism of the human C5a receptor may represent a realistic therapeutic target in a group of patients with ruptured AAA, which currently is associated with high mortality.

We thank Dr Joan Ivanov, PhD, Medical Statistician, University of Toronto, and Mr Sherwin Nicholson, BSc, Division of Vascular Surgery, Toronto General Hospital, for expert analysis of samples; and Uniquest Limited, Queensland, Australia, and Dr S. M. Taylor, Department of Physiology and Pharmacology, University of Queensland, Australia, for kind provision of the test agent.

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Submitted Apr 18, 2003; accepted Jul 21, 2003.