C5-blocking antibody reduces fluid requirements and improves responsiveness to fluid infusion in hemorrhagic shock managed with hypotensive resuscitation

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Peckham RM, Handrigan MT, Bentley TB, Falabella MJ, Chrovian AD, Stahl GL, Tsokos GC. C5-blocking antibody reduces fluid requirements and improves responsiveness to fluid infusion in hemorrhagic shock managed with hypotensive resuscitation. J Appl Physiol 102: 673–680, 2007. First published October 26, 2006; doi:10.1152/japplphysiol.00917.2006.—Hypotensive resuscitation strategy aimed at restoring normal hemodynamic parameters. Aggressive resuscitation with the goal of restoring normal blood pressure may be inappropriate or unfeasible in some situations, however (2, 3, 24, 30). Furthermore, logistical constraints and delayed medical evacuation may limit the supply of resuscitation fluid available in military or remote environments. Given these limitations, we and others have focused on strategies involving permissive hypotension (7, 11, 26). Under such strategies, small fluid volumes are administered as needed with a goal of maintaining central organ perfusion while minimizing additional hemorrhage. Current PreHospital Trauma Life Support guidelines for tactical combat casualty care embrace application of a hypotensive resuscitation strategy pending definitive hemorrhage control (25a).

Numerous lines of evidence implicate complement in the pathogenesis of ischemia-reperfusion injury and hemorrhagic shock. Human and animal studies suggest that complement activation occurs in trauma and hemorrhage and may correlate positively with outcome (15, 31). Previous studies have demonstrated beneficial effects of complement depletion or inhibition in animal models of hemorrhage (8, 13, 14, 17, 21, 28, 37). A recent study using C5a receptor antagonist demonstrated decreased lung and intestinal permeability in a model of hemorrhage and mesenteric ischemia-reperfusion (14). In another study of hemorrhage and mesenteric ischemia-reperfusion, administration of C5-blocking antibody decreased resuscitation fluid requirements (13). No studies have addressed the potential role for complement inhibition in a model of hypotensive resuscitation, however.

Based on the aforementioned data, we hypothesized that complement inhibition would decrease resuscitation fluid requirements and improve hemodynamic responsiveness in hemorrhagic shock managed with a hypotensive resuscitation strategy. We chose these two end points because of their direct relevance to the care of trauma patients and combat casualties. We studied clone 18A (anti-C5), a monoclonal antibody that blocks cleavage of rat C5. We chose clone 18A because a related anti-human C5 variant, pexelizumab (h5G1.1-SC), has demonstrated safety and mortality benefit in phase III clinical trials of patients undergoing coronary artery bypass grafting plus aortic valve replacement (4, 34). Pexelizumab has been shown to inhibit complement-mediated hemolysis for ~12 h following a single dose, a time frame relevant to trauma medicine (36). We studied both lactated Ringer’s (LR) and Hextend (Hex) because these fluids are used to treat trauma patients. LR is perhaps the most widely used resuscitation fluid in trauma settings, and Hex has been recommended for use in tactical combat casualty care (25a). We report that treatment with anti-C5 leads to a significant reduction in total resuscitation fluid requirements and improved mean arterial pressure (MAP) response to fluid infusion in hemorrhagic shock managed with a hypotensive resuscitation strategy.

MATERIALS AND METHODS

This research was conducted in compliance with the Animal Welfare Act and other Federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals. All costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
procedures were reviewed and approved by the Institute’s Animal Care and Use Committee and were performed in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International. Before experimentation, the animals underwent at least 1 wk of environmental stabilization in the Walter Reed Army Institute of Research animal care facility, with access to food and water ad libitum.

Surgical preparation. Male Sprague-Dawley rats (Charles River Laboratories, Raleigh, NC) weighing 394.4 ± 4.6 g underwent surgical catheter placement (Renapulse; Braintree Scientific, Braintree, PA) under sterile conditions into the femoral artery and vein under isoflurane anesthesia (5% induction, 2% maintenance; Minrad, Buffalo, NY). Immediately after placement, the catheters were flushed with a total of 0.3 ml of 1% heparin solution to maintain catheter patency. No further heparin was administered during the experiment or observation period. The cannulas were tunneled subcutaneously, exteriorized, and secured at the posterior neck exit site via a flexible button cannula guide sutured to the skin. The catheters were then connected to the corresponding fluid reservoir and blood pressure monitor (BPA-400; Micro-Med, Louisville, KY) through a two-channel fluid swivel. This allowed the animal free movement after recovery from the surgical preparation and prevented the animal from biting or twisting the cannulas, thus enabling the experiments to be conducted in awake (unanesthetized) rats.

Experimental design. After surgical preparation, all animals were fully recovered from anesthesia for a minimum of 1 h. At the time of experimentation, each enrolled animal was noted to be awake, alert, and without evidence of discomfort. No further anesthesia was administered during the experimental protocol. The animals were enrolled in one of five experimental groups: sham hemorrhage (Sham; n = 2), LR (n = 7; Baxter, Deerfield, IL), LR plus anti-C5 (LR + anti-C5; n = 5), Hex (n = 8; 6% hydroxyethyl starch in a balanced salt solution, Abbott Laboratories, Abbott Park, IL); Hex plus anti-C5 (Hex + anti-C5; n = 8). Sham animals underwent identical anesthetic and surgical procedures but were not hemorrhaged or resuscitated. Blood samples (1 ml) from animals in the sham-hemorrhage group were obtained at baseline and again 265 min later. It should be noted that we used only l-isomeric LR. The hemorrhage model used in this study has been described and was previously shown to produce an approximate LD 50% at 24 h (11). The experimental hemorrhage and resuscitation were conducted in a computer feedback and control program written in LabVIEW (National Instruments, Austin, TX) to control a low-flow peristaltic pump (model P720; Instech, Plymouth Meeting, PA). Blood was withdrawn, and resuscitation fluids were given via the venous cannula. Shed blood and resuscitation fluids were held in separate reservoirs placed on a balance with fluid weights recorded every 5 s. Fluid volume was calculated from weight based on measured fluid density. MAP, shed blood volume, and intravenous fluid volume were continuously monitored and recorded every 5 s. Five-minute block averages of hemodynamic data were calculated and tabulated for analysis using Excel (Microsoft, Redmond, WA). The experimental hemorrhage was initiated after a 20-min control period (Fig. 1). Blood was withdrawn at a rate necessary to lower the MAP to 40 mmHg over a period of 15 min, followed by withdrawal of additional blood as needed to maintain a MAP ceiling of 40 mmHg for a period of 30 min. At no time during the experiment was blood reinfused. After the hemorrhage phase, animals assigned to receive anti-C5 were given an infusion of anti-ri-C5 monoclonal antibody clone 18A, diluted in normal saline, at 20 mg/kg body wt over 5 min. Clone 18A has been previously described and has been shown to decrease myocardial infarct size in rats at a dosage of 20 mg/kg (33). Clone 18A hybridoma was a kind gift from Alexion Pharmaceuticals and was cultured and purified as previously described (35). Animals assigned to the control groups received an equal volume of normal saline or polyclonal rat IgG (Jackson ImmunoResearch, West Grove, PA) administered over 5 min. IgG was used to control for any complement inhibitory effects unrelated to specific blockade of C5 cleavage. Animals then received an infusion of either LR or Hex as needed to raise the MAP to 60 mmHg over 10 min followed by infusion, as necessary, to maintain the MAP at a minimum of 60 mmHg for a period of 3 h. During this hypotensive support period, fluid was infused only if the MAP fell below 60 mmHg. No additional blood was withdrawn if the MAP rose above 60 mmHg. At the end of the hypotensive support period, the animals received an infusion of resuscitation fluid to raise the MAP to a target of 80 mmHg over 15 min followed by infusion as needed to maintain a MAP at or above 80 mmHg for an additional 10 min. Following this final resuscitation, the animals were euthanized. To calculate MAP response per unit fluid infused, we plotted the cumulative fluid infused during the resuscitation from 60-mmHg threshold to the 80-mmHg threshold against the 5-min-block-averaged MAP at the beginning and end of this resuscitation. Arterial blood was sampled at the end of the 20-min control period (baseline blood sample) and at the end of the experiment. Blood chemistries, hemoglobin, and arterial blood gas parameters were measured using a Radiometer model 735 (Radiometer, Westlake, OH).

Total hemolytic complement assay. Prehemorrhage (baseline) and postresuscitation blood samples were used for total hemolytic complement (CH50) assays. Postresuscitation CH50 values are reported as a percentage of the baseline value. We used Mayer’s CH50 assay modified for application in 96-well plates (25). Briefly, plasma samples were diluted in gelatin veronal buffer (GVB) to 1:20, 1:40, 1:70, and 1:100; 25 μl were added in quadruplicate to a 96-well plate. Positive and negative controls were wells with 25 μl of 10% Triton X-100 and 25 μl of GVB added, respectively. Sheep red blood cells were diluted in GVB for a final concentration of 3.3 × 10⁷/ml and sensitized with 1/1,000 anti-sheep red blood cell immunoglobulin at room temperature for 30 min. Sensitized sheep red blood cells (200 μl) were added to each well and incubated at 37°C for an additional 30 min. The plates were centrifuged at 2,000 rpm for 8 min, and the supernatant was transferred to an empty plate. Absorbance at 405 nm was read using an optical plate reader. Hemolysis was determined as % = (ODsample − ODspontaneous hemolysis/OD100% lysis − ODspontaneous hemolysis) × 100, where OD is optical density.

Statistical analysis. Animals that died during surgical preparation or before completion of the hemorrhage portion of the experiment were excluded from analysis. Individual biochemical parameters, fluid requirements, and hemodynamic variables were compared using one-way ANOVA. When significant differences were identified among treatment groups by ANOVA, Bonferroni’s post hoc test was per-
formed by comparing each group to its respective control. To stabilize variance, log10 transformed data were used for the analysis of fluid requirements and for the analysis of MAP response curve slopes. The antilogarithm was calculated and results expressed in original units as a point estimate and 95% confidence interval for the ratio of group medians. CH50 assay results were compared using one-way ANOVA tests resulting in P values of < 0.05. Unless otherwise noted, results are expressed as means ± SE. Statistical analyses were performed using Minitab version 14 (Minitab, State College, PA) and GraphPad Prism (GraphPad Software, San Diego, CA).

**RESULTS**

**Baseline characteristics.** In this study, we investigated the effects of a clinically relevant C5 inhibitor administered in a rat model of hemorrhagic shock. We subjected male Sprague-Dawley rats to controlled hemorrhage followed by a period of hypotensive resuscitation with either Hex or LR (Fig. 1). We studied unanesthetized animals and administered anti-C5 at the time of resuscitation. We analyzed resuscitation fluid requirements as well as hemodynamic and metabolic variables.

Tables 1 and 2 provide the baseline characteristics of each group. The groups did not differ significantly with respect to weight, hemoglobin concentration, arterial pH, PO2, PCO2, glucose, or lactate levels. The mean prehemorrhage MAP was statistically significantly higher in the Hex + anti-C5 group compared with the Hex group (106.6 ± 1.8 vs. 100.6 ± 1.2 mmHg; P < 0.05). Conversely, there was a trend toward a higher prehemorrhage MAP in the LR group compared with the LR + anti-C5 group (107.1 ± 1.8 vs. 101.7 ± 2.0 mmHg; P = not significant). The groups did not differ with respect to average MAP during the 40-mmHg ceiling hemorrhage phase of the experiment. Likewise, the groups did not differ significantly with respect to maximum shed blood volume as a percentage of body weight.

**Complement activation and inhibition.** Previous work established the ability of clone 18A to block C5 cleavage (33). To confirm this, we performed CH50 assays. Mean final CH50 values for animals subjected to hemorrhage and resuscitated with Hex (43.1 ± 7.2%; P < 0.001) or LR (58.1 ± 6.2%; P < 0.01) declined from baseline and were significantly lower than mean final CH50 values in the sham group. This observation is consistent with hemorrhage-induced complement activation. CH50 results for the Hex group did not differ significantly from the CH50 results for the LR group. In the Hex + anti-C5 and LR + anti-C5 groups, final CH50 values were 1.7 ± 0.5 and 7.4 ± 1.2% of baseline, respectively. These values did not differ significantly from each other (P = 0.9) but were significantly less than CH50 values in the sham group or respective control group (P < 0.001 in all cases). These observations are consistent with prior data demonstrating inhibition of C5 cleavage by clone 18A (33).

**Metabolic data.** To evaluate metabolic responses to hemorrhage and resuscitation, we performed arterial blood gas and electrolyte analysis. There were no significant differences be-

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<th>Table 2. Pre- and postresuscitation metabolic data</th>
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Values are means ± SE; n, no. of animals. Arterial blood gas samples were obtained at the beginning and end of the experiment. Group data were compared using one-way ANOVA with P values of < 0.05 considered significant.
differences in resuscitation fluid requirements, we evaluated the average MAP in the experimental groups during various phases of the experiment (Fig. 4A). During the hypotensive support phase, average MAP in the anti-C5 treated groups was not significantly different from that of respective controls (Hex + anti-C5 = 80.4 ± 1.5 mmHg, Hex = 77.6 ± 1.0

Fig. 2. Anti-C5 (αC5) prevents complement-mediated cell lysis. Total hemolytic complement (CH50) assays were performed as described in MATERIALS AND METHODS and expressed as a percentage of baseline. Mean CH50 values for animals in the sham group (n = 2) were 97.6 ± 5.9%, for Hextend (Hex; n = 6) 43.1 ± 7.2%, lactated Ringer’s (LR; n = 6) 58.1 ± 6.2%, Hex + anti-C5 (n = 6) 1.7 ± 0.5%, and LR + anti-C5 (n = 6) 7.4 ± 1.2%. There was no significant difference when comparing the Hex and LR groups or the Hex + anti-C5 and LR + anti-C5 groups. Groups were compared using one-way ANOVA followed by Tukey’s post hoc test, with P < 0.05 considered significant.

Fig. 3. Anti-C5 treatment decreases resuscitation fluid requirements. A: 5-min-block averages for cumulative fluid infusion over time. B: total resuscitation fluid requirements for individual animals (*) as well as mean fluid requirements (lines) for each group. Natural logarithm transformed data were compared using one-way ANOVA followed by the Bonferroni post hoc test when significant differences were identified. C: the antilogarithm was calculated, allowing results to be expressed in original units as the point estimate and 95% confidence interval for the relative reduction in fluid volume with anti-C5 treatment. The reduction in fluid was significant for the LR + anti-C5 group (n = 8) vs. LR group (n = 7) and for the Hex + anti-C5 (n = 7) vs. Hex (n = 8) comparisons.

Fluid requirements. Previous work using anti-C5 in a model of combined hemorrhage and ischemia-reperfusion demonstrated decreased resuscitation fluid requirements (13). We asked whether treatment with anti-C5 could decrease resuscitation fluid requirements when applied in conjunction with a hypotensive resuscitation strategy for the management of hemorrhage. Over the course of the experiment, we observed decreased fluid requirements in the anti-C5 treated groups vs. respective controls (Fig. 3A). Mean total resuscitation fluid requirements in each group were Hex = 2.89 ± 0.65, Hex + anti-C5 = 1.16 ± 0.32, LR = 6.47 ± 0.97, LR + anti-C5 = 2.89 ± 0.70 ml/100 g body wt (Fig. 3B). Treatment with anti-C5 resulted in an estimated 62.3% (95% confidence interval: 22.9–81.6%; P < 0.01) reduction in total resuscitation fluid in the Hex + anti-C5 group and an estimated 58.5% (95% confidence interval: 15.2–79.7%; P < 0.05) reduction in total resuscitation fluid in the LR + anti-C5 group compared with respective controls (Fig. 3C). During the hypotensive support phase, there was a trend toward reduced resuscitation volume requirements in both of the anti-C5 groups compared with respective controls. For the Hex + anti-C5 group, there was an estimated 35.9% reduction in volume required (95% confidence interval: 27.5–67.8%; P = not significant), and for the LR + anti-C5 group there was an estimated 38.4% reduction in volume required (95% confidence interval: 22–69.1%; P = not significant). Since colloid based resuscitation fluids (e.g., Hex) expand and maintain intravascular volume more than comparable amounts of isotonic crystalloid solutions (e.g., LR), we did not compare the Hex groups with the LR groups. These data demonstrate that treatment with anti-C5 can reduce resuscitation fluid requirements in hemorrhagic shock.

Hemodynamics. We used a pressure-targeted model of hemorrhage and resuscitation. Therefore, to help understand the
mmHg; $P = 0.05$ was considered significant. ns, Not significant.

DISCUSSION

The resuscitation of trauma patients may require large fluid volumes. Large-volume resuscitation can be logistically impractical and potentially harmful to the patient. Hypotensive or limited volume resuscitation strategies may be particularly relevant in such circumstances. A small number of studies in animal models of hemorrhage have demonstrated benefits from modulation of the complement system. Soluble complement receptor 1 can limit endothelial dysfunction and preserve mesenteric blood flow in hemorrhaged rats (8, 28). In another study, also in rats, complement depletion with cobra venom factor before hemorrhage resulted in improved postresuscitation survival and outcome (8, 28). In the present study, we investigated the role of anti-C5 treatment on resuscitation requirements in hemorrhage. Anti-C5 treatment was associated with a significant increase in MAP response following hemorrhage and resuscitation (Fig. 5B). These data indicate that, in animals whose average MAP increased over the time period of interest, anti-C5 enhanced responsiveness to fluid infusion following a period of prolonged hypotension.
tion MAP (37). Administration of C1-esterase inhibitor led to decreased mesenteric leukocyte adhesion in another rat study of hemorrhagic shock (17). More recently, using a model of combined hemorrhage and frank mesenteric ischemia-reperfusion (aortic cross-clamping), Harkin et al. (13, 14) demonstrated decreased resuscitation volume requirements, improved MAP, and decreased gut and lung injury in rats treated with C5-blocking antibody or C5α receptor antagonist.

Two main findings emerge from our study. First, we observed a significant decrease in total resuscitation fluid requirements and a trend toward reduced resuscitation fluid requirements during the hypotensive phase in both of the anti-C5 treated groups. The magnitude of reduction in total fluid required was remarkable (~60%), similar in both groups, and comparable to published data in a model of ruptured abdominal aortic aneurysm (13).

Our findings have practical implications. Large-volume resuscitation may worsen trauma-related complications such as abdominal compartment syndrome and dilutional coagulopathy (20, 22). Starch-containing fluids such as Hex may also cause derangements in coagulation, and, for this reason, some authorities advocate limiting the volume of Hex administered during resuscitation (12, 25a, 27). Furthermore, identification of volume-sparing adjuncts has particular relevance for situations such as forward military environments with delayed evacuation or mass casualty scenarios where the supply of resuscitation fluid may be limited. Last, reduction in resuscitation fluid requirements is directly relevant to battlefield

Fig. 5. Anti-C5 improves MAP response to fluid infusion following prolonged hypotension. A: for each animal, the amount of fluid infused during the resuscitation from the 60-mmHg threshold to the 80-mmHg threshold was plotted against the 5-min-block-averaged MAP at the beginning and end of this time period. The slope of the resultant line served as an index of MAP responsiveness. B: natural logarithm transformed data for the Hex (n = 6), Hex + anti-C5 (n = 5), LR (n = 4), and LR + anti-C5 (n = 6) groups were compared with one-way ANOVA followed by the Bonferroni post hoc test if significant differences were identified. The antilogarithm was calculated, allowing results to be expressed in original units as point estimate ± 95% confidence interval for the ratio of group medians for anti-C5 treated vs. untreated groups.
medicine where first-responders physically transport medical supplies and the weight of resuscitation fluid is a consideration.

The second main finding in our study is improved MAP responsiveness to fluid infusion following a period of prolonged hypotension in the anti-C5-treated groups. In both of the anti-C5-treated groups, we documented an estimated fourfold increase in MAP response per unit fluid infused. This is an important finding because massive hemorrhage or prolonged hypotension may lead to vascular hyporesponsiveness and “irreversible shock” (23, 32, 38). It is important to note that we did not include all animals in this analysis and thus cannot fully generalize our results. Specifically, animals whose MAP declined over the time period of interest were not included. It would have been methodologically inappropriate to include these animals because, for any given decline in MAP over time, a small associated volume infusion would generate a line with a highly negative slope, whereas a large volume infusion would generate a line with a less negative slope. As such, inclusion of these animals would have led to erroneous conclusions.

Our study does not define the mechanism of the observed benefits of anti-C5 treatment. Numerous possible explanations exist. In the simplest scenario, anti-C5 therapy could have a generalized vasopressor effect. This explanation is unlikely as we observed no significant difference in average MAP between anti-C5-treated groups and their respective controls during the hypotensive resuscitation phase. Furthermore, absent adequate fluid administration, a systemic vasopressor would likely result in inadequate resuscitation and a greater physiological insult; we detected no differences in arterial pH or lactate to suggest this. Complement likely plays a multifaceted role in the pathophysiology of hemorrhage. C5a promotes vascular leak, and the membrane attack complex impairs endothelium-dependent arterial relaxation, and both molecules possesses numerous proinflammatory properties (10, 16, 29). Studies in relevant animal models demonstrated decreased pulmonary and intestinal permeability and preserved vascular endothelial function with inhibition of complement (8, 14, 13). We speculate that the observed effects of anti-C5 stem from a combination of preserved vascular endothelial function, decreased vascular leak, and early attenuation of the inflammatory response.

Lactic acidosis may develop in hemorrhagic shock. Why a mild alkalosis developed in all groups in our study is unclear. A compensatory hyperventilation has been observed in early hemorrhage (J. L. Atkins, unpublished data), and we speculate that hyperventilation may account for the observed alkalosis. Because we did not directly measure serum bicarbonate concentrations, we cannot be certain.

As anticipated, we observed a decline in CH50 values in all groups except the sham-hemorrhage group. CH50 results from the anti-C5-treated groups are consistent with previously published data in which clone 18A was shown to block C5b-9-mediated cell lysis and C5a-induced neutrophil chemotaxis (33). The decline in CH50 values we observed for the Hex and LR groups likely reflects combined consumptive and dilutional effects of hemorrhage/resuscitation. Because complement-mediated hemolytic activity can be affected by consumption, dilution, or inhibition of complement, the CH50 assay does not differentiate the relative contributions from each of these factors.

During the prehemorrhage phase of the experiment, the average MAP in the Hex + anti-C5 group was somewhat greater than the average MAP in the Hex group. It is unlikely this difference biased our results because the two groups were otherwise well matched and we observed similar outcomes in both of the groups receiving anti-C5.

Our study has limitations. To prevent catheter thrombosis, all animals received heparin. Heparin can inhibit complement and is not an ideal agent for use in studies evaluating complement inhibitors (9). The use of heparin could have biased our results in either direction. We considered using other anticoagulants such as hirudin or sodium citrate, but each has associated limitations. Hirudin, like heparin, can inhibit complement activation, and sodium citrate binds calcium, an ion essential for complement activity (6, 19). Because animals in all groups received heparin, our study was adequately controlled.

A number of questions regarding anti-C5 therapy in hemorrhage remain unanswered. It is not known whether anti-C5 therapy would change overall mortality following hemorrhage. Specifically, we do not know the effect of anti-C5 therapy on complications of hemorrhage such as systemic inflammatory response syndrome and infection in the long term. Our results suggest anti-C5 therapy helps preserve hemodynamic responsiveness following prolonged hypotension. Whether anti-C5 might prevent, delay, or serve as rescue therapy from decompensated shock is a question that deserves further study.

In summary, we have shown that C5-blocking antibody administered during resuscitation leads to decreased fluid requirements in an awake rat model of hemorrhagic shock managed with a hypotensive resuscitation strategy. We have also shown that anti-C5 can improve MAP response to fluid infusion, an index of hemodynamic responsiveness, following a prolonged period of hypotension.

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