J Appl Physiol
90: 441–446, 2001.

Detrimental effects of complement activation in hemorrhagic shock

JOHN G. YOUNGER,[1] NOBUYOSHI SASAKI,[2] MICHAEL D. WAITE,[3] HOLT N. MURRAY,[3] EDWARD F. SALEH,[1] ZACHARY A. RAVAGE,[4] RONALD B. HIRSCHL,[5] PETER A. WARD,[4] AND GERD O. TILL[4]

Departments of [1]Emergency Medicine, [4]Pathology, and [5]Surgery, University of Michigan, Ann Arbor, Michigan 48109-0303; [3]Department of Emergency Medicine, Ohio State University, Columbus, Ohio 43210; and [2]Department of Anesthesiology, Jikei University, Tokyo 105, Japan

Received 15 May 2000; accepted in final form 25 August 2000

Younger, John G., Nobuyoshi Sasaki, Michael D. Waite, Holt N. Murray, Edward F. Saleh, Zachary A. Ravage, Ronald B. Hirschl, Peter A. Ward, and Gerd O. Till. Detrimental effects of complement activation in hemorrhagic shock. *J Appl Physiol* 90: 441–446, 2001.—The complement system has been implicated in early inflammatory events and a variety of shock states. In rats, we measured complement activation after hemorrhage and examined the hemodynamic and metabolic effects of complement depletion before injury and worsening of complement activation after hemorrhage and resuscitation [with a carboxypeptidase N inhibitor (CPNI), which blocks the clearance of C5a]. Rats were bled to a mean arterial pressure of 30 mmHg for 50 min and were then resuscitated for 2 h. Shock resulted in significant evidence of complement consumption, with serum hemolytic activity being reduced by 33% ($P < 0.05$). Complement depletion before injury did not affect hemorrhage volume (complement depleted = 28 ± 1 ml/kg, complement intact = 29 ± 1 ml/kg, $P = 0.74$) but improved postresuscitation mean arterial pressure by 37 mmHg ($P < 0.05$) and serum bicarbonate levels (complement depleted = 22 ± 3 meq/ml, complement intact = 13 ± 8 meq/ml, $P < 0.05$). Pretreatment with CPNI was lethal in 80% of treated animals vs. the untreated hemorrhaged group in which no deaths occurred ($P < 0.05$). In this model of hemorrhagic shock, complement activation appeared to contribute to progressive hypotension and metabolic acidosis seen after resuscitation. The lethality of CPNI during acute blood loss suggests that the anaphylatoxins are important in the pathophysiological events involved in hemorrhagic shock.

anaphylatoxin; C5a; traumatic shock; carboxypeptidase N; cobra venom factor; ischemia-reperfusion

THE COMPLEMENT SYSTEM IS A cascade of over 30 activating, effector, and regulatory proteins that enhance the ability of the humoral immune system to opsonize or destroy a variety of pathogens. The cascade also contributes to cellular immunity by producing peptides that promote capillary leak and leukocyte migration at sites of infection or tissue damage. Several investigators have noted that, in humans, acute blood loss and trauma activate the complement cascade and that the degree of activation correlates with injury severity, development of multiple organ failure, and death (3, 5–8, 12, 17, 21). Similar observations have been made in animal studies, where systemic complement activation has been described in soft tissue trauma, burn injury, and models of myocardial, intestinal, and skeletal muscle ischemia (6, 9, 11, 18).

Although activation occurs in the setting of severe blood loss, data implicating complement as an effector of tissue injury are limited (4). In the present studies, we used a rat model to examine hemodynamic and metabolic recovery from an episode of prolonged, profound hemorrhagic hypotension. We found that hemorrhage and resuscitation appeared to cause complement consumption and that prior depletion of circulating complement affords protection from shock as measured by mean arterial blood pressure and metabolic acidosis. Furthermore, intensifying complement activity in this setting, either by administration of an exogenous complement activator or by inhibition of serum carboxypeptidase N activity, exacerbates injury.

METHODS

Rat model of hemorrhagic shock. A fixed-pressure hemorrhagic shock model was used in all experiments and has been described previously (19, 20). Male, specific pathogen-free Sprague-Dawley rats were anesthetized with subcutaneous ketamine hydrochloride (50 mg/kg) and xylazine hydrochloride (10 mg/kg). With animals in a supine position, a tracheostomy was performed, and left jugular venous and bilateral carotid arterial catheters (PE-50, Becton Dickinson, Sparks, MD) were placed. Body temperature was monitored with a rectal thermometer and maintained between 37 and 39°C with a heating lamp. Mean carotid arterial pressure was continuously measured (neonatal monitor model 78901A, Hewlett-Packard, Andover, MD). Animals were mechanically ventilated with a piston ventilator (model 683 rodent ventilator, Harvard Apparatus, South Natick, MA) set to deliver a fraction of inspired O_2 of 1.0, a rate of 70 breaths/min, a tidal volume of 8 ml/kg, and 2 cmH_2O positive end-expiratory

Address for reprint requests and other correspondence: J. G. Younger, Dept. of Emergency Medicine, Taubman Center B1354, 1500 East Medical Center Drive, Ann Arbor, MI 48109–0303 (E-mail: jyounger@umich.edu).

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pressure. The local animal use committee approved all surgical procedures and experimental protocols, which were in compliance with federal guidelines.

To induce hemorrhagic shock, animals were bled from the carotid artery at 1.5 $ml \cdot kg^{-1} \cdot min^{-1}$ into a citrate-anticoagulated syringe (anticoagulant citrate phosphate dextrose solution, Abbott Laboratories, North Chicago, IL) until the mean arterial pressure (MAP) reached 30 mmHg. Additional blood was removed whenever the MAP exceeded 35 mmHg. Hypotension was thus maintained for 50 min, after which the total hemorrhage volume was recorded. Except where noted, resuscitation began with the reinfusion of all shed blood and an additional 30 ml/kg of saline over 40 min. The animals were then observed for 80 min, making the entire protocol 170 min in duration. In each set of experiments, control animals that were instrumented and ventilated in an identical fashion but neither bled nor resuscitated were included.

Measurement of metabolic parameters. Arterial blood-gas analysis was performed at relevant time points throughout the experiment (ABL 505, Radiometer America, Westlake, OH). Divalent cations are required cofactors for several steps in complement activation. Assembly of the C3 convertases of both the classic and alternative pathways requires Mg^{2+}. Similarly, Ca^{2+} is needed for assembly of the three protein components of C1, the initiator of the classic pathway activation. Because citrate was used as the anticoagulant for shed blood in our model, whole blood ionized calcium levels were also measured at each time point with the same blood-gas analyzer described above to confirm that excessive citrate exposure was not occurring.

Measurement of circulating complement activity. To assess complement activation, total serum hemolytic activity and serum C3 titer were measured at baseline, before and after resuscitation, and at the conclusion of the experiment. Total serum hemolytic activity was measured by using the total hemolytic complement (CH50) technique (15). Serial dilutions of serum were incubated for 1 h at 37°C with sheep erythrocytes (Colorado Serum, Denver, CO) that had been sensitized with rabbit anti-sheep hemolysin (Colorado Serum). The reciprocal of the serum dilution that resulted in 50% erythrocyte hemolysis was recorded as the CH50 value. C3 titers were determined by double immunodiffusion in phosphate-buffered 2% agarose gel. Serial dilutions of serum from experimental animals were placed around a central well containing goat anti-rat C3 IgG (United States Biochemical, Cleveland, OH). After incubation in a humidified chamber for 24 h at 4°C, the gels were washed and then stained with Coomassie blue. The highest dilution with a visible immunoprecipitation band was recorded as the C3 titer.

Depletion of circulating complement. To confirm the relevance of complement activation to hemorrhagic shock, cobra venom factor (CVF) was used to produce complement depletion in some animals before injury. Whole venom (Naja naja atra, Sigma Chemical, St. Louis, MO) was dialyzed for 24 h against 40 mM phosphate-buffered saline and then fractionated by anion-exchange chromatography on a diethylaminoethyl cellulose column (DE52, Whatman, Kent, UK) (1). Animals were given intraperitoneal injections of 25 units of CVF in 1.0 ml of saline 36, 24, and 12 h before hemorrhage. Serum CH50 and C3 levels at the onset of hemorrhage were used to confirm complement depletion. In these animals, serum hemolytic activity was undetectable, and C3 titers were significantly reduced ($\leq$1:160, with normal being $>$1:640 in our laboratory).

Augmentation of complement activation. In some experiments, we examined the effect of intensified complement activation after hemorrhage and resuscitation. In these animals, 5 units of CVF were administered intravenously at the conclusion of the resuscitation phase of the protocol (the 120-min time point). Control animals received an equal dose of CVF after instrumentation and 120 min of mechanical ventilation. End points included mortality, change in arterial oxygenation and HCO_3^- concentration 1 h postinjection, and fall in MAP (from the time of CVF injection to the nadir blood pressure, which was reached in all cases 20 min after injection).

Inhibition of carboxypeptidase N. On their generation, C3a and C5a are quickly metabolized to limit their biological activity. To study the importance of C3a and C5a conversion to C3a des Arg and C5a des Arg, respectively, a series of experiments was conducted in animals that were given a carboxypeptidase N inhibitor (CPNI) before hemorrhage. After instrumentation, animals received 100 mg/kg of DL-2-mercaptomethyl-3-guanidinoethylthiopropanoic acid (Calbiochem, La Jolla, CA) intraperitoneally. This agent has demonstrated specificity for carboxypeptidase N and reduces serum enzyme activity by $>$90% for $>$3 h (10, 13). Control animals also received CPNI after instrumentation.

Preconditioning effect of complement activation. Whereas serial intraperitoneal injections of CVF deplete serum complement activity and are associated with protection against numerous inflammatory events, the possibility that CVF administration might convey protection through complement-independent means has not been thoroughly examined. One mechanism by which CVF might protect animals against hemorrhagic shock is by preconditioning the cardiovascular system such that hemorrhage and resuscitation are better tolerated. If preconditioning occurred, then any systemic protection seen with serial CVF injections might erroneously be attributed to complement depletion. To better quantify cardiovascular preconditioning induced by complement activation, we looked for evidence of protection from ischemia and reperfusion in isolated rat hearts after intravenous CVF injection. A series of rats were administered 25 U/kg CVF or vehicle intravenously and observed for 30 min. After death, the hearts were excised, placed on a Langendorff apparatus, perfused in a retrograde fashion with warm Krebs-Henseleit buffer containing 5.5 mM glucose and 0.2 mM caprylic acid, and preoxygenated with 95% O_2-5% CO_2. A left ventricular (LV) balloon was placed to measure intraventricular pressures, and the heart was allowed to stabilize for 15 min before baseline measurements of LV function were taken. These included heart rate, developed pressure (the systolic-diastolic ventricular pressure difference), change in pressure over time (dP/dt; as a measure of contractility), $-dP/dt$ (as a measure of myocardial relaxation), and rate-pressure product. Next, the hearts were subjected to 20 min of warm, no-flow ischemia by stopping the flow of perfusate. The organs were then reperfused for 30 min, and repeat measurements of function were taken.

Statistical analysis. MAP, blood-gas, and ionized calcium results are reported as means ± SD and were compared between groups by using unpaired *t*-tests or repeated-measures analysis of variance where appropriate. The Tukey method of post hoc comparison was used to examine differences between groups at individual time points. CH50 and C3 titers, although also reported as means ± SD, were compared by using the Kruskal-Wallis method. Differences in mortality in the posthemorrhage CVF injection and CPNI studies were compared with Fisher's exact test. In experiments performed on isolated perfused hearts, comparisons between control and CVF-treated groups were made at baseline and after ischemia and reperfusion with two-tailed

Table 1. *Loss of total serum hemolytic ability after hemorrhage and resuscitation*

Group	CH50, %Baseline: Posthemorrhage (50 min)	Postresuscitation (110 min)	End of experiment (170 min)	*P* Value
Control ($n = 5$)			95 ± 1	<0.05
Hemorrhage ($n = 5$)	67 ± 8	80 ± 15	71 ± 12	<0.05

Values are means ± SD; *n*, no. of animals. Control animals had total hemolytic complement (CH50) activity checked only at the conclusion of 170 min of observation and mechanical ventilation. *P* values are in comparison to baseline CH50.

t-tests. All analyses were performed by using the software SAS 6.12 (SAS Institute, Cary, NC).

RESULTS

Complement activation during shock. Compared with control animals, CH50 levels dropped significantly within 1 h of the onset of shock (Table 1). After resuscitation and during the remainder of the protocol, these levels were noted to rebound, although not in a statistically significantly fashion. Shed blood, the reinfusion of which constituted the first phase of resuscitation, possessed 71 ± 12% of the measured baseline hemolytic ability, indicating that some complement activation occurred in the hemorrhage reservoir during the experiment. C3 titers were not measurably reduced after hemorrhage and remained >1:640 at the conclusion of hemorrhage in each of five animals tested.

Hemodynamic and metabolic significance of complement activation during hemorrhage and resuscitation. The hemorrhage volume needed to maintain a MAP of 30 mmHg for 50 min was not affected by prior complement depletion (complement intact = 29 ± 1 ml/kg, complement depleted = 28 ± 1 ml/kg, $P = 0.74$). However, complement-depleted animals demonstrated an improved response to resuscitation and, at the conclusion of the experiment, had a MAP that was on average 37 mmHg higher than that of complement-intact animals (complement-intact hemorrhage = 48 ± 25 mmHg, complement-depleted hemorrhage = 85 ± 27 mmHg, $P < 0.05$; Fig. 1).

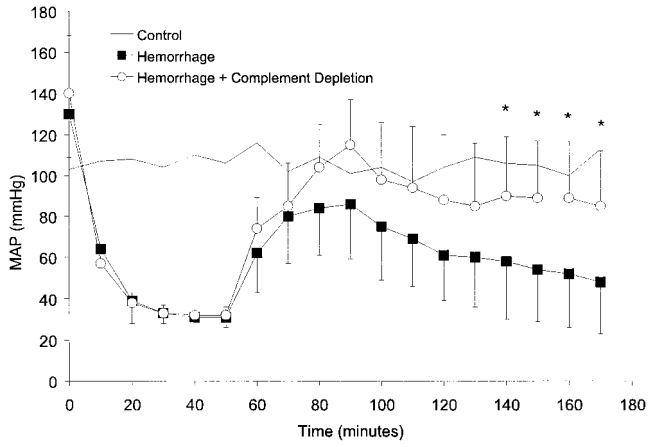

Fig. 1. Effect of complement depletion on hemodynamic recovery after hemorrhage and resuscitation. Values represent means ± SD of carotid artery mean arterial pressures (MAPs) during the course of the experiment. ■, Complement-intact animals ($n = 10$); □, complement-depleted animals ($n = 10$). The MAP of control animals ($n = 5$) is shown without error bars for the purpose of clarity. *Time points where the complement-intact and complement-deficient groups were statistically different as determined by Tukey's post hoc comparisons of repeated-measures analysis of variance ($P < 0.05$).

Table 2. *Metabolic measurements during acute blood loss and resuscitation*

Parameter	Control ($n = 5$)	Hemorrhage Complement Intact ($n = 10$)	Hemorrhage Complement Depleted ($n = 10$)	*P* Value
Pa_{O_2}, Torr				
Baseline	494 ± 23	452 ± 82	495 ± 44	0.25
Posthemorrhage	476 ± 19	435 ± 56	446 ± 104	0.63
Postresuscitation	412 ± 126	439 ± 118	458 ± 98	0.37
End of study	462 ± 78	416 ± 97	450 ± 121	0.39
$[HCO_3^-]$, meq/l				
Baseline	23 ± 3	23 ± 2	26 ± 3	0.04
Posthemorrhage	21 ± 2	9 ± 3	13 ± 2	<0.01*
Postresuscitation	20 ± 1	13 ± 4	22 ± 3	<0.01*
End of study	22 ± 3	13 ± 8	22 ± 3	<0.01*
$[iCa^{2+}]$, meq/l				
Baseline	2.3 ± 0.3	2.4 ± 0.1	2.3 ± 0.3	0.52
Posthemorrhage	2.2 ± 0.4	2.1 ± 0.4	1.6 ± 0.4	0.02
Postresuscitation	2.1 ± 0.3	1.8 ± 0.3	1.8 ± 0.2	0.12
End of study	2.3 ± 0.3	2.3 ± 0.3	2.0 ± 0.4	0.17

Values are means ± SD; *n*, no. of animals. Pa_{O_2}, arterial P_{O_2}; $[HCO_3^-]$, concentration of HCO_3^-; $[iCa^{2+}]$, whole blood ionized calcium concentration. *P* values represent repeated-measures analysis of variance for comparison of all 3 groups. *Instances where post hoc comparisons between complement-intact and complement-depleted hemorrhage groups were statistically significant ($P < 0.05$).

CVF pretreatment also ameliorated metabolic acidosis after hemorrhage. Complement-depleted animals experienced a less severe metabolic acidosis after blood loss and more quickly and completely resolved their acidosis on resuscitation (Table 2). No significant

Table 3. *Effects of intravenous CVF administration after resuscitation*

Parameter	Control ($n = 5$)	Hemorrhagic Shock ($n = 5$)	*P* Value
Pa_{O_2}, Torr			
Pre-CVF	466 ± 41	509 ± 47	
Post-CVF	289 ± 109	267 ± 162	
Difference	177 ± 126	242 ± 202	0.62
$[HCO_3^-]$, meq/l			
Pre-CVF	23 ± 3	22 ± 2	
Post-CVF	14 ± 5	20 ± 2	
Difference	9 ± 7	2 ± 3	0.17
MAP, mmHg			
Pre-CVF	102 ± 19	83 ± 23	
Post-CVF	44 ± 6	50 ± 13	
Difference	58 ± 24	33 ± 21	0.12
Deaths	1	2	0.51

Values are means ± SD; *n*, no. of animals. Pa_{O_2} $[HCO_3^-]$, and mean arterial pressure (MAP) values reflect only animals that survived for 1 h after cobra venom factor (CVF) administration. *P* values represent *t*-tests or Fisher's exact test, where appropriate.

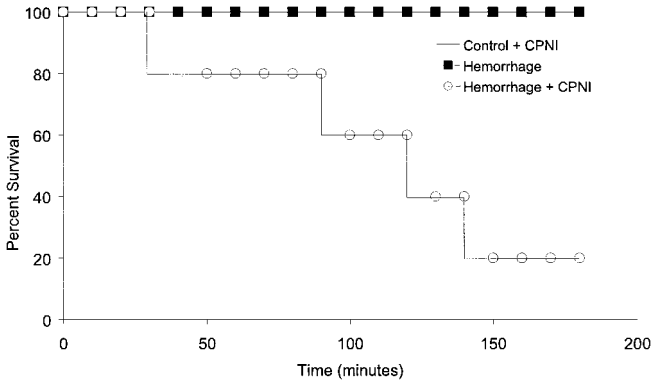

Fig. 2. Mortality associated with carboxypeptidase N inhibition after hemorrhagic shock. Control animals were instrumented and subjected to 170 min of mechanical ventilation. Injured animals were subjected to the hemorrhagic shock and resuscitation protocol described in the text. CPNI, carboxypeptidase N inhibitor. $P < 0.05$ by Fisher's exact test.

change in arterial oxygenation was seen in any group at any time point, consistent with our laboratory's prior observations in this model (19, 20). Baseline ionized calcium concentrations were similar between control and hemorrhaged animals. After hemorrhage, complement-depleted animals had lower ionized calcium concentrations than either control animals or complement-intact animals that had been bled, although the levels in depleted animals were still within the physiological range. The explanation for this finding is not obvious, but as the posthemorrhage data point was measured before resuscitation (i.e., before animal exposure to the citrate-containing reservoir), the relative hypocalcemia seen with complement depletion seems real. There were no differences in whole blood ionized calcium concentration between complement-intact and complement-depleted rats at the later time points, suggesting that the difference in recovery between these two groups was not a result of an inadvertent difference in citrate exposure.

Effects of induced complement activation after resuscitation. Intravenous injection of 5 units of CVF in control animals after 120 min of observation produced a decrease in arterial P_{O_2}, HCO_3^- concentration, and MAP, as well as one death (Table 3). CVF produced two deaths and otherwise similar results in animals that had been subjected to hemorrhage and resuscitation, and no statistically significant differences were seen between groups. The unexpectedly high serum HCO_3^- levels seen in animals subjected to hemorrhage, resuscitation, and CVF treatment may reflect censoring of the data, in that two of the five animals in this group, likely with very low serum HCO_3^- levels, expired.

Effect of carboxypeptidase N inhibition. Pretreatment of control animals with CPNI produced no apparent hemodynamic or metabolic effect. However, enzyme inhibition in animals subjected to acute blood loss and resuscitation uniformly resulted in severe hypotension and was lethal in four of the five animals studied ($P < 0.05$, Fig. 2), suggesting that preservation of C3a and C5a under these experimental conditions is detrimental.

Effect of CVF injection on ex vivo myocardial response to ischemia and reperfusion. After isolation and 15 min of stabilization, there were no baseline differences between control and CVF-treated hearts (Table 4). After warm no-flow ischemia and reperfusion, all functional parameters except heart rate fell in both groups ($P < 0.05$). Trends toward better postreperfusion function in all measured parameters were seen in the hearts of CVF-treated animals. However, performance in both groups was very similar after reperfusion, and only $-dP/dt$ and the percent recovery of rate-pressure product were statistically significant.

DISCUSSION

The relationship in humans of acute injury to complement activation (as measured by a decrease in circulating activity or the appearance in blood of activation products) has been noted repeatedly (3, 5–8, 12, 17, 21). These observations have also been made in laboratory models of blood loss or extensive tissue injury (6, 16, 18). In the present studies, we too found that severe blood loss in rats was associated with complement activation. That exposure to CVF before hemorrhage improved systemic markers of injury, whereas strategies exacerbating complement activation produced hypotension, worsening acidosis, and death, is further evidence for the contribution of this pathway to the systemic manifestations of severe blood loss and resuscitation. These findings bring hemorrhagic shock into the list of clinically important nonin-

Table 4. *Effect of CVF on left ventricular myocardial functional recovery from warm ischemia ex vivo*

Parameter	Control (n = 10)		CVF (n = 10)		95% CI for Difference
	Preischemia	Postischemia	Preischemia	Postischemia	
Heart rate, beats/min	251 ± 22	229 ± 20	254 ± 20	230 ± 35	24, −28
LVdP, mmHg	130 ± 13	76 ± 29	120 ± 6	92 ± 11	8, −33
dP/dt, mmHg/s	3,663 ± 797	2,407 ± 1,076	3,620 ± 592	3,129 ± 598	96, −1,539
$-dP/dt$, mmHg/s	2,295 ± 225	1,456 ± 571	2,282 ± 130	1,871 ± 216	−9, −820
RPP	32,574 ± 1,630	17,478 ± 6,754	30,603 ± 2,861	21,026 ± 3,001	1,362, −8,458
%Recovery of RPP		53 ± 20		68 ± 9	0, −30*

Values are means ± SD; n, no. of animals. LVdP, systolic-diastolic ventricular pressure difference; dP/dt, change in pressure over time; RPP, rate-pressure product; CI, confidence interval. 95% CI for difference is by 2-tailed t-test. Values represent 95% CI for control postischemia − CVF postischemia. *P = 0.048.

fectious conditions that are associated with, and potentially exacerbated by, systemic complement activation.

The complement cascade may worsen hemorrhagic shock in a variety of ways. Whereas evidence exists for a direct role of the membrane attack complex in models of ischemia (14), data from other models suggest a pivotal role for C5a (2). Confirmation of the relevance of C5a in this model may only come with the availability of anti-rat C5a antibodies. Given that C5a is an 8-kDa fragment of the much larger C5 protein, antibodies that effectively neutralize C5a without affecting the activity of the parent molecule C5 (and thus interfering with the assembly and function of the membrane attack complex) have been very difficult to develop. Until such tools are perfected and available in quantities sufficient to conduct in vivo experiments, evidence for the importance of C5a remains indirect. However, amelioration of C5a-mediated capillary leak and vasoconstriction by pretreatment with CVF (thus increasing intravascular volume and augmenting regional perfusion) would explain the improved MAP and acidosis seen 2 h after resuscitation. These changes might also be affected by CVF indirectly, by decreasing C5a-dependent neutrophil activation or mast cell degranulation. The lethality of carboxypeptidase N inhibition seen in our experiments further indicts the anaphylatoxins as relevant mediators.

As more is learned of the role of complement in the development of hemorrhagic shock, this cascade may become a therapeutic target. Soluble complement receptor 1 improves microvascular function in rats after hemorrhage (4). Recently, blockade of C5a (without interfering with the more proximal bacterial opsonizing function of complement or the later lytic function) was shown to decrease the rate of bacteremia and improve survival in a rat model of cecal ligation and puncture (2). Based on our findings, similar experiments are warranted in hemorrhage shock.

The primary limitation of these experiments is their applicability to clinical experience. Animals were resuscitated with shed blood that had undergone a degree of complement activation, as documented by the reduced CH50 in the hemorrhage reservoir. The impact of injecting activated whole blood, although consistent with many models of hemorrhage and resuscitation, deserves closer examination. To maximize the hemodynamic and metabolic differences between hemorrhage and control groups (and thus detect the effect of complement activation), animals were resuscitated with only 30 ml/kg of saline after the reinfusion of shed blood. Although this too is consistent with clinical practice, the persistent and progressive hypotension seen in hemorrhaged animals suggests that they were underresuscitated; the clinically measurable effects of complement activation might be overcome to a greater degree by larger volume fluid resuscitation.

In experiments using isolated rat hearts, we found only partial evidence for preconditioning after a single intravenous injection of CVF. These results suggest that preconditioning plays a minor role, if any, in the protection from shock that is afforded by CVF administration. However, the present experiments did not address the effects of repeated exposure to low doses of intraperitoneal CVF, and further study is needed to completely address the phenomenon of preconditioning by complement activation.

Lastly, the relatively short duration of the experimental protocol emphasizes the harmful circulatory and metabolic effects of complement but does not address what may be a very important role for this pathway in preventing postshock bacteremia and sepsis.

In conclusion, in a rat model of hemorrhage shock, complement activation appeared to contribute to progressive hypotension and metabolic acidosis seen after resuscitation. The lethality of carboxypeptidase N inhibition suggests that the anaphylatoxins are in part responsible for this effect.

We thank Chris W. Seder for assistance in conducting the hemorrhagic shock experiments.

This project was funded in part by a Career Development Award, by a Center of Excellence Award from the Emergency Medicine Foundation, and by National Heart, Lung, and Blood Institute Grant HL-03817.

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