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ATTENUATION OF THE EFFECTS OF RAT HEMORRHAGIC SHOCK WITH A REPERFUSION INJURY-INHIBITING AGENT SPECIFIC TO MICE

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Abstract

Death after hemorrhagic shock (HS) may be caused by a generalized reperfusion injury, particularly noticeable in the gut. A period of tissue ischemia followed by reinstitution of perfusion produces severe inflammation that can be blocked in mice by preventing the binding of a pathogenic natural immunoglobulin M (IgM) of defined specificity to antigens in reperfused tissue by using a soluble peptide analogue of the IgM tissue target. We hypothesize that this agent can improve end points of rat HS: death, intestinal injury, and lung injury. Male Sprague-Dawley rats were anesthetized; 50% of calculated blood volume was removed for 120 min, shed blood, then returned; and animals were sacrificed at 72 h. One group of rats received i.v. analogue ([N2] 300 µg) with the return of shed blood. Small intestine and lung were evaluated by histological examination and immunohistochemistry. Lung edema was assessed by Evans blue extravasation and histological examination. I.v. N2 decreased experimental mortality from 62% to 12% ($P < 0.05$). Associated with this was diminution of gut injury score from $57.8\% \pm 5.5\%$ to $19.5\% \pm 2.5\%$ ($P < 0.05$), lung injury from 21.4 ± 1.5 to 14.8 ± 1.3 polymorphonuclear leucocytes per high-power field $\times 400$ ($P < 0.05$), and Evans blue extravasation index from 0.61 ± 0.14 to 0.18 ± 0.06 ($P < 0.05$). As well, the deposition of IgM and C3 that is seen in intestinal villi from HS was not present in N2-treated rats. The N2 peptide agent that blocks reperfusion injury in mice prevents death from rat HS, as well as attenuates gut reperfusion injury and its remote target injuries. These data suggest that death from HS is caused by reperfusion injury, and that an agent derived from mice is effective in rats when given in real therapeutic time.

Keywords

Ischemia reperfusion; hemorrhagic shock; survival; peptide; IgM

INTRODUCTION

The response of the hormonal and inflammatory mediator systems in hemorrhagic shock (HS) seems to represent a distinct set of responses that is different from those of other forms of shock (1,2). The classic neuroendocrine response to hemorrhage attempts to preserve homeostasis by maintaining perfusion to the heart and brain, often at the expense of other organ systems, such as gut and lungs. The resulting ischemic episode to critical organs alone may not be sufficient to lead to histological evidence of injury. But, with resuscitation, the

resulting reperfusion of ischemic organs may produce an intense inflammation (reperfusion injury) in the vascular beds that were sacrificed to maintain the heart and the obligate glucose metabolism of the brain. The violent inflammation from a global reperfusion injury could be an important feature in the pathophysiology of recovery from HS (3–5).

During ischemia, cells undergo specific changes in enzymatic activities, mitochondrial function, and antioxidant defenses that are thought generally to underlie the cellular derangements of reperfusion injury. However, in 1999, Weisman et al. (6) proposed that the acute inflammatory attack after reperfusion was at least as important and depended on activation of the serum complement system. Pretreatment of animals with a soluble inhibitor of complement C3b (sCR1) significantly reduced reperfusion injury in rat myocardial infarction. Multiple additional animal models of reperfusion injury have shown the same salutary effect of sCR1. Other investigations have noted that the complement cascade is activated during clinical reperfusion events, injury, and shock, and that the degree of activation correlates with injury severity, development of organ failure, and death (7–9).

Studies in knockout mice have revealed the source of complement activation in reperfusion injury: the binding of a preexisting natural immunoglobulin M (IgM) with specificity for injury antigens on reperfused tissue (10,11). There may be other sources of complement activation such as interaction of reperfused tissue with the mannose-binding lectin pathway (12–14). However, the discovery in both skeletal muscle (15) and gut (16) reperfusion injuries that immunoglobulin-deficient mice were protected from injury and could be reconstituted only with IgM from normal mice (not IgG) suggested that natural IgM was the source of complement activation. Natural IgM circulates in all normal mammals and has binding-site specificity that arises from gene encoding and not recombination. As it is a product of a class of lymphocytes with a defined phenotype and that is primarily resident in the body cavities (B1 lymphocytes), cloning of such antibodies should be possible. Indeed, B1 cell cloning in the mouse produced an IgM clone that was capable by itself of reconstituting reperfusion injury in otherwise protected immunoglobulin-deficient mice (10,11); other IgM clones were ineffective. The same dependency on natural antibody was seen after adoptive transfer of B1 cells from the peritoneal cavity of a normal mouse to that of an IgM-deficient mouse (17). A combination of immunoprecipitation and phage display library techniques led to the identification of the antigen in the injured tissue to which the pathogenic IgM binds (nonmuscle myosin heavy chain IIC) and the exact epitope (a 12–amino acid segment of the hinge region) (18,19). Knowledge of the hinge region amino acid sequence was used to develop a series of 12–amino acid peptide mimics or mimotopes. These were able to block virtually all evidence of injury in gut and skeletal muscle when given i.v. at the time of reperfusion in skeletal muscle and gut models (18,19) or when given topically to a deep second-degree burn (20). The exact sequence from the hinge region—LMK NMDPLNDNV—was used for the experiments described below and is designated *N2*.

The aim of this study was to (1) demonstrate the involvement of IgM and complement in HS, and (2) determine whether administration of this peptide mimic would improve parameters of HS. We chose this method, rather than studies in knockout mice, as we had previous data suggesting that different strains had radically different death rates from shock only, before resuscitation had ever taken place (21). The beneficial effects that we have observed in the experiments below strongly suggest that reperfusion injury produces significant mortality and morbidity in HS, and that rats possess the same natural IgM specificity and injury neo-antigen as do mice.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (125 – 200 g) were purchased from Charles River (Boston, Mass). A 12-h light/dark cycle was instituted, and food and water were provided. Animals were fasted overnight before the procedure. Animals in this study were maintained in accordance with the protocol of the Committee on Animals of Harvard Medical School and those prepared by the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (Department of Health, Education and Human Services, Publication no. 85–23 [National Institutes of Health], revised 1985).

Hemorrhagic shock

Animals were anesthetized with intraperitoneal pentobarbital (50 mg/kg). The right femoral artery was cannulated using a 24-gauge i.v. cannula (BD Insyte, Becton Dickson, Sandy, Utah). Over 10 min, experimental animals were bled to 50% of total blood volume (calculated by weight: $\text{weight [g]} \times 0.03 + 0.7 \text{ mL}$) into heparinized syringes (heparin sodium, 1,000 U/mL). Sham animals were cannulated but were not subjected to hemorrhage. At the end of the shock period (120 min), shed blood was returned slowly over 15 to 30 min. The catheter was removed, the incision was closed and anesthetized with 1 mL s.c. 0.125% bupivacaine, and animals recovered. In one experiment, animals were observed for survival for 72 h and then sacrificed. In other experiments, blood, lungs, and intestines (terminal ileum) were harvested at 4 and 72 h after shock. For N2 experiments, one group of rats received i.v. peptide N2 (sequence: LMKNMDPLNDNV, 300 μg ; New England Peptide, Mass) or control peptide (300 μg , see later) with the shed blood. The control peptide was a 12-amino acid peptide closely related to N2 and is the comparable hinge region for skeletal muscle myosin heavy chain (LDKNKDPLNETV). For cobra venom factor (CoVF) experiments, animals were complement depleted by intraperitoneal injections of 25 U CoVF (Quidel, Santa Clara, Calif) at 36, 24, and 12 h before shock. Complete complement depletion was confirmed by an undetectable serum CH50 in CoVF-treated rats (22).

Tissue edema

In a separate experiment, at 72 h, i.v. Evans blue (20 mg/kg) was injected 30 min before sacrifice (23). Bronchoalveolar lavage was performed via tracheostomy, 7 mL of sterile isotonic saline three times into lungs. Lavage fluid was centrifuged to remove cells and debris, after which its optical density was determined at 620 nm. The Evans blue tissue edema index for each animal was calculated as a ratio of bronchoalveolar lavage/serum Evans blue concentration.

Histological analysis

Harvested intestine and lungs were flushed and fixed in 4% paraformaldehyde for 24 h, sectioned, and stained using hematoxylin and eosin (H&E). Ten random areas of each lung specimen were blindly scored for the number of polymorphonuclear leucocytes (PMNs) per 400 $\times$ field. Intestinal injury was scored based on the following calculation: % injury = $(\text{number of villi with subepithelial space} / \text{number of total villi}) \times 50\% + (\text{number of villi with epithelial disruption} / \text{number of total villi}) \times 100\%$. Villi with the following two morphological changes were considered pathologically damaged and were counted separately: (a) presence of a subepithelial space, defined as an acellular space under a continuous epithelial layer and milder form of damage; and (b) epithelial disruption, defined as discontinuity of the epithelial layer of villus and more severe form of damage (24).

Immunohistochemistry

For IgM deposition, paraffin sections were blocked in 5% horse serum for 1 h incubated in 1/100 dilution of horseradish peroxidase–labeled goat antirat IgM (Sigma Chemical) overnight at 4°C, and developed with diaminobenzidine.

For complement deposition, sections were blocked with 5% rabbit serum, incubated with mouse antirat C5b-9 (gift from Dr Lester Kobzik) overnight at 4°C, and horseradish peroxidase–labeled horse antimouse IgG 1/100 (Sigma Chemical) was added and developed 1 h later (25). In many sections from shock animals, so many villi were sloughed that analysis of deposition was impossible. Whereas H&E analysis of injury used random sections, this analysis used sections with lesser degrees of injury and, therefore, more villi present for analysis.

Statistics

Data are expressed as the mean $\pm$ SE of mean in the text and figures. Groups were subjected to one-way ANOVA, and when significance was found, Student *t* test with the Bonferroni correction for multiple comparisons was applied. Kaplan-Meier method was used to analyze survival data. $P < 0.05$ was considered statistically significant.

RESULTS

The first sets of experiments were designed to determine whether survival in this rat model of HS and resuscitation was complement dependent. For this, rats were depleted of complement with CoVF for several days, subjected to HS and resuscitation, followed for 72 h, and percentage survival was determined. Complement depletion by this method improved survival at 72 h from 33% (2 of 6) in shocked nondepleted rats to 83% (5 of 6) in shocked complement-depleted animals ($P < 0.05$; data not shown). This demonstrated the possibility of complement-dependency similar to that seen in other reperfusion injury models, but the CoVF method also produces systemic neutrophil activation (and secondary failure of chemotaxis) by generation of complement anaphylatoxins. Thus, results using CoVF could also be interpreted to show granulocyte dependency instead.

Histological analysis was then used to define this issue further. This focused primarily on rat small intestine, where a pronounced injury to intestinal villus morphology was evident (Fig. 1). Gut injury score at 72 h after resuscitation was increased from $5.6\% \pm 4\%$ in sham-injured animals to $57.8\% \pm 5.5\%$ ($P < 0.05$, $n = 6$) in rats subjected to HS.

Immunohistochemical analysis at 4 h demonstrated evidence of complement C5b-9 deposition on the relatively intact nonsloughed villi of shocked animals compared with an absence in sham-injured animals (Fig. 2). This was also true for villus deposition of IgM, which was seen only in the animals undergoing shock and resuscitation (Fig. 3). Thus, the gut of the rats subjected to this HS preparation showed the same pattern of inflammatory protein deposition that had been seen in the complement-dependent mesenteric reperfusion injury models (24). Furthermore, these sections demonstrate little, if any, neutrophil infiltration at the 4-h time point. Together, this data points to complement, rather than neutrophil, dependency and a strong parallelism to pure reperfusion injury findings.

A second feature of mesenteric reperfusion injury is a pronounced secondary pulmonary injury. This model of shock and resuscitation was assessed for pulmonary PMN infiltration and pulmonary capillary permeability at 72 h (Figs. 4–6). Animals subjected to the shock preparation demonstrated marked neutrophil infiltration compared with sham animals: 21.4 ± 1.5 PMN per high-power field $\times 400$ vs. 12.8 ± 1 in sham animals ($P < 0.05$, $n = 6$; Figs. 4 and 5). As well, pulmonary capillary permeability increased from a permeability index of 0.07 ± 0.02 in sham animals to 0.61 ± 0.14 in shocked animals ($P < 0.05$, $n = 6$; Fig. 6).

Finally, plotting the data from all animals in which both intestinal injury had been scored and permeability index had been calculated at 72 h killing ($n = 14$), a linear correlation of increasing intestinal injury to increasing pulmonary injury was noted, $r = 0.72$ ($P < 0.05$; Fig. 7). Thus, many of the features of mesenteric reperfusion injury were recapitulated in this model of HS.

We then tested the ability of the soluble 12-amino acid mimic, N2, to change these parameters of HS. This agent is the exact sequence of the binding site of the pathogenic natural IgM to ischemic tissue and prevents binding of IgM by occupying the IgM ligation sites in the fluid phase (18,19), in effect, a decoy ligand. The agent, thus, is specific for reperfusion injury. Three hundred micrograms was selected as the dose based on the effective dose in mice (18,19) multiplied by mass of rat/mass of mouse, as if a rat were a large mouse. Although smaller doses of N2 are known to be effective pretreatment for reperfusion injuries (18,19) and burns (20), this timing of administration was chosen to mimic the clinical scenario of resuscitation from trauma. Administration of 300 μ g N2 peptide with the i.v. return of shed blood at the end of the shock period improved survival at 72 h from 38% (3 of 8) to 88% (7 of 8, $P < 0.05$; Fig. 8). In addition, gut injury score at 72 h improved from $57.8\% \pm 5.5\%$ in untreated animals to $19.5\% \pm 2.5\%$ in N2-treated animals ($P < 0.05$; Fig. 1). The prominent IgM and C5b-9 deposition seen in shocked animals was no longer visible in N2-treated animals (Figs. 2 and 3). Likewise, pulmonary injury was attenuated by N2 treatment, with reduced neutrophil infiltration (21.4 ± 1.5 PMN per high-power field in untreated animals to 14.8 ± 1.3 in N2-treated animals [$P < 0.05$]; Fig. 5) and reduced capillary permeability (permeability index, 0.61 ± 0.14 in untreated animals to 0.18 ± 0.06 in treated animals [$P < 0.05$]; Fig. 6). Finally, we tested 300 μ g of a control 12-amino acid peptide [Skeletal Muscle Myosin (SMM)] and saw only a slight effect on survival, gut injury score, or pulmonary permeability index. This peptide was, in fact, the sequence for the hinge region of skeletal muscle myosin, comparable in other respects to nonmuscle myosin. This lack of activity indicates a high degree of specificity to the action of N2 peptide (Figs. 1, 4–6, and 8).

DISCUSSION

We have chosen a simple model of resuscitated HS to test an agent specific for reperfusion injury and determine whether death and other effects caused by resuscitated shock is caused by global reperfusion injury. Another model of rodent HS using hemorrhage to a defined arterial pressure is more commonly in use because it produces measurable effects without death, the basic end point that we wished to examine. To that end, we titrated the shock time to produce 50% mortality at 72 h after resuscitation but no mortality during the shock period itself. It is important not to have loss of experimental animals during the injury phase of an experiment involving inflammation, as the animals lost could be those individuals with the most active proinflammatory systems. This is why we eliminated knockout mice as the method of study, as we have found significant differences in mortality from just the shock between relevant experimental strains (21); to study shock and resuscitation, the clinically relevant issue, all animals need to survive to resuscitation.

The findings on complement depletion by CoVF suggested that mortality in our model was influenced by the presence or absence of an intact complement system. However, a by-product of this depletion method is systemic generation of the complement anaphylatoxins, an event known clinically to impair neutrophil function (26,27). Thus, results with this method of depletion can also be interpreted as reflective of neutrophil involvement. The sCR1 (6) is an agent, now in commercial development (TP10; Avant Therapeutics, Needham, Mass), that is an inhibitor of complement C3b, C4b, and C1q. It was used extensively in the early studies of reperfusion injury (6, 24, and many others) to prove the

central role of complement in the pathogenesis of reperfusion injury. However, for our purposes, because sCR1 prevents generation of complement anaphylatoxins and, therefore, secondary pulmonary injury, use of this agent might not allow us to discriminate whether death from HS was caused by reperfusion injury or its secondary effects.

We chose instead to determine whether HS and resuscitation produced the typical appearance of a reperfusion injury in an organ, the intestine, thought to be particularly susceptible to such an injury. We found evidence of gut injury (Fig. 1) at 72 h in shocked animals, as quantitated by a scoring system that grades disruption of villi and lifting of the intestinal epithelium off of the mucosa. For this determination, 10 random sections in each animal were assessed. We also found that there was both complement (C5b-9) and IgM deposition on the villi of shocked animals (Figs. 2 and 3). Few neutrophils were evident. For this analysis, a 4-h time point was selected because later tissue necrosis may produce amplifying C3 and IgM deposition or loss of the target tissue altogether. In addition, less injured sections were used because it is impossible to assess deposition on villi that have completely disintegrated. Nevertheless, partial disruption of the tips of villi is visible clearly in the sections from shocked animals, a feature not present in sham-treated animals. This analysis strengthens the hypothesis from the CoVF experiment that complement-generated injury plays a role in HS. Furthermore, the presence of IgM, a hallmark of early reperfusion injury, indicated that the source of complement activation might be IgM deposition as a result of reperfusion injury (28). Thus, HS produces the typical features of reperfusion injury in the gut.

A second indication that a reperfusion injury might be present after HS is the development of a secondary pulmonary injury, a prominent feature of mesenteric reperfusion injury. Indeed, in this HS model, pulmonary neutrophil sequestration was produced (Figs. 4 and 5), as was increased pulmonary capillary permeability (Fig. 6). We observed a direct correlation between the severity of the gut injury and the severity of the pulmonary capillary leak (Fig. 7), suggestive of a causal link. Thus, HS might produce pulmonary injury as a result of reperfusion injury to the gut. However, this conclusion must be tempered by several other considerations: (a) We are reporting studies on only one zone of potential reperfusion injury. Other organs might be more severely affected and causing the pulmonary injury. (b) It is conceivable that the pulmonary injury itself is primary reperfusion injury and that the correlation represents the overall severity of HS on an individual basis.

The final indication that a reperfusion injury might be present is the inhibition of injury parameters caused by an agent specific to reperfusion injury. The development of this agent arose from a series of observations. First, gut and skeletal muscle reperfusion injuries were absent in antibody-deficient animals and were present if animals were reconstituted with IgM purified from normal mice (15,16). Second, mice lacking in natural IgM antibody repertoire were as protected from reperfusion injury as were the completely antibody-deficient mice. Restoration of injury was possible by either reconstitution with normal IgM or transplantation of peritoneal B-1 lymphocytes (17,29). Third, cloning of B-1 lymphocytes, the source of natural IgM, produced a single clone of IgM that was capable of restoring reperfusion injury in antibody-deficient mice (10,11). This implied that there was a specific antigenic target that appeared on ischemic tissue. This target was identified by interacting the pathogenic IgM clone with a 12-mer peptide phage display library and with the ischemic tissue itself. This yielded both the protein with which the IgM interacted and the site within the protein that was the antigen, nonmuscle myosin heavy chain. A series of similar 12-amino acid peptides was synthesized and tested for the ability to inhibit the interaction. The peptide that corresponds to the exact sequence in the parent protein, N2, inhibited reperfusion injury, whereas random peptides and even the peptide representing the hinge region from muscle myosin heavy chain did not (18,19). Thus, N2 peptide acts at the

initiation of reperfusion injury and not in its inflammatory amplification. As such, it is a highly specific inhibitor. We chose to administer N2 peptide in this HS model in real clinical time, that is, with resuscitation. Given in this way, N2 peptide prevented or nearly prevented all of the features that accompany HS that we assessed. Death rates were reduced (Fig. 8). Intestinal mucosal injury was reduced (Fig. 1), as was C5b-9 deposition and IgM deposition, the anticipated mechanism of action (Figs. 2 and 3). Pulmonary neutrophil sequestration and capillary permeability increases were attenuated (Figs. 4–6). Thus, an agent that is highly specific for reperfusion injury prevents death and stigmata of HS, suggesting that reperfusion injury causes the adverse consequences of HS in this model. The hypothesis that relates reperfusion injury to HS is not novel (3) nor is the attempt to prove this linkage or treat HS with agents developed from the study of reperfusion injury (30,31). The novelty of these findings is the simplicity of the agent (12-amino acid peptide), the high degree of specificity for the pathway involved in generating a reperfusion injury, and the ability to treat in real clinical time (at resuscitation).

There is a contrast in effects noted between the treatment of mesenteric reperfusion injury with complement inhibition (sCR1) (24) and the treatment of HS with N2 peptide. In the former, although sCR1 prevented increased pulmonary capillary permeability, it did not prevent increased pulmonary leukosequestration, as assessed by tissue content of myeloperoxidase. The interpretation was that pulmonary injury, as manifested by permeability changes, required both pulmonary endothelial activation and neutrophil activation. The sCR1 treatment, by preventing the generation of complement anaphylatoxins systemically, prevented the pulmonary endothelial activation. But the neutrophils continued to be activated by passage through the vasculature of injured gut, resulting in leukosequestration without injury. However, in this HS model, N2 peptide prevented both effects. This could indicate that the pulmonary injury in this HS model is not purely secondary to reperfusion injury in other susceptible organs and that the N2 peptide suggests that there is a primary pulmonary reperfusion injury as a result of HS.

As can be seen in Figure 1, not every animal is equivalently injured by HS nor protected by N2. In particular, there is a single HS animal without N2 treatment that has near-sham levels of lung permeability and intestinal injury score. There are also two N2-treated animals with limited protection against intestinal injury score but protection from increased lung permeability. Finally, in no case, with the possible exception of burn injury (20), have we ever observed a complete absence of injury in N2-treated animals. All these observations also apply to studies in inbred mouse experiments, where variability should be reduced. Although this is easily attributable to unnoticed technical variations or difficulties in challenging i.v. drug administration, it is possible that there is a biological explanation for this variation. First, there is likely to be some component of the injury that is purely metabolic and will remain after the amplification of the inflammatory system is removed. In that respect, the titration of our models has been based on maximal injury as reflected in mortality, rather than optimized for a measurement end point. Second, the titers of natural IgM are known to vary, even in inbred mice raised in the same cage, for unknown reasons (32). Animals with low titers of the pathogenic antibody, in theory, would be protected from reperfusion injury and, perhaps, HS. Likewise, high-titer animals might require higher N2 doses. Thus, premorbid knowledge of anti-nonmuscle myosin heavy chain IgM antibodies might predict susceptibility to injury. Finally, within a given otherwise homogeneous tissue, reperfusion injury in terms of cell necrosis, IgM deposition, or complement deposition is not uniform (33). The exact basis of individual, individual organ and individual cell-type susceptibility to reperfusion injury has yet to be clarified.

Finally, it should be noted that the N2 peptide was developed as a result of murine protein and antibody studies. This HS study is in rats. The sequence of the hinge region of

nonmuscle myosin heavy chain is identical throughout all species studied from yeast to humans. Thus, N2 is potentially effective in many species, as long as the basic mechanism of injury causing exposure of this antigenic region to circulating antibodies is maintained.

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GLOSSARY

IgM	Immunoglobulin M, a 750,000-kd pentameric antibody with 10 antigen-binding sites. Serum concentration 0.5 to 1 mg/mL.
Natural IgM	A subclass of IgM produced by B1 lymphocytes. The antigen specificity is gene encoded present at birth, and innate to each individual and species.
IgG	Immunoglobulin G, a 150,000-kd antibody with 2 antigen-binding sites. Antigen specificity and affinity increase with exposure to antigen through a multistep multicellular process. Produced by B2 lymphocytes of lymph nodes, spleen, and other organs.
B1 lymphocytes	Responsible for natural IgM production. CD5 ⁺ lymphocytes, identifiable by cell surface antigens surface IgM and CD11b. Primary population is self-replicating and resides in body cavities.
C3	Major complement protein involved in all three activation pathways.
C3b	C3 fragment that covalently binds to complement activators. Serves as scaffold for formation of lytic complement complex and amplified C3 cleavage. Serves as receptive antigen for immunological cell complement receptors. Stable.
C4b	Fragment of classical complement pathway C4 protein that binds to complement activators. Serves as scaffold for formation of C3-cleaving enzymes. Labile.
C1q	Collagenous protein that interacts with IgG or IgM to produce activation of the classical complement pathway.
C5b-9	Complement cell lytic structure that inserts into complement-activating membranes.
sCR1	Soluble form of complement receptor 1, the C3b receptor of immunological cells. Has potent complement inhibitory activity.
N2	a 12-amino acid peptide that is identical to the highly conserved hinge region of nonmuscle myosin heavy chain IIC: LMKNMDPLNDNV. Previously shown to prevent reperfusion injury.
SMM	a 12-amino acid peptide that is identical to the hinge region of skeletal muscle myosin heavy chain: LDKNKDP LNETV. Used as a control in these experiments.

FIG. 1. Intestinal injury score of rat small intestine at 72 h after shock and resuscitation, as assessed by grading of 10 random sections

Sham: animals undergoing cannulation, anesthesia, but no HS. Shock: animals undergoing withdrawal of 50% blood volume for 2 h, followed by return of shed blood, and observation for 72 h before sacrifice. N2: HS animals that received 300 μ g N2 peptide (nonmuscle myosin heavy chain sequence) with the return of shed blood. SMM: HS animals that received 300 μ g of control peptide (skeletal muscle myosin heavy chain sequence) with the return of shed blood. Error bars are SEM, $n = 6$ in each group. * $P < 0.05$ compared with sham. ** $P < 0.05$ compared with shock. The difference between N2 and SMM was significant ($P < 0.05$).

FIG. 2. Representative sections (H&E, original magnification $\times 100$) taken from rat small intestine 4 h after shock and resuscitation

A, C, and E, Sections reacted with mouse antirat complement C5b-9 neoantigen and developed by immunoperoxidase. B, D, and F, Sections stained with H&E. Intestines from rats undergoing HS showed villus disruption (B) and deposition of C5b-9 (A). This intestine was chosen to be relatively uninjured in terms of villus loss. Intestine from sham-treated rats showed no villus disruption (F) and no deposition of C5b-9 on villi (E). Intestines from HS rats that received N2 peptide also showed little villus disruption (D) and complement deposition (C).

FIG. 3. Representative sections (H&E, original magnification $\times 100$) taken from rat small intestine 4 h after shock and resuscitation

A, C, and E, Sections reacted with goat antirat IgM and developed by immunoperoxidase. B, D, and F, Sections stained with hematoxylin and eosin. Intestines from rats undergoing HS showed villus tip disruption (B) and villus deposition of IgM (A). This intestine was chosen to be relatively uninjured in terms of villus loss. Intestine from sham-treated rats showed no villus disruption (F) and no deposition of IgM on villi (E). Intestines from HS rats that received N2 peptide also showed little villus disruption (D) or IgM deposition (C). Panel (A) from an HS rat also showed a prominent vascular wall deposition of IgM, a feature also seen in pure I/R injury.

FIG. 4. Representative sections (H&E, original magnification $\times 100$) of lung 72 h after HS
Lung sections from rats undergoing HS (A) showed diffuse increase of interstitial cellularity, neutrophil infiltration, interstitial edema, and vascular congestion. Lung sections from sham-treated rats showed no infiltration (D). Lung samples from HS rats that received N2 peptide also showed little inflammation and infiltration (C). The HS animals treated with control peptide (SMM) showed congestion and infiltration (B).

FIG. 5. Pulmonary neutrophil sequestration in animals 72 h after HS and resuscitation, as assessed by mean number of PMN/400 × field from 10 sections/animal and six animals per group Sham: animals undergoing cannulation, anesthesia, but no HS. Shock: animals undergoing withdrawal of 50% blood volume for 2 h, followed by return of shed blood, and observation for 72 h before sacrifice. N2: HS animals that received 300 μg N2 peptide with the return of shed blood. SMM: HS animals that received 300 μg of control peptide (skeletal muscle myosin) with the return of shed blood. Error bars are SEM. * $P < 0.05$ compared with sham. ** $P < 0.05$ compared with shock. The difference between N2 and SMM was significant ($P < 0.05$).

FIG. 6. Pulmonary capillary permeability index in animals 72 h after HS and reperfusion, as calculated from extravasation of Evans blue dye

Evans blue index calculated by ratio of bronchoalveolar lavage over serum concentration of Evans blue 30 min after i.v. injection of Evans blue. The N2-treated animals showed lower index than shock animals ($P < 0.05$). SMM: HS animals that received 300 μ g of control peptide (skeletal muscle myosin) with the return of shed blood. The difference between N2 and SMM was significant ($P < 0.05$).

FIG. 7. Correlation of intestinal injury score to logarithm of pulmonary permeability index in animals subjected to HS and killed at 72 h, $r = 0.72$, $P < 0.05$

Circles are sham animals. Squares are animals undergoing HS. Triangles are animals undergoing HS with N2 treatment.

FIG. 8. Kaplan-Meier survival curve for HS mortality in the hours after resuscitation with shed blood (time 0)

Sham: animals undergoing cannulation, anesthesia, but no HS. Shock: animals undergoing withdrawal of 50% blood volume for 2 h, followed by return of shed blood, and observation at time 0. N2: HS animals that received 300 μ g N2 peptide with the return of shed blood. SMM: HS animals that received 300 μ g of control peptide (skeletal muscle myosin) with the return of shed blood. $n = 6$ in each group. * $P < 0.05$ compared with sham. ** $P < 0.05$ compared with shock. The difference between N2 and SMM was significant ($P < 0.05$).