Sepsis and septic shock are among the most life-threatening conditions managed by emergency physicians. The incidence of this condition in the United States has been estimated to be 751,000 cases per year, roughly three cases per 1,000 population.1 Many with the illness will die. Although traditionally deemed a disease of intensive care units, a recent prospective study has underscored the fundamental importance of early recognition and aggressive therapy of this syndrome in the emergency department (ED).2 A fundamental component of therapy in that trial was the early use of mixed-venous oxygen saturation to gauge oxygen kinetics in critically ill patients.

Oxygen-related metabolic abnormalities are common in life-threatening infection and occur at multiple scales throughout the host.3,4 Infection prompts the proliferation of hematopoietic cell lines renowned for their respiratory burst. Activated neutrophils and monocytes/macrophages produce superoxide, hydrogen peroxide, hypochlorous acid, nitric oxide, and peroxynitrite, each consuming oxygen.5 Widespread endothelial injury leads to loss of capillary integrity and tissue edema, lengthening the diffusion path from red blood cells to the mitochondrial inner membrane of peripheral cells. Mitochondria display a number of abnormalities that have been reviewed extensively.6 At the organ level, oxygen delivery (DO2) may be impaired by anemia, myocardial depression, maldistribution of splanchnic blood flow, and widened ventilation–perfusion inequality in the acutely injured lung. At the broadest scale, sepsis impacts central homeostatic mechanisms regulating physical activity and the wake–sleep cycle, both of which are major determinants of a host’s oxygen consumption during acute illness.7 To further complicate matters, in many septic patients, a so-called hypermetabolic state may eventually occur, although differences in oxygen consumption between septic patients with and without hypermetabolic features are subtle (about twofold) compared with differences between septic patients and normal humans performing daily activities such as exercise (about 12-fold).8

The net abnormality under these circumstances is usually decreased oxygen content in blood returning to the heart from distal vascular beds. As the utility of monitoring mixed or central venous oxygen content and devising therapies capable of improving it undergoes intense scrutiny in clinical trials, much remains unknown about the events leading to abnormal oxygen kinetics. It has long been recognized that lipopolysaccharide (LPS, an important structural...
constituent of the outer leaflet of the outer membrane of gram-negative bacteria) and, to a lesser extent, lipoteichoic acid (a similar part of the gram-positive cell wall) can precipitate many of the clinical features of sepsis in the absence of actual infection. These and other molecules set into motion a cascade of events that ultimately produce the clinical phenomenon of sepsis, including impaired oxygen kinetics. Yet the intermediate steps in this disease pathway remain obscure.

In the current study we explored the relationship between impaired oxygen kinetics and life-threatening infection using a mouse model of polymicrobial abdominal sepsis, namely, cecal ligation and puncture (CLP). Specifically, we studied the contribution of the complement cascade as a link in the development of abnormal oxygen kinetics. The complement cascade and, in particular, the mannose-binding lectin and alternative pathways are constitutively expressed and activated within seconds of exposure to gram-negative or gram-positive bacteria. Central features of complement activation in this setting are rapid opsonization of bacterial surfaces by activated C3 (needed for optimal phagocytosis by neutrophils and macrophages), assembly within the bacterial cell wall of the membrane attack complex (MAC), which, in some instances, may be directly bactericidal, and release of small peptides (C5a and C3a) that serve as potent activators of cellular immunity. The role of complement in sepsis has been described in several recent reviews.

Although many proteins contribute to complement activation, C5 occupies a pivotal position. Cleavage of C5 produces C5b, which initiates assembly of bactericidal MAC, and C5a. The role of C5a in life-threatening infection is complex. In the setting of gram-negative pneumonia, failure to generate C5a or the absence of the C5a receptor leads to an abnormal inflammatory response and increased mortality. But in models of abdominal sepsis, C5a production may be so robust as to harm the host. Widespread, dysregulated complement activation has been documented to produce deleterious effects on oxygen-dependent systems at the subcellular, cellular, and organ levels. Intravascular complement activation causes hypotension, metabolic acidosis, and loss of endothelial integrity in the lung and the gut. Strategies that block C5a’s effects have been shown to preserve neutrophil function, correct coagulation abnormalities, and improve survival in polymicrobial sepsis.

The specific contribution of complement, or to the best of our knowledge, any innate immune pathway, to oxygen kinetics during sepsis has not been demonstrated, although it is reasonable to suspect a connection. A link between immunologic events and clinical phenomena such as altered oxygen demand may lead to new therapies or generate further enthusiasm for strategies under study (such as anti-C5a treatment). In this study we examined the relationship between C5 and abnormal oxygen kinetics following CLP in mice. Using an open-circuit calorimeter, we characterized oxygen consumption, carbon dioxide production, and the respiratory exchange ratio (VO₂, VCO₂, and RER, respectively) after CLP and correlated changes with seven-day mortality. Next, the role of C5 in these metabolic changes was considered. We argued that if C5 was involved, a distinct oxygen kinetics phenotype might be observed in animals genetically deficient in C5. Specifically, if C5 linked peritoneal contamination with the development of oxygenation abnormalities, C5-deficient animals might demonstrate less severe abnormalities. Furthermore, an argument in support of a role of C5 would be strengthened if, in the absence of fecal contamination of the abdomen, sterile intraperitoneal complement activation reproduced the oxygen kinetic abnormalities seen in sepsis. Lastly, to specifically address differential contributions of C5a and C5b, we also studied animals in which a function-blocking antibody directed against C5a had been administered at the time of injury.

METHODS

Study Design. This was a laboratory investigation of abdominal sepsis using a well-established murine model. Sepsis was induced by CLP. Animal protocols were approved by the institutional committee on the use and care of animals, and animal handling conformed to existing regulatory policies on the use of animals in science.

Animal Preparation. Specific-pathogen-free ICR-strain outbred mice were obtained from Harlan Sprague Dawley, Indianapolis, Indiana. C5-deficient mice, B10.D2-He<sup>1</sup> H<sup>2</sup>d<sup>1</sup> H<sup>2</sup>-T18<sup>+/−</sup>/JSnJ and their control strains, B10.D2-He<sup>1</sup> H<sup>2</sup>d<sup>−</sup> H<sup>2</sup>-T18<sup>+/−</sup>/JSnJ (hereafter referred to as C5<sup>−−</sup> and C5<sup>+/−</sup>) were obtained from Jackson Laboratories, Bar Harbor, Maine. The C5<sup>−−</sup>-strain is homozygous for a naturally occurring two base-pair deletion in the 5’ end of the C5 open reading frame resulting in a premature stop codon and a nonfunctional protein. Reagents were obtained from Sigma Chemical Co., St. Louis, Missouri, unless otherwise noted.

Study Protocol.

Metabolic Measurements. The VO₂ and VCO₂ were measured using an indirect open-circuit calorimeter (Oxymax Deluxe, Columbus Instruments, Columbus, OH) that contained eight sealed cages and provided measurements every 30 minutes for each cage. The VO₂ and VCO₂ were determined by the difference in contents of each gas between the cage inlet and exhaust, with an electrochemical method used for
measurement of O₂ and an infrared spectroscopic method for CO₂. The RER was calculated as VCO₂/V O₂. Mice were weighed and placed in individual chambers with rodent chow (Labadhchow 5001, Purina Mills, Richmond, IN) and a water substitute (Napa Nectar, SE Lab Group, Maiden, NC) the day prior to the experiment. A six-hour initial measurement served as a baseline control for each animal. Experiments were always started in late afternoon to control for circadian fluctuations in activity and metabolism.

Cecal Ligation and Puncture (CLP). Mice were anesthetized with isoflurane in oxygen (Abbott Critical Care, North Chicago, IL). A 1-cm incision was made in the abdomen. The cecum was exteriorized and ligated below the ileocecal valve, and a 20-ga needle was passed through and through. The cecum was returned and the abdomen was closed in two layers. This produced a seven-day median lethal dose (LD₅₀) returned and the abdomen was closed in two layers.

Anti-C₅a Antibody. Affinity-purified polyclonal goat immunoglobulin G (IgG) raised against the C-terminus of rat C₅a (CTIADKIRKESHHKGMLLGR) was produced as described. Cross-reactivity with murine C₅a has been shown previously in a mouse CLP model. Mice receiving treatment with the antibody were administered 40 μg in 100 μL of saline via tail-vein injection at the time of CLP.

Sterile Peritoneal Inflammation. In some experiments, cobra venom factor (CVF) was used to produce intra-abdominal C₅ activation in the absence of fecal contamination of the peritoneal cavity. CVF, which forms a stable C₃/C₅ convertase with factor B and catalyzes sustained and unregulated cleavage of C₃ and C₅, was purified from raw venom using anion exchange chromatography as described. Contaminating LPS in the purified protein was reduced using a polymyxin-B column (Detoxigel, Pierce, Rockford, IL). Residual LPS content was measured with a Limulus assay (Associates of Cape Cod, Falmouth, MA) and found to be 0.4 ng/unit CVF. To produce complement activation, the animals received 30 units/kg CVF in 90 μL of saline intraperitoneally, a dose known to produce extensive activation in mice. As trace amounts of LPS were detectable in this material, an LPS-balanced vehicle was prepared using equal amounts of LPS in saline.

In additional experiments, sterile endotoxic peritonitis was induced with intraperitoneal injections of 80 or 400 μg of LPS in sterile saline.

Data Analysis. Values were reported as mean ± standard deviation. Changes in VO₂, VCO₂, and RER over time within each group were analyzed with analysis of variance (ANOVA) followed by Dunnett’s test for comparison of each time point with the time 0 value. Differences between groups (e.g., VO₂ trends between C₅⁺/⁺ and C₅⁻/⁻ animals) were analyzed using repeated-measures ANOVA with post-hoc comparisons made using the Tukey multiple comparisons correction. Survival analysis was done with a proportional hazards model as we have described previously.

RESULTS

VO₂, VCO₂, and RER Are Significantly Depressed by CLP. All measured oxygen kinetic parameters decreased significantly within three to six hours of CLP (p < 0.01, Figure 1). In particular, RER decreased from 0.85 to approximately 0.7, suggesting a change in metabolic substrate from carbohydrate to lipid early in the course of illness. While no death was seen in the first 24 hours, eight of 20 ICR-strain mice died within seven days of injury. When the animals were stratified by seven-day survival, no difference in VO₂, VCO₂, or RER over the first 24 hours was observed. Thus, although CLP produced rapid derangement in oxygen kinetics, the severity of these changes over the first 24 hours did not predict survival.

C₅ Deficiency Worsens Oxygen Kinetics and Increases Lethality. When C₅⁺/⁺ and C₅⁻/⁻ mice (both on the B10.D2, rather than ICR, genetic background) were subjected to CLP, both groups displayed more severe early abnormalities than similarly injured ICR mice (Figure 2). The VO₂ and VCO₂ were significantly worse in ill C₅-deficient animals. Importantly, 75% of the C₅⁻/⁻ animals died during the first 24 hours of injury, compared with no death in the C₅⁺/⁺ group (p < 0.05). Accordingly, most of the animals in the C₅⁻/⁻ group were moribund before the conclusion of the 24-hour data-collection period. This may explain swings in observed RER in this group, with some individuals demonstrating an RER > 1.0. This difference in response to infection underscores the importance of C₅ in early host defense. Rather than ameliorating oxygen kinetic abnormalities during abdominal sepsis, the absence of C₅ led to an accelerated course with greater abnormalities in VO₂, VCO₂, and RER.

Anti-C₅a Antibody Has Only a Modest Effect on Calorimetry during Sepsis. In parallel experiments using ICR-strain mice treated with function-blocking polyclonal IgG directed against the C-terminal of C₅a, no difference in VO₂ or VCO₂ was noted between the treated and untreated mice with sepsis. However, the anti-C₅a-treated mice demonstrated a statistically detectable but transient diminution in RER when compared with the untreated mice (Figure 2). The significance of this observation is uncertain, given that no change in VO₂ or VCO₂ was statistically detectable.
Intraperitoneal Complement Activation Does Not Mimic CLP.

Intraperitoneal activation of C5 using the C3:C5 convertase CVF produced changes in oxygen kinetics distinct from CLP (Figure 2).

In summary, the complement-related experimental results indicate a central role for C5 in survival during CLP. Rather than leading to worsened oxygen utilization in early sepsis, the presence of C5 somewhat ameliorated these abnormalities and was highly protective against early mortality. As anti-C5a treatment did not increase mortality or dramatically affect oxygen kinetics, these data point to C5b (and, by inference, the MAC) as being the predominant protective component of C5 in the first hours of injury.

Sterile LPS-induced Peritonitis Produces Calorimetric Abnormalities Similar to CLP.

Unlike sterile intra-abdominal complement activation, intraperitoneal administration of LPS was associated with a rapid fall in VO2, VCO2, and RER that closely resembled the changes seen following CLP, particularly at the 400-μg dose (Figure 3). We interpret these findings as indicating that while intraperitoneal complement activation was not observed to mimic the changes in oxygen kinetics seen during intra-abdominal infection, other immune pathways (in this case, presumably the LPS-binding protein/CD14/Toll-like receptor system) were capable of reproducing the effects of peritoneal soilage.

DISCUSSION

Innate recognition of extraluminal bacteria is an important early step in host defense against life-threatening intra-abdominal infections, and a number of strategies are constitutively in place to carry out this task. In the current study, we hypothesized that important global physiologic responses to infection, namely, changes in VO2, VCO2, and RER, might be linked to triggers in the complement cascade. The picture that emerges from the data is complex. C5 is a crucial component of host defense early in the course of injury. Its absence worsens the oxygen kinetic abnormalities produced by the model and, more importantly, markedly increases the lethality of CLP. As C5 plays two roles in host defense—initiation of MAC formation and release of C5a—we were interested in whether the defect in the C5-deficient animals could be tied to one role or the other. We found that treatment with a polyclonal function-blocking IgG directed against the C-terminal of C5a had only very modest effects on the oxygenation parameters and did not lead to early deaths, suggesting that the other central function of C5—assembly of MAC—is predominant.

Our results demonstrated striking changes in oxygen kinetics within hours of intra-abdominal injury. A clear role for C5 emerged, but not as expected; the C5-deficient mice experienced significantly greater mortality and had more severe derangements in oxygen kinetics than the C5-sufficient mice after injury. These abnormalities were distinctly different from those induced by sterile intraperitoneal complement activation. The differential responses to injury among normal mice and those treated with anti-C5a were modest and not sustained, suggesting that C5b, and not C5a, was necessary for host survival.

The current data reinforce the importance of distinguishing between C5b and C5a when evaluating...
models of infection. C5b is required for assembly of a directly antibacterial defense (MAC). C5a is a chemokine and proinflammatory mediator and thus impacts bacterial killing only indirectly by attracting and stimulating professional phagocytes. The contributions of C5a to defense may differ by site. The beneficial effects of C5 in life-threatening infection has been demonstrated by our group and others in studies of gram-negative pneumonia caused by Pseudomonas aeruginosa. Yet in models of intra-abdominal infection, presumably polymicrobial in origin and elicited by normal flora allowed access beyond their normal confines, anti-C5a therapy has been shown to improve a number of parameters in murine abdominal sepsis, most importantly, survival. These disparate findings may reflect differences between the bacterial adversaries present in the pneumonia and abdominal sepsis models or more fundamental differences between immunity within the peritoneum and at a mucosal surface.

Greater understanding of the role of C5 in life-threatening infections and in shock has recently received significant interest due to ongoing development of anticomplement strategies currently in clinical trials. Of particular interest to our group are those studies examining anti-C5 treatment in ischemia–reperfusion-associated conditions and cardiopulmonary bypass (CPB). CPB shares some common features with sepsis as a result of extensive activation of innate humoral immune pathways by blood contacting artificial surfaces within the perfusion apparatus. Recent work with pexelizumab, a monoclonal antibody that conceals the C5a–C5b cleavage site on the C5 molecule and thus prevents its activation, indicates a reduced risk of 30-day perioperative myocardial infarction and mortality following CPB. Clinical studies with this and other agents taking aim at the complement system are currently under way for a number of inflammatory conditions. A central issue will be how best to balance the potent anti-inflammatory effects of these agents with consequences they may have on host defense. This challenge is likely to be particularly difficult in the emergency setting, where inflammatory and infectious diseases may exhibit considerable clinical overlap and may be difficult to rapidly distinguish from one another. How targeted interference with the complement cascade, in infectious and noninfectious inflammatory conditions, ultimately finds its way into the emergency medicine arsenal is a question likely to see preliminary answers in the next five years.

Figure 2. Role of complement C5 in oxygen kinetic changes in sepsis. A. Metabolic response to sterile intra-abdominal complement activation using cobra venom factor (CVF). C5 activation did not produce the characteristic decline in each parameter seen with cecal ligation and puncture (CLP) (#p < 0.05 for vehicle-treated animals compared with baseline). i.p. = intraperitoneal. B. Oxygen kinetics in C5-sufficient (C5+/+) and C5-deficient (C5−/−) animals following CLP. Compared with the C5-sufficient animals, the C5-deficient mice displayed significantly worse oxygen consumption (VO2) and carbon dioxide production (VCO2). The wide swings seen in the respiratory exchange ratio (RER) in the C5−/− animals were likely related to the fact that 75% of these animals became moribund and died over the observation period (p < 0.05 by proportional hazards analysis). (p < 0.05 compared with baseline for C5+/+; #p < 0.05 for C5−/−). C. Effect of anti-C5a antibodies. Anti-C5a was associated only with a small difference in RER between the untreated and treated mice. (p < 0.05 compared with baseline for untreated control animals; #p < 0.05 for mice treated intravenously with anti-C5a polyclonal immunoglobulin G prior to injury.) n ≥ 4 animals per condition in all experiments.
LIMITATIONS

To the best of our knowledge, this is the first report to consider immune system genetic determinants of calorimetric behavior during sepsis in mice. Indirect calorimetry is a useful technique for evaluating metabolic changes in rodent models of life-threatening disease during which the prolonged anesthesia needed for invasive methods may be harmful. A limitation of the method is the difficulty with which a relatively hypermetabolic state might be identified if present in septic animals. Calorimetric measures in unanesthetized mice will reflect not just changes in metabolism, but also changes in activity (although the observed changes in RER in the current study indicate that the measured phenomenon was more than an indirect assessment of the animals’ tendency to huddle during acute illness). In many septic patients, an early fall in VO₂ may be followed by a relative hypermetabolic state. The increased oxygen consumption occurring in this state may, particularly when acute lung injury is also present, pose a significant challenge to caregivers. However, regardless of the presence or absence of hypermetabolism, the amount of oxygen consumed in sepsis is much smaller than is consumed in many activities performed in daily life. In the experimental conditions imposed by indirect calorimetry, particularly when uninjured animals serve as controls, relative hypermetabolic conditions are difficult to detect, and our methodology could not evaluate the role of complement in the hypermetabolic response that is often seen in sepsis and septic shock.

CONCLUSIONS

Cecal ligation and puncture in mice produces a pathologic signature of abnormalities in VO₂, VCO₂, and RER as measured with an open-circuit indirect calorimeter. The role of C5 in these changes in this mouse model appears to be related to protective effects of C5b rather than to aggravating effects of C5a activation.

The authors thank Janet Hoff and the Center for Integrative Genomics at the University of Michigan for their assistance with the calorimeter.

References


