Inhibition of C5 or absence of C6 protects from sepsis mortality

Jon A. Burasa, Lauren Ricea, Daniel Orlowa, Stavros Pavlidesa, Wende R. Reenstraa, Kathleen Ceonzob, Gregory L. Stahlb

aDepartment of Emergency Medicine, Beth Israel Deaconess Medical Center, Boston, MA 02215, USA
bCenter for Experimental Therapeutics and Reperfusion Injury, Department of Anesthesia, Brigham and Women’s Hospital, Boston, MA 02215, USA

Received 2 August 2004; accepted 7 September 2004

Abstract

Inhibiting complement anaphylotoxin C5a during sepsis may prevent sepsis mortality. Although human anti-C5 antibodies exist, their therapeutic use in microbial sepsis has been avoided because of the hypothesis that inhibiting C5b will prevent formation of the bactericidal membrane attack complex (MAC) and worsen clinical outcome. We wished to test the hypothesis that inhibition of C5 would improve outcomes in sepsis. Sepsis was induced in rats by laparotomy and cecal ligation and puncture (CLP) by an IACUC-approved protocol. Sham animals underwent laparotomy without CLP. Following CLP rats were randomized to receive a single IV dose of purified IgG ant-C5 antibody (Ab) or control IgG Ab. Anti-C5 Ab treated rats (n = 20) had significantly lower mortality vs. controls (n = 21); 20% vs. 52% (P = 0.019; log-rank). Analysis of bacterial load by culture of spleen and liver homogenates showed a reduction in colony forming units in anti-C5 Ab treated rats vs. control IgG (P = 0.003 and 0.009, respectively). Anti-C5 treatment reduced lung injury as measured by total MPO content of lung tissue (P = 0.024). Finally, rats genetically deficient in C6 production, unable to form MAC but capable of producing C5a and C5b, were protected from CLP-induced sepsis mortality. Our results show that an anti-C5 antibody therapy prevents CLP sepsis-induced mortality and improves lung injury. Inhibition of the complement MAC does not increase bacterial load or mortality, therefore, the use of anti-C5 therapy may be beneficial rather than detrimental in sepsis.

Keywords: Complement; C5b-9; C5a; Antibody; Therapy

Introduction

Sepsis remains a significant cause of morbidity and mortality affecting greater than 500,000 people annually (Hotchkiss and Karl, 2003). Despite multiple years of research, there are few proven therapies available for sepsis. Recently, complement inhibition has been identified as a potential sepsis therapy (Ward, 2004; Gerard, 2003). Experimental studies have shown that dysregulated overproduction of the anaphylatoxin C5a may be detrimental in sepsis (reviewed in Ward, 2004). Limited human study suggests that elevated C5a serum levels may correlate with mortality in sepsis (Nakae et al., 1996). Direct inhibition of C5a via blocking antibodies or via C5a receptor (C5aR) has reduced mortality from sepsis (Czermak et al., 1999a; Strachan et al., 2000). The mechanism of anti-C5a therapy in sepsis is mediated in part through improved neutrophil phagocyte function and enhanced NADPH oxidase assembly (Huber-Lang et al., 2002).
Inhibition of C5a rather than C5 is the suggested point of complement inhibition during sepsis as this strategy allows production of the C5 split product C5b (Gerard, 2003). C5b is required for optimal formation of the multimeric protein C5b-9 terminal membrane attack complex (MAC). The MAC is assumed to have a protective role in sepsis through its potential ability to directly lyse bacteria in vivo (Bloch et al., 1997). There is also some evidence supporting a role for C5b in clearance of Pseudomonas aeruginosa during experimental lung infection (Younger et al., 2003). However, direct evaluation of MAC function during sepsis has not been fully described. There is some evidence suggesting that C5a and C5b-9 are not always required for host responses in sepsis. Experiments employing genetically C5-deficient B10 mice demonstrated prolonged survival time and reduced lung injury following sepsis-induced cecal ligation and puncture (CLP) (Olson et al., 1985). Also, C5 and its split products were not required for pulmonary clearance Bordetella bronchiseptia in vivo (Pishko et al., 2004). Furthermore, genetically C5-deficient mice are less susceptible to mortality and organ damage induced by zymosan (Nieuwenhuiizen et al., 1995; Mahesh et al., 1999). These studies suggest that C5 may not be essential to all host responses during inflammation and sepsis. However, these studies are limited as the genetically deficient C5 animal model has the potential for unrecognized adaptive compensatory responses. Furthermore, study of C5 deficiency does not discriminate between the specific contributions of C5a and C5b to the host sepsis response. Acute inhibition of C5 or isolated inhibition of MAC in the presence of functional C5a during sepsis has not been reported. The first objective of this study was to evaluate whether acute inhibition of C5 represents a potential target in treatment of polymicrobial sepsis. A second study objective was to determine whether C5 inhibition altered bacterial clearance as a consequence of reducing C5a and C5b/MAC levels. The final study objective was to determine the relative contribution of MAC to sepsis outcome in the presence of functional C5a.

Material and methods

Antibody preparation

Creation and preparation of the mouse anti-rat monoclonal anti-C5 antibody 18A was described in Vakeva et al. (1998).

CLP-sepsis model

Specific pathogen free male PVG rats were purchased from Taconic (Germantown, NY). The C6-deficient PVG rat strain has been previously described (Brauer et al., 1993). All procedures were conducted under an IACUC-approved protocol. Anesthesia was induced with isoflurane and oxygen provided via a nosecone mask. A 2 cm vertical midline incision was made in the lower abdomen, carried to the peritoneum using blunt dissection, and cecum identified. The cecum was lighted below the ileo-cecal valve using 4-0 vicryl suture. The ligated cecum was punctured through-and-through using a 21-gauge needle and compressed to express a small amount of fecal material to ensure patency of the puncture sites. The cecum was returned to the abdominal cavity and peritoneum and abdominal muscles closed with 5-0 vicryl sutures and the overlying skin approximated with surgical staples. Rodents received a 5 ml subcutaneous dose of normal saline for fluid resuscitation. Twenty minutes prior to closure of the skin, rats received a subcutaneous injection of 1.6 mg/kg of buprenorphine for analgesia. Following surgery, animals received additional buprenorphine at 12 h intervals.

Bacterial load

At necropsy, spleens and livers were harvested and equal wet tissue weights were homogenized and briefly centrifuged to remove gross particulate matter. Serial log dilutions of tissue homogenates were applied to chocolate agar plates and incubated at 37°C for 24 h under aerobic conditions. Plates were subsequently analyzed by colony counts and expressed as CFU/mg wet tissue weight.

ELISA

Peritoneal fluid (PF) was collected immediately following euthanasia. Briefly, following a midline incision the peritoneal cavity was washed with 5 ml of normal saline. Recovered PF was briefly centrifuged to remove cellular debris and stored at -80°C until analysis. Serum was obtained from blood collected by cardiac puncture at necropsy, allowed to clot at room temperature for 1 h, centrifuged at 25,000g, and stored at -80°C until analysis. ELISA analysis of IL-6 (R&D Systems) was performed according to the manufacturer’s specifications. Sample ODs were read using a microtiter plate spectrophotometer (Spectramax 190, Molecular Devices) and data analyzed using the Softmax Pro software package (Molecular Devices).
Tissue MPO activity

Measurement of tissue MPO was performed as described in Vakeva et al. (1998). Briefly, lung tissue was weighed and homogenized in 4 ml of buffer containing 0.25 M sucrose and 0.1 mM EDTA. Samples were centrifuged at 30,000 g at 4 °C for 30 min. Pellets were subjected to freeze/thaw and sonication for three cycles. Tissue was homogenized in 0.5% hexadecyltrimethyl ammonium bromide in 500 mM potassium phosphate buffer (pH = 6.0), and then centrifuged at 12,500 g at 4 °C for 15 min. Supernatants were collected and reacted with 0.167 mg/ml 0-dianisidine dihydrochloride and 0.0005% H₂O₂ in 50 mM Potassium phosphate buffer (pH = 6.0). Changes in absorbance were measured on a microtiter plate reader (Spectromax 190, Molecular Devices) at 460 nm over 10 min. Linear slope of absorbance change was determined for each sample. One unit of MPO activity was defined as that quantity of MPO that hydrolyzed 1 μM H₂O₂/min at 25 °C. Final activity amounts were expressed as units/gram wet tissue weight.

Statistical analysis

Data is expressed as mean +/- standard deviation unless otherwise noted. Statistical analysis was performed using ANOVA with the exception that survival analyses were performed by log rank test. Significance level was set at P value < 0.05. Statistical analyses were performed using the Statview and JMP software packages (Abbacus/SAS Inc., CA).

Results

C5 inhibition prevents sepsis mortality

We wished to determine whether acute inhibition of C5 was beneficial or detrimental to mortality outcome in sepsis. Long Evans rats were subjected to CLP sepsis and randomized to receive either control IgG Ab or 18A Ab by tail vein injection upon conclusion of the surgical procedure. A 20 mg/kg dose was chosen based on prior study documenting functional C5 inhibition in vivo through 8 h (Vakeva et al., 1998). Administration of 18A conferred protection from mortality during sepsis as compared to control IgG over the study period (Fig. 1, P = 0.019).

C5 inhibition reduces bacterial load

To determine whether acute inhibition of C5 affected bacterial clearance during CLP sepsis, Long Evans rats were subjected to CLP sepsis and randomized to receive either control IgG Ab or 18A by tail vein injection upon conclusion of the surgical procedure. At 24 h following CLP, bacterial load was assessed in spleen and liver homogenates. As shown in Fig. 2, CLP induced significant increases in splenic and liver bacterial load vs. sham controls (P = 0.003 and 0.016, respectively). Treatment with anti-C5 mAb resulted in a significant reduction in both splenic and liver bacterial load vs. treatment with control IgG Ab (P = 0.003 and 0.009, respectively). While splenic bacterial load in anti-C5 mAb-treated rats trended higher than sham rats, the difference was not statistically significant (P = 0.452). These results suggest that neither C5a nor C5b-9 is acutely required for bacterial clearance during the development of CLP sepsis.

C5 inhibition reduces CLP-induced lung injury

Previous study has shown that C5a inhibition reduces sepsis-induced lung injury (Czermak et al., 1999b). We wished to determine whether inhibition of C5 was also capable of reducing secondary lung inflammation induced by CLP. Following CLP and treatment with either 18A or IgG, lungs were analyzed for total MPO content. Fig. 3 shows that CLP induced a significant increase in total lung MPO content following 24 h of sepsis (P = 0.013). Treatment with 18A reduced total lung MPO content compared to IgG-treated animals after 24 h of CLP sepsis (P = 0.024).

C5 inhibition increases local and reduces systemic IL-6 levels following CLP sepsis

Enhanced IL-6 levels may correlate with poor outcomes in human sepsis (Calandra et al., 1991; Gardlund et al., 1995). Inhibition of C5a during sepsis leads to a reduction of IL-6 production (Hopken et al., 1996).
However, profound inhibition of IL-6 during CLP sepsis is detrimental to survival (Leon et al., 1998; Riedemann et al., 2003). To determine whether inhibition of C5 affected IL-6 expression during CLP, we evaluated IL-6 levels in plasma and peritoneal fluid from rats following CLP and treatment with either IgG or 18A. Following CLP, C5 inhibition increased IL-6 levels at 24 h in peritoneal fluid as compared to IgG treatment (Fig. 4, \( P = 0.047 \)). Conversely, serum levels of IL-6 at 24h were reduced by C5 inhibition (Fig. 5, \( P = 0.039 \)).

C6-deficiency protects from CLP sepsis mortality

The specific contribution of MAC to the host sepsis response is unknown. To determine the effect of MAC inhibition in the presence of functional C5a during sepsis, CLP sepsis was induced in C6-deficient and background PVG rat strains. Fig. 6 shows that genetic deficiency of C6 was protective from CLP-induced mortality (0% vs. 80%, \( P = 0.003 \), log rank test). As C6-deficient rats are capable of producing C5a, but not MAC, this data suggests that MAC may actually generate deleterious effects during the host sepsis response.

Discussion

We have shown that acute inhibition of C5 with an anti-C5 mAb protects from mortality following CLP.
Previous study has shown that the C5-deficient mouse strain B.10 exhibited a longer survival than the control strain, yet, all animals eventually succumbed to sepsis in that study (Olson et al., 1985). These data suggest that inhibition of both C5a and C5b affords some protection during the sepsis host response. However, it is unknown whether this observation is due to adaptive responses of the mutant strain or whether such protection is afforded to different species. Acute antibody-mediated inhibition of C5 confirms the protective effect of reduced C5 levels during CLP sepsis without additional confounding variables of genetic adaption.

Inhibition of C5a by antibody or receptor antagonists has been successful in reducing experimental sepsis mortality and tissue injury (Czermak et al., 1999a; Strachan et al., 2000; Huber-Lang et al., 2001). Previous hypotheses regarding anti-complement therapy strate-
gies for sepsis suggested that the ideal agent would inhibit only C5a and allow C5b function intact for its presumed direct bactericidal functions (Gerard, 2003). Our data suggest that C5 inhibition of both C5a and C5b is not detrimental to outcome from polymicrobial peritonitis-induced sepsis. The reduced bacterial load following C5 inhibition observed in our study suggests that MAC does not actively contribute to early bacterial clearance from the peritoneum. The lack of requirement for functional MAC in bacterial clearance of B. bronchoseptia in vivo supports the notion that MAC is not universally needed for survival from all bacterial infections (Pishko et al., 2004). This is further supported by the observation that human subjects deficient in MAC components suffer only from an increased incidence of neisserial infections rather than all bacterial species (Ross and Densen, 1984).

It is possible that the reduction in mortality observed in our study is due to the inhibition of C5a production. However, the protection from mortality observed in the C6 deficient rats suggests that C5a is not the only detrimental complement product produced during the host sepsis response. The C6-deficient rats possess the ability to make functional C5a and C5b, yet lack the ability to create functional MAC. Improved survival of the C6 deficient rats in our study suggests that the MAC itself may be some how detrimental to the host response during sepsis. Again, this possibility is supported by the observation that human subjects deficient in MAC components may suffer less severe forms of disseminated neisserial infections despite greater disease incidence in this population (Ross and Densen, 1984).

The MAC mediates cellular damage and apoptosis during ischemia/reperfusion injury, however, the role of MAC in sepsis is less well defined (Hughes et al., 2000; Zhou et al., 2000). It is possible that MAC induces apoptosis and cellular injury during sepsis via similar mechanisms, but this hypothesis is untested at present. Prior study has shown that MAC may stimulate procoagulant activity of the endothelium (Weidmer et al., 1986). Recently, the administration of activated Protein C has improved sepsis mortality outcome in humans (Bernard et al., 2001). It is attractive to postulate that a reduction in MAC exerts a beneficial effect on the endothelium and coagulation system similar to activated Protein C. Finally, it is possible that the C6-deficient rats have developed compensatory adaptive responses that account for the observed response to sepsis. Further study using agents to acutely inhibit MAC function during sepsis in a normal genetic background should clarify this question.

Inhibition of MAC had opposite effects upon IL-6 production in peritoneal and vascular compartments. We found a similar reduction in serum IL-6 expression with C5 inhibition as has been reported with C5a

![Fig. 5. C5 inhibition reduces serum IL-6 levels. Serum IL-6 levels were determined following 24h of sham treatment, CLP +18A, and CLP + IgG Ab. Sham, n = 3; CLP + IgG, n = 4; CLP + 18A, n = 5. IL-6 pg/ml: sham, 55 ± 44; CLP + IgG, 811 ± 334; CLP + 18A, 284 ± 391.](image)

![Fig. 6. C6-deficient rats are protected from sepsis mortality. Several in PVG rats deficient in C6 n = 10 (dashed line) vs. wild type PVG strain n = 20 (solid line). P<0.003, log rank test.](image)
inhibition (Riedemann et al., 2002; Hopken et al., 1996). The trend of greater IL-6 expression in the peritoneal compartment as compared to control animals was unexpected. However, previous study in murine systems suggests that IL-6 levels may not always correlate directly with the severity of sepsis induced by CLP (Walley et al., 1996). Previous work has suggested that enhanced serum IL-6 levels may correlate with mortality in humans (Calandra et al., 1991; Gardlund et al., 1995). However, rodent studies have shown that IL-6 expression is required for survival from CLP sepsis (Leon et al., 1998). It is possible that inhibition of C5 and MAC allows differential expression of IL-6 depending on the compartment of infection. The reduction of serum IL-6 expression may also be an indirect result of the containment of infection within the peritoneum and prevention of sepsis development.

We wished to determine whether C5 inhibition reduced distal lung inflammation which may be induced by CLP. The link between CLP and lung injury is variable in the literature. CLP induction of lung injury and tissue MPO may depend on the exact CLP model used. Some have shown enhancement of lung injury and lung MPO at 24h following CLP (Goya et al., 1992; Matsukawa et al., 2001). Others have suggested that a secondary insult may be required for development of lung injury following CLP (Czermak et al., 1999b). The severity of the CLP-induced sepsis model may account for these conflicting results. Previous reports have shown that increasing the needle gauge and puncture size will alter sepsis pathogenesis and significantly enhance mortality (Baker et al., 1983; Walley et al., 1996). CLP sepsis-induced lung injury may be prevented by C5a inhibition (Czermak et al., 1999b). MPO may not have been induced by CLP alone as the model utilized was sub-lethal with a 36h mortality of 12% (Czermak et al., 1999b). Our model induced a more severe form of sepsis as determined by comparing differences in the observed 36h mortality rates, 52% vs. 12%, respectively (Czermak et al., 1999b). It is likely that the observed level of lung inflammation is due to the enhanced severity of sepsis in our model system. The observed reduction of MPO content in lung following C5 inhibition is likely related to a similar indirect host response to the prevention of a lethal septic state.

Our data demonstrate that acute inhibition of C5 represents a successful strategy for improving host survival during polymicrobial peritoneal sepsis. Furthermore, our data suggest that C5 and its split products are not absolutely required for bacterial clearance during intra-abdominal infections. Further experimentation should determine whether anti-complement therapy is applicable to sepsis induced by other pathogens, such as gram-positive bacteria, and within other anatomic compartments. Finally, the improved survival of C6-deficient rats indicates a possible regulatory role of MAC in the pathogenesis of sepsis, suggesting that MAC alone could represent a therapeutic target.

Acknowledgements

This work was supported in part by the Department of Emergency Medicine, Beth Israel Deaconess Medical Center and NIH award AI62689 to JAB; and also in part by HL56086 and HL63927 to GLS. The authors wish to thank Russel Rother, Ph.D., Alexion Pharmaceuticals Inc., for helpful discussion and review of the manuscript.

References


