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Early antibiotic administration but not antibody therapy directed against IL-6 improves survival in septic mice predicted to die based upon high IL-6 levels

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Abstract

Elevated interleukin (IL)-6 levels correlate with increased mortality following sepsis. IL-6 levels >14,000 pg/ml drawn 6 hours following cecal ligation and puncture (CLP) are associated with 100% mortality in ND4 mice, even if antibiotic therapy is initiated 12 hours after the septic insult. The first aim of this study was to see if earlier institution of antibiotic therapy could improve overall survival in septic mice and rescue the subset of animals predicted to die based upon high IL-6 levels. Mice (n=184) were subjected to CLP, had IL-6 levels drawn six hours later and then were randomized to receive imipenem, a broad spectrum antimicrobial agent, beginning six or twelve hours post-operatively. Overall one-week survival improved from 25.5% to 35.9% with earlier administration of antibiotics (p<0.05). In mice with IL-6 levels >14,000 pg/ml, 25% survived if imipenem was started at 6 hours, while none survived if antibiotics were started later (p<0.05). Based upon these results, we examined whether targeted antibody therapy could improve survival in mice with elevated IL-6 levels. A different cohort of mice (n=54) had their blood drawn six hours after CLP and then were randomized to receive either monoclonal anti-IL-6 IgG or irrelevant rat IgG. Anti-IL-6 antibody failed to improve either overall survival or outcome in mice with IL-6 levels >14,000 pg/ml. These results demonstrate that earlier systemic therapy can improve outcome in a subset of mice predicted to die in sepsis, but we are unable to demonstrate any benefit in similar animals using targeted therapy directed at IL-6.

Keywords

Cecal ligation and puncture; cytokine; imipenem; critical illness

INTRODUCTION

Despite aggressive treatment, between 120,000 and 210,000 people die of sepsis annually in the United States (2,15). Early goal-directed therapy in sepsis is associated with a marked improvement in overall patient outcome (22) although there is currently no way to know which specific patients will have the most favorable response to this therapy. The pro- and anti-inflammatory cytokine interleukin (IL)-6 is associated with increased mortality in patients with sepsis (7,10,11,17,23). In addition, IL-6 levels drawn 6 hours after the onset of sepsis predict

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mortality with high specificity in mice subjected to either cecal ligation and puncture (CLP) (20,26,27,29) or *Pseudomonas aeruginosa* pneumonia (5).

While IL-6 levels correlate with mortality in sepsis, its role as a mediator of the septic response is more complicated. Mice have improved survival if given anti-IL-6 antibody immediately after CLP (21), or 2 or 4 hours following gavage with *Escherichia coli* and thermal injury (9). These effects are time-dependent since mortality is unaffected if anti-IL-6 is given 4 hours following CLP (21) or 8 hours after bacterial gavage and burn (9). Further, model-dependent results have been seen in IL-6 knockout mice, which have similar survival to wild type animals subjected to CLP or given LPS (6,14,19), die earlier (but have similar overall outcomes) if infected with *Trypanosoma cruzi* (8), and have increased mortality if given *Escherichia coli* (6). Of note, IL-6 has been shown to modulate some physiologic responses following CLP (such as weight gain, thermoregulation, and white blood cell count) even though mortality is similar between wild type and IL-6 knockout mice (19).

Outbred ND4 mice with IL-6 levels >14,000 pg/ml have a 100% mortality following CLP regardless of whether they are treated with antibiotic therapy beginning 12 hours following the onset of sepsis (26). This timing of antibiotic administration is clinically relevant since earlier antibiotic administration is associated with improved survival in septic patients (12,13) but it has been proposed that the abdomen must be exposed to infectious material for at least 12 hours for an infection to develop (16).

Whether administration of antibiotic therapy prior to 12 hours after the onset of sepsis improves survival is unclear. We therefore studied the effect of antibiotic administration begun 6 hours after CLP on survival in ND4 mice. A pre hoc study design decision was made to separately analyze mice with IL-6 levels >14,000 pg/ml since this subset of animals (representing approximately 20% of all septic mice) has a markedly worse outcome than genetically similar animals subjected to the same insult. After seeing whether a nonspecific systemic therapy would change outcome in mice with markedly elevated IL-6 levels, we examined whether targeted antibody therapy designed to decrease these levels would improve survival.

MATERIALS AND METHODS

Sepsis Model

Six to ten week old male ND4 mice (Harlan, Indianapolis, IN) were made septic by CLP by the methods of Baker et al and as previously described (3,25). Briefly, anesthesia was induced with 5% halothane and maintained with 2% halothane. Following a midline laparotomy, the cecum was exteriorized and ligated distal to the ileocecal valve without causing intestinal obstruction. The cecum was then punctured twice with either an 18 or 21 gauge needle, and stool was gently extruded. The abdomen was closed in layers, and 1 ml of 0.9% NaCl was injected subcutaneously to compensate for insensible fluid losses. All animals were allowed to acclimatize for one week prior to surgical manipulation and were maintained on 12 hour light-dark cycles with free access to food and water at all times. All studies were approved by the Washington University Animal Studies Committee in accordance with the National Institutes of Health guidelines for the use of laboratory animals.

IL-6 determination

A total of 70 μ l of blood was harvested from the tail veins of each animal either 6 or 12 h after CLP and centrifuged at 3000g for five minutes to separate plasma. Unless otherwise specified, IL-6 levels were always drawn prior to the initiation of antibiotic therapy or administration of anti-IL-6 antibody. IL-6 levels were measured using the enzyme-linked immunosorbent assay via a commercially available kit (R&D Systems, Minneapolis, MN) according to manufacturer

specifications. IL-6 levels were measured in duplicate, and the reported results represent an average of these readings.

Antibiotic therapy

All animals received subcutaneous injections of Imipenem (25 mg/kg, Merck, West Point, PA) beginning either 6 or 12 hours following CLP. Antibiotics were given every 12 hours and continued for 5 days or until death.

Anti IL-6 antibody

Mice were given an intraperitoneal injection of monoclonal rat anti-mouse anti-IL-6 IgG or irrelevant rat IgG (BD PharMingen, San Jose, CA) six hours following CLP. The antibody was chosen because of published data demonstrating its success in improving survival if given immediately after CLP in B10D2 nsnJ mice (21).

Survival studies

All animals were followed 7 days postoperatively for survival unless otherwise specified. When designing this study, a pre hoc decision was made to evaluate both overall survival and survival in a subset of animals with IL-6 levels >14,000 pg/ml since previous studies have shown these animals have 100% mortality following CLP, regardless of the type of injury used or if antibiotic therapy is initiated 12 hours postoperatively (26).

Statistics

Differences in survival were analyzed using the Log-Rank test. IL-6 levels were compared using one way analysis of variance for group comparisons and unpaired t-test for pairwise comparison. The percentage of mice with IL-6 levels > 14,000 pg/ml that survived when antibiotics were initiated 6 hours after CLP and the percentage that survived when antimicrobial therapy was started 12 hours after CLP were compared using Fisher's exact test. Data analysis was performed using GraphPad Prism 3.0 (GraphPad Software, San Diego, CA). P values < 0.05 were considered to be statistically significant.

RESULTS

IL-6 levels >14000 pg/ml predict mortality

Mice (n=63) were subjected to double puncture CLP with an 18 gauge needle and subsequently had IL-6 levels drawn six hours post-operatively and antibiotic therapy initiated 12 hours post-operatively. Animals were followed seven days for survival and retrospectively analyzed to see if all those with IL-6 levels > 14,000 pg/ml died. Overall survival was 15.9 percent. All 14 mice with IL-6 levels >14,000 pg/ml died.

Earlier administration of antibiotics improves overall survival following CLP

Based upon the high mortality seen in the above pilot experiments, all additional experiments were performed using double puncture CLP with a 21 gauge needle. The next cohort of animals was randomized to receive imipenem beginning either six hours (n=78) or 12 hours (n=43) post-operatively. IL-6 levels drawn six hours after CLP (i.e. before antibiotic therapy was begun in either group) were similar in the two groups (p=n.s.). Earlier antibiotic therapy was associated with increased survival – 35.9% (28/78) for those treated with imipenem begun 6 hours following CLP compared to 25.5% (11/43) for those whose antibiotics were begun 12 hours after the onset of sepsis (p<0.01, Fig. 1).

Earlier administration of antibiotics rescues a subset of animals with IL-6 levels >14,000 pg/ml following CLP

Survival was compared between mice that received antibiotics starting 6 or 12 hours following CLP with IL-6 levels >14,000 pg/ml. Survival was 25% in mice that had antibiotics started at 6 hours (4/16) compared to 0% in animals where antibiotics were started at 12 hours (0/18), suggesting that earlier antibiotic therapy can rescue a subset of mice that would have been predicted to die based upon elevated IL-6 levels (Fig. 2, $p<0.05$). When the data in figure 2 was analyzed by examining IL-6 levels in increments of 1,000 pg/ml, earlier antibiotic therapy was noted to improve survival in mice with IL-6 levels <9,000 pg/ml or >14,000 pg/ml, but was not predictive of survival in mice with IL-6 levels between these values (Fig. 3).

Anti-IL-6 antibody fails to improve either overall mortality or survival in the subset of animals with IL-6 levels >14,000 pg/ml following CLP

Since the above results demonstrated that generalized systemic therapy could improve survival in a subset of mice with IL-6 levels >14,000 pg/ml, we next examined whether targeted therapy with a monoclonal antibody directed against IL-6 could improve survival in this subset of mice. A new cohort of animals ($n=54$) was randomized to receive monoclonal rat anti-mouse anti-IL-6 IgG at doses of 1.33 mg/kg or 2.66 mg/kg (doses chosen based upon reference (21)) or irrelevant rat IgG six hours following CLP. All animals had IL-6 levels drawn six hours post-operatively (i.e. immediately prior to administration of anti IL-6 antibody), and each received imipenem beginning 12 hours following CLP. IL-6 levels drawn at 6 hours before antibody administration were similar in all groups (Fig. 4A, $p=n.s.$) although retrospective analysis demonstrated that mice with higher IL-6 levels following sepsis eventually had a higher mortality (Fig. 4B, $p<0.005$). However, anti-IL-6 antibody had no impact on either overall survival (Fig. 5) or survival in the subset of animals with IL-6 levels >14,000 pg/ml, which all died regardless of treatment (Fig. 6).

To verify that anti-IL-6 antibody was functionally active, an additional cohort of mice ($n=22$) was examined to determine its effectiveness in blocking IL-6. Mice were subjected to CLP, randomized to receive either 2.66 mg/kg anti-IL-6 IgG or irrelevant rat IgG at six hours, and then had blood drawn for IL-6 levels twelve hours post-operatively. IL-6 levels decreased greater than five-fold six hours following targeted antibody therapy (Fig. 7). Of note, this is the only experiment described where IL-6 levels were not drawn six hours following CLP and the only time IL-6 levels were drawn after pharmacologic intervention.

DISCUSSION

This study demonstrates that starting antibiotics 6 hours after the onset of sepsis yields an improvement in overall survival compared with initiating antimicrobial therapy at 12 hours. Importantly, early administration of antibiotics appears to rescue a subset of animals that would otherwise have been predicted to die. While ND4 animals with IL-6 levels >14,000 pg/ml 6 hours after CLP have a 100% mortality if antimicrobial therapy is initiated at 12 hours, 25% of these mice survive if imipenem is begun at 6 hours. However, targeted antibody therapy, which decreases IL-6 levels greater than five-fold, fails to increase overall survival or change outcome in the subset of mice with markedly elevated IL-6 levels.

It is clear that elevated IL-6 levels in both patients and animals are associated with increased mortality (5,7,10,11,17,20,23,26–29). In fact, a recent prospective randomized phase III clinical trial was designed to see if a pharmacologic intervention would improve outcomes in patients with elevated serum IL-6 levels (18). However, the physiologic significance of elevated IL-6 levels remains unclear. Numerous studies using anti IL-6 antibodies, exogenous IL-6 or IL-6 knockout mice fail to give a complete picture as to whether this cytokine represents

a marker or a mediator in sepsis (19,20,21). The picture is further clouded by the fact that even if IL-6 levels are increased in sepsis, this does not necessarily correlate to IL-6 activity, which is actually decreased following CLP in the liver (1). In addition, genetic variability appears to play a major role in IL-6 levels in patients (28), and IL-6 levels may be similar in different inbred strains of mice despite widely varying mortalities (24).

To address the functional significance of increased IL-6 levels 6 hours after the onset of murine sepsis, the interventions performed in this study were initiated *after* IL-6 levels were obtained. This is a physiologically relevant method for examining the effects of the interventions studied since animals could be identified as having markedly elevated IL-6 levels prior to administration of antibiotics or anti-IL-6 antibody. This is also clinically relevant since it frequently takes greater than 6 hours to initiate therapy in a septic patient. For instance, mediator therapy that improves survival in septic patients can be started up to 24 hours after the onset of organ failure (4) and initiation of antibiotics for pneumonia is not considered to be delayed unless a greater than 24 hours delay exists in treating an infected patient (12).

The fact that earlier administration of antibiotics rescues mice predicted to die based upon elevated IL-6 levels is an important observation. Between the results presented herein and a previous publication from our lab (26), 38 ND4 mice receiving either no antibiotics or antimicrobial therapy beginning 12 hours after CLP have had IL-6 levels >14,000 pg/ml, and all 38 have died (23 received imipenem, 15 did not). Further, IL-6 levels above this threshold predict death with certainty regardless of the severity of injury used (5 different needle gauges or number of punctures have been used in our experiments). Even though antibiotic therapy initiated 12 hours after CLP significantly improves survival compared to untreated animals, the subset of mice with markedly elevated IL-6 levels still has a 100% mortality if antimicrobial therapy is started at this timepoint, demonstrating these animals have a fundamentally different response to sepsis than their littermates. However, when antibiotics are initiated at 6 hours -- immediately after drawing IL-6 levels -- 25% of animals with markedly elevated IL-6 levels survive. This suggests that although this subgroup of mice will shortly go down a pathway that leads to inevitable death even if treatment is initiated at 12 hours, this outcome can be altered with earlier therapy.

It is also interesting to note that earlier antibiotic therapy improved overall survival of mice but that this survival correlated to IL-6 levels only if they were either very high (>14,000 pg/ml) or low to moderate (<9,000 pg/ml). Since we made a pre hoc decision to analyze mice with IL-6 levels >14,000 pg/ml because they are "destined to die," the majority of this manuscript focuses on this subset of mice. However, it is intriguing that IL-6 levels statistically predict survival at lower or very high levels while losing discrimination at moderate to high levels, and we believe this merits further study. Unfortunately, it is impossible to draw definitive conclusions based upon these data. First, this represents a retrospective analysis of survival between the two groups based upon examining the data in arbitrary IL-6 increments of 1,000 pg/ml and it is possible that any relationship seen is present by chance. Second, many more mice have IL-6 levels greater than a lower threshold (i.e. 175 mice had IL-6 levels > 3,000 pg/ml but only 40 mice had IL-6 levels >13,000 pg/ml). While this increases the statistical power to see a difference in survival between groups, we are unable to know if this correlates to a biologically meaningful result.

Our experiments do not distinguish whether markedly elevated IL-6 levels serve as a marker of death (perhaps by signifying a hyperinflammatory state) or whether they actually mediate this outcome. To study this question, we gave anti-IL-6 antibody to mice with known IL-6 levels. Targeted monoclonal therapy did not improve survival even in mice with markedly elevated IL-6 levels which could mean that IL-6 is not a mediator at this timepoint or that we gave an insufficient dose of antibody to see an effect on survival. Riedemann et al. have

previously shown that the survival benefit of anti-IL-6 antibody is dependent on both dose and time (21). Either too much (2.66 mg/kg) or too little (0.33 mg/kg) antibody led to some improvement in survival over control IgG but markedly less than the optimal dose (1.33 mg/kg) when given immediately after CLP. However, this optimal dose has no impact on mortality when given 4 hours later. When comparing our results to Riedemann et al., there are clear differences in experimental design: we studied a different strain of mice (IL-6 levels are approximately 30% higher in ND4 mice than B10/D2 nsnJ mice 6 hours following CLP with a similar injury) and examined effects of the antibody on IL-6 levels and survival at different timepoints. This was necessary since a predetermined endpoint of our study was to examine mice with markedly elevated IL-6 levels 6 hours following sepsis, and therefore we could not give a treatment that lowered cytokine levels until this timepoint had been reached. Further, a similar dosage of monoclonal antibody decreases IL-6 levels 40-fold when given immediately after sepsis and measured 6 hours later, but decreases IL-6 levels 5-fold when given 6 hours after CLP and measured 12 hours after the onset of sepsis. It is therefore unclear whether the lack of survival benefit from anti-IL-6 antibody in our study is related to the timing or the dosing of this agent. It is possible that IL-6 acts as a mediator immediately after the onset of sepsis (which explains the benefit when anti-IL-6 antibody is given very early) but has a differing role at a later timepoint. It is also possible that a higher dose of antibody which decreases IL-6 levels even further may have been beneficial.

This study has a number of limitations. We do not know why the subset of mice with IL-6 levels >14,000 pg/ml responds differently than littermates that receive the same injury. We speculate that these animals are more hyperinflammatory and so might have a different immune response to the same injury; however, we have not determined if their inflammatory profile is different from their septic littermates with lower IL-6 levels. An additional limitation is the fact that the data comparing mortalities of mice with IL-6 levels >14,000 pg/ml with antibiotics started at 6 or 12 hours is pooled between two different injuries (2×18 and 2×21 CLP). While the potential downside to pooling samples from two experiments is obvious, we feel this result is valid for a number of reasons. First, survival is statistically similar between 2×18 and 2×21 injuries in this study ($p=0.24$). Next, we have historic data on 20 mice with IL-6 levels >14,000 pg/ml with multiple different injuries as well as 14 mice from the 2×18 CLP in this study, all showing 100% mortality. Based upon the frequency of obtaining mice with IL-6 levels >14,000 pg/ml, in order to complete a contingency table showing statistical significance solely between mice receiving 2×21 CLP with antibiotics started at 6 or 12 hours, approximately 60 additional animals would have had to be subjected to CLP. We believe this cannot be ethically justified. We now cumulatively have 38 mice with IL-6 levels >14,000 pg/ml without a single animal surviving if antibiotics are started after 6 hours and believe the 25% survival of this subset of mice when antibiotics are started at 6 hours represents not only a statistically valid but also a biologically meaningful result.

Another limitation is that while we demonstrated that targeted antibody therapy decreases IL-6 levels, we did not examine whether it has the same effect on the subset of mice with IL-6 levels >14,000 pg/ml. In the experiments where we examined the effect of antibody treatment on cytokine levels, IL-6 levels were obtained at 12 hours, 6 hours after giving anti-IL-6 antibody. It is therefore unknown what their IL-6 levels were at 6 hours. We used this experimental design because we did not want to subject animals to two separate blood draws, one before giving anti-IL-6 antibody and one six hours later. While it is likely that antibody therapy decreases IL-6 levels by a similar percentage in all animals, it is possible that the overall decrease in IL-6 levels is not reflective of a similar decrease in mice with markedly elevated IL-6 levels at 6 hours.

Despite these limitations, our results demonstrate that a subset of mice that would be predicted to die with 100% accuracy based upon high IL-6 levels can be rescued with very early antibiotic

administration but not by targeted anti-IL-6 therapy. Further investigation into the pathobiology of mice with IL-6 levels >14,000 pg/ml should yield additional insights into why they have strikingly different outcomes than their littermates after seemingly similar septic insults.

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Fig. 1.

Survival curve of mice that received imipenem beginning 6 hours or 12 hours following double puncture CLP with a 21 gauge needle. Mice that receive earlier antibiotic therapy have improved overall survival. Despite the early administration of antibiotics in both groups, the survival benefit conferred is not apparent until 4 days following the onset of sepsis.

Fig. 2.

Earlier administration of antibiotics improves survival in mice with IL-6 levels >14,000 pg/ml. All mice (n=18) with IL-6 levels above this threshold die if antibiotics are initiated 12 hours after CLP. In contrast, 25% of mice (4/16) with markedly elevated IL-6 levels survive if antibiotics are begun 6 hours after the onset of sepsis. The solid horizontal line in each column represents mean IL-6 levels, which were drawn prior to instituting antibiotics in all groups.

IL-6 (pg/ml)	12 hours (alive/total)	6 hours (alive/total)	P value
1000	21/103 (20.4%)	28/76 (36.84%)	.0178
2000	21/103 (20.4%)	28/76 (36.84%)	.0178
3000	21/101 (20.7%)	28/74 (37.8%)	.0169
4000	18/94 (19.1%)	28/72 (38.8%)	.005
5000	14/86 (16.2%)	27/69 (37.5%)	.0017
6000	13/76 (17.1%)	25/65 (38.46%)	.0045
7000	12/69 (17.39%)	20/58 (34.48%)	.039
8000	11/61 (18.03%)	19/49 (38.77%)	.0178
9000	10/55 (18.18%)	16/44 (36.36%)	n.s.
10,000	7/42 (16.6%)	12/34 (35.29%)	n.s.
11,000	6/33 (18.18%)	8/27 (29.62%)	n.s.
12,000	5/29 (17.2%)	4/19 (21%)	n.s.
13,000	3/23 (13.04%)	4/17 (23.52%)	n.s.
14,000	0/18 (0%)	4/16 (25%)	.039

Fig. 3.

Relationship between IL-6 levels, timing of antibiotic administration, and survival. All animals that received antibiotics either 6 hours or 12 hours after CLP with IL-6 levels above the threshold specified were retrospectively assessed for mortality and compared for statistical significance.

Fig. 4. IL-6 levels 6 hours after CLP. All levels were drawn immediately prior to antibody therapy (given at 6 hours) and antibiotics (begun at 12 hours). A: Baseline IL-6 levels are similar regardless of whether animals were randomized to receive anti IL-6 antibody or control IgG. B: Pooled analysis of baseline IL-6 levels demonstrates that these are higher in animals that will die from sepsis.

Fig. 5.

Survival curve of mice that received either 1.33 mg/kg or 2.66 mg/kg anti-IL-6 antibody or control IgG beginning 6 hours following CLP. Monoclonal therapy directed against IL-6 does not alter survival when initiated at this timepoint.

Fig. 6.

Effect of anti-IL-6 antibody on survival in mice with IL-6 levels >14,000 pg/ml. All animals with IL-6 levels above this threshold at 6 hours die, despite initiation of targeted antibody therapy begun immediately after IL-6 levels were drawn.

Fig. 7.

Effect of anti-IL-6 antibody on IL-6 levels 12 hours after CLP. Mice were subjected to CLP, given monoclonal anti-IL-6 or control IgG at 6 hours and had their blood drawn at 12 hours. Anti-IL-6 antibody markedly reduces IL-6 levels. Of note, this is the only experiment in the manuscript where IL-6 levels were not drawn at 6 hours and the only experiment where IL-6 levels were drawn after an intervention.