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

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First published June 9, 2005; doi:10.1152/ajpregu.00312.2005.—Early antibiotic administration but not antibody therapy directed against IL-6 improves survival in septic mice predicted to die on basis of high IL-6 levels. A different cohort of animals predicted to die in sepsis, but we are unable to demonstrate any benefit in similar animals using targeted therapy directed at IL-6.

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 h after the onset of sepsis predict mortality with high specificity in mice subjected to either cecal ligation and puncture (CLP) (20, 26, 27, 29) or Pseudomonas aeruginosa pneumonia (5).

Although 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 h after gavage with Escherichia coli and thermal injury (9). These effects are time dependent, because mortality is unaffected if anti-IL-6 is given 4 h after CLP (21) or 8 h after bacterial gavage and burn (9). Furthermore, 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 E. coli (6). Of note, IL-6 has been shown to modulate some physiological 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 after CLP, regardless of whether they are treated with antibiotic therapy beginning 12 h after the onset of sepsis (26). This timing of antibiotic administration is clinically relevant because 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 h for an infection to develop (16).

Whether administration of antibiotic therapy prior to 12 h after the onset of sepsis improves survival is unclear. We therefore studied the effect of antibiotic administration begun 6 h 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 because this subset of animals (representing ~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 according to the methods of Baker et al. (3) and as previously described (25). Briefly, anesthesia was induced with 5% halothane and maintained with 2% halothane. After 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 1 wk before surgical manipulation and were main-
RESULTS

IL-6 levels >14,000 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 6 h postoperatively and antibiotic therapy initiated 12 h postoperatively. Animals were followed 7 days for survival and retrospectively analyzed to see whether all those with IL-6 levels >14,000 pg/ml died. Overall survival was 15.9%. All 14 mice with IL-6 levels >14,000 pg/ml died.

Earlier administration of antibiotics improves overall survival after CLP. Based on the high mortality seen in the 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 6 h (n = 78) or 12 h (n = 43) postoperatively. IL-6 levels drawn 6 h after CLP (i.e., before antibiotic therapy was begun in either group) were similar in the two groups (P = nonsignificant (NS)). Earlier antibiotic therapy was associated with increased survival: 35.9% (28/78) for those treated with imipenem begun 6 h after CLP compared with 25.5% (11/43) for those whose antibiotics were begun 12 h 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 after CLP. Survival was compared between mice that received antibiotics starting 6 or 12 h after CLP with IL-6 levels >14,000 pg/ml. Survival was 25% in mice that had antibiotics started at 6 h (4/16) compared with 0% in animals whose antibiotics were started at 12 h (0/18), suggesting that earlier antibiotic therapy can rescue a subset of mice that would have been predicted to die on the basis of elevated IL-6 levels (P < 0.05, Fig. 2). When the data in Fig. 2 were 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 or >14,000 pg/ml but was not predictive of survival in mice with IL-6 levels between these values (Table 1).

Anti-IL-6 antibody fails to improve either overall mortality or survival in a subset of animals with IL-6 levels >14,000 pg/ml after CLP. Because 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 or 2.66 mg/kg (doses chosen based on Ref. 21) or irrelevant rat IgG 6 h after CLP. All animals had IL-6 levels drawn 6 h postoperatively (i.e., immediately before administration of anti IL-6 antibody), and each received imipenem (25 mg/kg; Merck, West Point, PA) beginning either 6 or 12 h after CLP and centrifuged for 5 min to separate plasma. Unless otherwise specified, 6 h after CLP. Antibiotics were given every 12 h 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) 6 h after 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 this study was designed, 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, because previous studies have shown these animals have 100% mortality after CLP, regardless of the type of injury used or whether antibiotic therapy is initiated 12 h 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 h after CLP, and the percentage that survived when antimicrobial therapy was started 12 h 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.
penem beginning 12 h after CLP. IL-6 levels drawn at 6 h before antibody administration were similar in all groups (P NS, Fig. 3A), although retrospective analysis demonstrated that mice with higher IL-6 levels following sepsis eventually had a higher mortality (P < 0.005, Fig. 3B). However, anti-IL-6 antibody had no impact on either overall survival (Fig. 4) or survival in the subset of animals with IL-6 levels >14,000 pg/ml, which all died regardless of treatment (Fig. 5).

To verify that anti-IL-6 antibody was functionally active, we examined an additional cohort of mice (n = 22) 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 6 h, and then had blood drawn for IL-6 levels 12 h postoperatively. IL-6 levels decreased more than fivefold 6 h after targeted antibody therapy (Fig. 6). Of note, this is the only experiment described in which IL-6 levels were not drawn 6 h after CLP and the only time IL-6 levels were drawn after pharmacological intervention.

**DISCUSSION**

This study demonstrates that starting antibiotics 6 h after the onset of sepsis yields an improvement in overall survival compared with initiating antimicrobial therapy at 12 h. Importantly, early administration of antibiotics appears to rescue a subset of animals that would otherwise have been predicted to die. Whereas ND4 animals with IL-6 levels >14,000 pg/ml 6 h after CLP have a 100% mortality if antimicrobial therapy is initiated at 12 h, 25% of these mice survive if imipenem is begun at 6 h. However, targeted antibody therapy, which decreases IL-6 levels more than fivefold, 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 whether a pharmacological intervention would improve outcomes in patients with elevated serum IL-6 levels (18). However, the physiolog-

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### Table 1. IL-6 levels, timing of antibiotic administration, and survival

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<th>IL-6, pg/ml</th>
<th>Survival at Antibiotic Administration</th>
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<td>12 h, alive/total (%)</td>
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therapy that improves survival in septic patients can be started also is clinically relevant, because it frequently takes more than before administration of antibiotics or anti-IL-6 antibody. This could be identified as having markedly elevated IL-6 levels ining the effects of the interventions studied, because animals

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 (19–21). 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 after 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 h after the onset of murine sepsis, we initiated the interventions performed in this study after IL-6 levels were obtained. This is a physiologically relevant method for examining the effects of the interventions studied, because animals could be identified as having markedly elevated IL-6 levels before administration of antibiotics or anti-IL-6 antibody. This also is clinically relevant, because it frequently takes more than 6 h to initiate therapy in a septic patient. For instance, mediator therapy that improves survival in septic patients can be started up to 24 h after the onset of organ failure (4), and initiation of antibiotics for pneumonia is not considered to be delayed unless a >24-h delay exists in treating an infected patient (12).

The fact that earlier administration of antibiotics rescues mice predicted to die on the basis of elevated IL-6 levels is an important observation. Between the results presented in this report and a previous publication from our laboratory (26), 38 ND4 mice receiving either no antibiotics or antimicrobial therapy beginning 12 h after CLP have had IL-6 levels >14,000 pg/ml, and all 38 have died (23 received imipenem, 15 did not). Furthermore, 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 h after CLP significantly improves survival compared with untreated animals, the subset of mice with markedly elevated IL-6 levels still has a 100% mortality if antimicrobial therapy is started at this time point, demonstrating these animals have a fundamentally different response to sepsis than their littermates. However, when antibiotics are initiated at 6 h, immediately after IL-6 levels are drawn, 25% of animals with markedly elevated IL-6 levels survive. This finding 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 h, this outcome can be altered with earlier therapy.

It also is interesting to note that earlier antibiotic therapy improved overall survival of mice but that this survival corre-
lated to IL-6 levels only if they were either very high (>14,000 pg/ml) or low to moderate (<9,000 pg/ml). Because we made a pre hoc decision to analyze mice with IL-6 levels >14,000 pg/ml given that they are “destined to die,” the majority of this report 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 on these data. First, this represents a retrospective analysis of survival between the two groups based on examination of 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). Although this increases the statistical power to see a difference in survival between groups, we are unable to know whether 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 time point or that we gave an insufficient dose of antibody to see an effect on survival. Riedemann et al. (21) previously have shown that the survival benefit of anti-IL-6 antibody is dependent on both dose and time. 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 h later. When comparing our results to those of Riedemann et al. (21), there are clear differences in experimental design: we studied a different strain of mice (IL-6 levels are ~30% higher in ND4 mice than in ND4 mice given that they are “destined to die,” the majority of this report focuses on this subset of mice. However, it is impossible to draw definitive conclusions based on these data. First, this represents a retrospective analysis of survival between the two groups based on examination of 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). Although this increases the statistical power to see a difference in survival between groups, we are unable to know whether this correlates to a biologically meaningful result.

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![Image of IL-6 levels in sepsis](http://ajpregu.physiology.org/...)

**Fig. 6.** Effect of anti-IL-6 antibody on IL-6 levels 12 h after CLP. Mice were subjected to CLP, given monoclonal anti-IL-6 or control IgG at 6 h, and had their blood drawn at 12 h. Anti-IL-6 antibody markedly reduced IL-6 levels. Of note, this was the only experiment in the study in which IL-6 levels were not drawn at 6 h and the only experiment in which IL-6 levels were drawn after an intervention. *P < 0.05 compared between mice that survived or died after CLP.
time point. It also is possible that a higher dose of antibody that 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 from littersmates 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 whether their inflammatory profile is different from that of their septic littersmates 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 h are pooled between two different injuries (2 × 18 and 2 × 21 CLP). Although 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 on the frequency of obtaining mice with IL-6 levels >14,000 pg/ml, completing a contingency table showing statistical significance solely because we did not want to subject animals to two separate treatments is unlikely. Finally, we used strict inclusion criteria such as having IL-6 levels >14,000 pg/ml without a single animal surviving if antibiotics are started after 6 h and believe the 25% survival of this subset of mice when antibiotics are started at 6 h 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 in which we examined the effect of antibody treatment on cytokine levels, IL-6 levels were obtained at 12 h, 6 h after administration of anti-IL-6 antibody. It is therefore unknown what their IL-6 levels were at 6 h. We used this experimental design because we did not want to subject animals to two separate blood draws, one before administration of anti-IL-6 antibody and one 6 h later. Although 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 h.

Despite these limitations, our results demonstrate 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 in which we examined the effect of antibody treatment on cytokine levels, IL-6 levels were obtained at 12 h, 6 h after administration of anti-IL-6 antibody. It is therefore unknown what their IL-6 levels were at 6 h. We used this experimental design because we did not want to subject animals to two separate blood draws, one before administration of anti-IL-6 antibody and one 6 h later. Although 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 h.

Despite these limitations, our results demonstrate that a subset of mice that would be predicted to die with 100% accuracy on the basis of 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 littersmates after seemingly similar septic insults.

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GRANTS

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