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

Dinesh Vyas,1 Pardis Javadi,1 Peter J. DiPasco,2 Timothy G. Buchman,1,2,3 Richard S. Hotchkiss,1,2,3 and Craig M. Coopersmith1,2

Departments of 1Surgery, 2Anesthesiology, and 3Medicine, Washington University School of Medicine, St. Louis, Missouri

Submitted 3 May 2005; accepted in final form 29 May 2005


Address for reprint requests and other correspondence: C. M. Coopersmith, Washington Univ. School of Medicine, 660 South Euclid Ave., Campus Box 8109, St. Louis, MO 63110 (e-mail: coopersmithc@wustl.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Vyas, Dinesh, Pardis Javadi, Peter J. DiPasco, Timothy G. Buchman, Richard S. Hotchkiss, and Craig M. Coopersmith. 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. Am J Physiol Regul Integr Comp Physiol 289: R1048–R1053, 2005—Elevated interleukin (IL)-6 levels correlate with increased mortality following sepsis. IL-6 levels >14,000 pg/ml drawn 6 h after cecal ligation and puncture (CLP) are associated with 100% mortality in ND4 mice, even if antibiotic therapy is initiated 12 h after septic insult. Our first aim was to see whether earlier institution of antibiotic therapy could improve overall survival in septic mice and rescue the subset of animals predicted to die on the basis of high IL-6 levels. Mice (n = 184) were subjected to CLP, had IL-6 levels drawn 6 h later, and then were randomized to receive imipenem, a broad spectrum antimicrobial agent, beginning 6 or 12 h postoperatively. Overall 1-wk 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 h, whereas none survived if antibiotics were started later (P < 0.05). On the basis of 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 blood drawn 6 h after CLP, and then they 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.

cecal ligation and puncture; cytokine; imipenem; critical illness; interleukin-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 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).

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.

Fig. 1. Survival curve for mice that received imipenem beginning 6 or 12 h after cecal ligation and double puncture (CLP) with a 21-gauge needle. Mice that received earlier antibiotic therapy had improved overall survival. Despite the early administration of antibiotics in both groups, the survival benefit conferred is not apparent until 4 days after the onset of sepsis.

Fig. 2. Earlier administration of antibiotics improves survival in mice 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 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 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).

Statistical 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.

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
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-

---

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

<table>
<thead>
<tr>
<th>IL-6, pg/ml</th>
<th>12 h, alive/total (%)</th>
<th>6 h, alive/total (%)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,000</td>
<td>21/103 (20.4)</td>
<td>28/76 (36.4)</td>
<td>0.0178</td>
</tr>
<tr>
<td>2,000</td>
<td>21/103 (20.4)</td>
<td>28/76 (36.4)</td>
<td>0.0178</td>
</tr>
<tr>
<td>3,000</td>
<td>21/101 (20.7)</td>
<td>28/74 (37.8)</td>
<td>0.0169</td>
</tr>
<tr>
<td>4,000</td>
<td>18/94 (19.1)</td>
<td>28/72 (38.8)</td>
<td>0.005</td>
</tr>
<tr>
<td>5,000</td>
<td>14/86 (16.2)</td>
<td>27/69 (37.5)</td>
<td>0.0017</td>
</tr>
<tr>
<td>6,000</td>
<td>13/76 (17.1)</td>
<td>25/65 (38.46)</td>
<td>0.0045</td>
</tr>
<tr>
<td>7,000</td>
<td>12/69 (17.39)</td>
<td>20/58 (34.48)</td>
<td>0.039</td>
</tr>
<tr>
<td>8,000</td>
<td>11/61 (18.03)</td>
<td>19/49 (38.77)</td>
<td>0.0178</td>
</tr>
<tr>
<td>9,000</td>
<td>10/55 (18.18)</td>
<td>16/44 (36.36)</td>
<td>NS</td>
</tr>
<tr>
<td>10,000</td>
<td>7/42 (16.6)</td>
<td>12/34 (35.29)</td>
<td>NS</td>
</tr>
<tr>
<td>11,000</td>
<td>6/33 (18.18)</td>
<td>8/27 (29.62)</td>
<td>NS</td>
</tr>
<tr>
<td>12,000</td>
<td>5/29 (17.2)</td>
<td>4/19 (21)</td>
<td>NS</td>
</tr>
<tr>
<td>13,000</td>
<td>3/23 (13.04)</td>
<td>4/17 (23.52)</td>
<td>NS</td>
</tr>
<tr>
<td>14,000</td>
<td>0/18 (0)</td>
<td>4/16 (25)</td>
<td>0.039</td>
</tr>
</tbody>
</table>

All animals that received antibiotics either 6 or 12 h after cecal ligation and puncture with IL-6 levels above threshold specified were retrospectively assessed for mortality and compared for statistical significance.

---

**Fig. 3.** IL-6 levels 6 h after CLP. All levels were drawn immediately before antibody therapy (given at 6 h) and antibiotics (begun at 12 h). A: baseline IL-6 levels were 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 were higher in animals that would die from sepsis. *P < 0.05 compared between mice that survived or died after CLP.

---

**Fig. 4.** Survival curve for mice that received either 1.33 or 2.66 mg/kg anti-IL-6 antibody or control IgG beginning 6 h after CLP. Monoclonal therapy directed against IL-6 does not alter survival when initiated at this time point.

---

**Fig. 5.** 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 h died, despite initiation of targeted antibody therapy begun immediately after IL-6 levels were drawn.
unless a antibiotics for pneumonia is not considered to be delayed up to 24 h after the onset of organ failure (4), and initiation of therapy that improves survival in septic patients can be started 6 h to initiate therapy in a septic patient. For instance, mediator 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 in the effects of the interventions studied, because animals intervened 12 h after CLP significantly improves survival compared 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) 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. 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 B10/D2 nsJ mice 6 h after CLP with a similar injury) and examined effects of the antibody on IL-6 levels and survival at different time points. This was necessary, because a predetermined end point of our study was to examine mice with markedly elevated IL-6 levels 6 h after sepsis, and therefore we could not give a treatment that lowered cytokine levels until this time point had been reached. Furthermore, a similar dosage of monoclonal antibody decreases IL-6 levels 40-fold when given immediately after sepsis and measured 6 h later, but it decreases IL-6 levels 5-fold when given 6 h after CLP and measured 12 h 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...
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 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 whether their inflammatory profile is different from that of 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 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 between mice receiving 2 × 21 CLP with antibiotics started at 6 or 12 h would require ~60 additional animals 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/mg 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 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 littermates after seemingly similar septic insults.

ACKNOWLEDGMENTS

We thank Isaiah Turnbull for technical assistance.

GRANTS

This study was supported by National Institute of General Medical Sciences Grants GM-66202, GM-00709, GM-44118, and GM-55194.

REFERENCES


