Outcome Following Femur Fracture and Subsequent Cecal Ligation and Puncture in Endotoxin-Sensitive (C3H/HeN) and Endotoxin-Resistant (C3H/HeJ) Mice

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This study examined the effect of sepsis following trauma in a reproducible model of sepsis—cecal ligation and puncture (CLP)—in endotoxin-sensitive (C3H/HeN) and endotoxin-resistant (C3H/HeJ) mice. Studies used CLP with a 25-gauge needle at different time intervals following injury, as induced by femur fracture (FF), to determine the effects of sublethal sepsis on survival after trauma. There was a 3% mortality for FF alone in both groups. Mortality in C3H/HeJ mice was not significantly increased over FF alone except when CLP followed FF by 3 days (45%, P < 0.02, Chi-square). In contrast, C3H/HeN mice had significantly increased mortality rates (75 to 90%, P < 0.001) versus FF alone at all intervals between FF and CLP. Mortality for FF plus CLP was significantly greater for C3H/HeN compared to C3H/HeJ (P < 0.001) for all time intervals between FF and CLP. In conclusion, animals exposed to a septic episode following FF had significantly greater mortality than FF animals without a septic challenge. Endotoxin-sensitive mice had significantly higher mortality after CLP and significantly increased mortality when CLP followed FF (regardless of timing) compared to endotoxin-resistant mice.

INTRODUCTION

Systemic sepsis continues to plague critically ill and injured surgical patients, carrying a mortality of 50 to 60% [1]. Although deaths from surgical sepsis have been thought to be due to the failure of host defense mechanisms, positive blood cultures have only been found in a third of trauma patients with sepsis [2]. Recently the demonstration of translocation of bacteria and endotoxin across the gut has been advanced as a solution to this paradox [3]. Our laboratory has been interested for several years in the role of macrophage dysfunction in immunosuppression following trauma. Since the macrophage is activated by endotoxin [4], it is possible that abnormal responses to endotoxin may lead to early macrophage dysfunction after trauma.

This study was undertaken to evaluate the interaction of trauma and sepsis. Initial studies used a valid model of intra-abdominal sepsis (CLP) in endotoxin-sensitive (C3H/HeN) and resistant mice (C3H/HeJ) and developed a model system for studying the interaction of trauma and sepsis. These mice were chosen because of the differential response of their macrophage to endotoxin [8], in anticipation of subsequent studies on the mechanisms of immunologic dysfunction after trauma. The cecal ligation and puncture model in mice [5] was chosen for the sepsis model for the reasons previously outlined by Chaudry [6]. Open isolated femur fracture was used as the model for trauma as reported by Stone [7]. Using this model system of FF followed by CLP, the effects of sepsis at various times after injury on survival were investigated. The experimental protocol is outlined in Fig. 1.

MATERIALS AND METHODS

Animals

C3H/HeJ mice were obtained from Jackson Laboratories (Bar Harbor, ME) and C3H/HeN mice were purchased from NIH (Bethesda, MD). All mice used were pathogen-free 4- to 6-week-old males (25–30 g) housed in laminar flow cabinets. Animals were cared for in accordance with NIH and Yale Department of Animal Care Protocols.

Surgical Procedures

The cecal ligation and puncture (CLP) procedure was adapted to mice from the model described in rats by Chaudry [6]. Animals were anesthetized intramuscularly with a combination of xylazine (16 mg/kg) and ketamine.
Femur Fracture (FF) Alone $\rightarrow$ 96 hours
Mortality Assessed

Cecal Ligation and Puncture (CLP) $\rightarrow$ 96 hours
Mortality Assessed

Intervals at which CLP performed

2 Days
4 Days
6 Days
1 Day
3 Days
5 Days
7 Days

Mortality Assessed 96 hours after CLP

FIG. 1. The experimental protocol is outlined here, showing the intervals between FF and CLP.

damine (80 mg/kg). After shaving the anterior abdominal wall, a 15-mm midline incision was made and the cecum exposed and ligated at its base with 2-0 silk. The cecum was punctured with the appropriate gauge needle, and approximately 0.1 ml of feces were squeezed out in order to be certain that the puncture holes were open. The abdominal incision was closed with 4-0 Nylon, and the mice rehydrated with 1 ml of normal saline subcutaneously.

Our murine model of femur fracture (FF) was adapted from Stone et al. [7]. Mice were anesthetized and shaved in the left thigh region. A 10-mm incision was made lateral to the femur, which was dissected from the surrounding muscle. The femur was then sharply cut at its midpoint with scissors. The incision was closed with 4-0 Nylon, and the mice were resuscitated as above.

Data Analysis

All animals were assessed for survival twice daily for up to 10 days after the experimental procedure; increases in mortality, however, were not seen after 96 hr in mice undergoing CLP. When mice were subjected to CLP after FF, mortality was determined twice daily for 7 days and did not increase thereafter. Differences between groups were assessed using Chi-square analysis with the Yates continuity correction [9].

RESULTS

The Effect of Endotoxin Sensitivity on Outcome after CLP

The effect of endotoxin sensitivity on mortality at 96 hr after CLP for the two groups of mice is depicted in Table 1. In several hundred previous experiments, we have rarely seen deaths occurring at times greater than 96 hr following CLP, which was also the case in this study. As can be seen, CLP was lethal for C3H/HeN for all needle sizes except 25 gauge (45% mortality). The mortality for C3H/HeJ, however, was greatest for the 18-gauge needle (50%) and dropped to zero for the 25-gauge needle. Mortality for CLP in the endotoxin-sensitive C3H/HeN mice was significantly increased ($P < 0.001$, x², Yates correction) for all needle sizes compared to the endotoxin-resistant C3H/HeJ mice. In addition, mortality for CLP in the C3H/HeJ mice tended to parallel needle size.

Studies on the Combined Effect of FF and CLP

Studies on FF alone as a model of injury demonstrated a low mortality of 3% (N = 30 animals/group) in both strains of mice. In the experimental studies, FF was followed by sublethal sepsis (CLP with a 25-gauge needle) in order to evaluate the effect of sepsis following trauma in the two strains of mice. It should be recalled that mortality following CLP alone with a 25-gauge needle was 45% for C3H/HeN and 0% for C3H/HeJ.

The data for this set of experiments are summarized in Table 2 and Fig. 2. In general, the C3H/HeJ mice were more resistant to the combined effects of FF and CLP than the C3H/HeN mice—mortality in C3H/HeJ mice was increased over FF alone (expected 3%) and CLP alone (expected 0%) when CLP followed FF by 1 day and 3 days (35% and 45%, $P < 0.02$). There were no significant increases over expected mortality in C3H/HeJ mice, however, when the interval between FF and CLP exceeded 3 days. In the endotoxin-sensitive mice, however, the results were different. Mortality for FF and CLP for C3H/HeN was significantly increased compared to C3H/HeJ mice for all intervals between FF and CLP ($P < 0.001$). Mortality for FF plus CLP in C3H/HeN was also increased ($P < 0.001$) compared to FF alone in C3H/HeN (3%). Furthermore mortality for FF plus CLP was significantly increased ($P < 0.01$) over FF alone (3%) and CLP alone (47%) for all intervals but 1 day in the endotoxin-sensitive mice. Data are depicted graphically in Fig. 2 for FF followed by CLP at intervals of 1, 3, 5, and 7 days.
### DISCUSSION

As surgical and anesthetic management has improved for patients with multiple injuries and those undergoing major elective surgery, the clinical problems preventing survival in this setting have become systemic sepsis and multiple organ failure (MOF), which cause 75% of the late deaths after major trauma [10]. Unfortunately, many of the human studies have evaluated heterogeneous groups of patients, and most previous animal models of sepsis—e.g., injection of live bacteria [11] or endotoxin [12]—were inadequate analogs of human sepsis. The animal model of CLP used in these studies is a good analog of surgical sepsis, as pointed out by Chaudry et al. [6], and closely parallels the cardiovascular patterns of sepsis in man described by MacLean et al. [13]. As shown in Table 1 of this study, mortality in C3H/HeJ mice closely parallels needle size, suggesting that this model represents a graded form of sepsis in these animals. In addition, it appears that CLP with a 25-gauge needle can be used to study the effect of sublethal sepsis following an immunosuppressive challenge (e.g., trauma, thermal injury), paralleling the clinical events often seen in patients. This principle was actually utilized in a previous study by Kupper, in which the sublethal model of CLP with a 25-gauge needle was converted to a lethal model in animals that were immunosuppressed by injection with T cells from burned mice 20 hr prior to CLP [14]. In the current study, we evaluated the immunosuppression of trauma using FF as a trauma model, followed by CLP.

The results of the current study have demonstrated several findings. While endotoxin sensitivity does not seem to affect mortality after FF, clearly late mortality after CLP is significantly increased in C3H/HeN mice. In the experiments on the combined effect of FF and CLP, the timing of CLP appeared to play a role, especially in the C3H/HeJ mice, which only had increased mortality compared to FF alone when CLP followed FF by 1 or 3 days but not at longer intervals. These data suggest that there may be a window of time early after FF during which the endotoxin-resistant animals are more susceptible to a septic challenge. Conversely, the C3H/HeJ mice had a significantly decreased mortality compared to C3H/HeN (P < 0.001) when CLP followed FF at all intervals tested except 1 day. This parallels the differences between C3H/HeJ and C3H/HeN seen for CLP alone (Table 1), but the increased susceptibility of the endotoxin-sensitive mice to sepsis is more striking for FF plus CLP (Table 2). It should be pointed out that a positive control or sham was not performed in this study, namely FF followed by anesthesia and laparotomy alone. Part of the reason for this was a desire on the part of our Animal Care Protocol Committee to limit the number of animals in control groups. In previous experi-

### TABLE 1

<table>
<thead>
<tr>
<th>Strain of mice</th>
<th>Needle size (gauge)</th>
<th>18</th>
<th>19</th>
<th>20</th>
<th>21</th>
<th>23</th>
<th>25</th>
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<tbody>
<tr>
<td>C3H/HeJ (resistant)</td>
<td></td>
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<tr>
<td>C3H/HeN (sensitive)</td>
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</tbody>
</table>

* Significantly greater than mortality for C3H/HeJ (P < 0.001).

Within strains, mortality greater than mortality for 25-gauge needle (P < 0.001).

### TABLE 2

<table>
<thead>
<tr>
<th>Strain of mice</th>
<th>Interval between FF and CLP (days)</th>
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<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>C3H/HeJ (resistant)</td>
<td>35%</td>
</tr>
<tr>
<td>(N)</td>
<td>(20)</td>
</tr>
<tr>
<td>C3H/HeN (sensitive)</td>
<td>68.4%</td>
</tr>
<tr>
<td>(N)</td>
<td>(19)</td>
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</tbody>
</table>

* Significantly increased versus C3H/HeJ for same interval (P < 0.001, Chi-square with Yates correction).

* Significantly increased for FF/CLP in C3H/HeN versus FF alone in C3H/HeN (P < 0.001).
It is not possible to conclude from these studies that endotoxin is the causal factor that leads to the increased mortality in the C3H/HeN animals because endotoxin levels were not measured. Experiments to evaluate endotoxin levels, antigen recognition, macrophage-T cell interactions, and macrophage production of inflammatory mediators such as interleukin-1 and tumor necrosis factor are planned for the future. Nonetheless, two things can be concluded from this study. First, endotoxin-resistant mice have a better survival following a septic challenge (CLP), compared to endotoxin-sensitive mice, which is accentuated when sepsis follows trauma. Second, the interaction of trauma and sepsis can be appropriately studied by combining the models of femur fracture and cecal ligation and puncture.

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REFERENCES


