Sepsis remains a significant clinical problem (34) despite recent advances in early goal-directed therapy (35), the use of activated protein C (6), and the use of low-dose glucocorticoids (3). A recent review of discharge records showed that there were an estimated 751,000 cases per year with an overall mortality rate of 29% (2). While in-hospital mortality has declined, the total number of deaths continues to rise due to the increased incidence of sepsis (24). There remains a critical, unmet need to better understand the basic immunopathologic alterations present in individual septic patients in order to appropriately guide therapy.

Intra-abdominal infections may be a source for sepsis. These infections generate a peritoneal inflammatory response to polymicrobial organisms derived from the gastrointestinal tract (16, 38). Clinical peritonitis may originate from a defect in an abdominal viscus, such as an acute intestinal perforation (49), or overgrowth or excessive inflammation (33). Intra-abdominal infections may be a source for sepsis. These infections generate a peritoneal inflammatory response to polymicrobial organisms derived from the gastrointestinal tract (16, 38). Clinical peritonitis may originate from a defect in an abdominal viscus, such as an acute intestinal perforation (49), or excessive inflammation (33), but the later stages of sepsis are not as well described. The present study is particularly relevant to clinical care since mice in the chronic phase each harbor an intra-abdominal abscess. While some mice control the abscess without difficulty, others die in the later phases. In this work we investigated whether the late deaths were due to bacterial overgrowth or excessive inflammation.

### MATERIALS AND METHODS

#### Animals.
Female ICR mice (Harlan-Sprague Dawley, Inc., Indianapolis, IN) with an average weight of 22 g (range, 18 to 24 g) were maintained under standard laboratory conditions. The mice were rested at the institutional vivarium and were further acclimated to the laboratory environment for at least 24 h before surgery. After surgery, the mice were housed in a temperature-controlled room with a diurnal cycle consisting of 12 h of light and 12 h of darkness. The experiments were performed in accordance with the National Institutes of Health guidelines, and approval was obtained from the University of Michigan Animal Care and Use Committee.

#### CLP and cecal resection (CR) surgery.
CLP was performed as previously described (48), with some modifications. Mice were anesthetized by inhalation of 2 to 5% isoflurane (Baxter, Deerfield, IL) in 100% oxygen using anesthesia equipment (Surgivet/Anesco, Waukesha, WI). A 2-cm midline incision was made first through the skin and then through the linea alba. The cecum was located and was ligated with 3-0 silk, and then it was perforated twice with an 18-gauge needle. A small amount of stool was extruded to ensure that the wounds were patent. The cecum was then replaced in its original position within the abdomen,

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which was closed with sutures and wound clips (Becton Dickinson Primary Care Diagnostics, Sparks, MD). Each mouse received a subcutaneous injection of 1 ml of warm saline (37°C) and was then maintained in a closed room at 23°C. Two hours after surgery (and every 12 h up to 5 days), each mouse received a subcutaneous injection of imipenem (0.5 mg/mouse; reconstructed in 1 ml of lactated Ringers/D5W).

Previous work in our laboratory demonstrated that resection of the necrotic cecum could prevent subsequent abscess formation and improve survival (data not shown). Therefore, on day 4 (72 h after the initial CLP surgery, since the day of surgery was defined as day 1), surviving mice were randomly divided into two groups. One group was used as a control and continued to receive antibiotics, while the other underwent resection of the necrotic cecum under isoflurane anesthesia. Another 2-cm incision was made along the right side of the first incision. The cecum was isolated and removed at the end of the ligature, and the peritoneal cavity was flushed with saline. The abdomen was closed with sutures (muscular layer) and skin adhesive glue (Nexaband Liquid; Abbott Laboratories, North Chicago, IL). The mice that underwent surgical resection also received antibiotics for an additional 2 days.

Collection of samples and collection of data. The body weight was determined immediately prior to the CLP surgery, and this weight was used as the baseline for calculating the change in body weight in the postoperative period. EDTA-anticoagulated blood (20 μl) was collected from the tail vein at different times for measuring interleukin-6 (IL-6) levels or hematological analysis. The hematological analysis of whole-blood samples included obtaining a complete blood count with automated differentials, as well as counting red cells and platelets with a Hemavet 1500 (CDC Technologies, Oxford, CT).

Peritoneal lavage cytology and bacterial cultures. CLP mice with and without cecal resection were examined on a daily basis for 21 days. Dying mice in either the acute or chronic phase exhibited typical signs, including decreased physical activity, lethargy, and hypothermia (27). Mice that would die in the chronic phase typically lost 1 to 4 g of body weight over the 24 h prior to death. Based on previous studies, the mice with diminished physical activity, loss of body weight, and hypothermia would die within 1 to 3 days (27).

Using the criteria described above, mice were categorized as predicted to die (moribund) or predicted to live (healthy). Moribund mice were sacrificed, and corresponding mice predicted to live were also sacrificed. Each abscess was measured in three dimensions to calculate the total volume. After sacrifice, the abdomen was opened under sterile conditions, and the peritoneal cavity was flushed with 1.5 ml of 0.9% saline, taking care to avoid the abscess. The peritoneal lavage fluid was serially diluted with saline 1:10 to 1:1014, and 100-μl portions were cultured on 5% sheep blood agar plates (Fisher Scientific). The plates were incubated at 37°C for 24 h, and colonies were read by a trained microbiologist using routine microbiological procedures. The remaining washing fluid was centrifuged at 1,520 g for 5 min, and the cell pellets were reconstituted with 300 μl of saline, and a cytospin slide was prepared (Shandon Pittsburgh, PA) and stained with Diff-quick (Dade Behring Inc., Newark, DE).

Analysis of peritoneal cell function. Moribund and surviving mice were identified as described above during the chronic phase of sepsis. A complete blood count analysis was performed, and the peritoneal cavity was washed first with 1 ml of Hanks balanced salt solution and then more extensively with 25 ml of saline, and a cytospin slide was prepared (Shandon) and stained with Diff-quick (Dade Behring Inc., Newark, DE).

 Statistical analyses. Data were analyzed using GraphPad Prism (GraphPad Software, San Diego, CA). The results were expressed as means ± standard errors of the means. Differences between groups were compared by analysis of variance with Dunnett’s post hoc analysis, and a P value of <0.05 was considered significant.

RESULTS

Late cecal resection decreases mortality. CLP results in peritonitis secondary to growth of normal intestinal flora that gives rise to a polymicrobial infection (5, 19, 37). Based on mortality and physiological changes, the response may be divided into the acute phase, which occurs during the first 4 days, and the chronic phase, which occurs after day 4. For these experiments, mice were subjected to cecal ligation and puncture (n = 121 in five separate experiments). The mice that survived until day 4 were then randomly divided into two groups: (i) mice that received cecal resection plus antibiotics and (ii) mice that received antibiotics alone (i.e., no surgical treatment). Mice without CR developed abdominal abscesses and had a mortality rate of 33% between days 4 and 20. The mice with CR had a significantly lower mortality rate (5%) between days 4 and 20 (Fig. 1A).

We next examined both the natural history of abscess formation and whether there was a residual abscess following cecal resection. Groups of mice were sacrificed on either day 11 or day 21 after cecal ligation and puncture. Upon reexamination of the abdomen, the abscess was localized near the necrotic cecum. Cecal resection on day 4 significantly reduced the size of the abscess on either day 11 or day 21, and most mice had minimal remaining necrotic tissue. As shown in Fig. 1B, all the mice without CR had abscesses on day 11, whose volumes ranged from 24 to 9,000 mm3. The abscess volume on day 11 was greater than that on day 21, suggesting that the abscess reabsorbed over time. However, as mice continued to die between days 11 and 21, it is also possible that only the mice with smaller abscesses survived until day 21.

Change in body weight. We evaluated the effects of sepsis and cecal resection on the overall physical condition of an animal by determining the body weight each day. As previously reported, there was an initial decrease in the body weight for the animals that would survive, which represented the anticipated response to the stress of the initial surgery and infection (27). For mice that had cecal resection on day 4 there was a greater loss of weight over the next 24 h, which may have been related to the stress from the surgical procedure (Fig. 2A). Similar to our previous reports, the mice that died in the acute phase of infection exhibited a significant weight gain (32, 51). Mice that underwent CR began to recover their body weight by day 5, while mice that did not undergo CR did not begin to recover their body weight until day 9 (Fig. 2A).

While mice that died in the acute phase gained weight (32, 51), a different pattern of weight change occurred in the chronic phase. Mice that died after day 5 exhibited substantial loss of body weight 1 to 2 days prior to death. To illustrate this physiological response, we determined the weight up to 5 days prior to the day on which the animal died. Because the animals died at different times after CLP, the data are presented here relative to the day of death rather than relative to the number of days after CLP. Presenting the data in this manner allowed us to better correlate the change in body weight to the day of...
death. As shown in Fig. 2B, the early-death mice gained weight prior to death, and the late-death mice lost weight prior to death.

Hematological changes. We performed blood profile analyses throughout the study to closely document changes that occurred during both the acute and chronic phases. Within 24 h of CLP, significant leukopenia that affected primarily the lymphocytes developed (12) (Fig. 3). In Fig. 3, the values for time zero are the hematological values obtained prior to the cecal ligation and puncture surgery. There were significant differences in the hematological profiles between the mice that had resection of the necrotic cecum and the mice that did not have resection. In the mice without CR, there was an initial leukopenia, followed by an increase in the white blood cell count at day 10, which persisted until the end of the study. This increase was due to increases in lymphocytes (Fig. 3B), monocytes (Fig. 3C), and neutrophils (Fig. 4B) (see below).

In contrast, for the mice that underwent CR there was resolution of the hematological profiles after day 10. The mice without CR also developed anemia that became apparent by day 10 (Fig. 3). The mice were observed daily, and it became apparent that...
the neutrophil counts were also higher in the mice with grossly visible and palpable abscesses. When the abscesses were measured at the time of sacrifice (between days 11 and 21), the mice with abscesses whose volumes were >30 mm³ had significantly higher neutrophil counts (Fig. 4A). Following CLP, the neutrophil counts were higher for the dying mice than for the surviving mice, regardless of whether CR had been performed (Fig. 4B). The neutrophil count in the peritoneal cavity did not correlate with the peripheral blood neutrophil count.

**Bacterial overgrowth in the chronic phase of sepsis.** The data described above clearly indicate that cecal resection and removal of the focus for the abscess and associated necrotic tissue resulted in a significant increase in survival. We were specifically interested in determining the mechanism of death for the mice that did not have cecal resection. Examining these mice allowed us to determine if ongoing excessive inflammation accounted for the mortality or if there was a depressed immune state that allowed proliferation of bacteria and subsequent escape from the abscess. A third possibility was that there was bacterial proliferation that caused excessive inflammation and subsequent mortality. On the basis of physical examination, we could accurately determine if mice were likely to die or survive in the next 24 to 48 h (27). An experiment was designed to sacrifice mice that were predicted to die, as well as a healthy CLP mouse at the same number of days after CLP that was predicted to live. Following sacrifice, the peritoneal cavity was lavaged, and bacterial cultures were prepared from the fluid recovered. Care was taken to ensure that the residual abscess was not ruptured or disrupted in order to determine whether the bacteria had escaped from the abscess to seed the peritoneal cavity. For surviving mice, neutrophils, macrophages, and lymphocytes were observed in the lavage solution (Fig. 5A), as previously reported (12). The mixed inflammatory cell infiltrate was similar to that observed during the acute phase of infection. However, for the mice that were predicted to die, substantial numbers of bacteria were present in the lavage fluid, as well as within macrophages and neutrophils (Fig. 5B and 5C). Many of the bacteria were phagocytosed, indicating that the bacteria had leaked from the abscess prior to death and were not merely spilled into the peritoneum at the time of sacrifice. The number of bacteria within the peritoneal cavity was determined by examining cultures of the peritoneal lavage fluid. For the mice in which cecal resection was performed there were virtually no bacteria in the peritoneal fluid (Fig. 6), and cultures were positive for only 5 of 16 mice. For mice without cecal resection that were predicted to survive there were detectable bacteria within the peritoneal cavity, and positive peritoneal cultures were obtained for 25 of 35 mice.
However, for all of the mice predicted to die (17 of 17 mice) the peritoneal cultures were positive with significantly greater concentrations of bacteria (Fig. 6). The cultures were positive for a mixture of organisms, including *Escherichia coli*, *Enterococcus*, and some yeasts. These data demonstrate that in mice that die during the chronic phase of infection there is significant overgrowth of bacteria, indicating that the local proliferation of the infectious organism is not controlled.

**Plasma IL-6 levels for early and late mortality.** The data described above indicate that in mice that die in the chronic phase of sepsis there is bacterial overgrowth. It is still not known whether this bacterial growth induces a strong inflammatory response that causes death or whether the mice fail to
mount an inflammatory response and die in an immunocompromised state. We previously reported that after CLP, the plasma levels of IL-6 are substantially elevated 6 h after surgery in BALB/c mice (31, 32). These results and multiple other reports (42, 43) demonstrated that high levels of IL-6 in the early phases of sepsis in experimental animal models predict early mortality. In the present study with outbred ICR mice, the mice that would have died in the first 1 to 4 days had increased IL-6 levels (>18,000 pg/ml), but mice that survived until day 21 had substantially lower concentrations of IL-6 in plasma obtained 6 h after CLP (<5,000 pg/ml) (Fig. 7A). From day 2 to day 20, the plasma levels of IL-6 in the survivors became undetectable (Fig. 7A). We were curious if the plasma levels of IL-6 increased again prior to death in order to ascertain if elevated IL-6 levels during the chronic phase of sepsis could predict mortality, similar to the manner that IL-6 predicted death during the acute phase of sepsis. A clear answer did not emerge. During the first study 17 mice died during the chronic phase of sepsis, with frequent (24 to 48 h) blood sampling. For mice that would have died during the chronic phase either the IL-6 levels were undetectable (7 of 17 mice, 41% of late deaths) (Fig. 7B) or there was a sharp increase in plasma levels of IL-6 immediately prior to death (10 of 17 mice, 59% of late deaths) (Fig. 7C). For the mice in which the level of IL-6 did rebound, the typical pattern was an initial peak of the IL-6 level at 6 h, followed by a rapid decline by 24 h. The IL-6 level remained low for several days and then increased to 2,000 to 10,000 pg/ml for 1 to 3 days before death. However, an increase in the IL-6 level was not absolutely predictive of death since for two mice there were transient increases in the IL-6 level and the mice survived for more than 5 days after increases in the IL-6 level.

Assessment of cellular function. Experiments were designed to determine the capacity of the macrophages from the peritoneum to respond to an inflammatory stimulus. Mice identified as moribund 5 days after CLP were sacrificed, and peritoneal macrophages were obtained; macrophages were also obtained from healthy mice on the same days after CLP. Moribund mice were identified on days 7, 8, 12, 14, and 15 after CLP. The cells recovered from the peritoneal cavity included equivalent numbers of lymphocytes for both the moribund and healthy mice (Fig. 8A). The number of macrophages was not
significant differences between the two phases. While mice that die in either the acute or chronic phase succumb to sepsis, the mechanisms are almost certainly different. This information about the nature of the inflammatory response in different phases of sepsis may explain the failure of previous trials with cytokine inhibitors, since it was assumed that all septic patients died from an exuberant inflammatory response to infection (30). The immune status of a patient is constantly changing and may not be easily predicted based solely on the time when the initial infection began. This complex pattern was demonstrated in patients with meningococcal sepsis more than 16 years ago (46).

The CLP model of sepsis has previously been described to have at least two phases, a hypodynamic phase and a hyperdynamic phase (50, 52). The previous reports showed that adrenomeullin is responsible for the transition between the phases. Other studies defined the phases of the CLP model based on blood glucose levels (11, 21). For these studies, the first phase of sepsis occurred within 6 h, while the second phase was present at 18 h. There are important differences between these previous reports and the present work. In our study we examined a more chronic phase of sepsis, which is present after 5 days. This time frame (days rather than hours) is more typical of the clinical course in septic patients, who more frequently die over several days (18). Clearly, it is important to define immunopathologic events in the first hours of sepsis, but more closely determining the alterations during the later stages of sepsis, when most patients die, may have more clinical relevance.

As previously reported by us and other workers, plasma levels of IL-6 in the first 6 h could be used to predict the early deaths (32, 42). The plasma levels of IL-6 in the late phase of sepsis were not as predictive and also were not as elevated. For the early deaths the IL-6 levels at 6 h were more than 18,000 pg/ml in the nonsurvivors, while in the late stage of sepsis the

**DISCUSSION**

The murine model of CLP shares many features with human sepsis and has rapidly become a widely used tool for investigating immunopathological changes. Peritonitis in both animals and humans is polymicrobial, and the organisms include *E. coli* and *Bacteroides* species (16, 38). Antibiotic therapy has been proven to be effective for management of clinical intra-abdominal infections (1, 23) and improves survival in the CLP model of sepsis (28, 41, 42). Although the controversies concerning surgical management of intra-abdominal infections are actively argued (36), there is little debate about the conclusion that appropriate source control of the local infection, including incision and draining of an abscess, improves the outcome. Our data showed that excision of the necrotic tissue and removal of the abscess resulted in better survival. While this finding provides another piece of evidence concerning the utility of the CLP model, since it again parallels the human condition, the findings of the present study provide broader insights into the immunopathology of sepsis in the more chronic phase.

We defined our deaths as deaths that occurred early (in the first 5 days) or late (after day 5). While this is an arbitrary division, we believe that the data support the hypothesis that there are different mechanisms of death in the two phases. For mice that died in the early phase high circulating levels of IL-6, increases in body weight, and decreased numbers of circulating leukocytes were observed (32), while for mice that died in the chronic phase low levels of IL-6, decreases in body weight, and elevated numbers of circulating leukocytes were frequently observed. Using a different model of sepsis, it has been shown that the loss of body weight after day 5 is due to increased skeletal muscle proteolysis (44). On the basis of these simple, easy-to-measure parameters there are distinct differences between the two phases. While mice that die in either the acute or chronic phase succumb to sepsis, the mechanisms are almost certainly different. This information about the nature of the inflammatory response in different phases of sepsis may explain the failure of previous trials with cytokine inhibitors, since it was assumed that all septic patients died from an exuberant inflammatory response to infection (30). The immune status of a patient is constantly changing and may not be easily predicted based solely on the time when the initial infection began. This complex pattern was demonstrated in patients with meningococcal sepsis more than 16 years ago (46).
highest level was 12,700 pg/ml. A clinical study performed with 17 patients also demonstrated that IL-6 levels became elevated when the patients became moribund (25). Other recent clinical studies demonstrated that there were persistent elevated levels in patients with abdominal sepsis (20) or septic patients with renal failure (40). These data suggest that plasma levels of IL-6 need to be interpreted in light of the clinical information if the levels are to have strong predictive value. Additionally, 7 of 17 mice did not exhibit an increase in the IL-6 level prior to death, indicating that a negative value does not preclude a bad outcome.

It has been postulated by many investigators that an infection initiates the systemic inflammatory response syndrome (SIRS), which then progresses to the compensatory anti-inflammatory response syndrome (CARS) (8, 17). CARS is also described as a status of suppression, deficiency, or paralysis of the immune system (7). The data in this paper provide evidence that the proposed progression from SIRS to CARS is probably not a linear but rather constantly changing and that it requires close observation.

During CARS there is depression of the immune system with functional defects in monocyte antigen presentation, formation of oxygen species, and cytokine secretion (10, 45). These defects are illustrated by reports that stimulation of whole blood obtained from septic patients does not result in proinflammatory cytokine production (14, 15, 22). Several reports have also documented suppression of macrophage function following cecal ligation and puncture (4, 47). Suppressed macrophage function may allow an abscess to grow and also permit continued growth and release of bacteria into the peritoneal cavity.

While the concept of a SIRS-to-CARS transition is attractive, the natural history of sepsis frequently does not follow a clear path. The status of an individual patient at a specific time may be difficult to ascertain, and predicting the clinical trajectory of a patient is probably not linear but rather constantly changing and that it requires close observation.

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of injury severity but do not change mortality in mice with markedly elevated interleukin 6 levels. Shock 21:121–125.


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