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Modeling Sepsis in the Laboratory: Merging Sound Science with Animal Well-Being

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Despite impressive advances in biomedical research, few noteworthy breakthroughs have been made in the treatment of sepsis during the past several decades. This stalemate is primarily due to the intricate and heterogenic nature of the systemic immune responses characterized as the sepsis syndrome. In general, such complexity must be approached with in vivo models. Several animal models have been described, suggesting that none adequately address all of the pressing needs in sepsis research. The most clinically applicable models involve a localized infection, such as surgically induced polymicrobial sepsis, that gradually propagates a systemic immune response. Because relevant models must mimic a severe and chronic syndrome, animal well-being is often a concern in sepsis research. A balance between the needs of sepsis research and animal welfare can only be achieved through knowledge of the strengths and weaknesses of and alternatives to in vivo sepsis models.

Abbreviations: CASP, colon ascendens stent peritonitis; CLP, cecal ligation and puncture; LPS, lipopolysaccharide; TLR, Toll-like receptor

The term 'sepsis' embodies an intricate, systemic immune response to infection that can have serious consequences, including multiple organ failure and death. Historically, the sepsis syndrome has been responsible for countless deaths associated with infectious disease epidemics and battlefield injuries. The number of sepsis-related mortalities sharply declined with the discovery of antimicrobial agents in the mid-1900s. However, little improvement in the treatment of sepsis has been accomplished since that time. In the United States alone, sepsis is responsible for more than 200,000 deaths each year, and the incidence of sepsis steadily increases annually[4]. Antimicrobial resistance accounts for some of this increase but the population at risk for sepsis has also grown. With medical progress, physicians have gained the skills to perform sophisticated interventions in patients with risk factors such as advanced age, compromised immune function, and concurrent disease. Ironically, successful biomedical research has increased the need to understand the sepsis syndrome.

Considering the complexities of sepsis, research investigators rely heavily on in vivo models. However, many of these models are controversial. In some cases, the actual suitability of the model for sepsis research has been questioned. In fact, the use of inaccurate animal models or the misinterpretation of preclinical results is believed to be the root cause for some treatment failures in clinical sepsis trials.[22,28,77] In addition, sepsis models are controversial with regard to key animal welfare issues, primarily because of the inherent severity of the disease. To optimize animal welfare and scientific pursuits, the strengths and limitations of each animal model of sepsis must be understood. We therefore discuss herein the primary objectives of sepsis research and the characteristics of currently available models, with special attention to animal care concerns.

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What Is Sepsis?

An evaluation of the animal models of sepsis requires an understanding of a multifaceted syndrome. In human medicine, sepsis is defined as the manifestation of the systemic inflammatory response syndrome in the presence of infection. Consequently, standard clinical diagnosis of sepsis requires finding a focus of infection that is accompanied by at least 2 signs of the systemic inflammatory response syndrome. These systemic signs include aberrations in body temperature (greater than 38 or less than 36 °C), heart rate (greater than 90 beats/min), respiration (greater than 20 breaths/min or arterial partial pressure of CO_2 less than 32 mm Hg) and white blood cell counts (greater than $12 \times 10^3/mm^3$, less than $4 \times 10^3/mm^3$, or greater than 10% bands).[9] The term 'severe sepsis' denotes sepsis that is accompanied by major organ failure, whereas 'septic shock' refers to the added complication of unresponsive cardiovascular collapse.[9] In veterinary medicine, the definition of sepsis is not well-defined with regard to clinical diagnosis. For in vivo models, sepsis is achieved when an animal has an infection and demonstrates 'toxicity' (fever, anorexia, weight loss, or other signs of illness).[95] More importantly, appropriate animal models of sepsis must demonstrate aspects of the complex pathophysiology resulting from overwhelming infection.

Classically, sepsis is believed to be the result of a dysregulated inflammatory response. Initial theories suggested that exuberant production of proinflammatory cytokines and other soluble mediators was ultimately responsible for sepsis-related multiple organ failure and death. However, numerous clinical trials failed to confirm the efficacy of various anticytokine[1,34,68] and other antiinflammatory therapies.[8,82] Furthermore, antiinflammatory responses and even immune paralysis have been detected dur-

ing chronic infection.[72] Current studies suggest that the systemic inflammatory and compensatory antiinflammatory response syndromes occur simultaneously.[69] An unbalanced relationship between the 2 syndromes is thought to initiate a cascade of cellular signaling events producing severe effects in distant organs. Dramatic changes occur in the cardiovascular system as the initial hyperdynamic response (high cardiac output, low vascular resistance) seen in early sepsis reverts to the hypodynamic state of septic shock. Within the pulmonary system, secondary inflammation may cause progressive lung injury that leads to acute respiratory distress syndrome and organ failure. In addition, inflammation and vascular collapse can lead to gastrointestinal dysfunction, compromised gut barriers, and bacterial translocation. Sepsis-associated inflammation has been linked to functional changes in liver, kidney and endocrine organs. Unchecked, the result of overwhelming sepsis is progressive, system-wide organ failure and death.[43]

Sepsis and Research Goals

To identify effective therapeutic targets, sepsis research must answer a single question: What causes multiple organ failure and death in sepsis?[43] Because sepsis is an inflammatory syndrome, a large portion of the associated research evaluates aspects of the innate immune response. However, the recognition of immunosuppression in chronic sepsis has spurred studies on antigen presentation, lymphocyte function, and changes in acquired immunity.[44,45,57,91] In addition, independent experiments examine the relationships of these immune responses with organ failure in all body systems.[20,24,33,37,47,60,61] These studies must address localized cellular responses as well as the mediators that drive more distant effects. Multiple mediators are being investigated, including cytokines, complement, eicosinoids, and signal transduction molecules.[16,29,31,43] The recent approval of activated protein C, which reduces thrombin production, as a therapeutic agent has revitalized interest in the role of coagulation in sepsis.[7] In addition, novel research is investigating the control of immune function by neurologic and endocrine mechanisms.[3,15,59,91,99] The primary goal of these sepsis studies is to identify targets for intervention and evaluate therapeutic responses.

Although understanding complex immune responses will identify therapeutic targets, an appreciation for the heterogeneity of these responses ultimately will govern successful treatment. Host responses to infection apparently vary from patient to patient and are influenced by a number of parameters.[3,24] Factors such as age, sex, and environment definitely influence cell function, cytokine production and outcome during sepsis.[3,19] The role of concurrent conditions and the timing of additional insults during the development of sepsis are additional, clinically relevant concerns.[28] In addition, genetic background has an important role in governing the immune responses to an inflammatory insult.[19] Currently, these factors and their effects on the immune response to sepsis are the focus of intensive investigations. Because so many factors can influence the immune response to sepsis, optimal treatment likely must be tailored to the subject. This need is the motivation for studies aimed at identifying biomarkers that predict the severity of sepsis and allow customized treatment for each patient.[75] Considering the vast goals and complex nature of sepsis research, appropriate animal models appear to be crucial to the success of these studies.

Animal Models of Sepsis

An ideal animal model of sepsis would consistently translate relevant information from experimental animals to the human condition. Such success requires replication of the pathophysiology of sepsis, with particular emphasis on patterns of inflammation and cardiovascular parameters. Because invasive monitoring techniques and intensive care are part of the clinical scenario in humans, an ideal sepsis model similarly would accommodate these interventions. In addition, an ideal sepsis model would be low-cost and cause no distress to the animals involved.[22,28,32,35,46] Based on these criteria, the ideal model of sepsis does not exist.

Species selection. In attempting to meet some of the criteria for an ideal model of sepsis, the choice of species is one of the most important factors. The vast majority of sepsis studies are performed in rodents. Rodent species tend to be popular because they are small and relatively inexpensive. These traits make them useful for studies that require large numbers of animals.[22,32] The defined genetic characteristics of various inbred strains, as well as the availability of specific knock-out and transgenic strains of mice, have made rodents invaluable in the study of the biology of sepsis.[22] In addition, a wider array of reagents is available for immunologic studies in rodents than any other species. However, rodent models offer some disadvantages, particularly with regard to size. Parameters such as cardiac output and pulmonary artery pressure are measured more easily in larger species.[32] Furthermore, large volumes of blood cannot be collected from rodents without exsanguination, and obtaining numerous repeat samples in these species requires fluid replacement. These drawbacks complicate running some assays and analyses[32] and performing longitudinal analyses of parameters of interest.

In addition to their size, large animal models offer another distinct advantage in that they are more likely to reproduce the gradual pathophysiologic changes seen in humans with sepsis.[48,49] Furthermore, large animal species present as a relatively heterogeneous genetic population that is more representative of the human condition than is afforded by laboratory rodents.[22] Therefore, several models use dogs, cats, pigs, sheep, rabbits, and various species of primate in sepsis research. Dog and cat models have largely been replaced by pigs and sheep, due to heightened public concerns about these companion animal species.[22,32] Because of their docile nature, sheep often are used in chronic, unanesthetized models, particularly in studies investigating pulmonary pathophysiology.[22,32] The similarities between humans and pigs with respect to renal, cardiovascular, and gastrointestinal anatomy and physiology make pigs excellent experimental models.[22,32] Nonhuman primates, including baboons, cynomolgus macaques, and rhesus macaques, are used in some sepsis studies to closely replicate the human inflammatory response.[49] However, the use of nonhuman primates generally is reserved for preclinical studies or those in which a small sample size is sufficient, in light of the ethical imperative to use the least sentient species possible. The substantial expense and potential for zoonotic disease transmission must also be considered when using primate models of sepsis.[32]

None of the large or small animal species reproduce all of the physiologic and immunologic consequences of sepsis. Regardless, valuable information can be derived from each model when data are interpreted with regard for the limitations of that model. In fact, full evaluation of promising treatments for sepsis may require assessment using a series of animal models of increasing

complexity.[71] This type of approach may facilitate translational research and avoids the failures previously seen in clinical sepsis trials. Accordingly, we now discuss the applications, strengths, and weaknesses of several animal models of sepsis.

Nonsurgical models. Animal models that do not require surgery offer distinct advantages with regard to cost and may present fewer concerns with regard to animal welfare than do models that require surgery. These models generally create a systemic inflammatory response by means of parenteral injection of an agent that rapidly disseminates throughout the body. However, systemic signs also may develop in response to localized infections, such as subcutaneous abscesses and lung infections. Because they are used more commonly to study pulmonary defenses than sepsis, pneumonia models will not be reviewed here. Readers are encouraged to consult other sources for information on pneumonia models.[50]

Toxemia models. Toxemia models often are used to study the basic biology of sepsis. These models involve exogenous administration, either by intraperitoneal or intravenous injection, of a Toll-like receptor (TLR) agonist into a laboratory animal to produce inflammation and a shock-like state. Several injectable stimulatory agents have been used, including lipopolysaccharide (LPS) from the outer membrane of a gram-negative bacterium, CpG DNA, synthetic lipopeptides, zymosan, and others.[11] Typically, these models involve a one-time, high-dose injection of a compound into a rat or mouse with no additional supportive care. However, models that more closely mimic the clinical situation have been developed by introducing variations, such as fluid resuscitation, pharmacologic therapies, and continuous, low-dose infusions of TLR agonists.[32]

A single injection of endotoxin, or LPS, is the most commonly used toxemia model. Compared with humans, laboratory animals appear relatively insensitive to LPS and, therefore, require higher doses of LPS to produce a shock-like state. In a study directly comparing the effects of LPS, the dose required to produce similar cytokine responses was 250 times higher in mice than in humans.[16] A single dose of LPS (approximately 2 ng/kg) could produce profound physiologic effects in humans (for example, increases in body temperature, systolic blood pressure, and heart rate), whereas similar signs were not seen in mice despite a much higher dose of LPS (500 ng/kg).[16] The amount of LPS used to produce systemic signs in murine models varies widely, depending on the origin of LPS and mouse strain, but doses of approximately 10 mg/kg or higher are not atypical. Alternatively, doses may be reduced by sensitizing animals to LPS by coinjection of D-galactosamine.[11]

After LPS administration in mice, the onset of systemic clinical signs, including reduced motor activity, lethargy, shivering, and piloerection, occurs quickly and usually progresses rapidly.[76] In general, doses of LPS that are used in models of sepsis result in hypothermia, probably due to the rapid progression of the disease state and the large surface-area-to-volume ratio of these small animals. However, fever can be induced with sublethal doses of LPS (for example, *Escherichia coli* O111:B4 at 1 to 3 mg/kg) when mice are housed under thermoneutral conditions (27 to 31 °C).[52,59,66] In addition to these physical signs, bolus injection of high-dose LPS quickly produces a hypodynamic cardiovascular state, characterized by decreased cardiac output and increased peripheral vascular resistance.[10,24] Complete blood counts reveal decreased total white blood cell numbers, with reduction of lymphocytes and neutrophils.[76] In terms of effects on immune parameters, bolus injection of high-dose LPS induces a very rapid, but transient, increase in systemic cytokine levels.[11] Cytokine production exhibits a bell-shaped curve over an 8-h period after injection, with levels reaching a peak between 1.5 to 4.5 h and then declining.[76] The injection of LPS can produce a high mortality rate that varies with the LPS dose, type of LPS, animal strain, and age of the animal.

Endotoxin models are popular because they are convenient and reproducible. LPS is a relatively pure compound that is reliably measured, and its use can easily be standardized in experimental studies.[32] In addition, low-dose injection of endotoxin into healthy human volunteers produces pathophysiologic alterations similar to those reported in patients with sepsis, suggesting the value of endotoxin in studying sepsis.[32] However, studies of antisepsis agents that appeared promising in endotoxin models have not proven efficacious in human clinical trials.[11,22] Some of these failures have been attributed to the fact that administration of LPS in laboratory animals may not accurately replicate many important features of human sepsis. After high-doses of LPS, rodents exhibit the hypodynamic cardiovascular state without demonstrating the initial hyperdynamic cardiovascular state seen in humans with sepsis.[11,22,32] Likewise, the cytokine responses seen in LPS models are more rapid and several orders of magnitude higher than those seen in humans.[11,22] Overall, the clinical course and progression of disease in rodent LPS models is much faster than that seen in human sepsis.[11,22,42] Furthermore, the agent used for sensitization in some models, D-galactosamine, may cause hepatotoxicity.[36] Some of these criticisms have been addressed by variations of the model. Fluid resuscitation, bolus injection of lower doses, and continuous LPS infusion will produce models that tend to show the hyperdynamic cardiovascular state and more sustained physiologic responses.[11,22,32] Still, the application of endotoxin models is often questioned, and additional models are often used to demonstrate clinical relevance.

Live bacteria models. As an alternative to endotoxicosis models, live bacteria can be used in sepsis models. These models vary widely with regard to route of infection (blood, peritoneal cavity, subcutis, lung), frequency of administration (bolus, continuous infusion, osmotic pump), bacterial strain, and size of innoculum. All of these parameters can affect progression and outcome.[11,22,32] For example, in a porcine model,[17] continuous IV infusion of *Staphylococcus aureus* resulted in minimal hemodynamic and pulmonary changes, whereas infusion of *E. coli* or *Pseudomonas aeruginosa* resulted in hypodynamic shock and acute respiratory failure.[20] IV inoculation of *E. coli* results in rapid increases in serum cytokine levels; however, inoculation of the same dose into the peritoneal cavity does not generate a robust serum cytokine response.[29,97] In general, a single, large-dose bolus of bacteria tends to produce effects similar to those seen after IV injection of a high dose of LPS. The clinical course is rapid, consisting of a hypodynamic cardiovascular state, exuberant rise in serum cytokine levels and progression toward death within 12 to 24 h.[11,22]

Sepsis models using live bacteria have several advantages. The strain and infecting dose of bacteria can easily be standardized. In addition, the host immune response is directed at the whole microbe. However, these models have been criticized widely for not mimicking many of the important features of human clinical sepsis.[11] Similar to the endotoxicosis models, bolus injection of high doses of viable bacteria leads to a very different clinical course from that seen in human sepsis. In fact, live bacteria

models may be endotoxicosis models rather than true models of infection,[11,18,22] due to the rapid lysis of the bacteria by complement.[11,22] Another criticism of bacterial models is that the sudden administration of a single species of microbe is not relevant to the human situation, in which a septic focus typically seeds the body over time.[22,32] As with endotoxin models, bacterial infection models can be manipulated to produce more clinically relevant results. Irrespective of limitations, these models have proven useful and provide insights into mechanisms of the host response to pathogens.

Surgical models. In general, surgical models have become the most relevant type of sepsis model, because they create a specific focus of infection that can disseminate or cause a systemic immune response.[11,13,22] These models generally involve abdominal surgery to create peritonitis. Some of these models entail implantation of contaminated materials, whereas others involve breaching normal gastrointestinal barriers to create a slow exposure to a mixed population of bacteria. A common criticism of animal models of peritonitis is that, unlike in human peritonitis, animal models rarely include surgical intervention.[22] However, these models are still considered to most closely represent the clinical scenario with regard to the onset and progression of the sepsis syndrome.[11,46] By their nature, surgical models tend to be more expensive than nonsurgical models and involve special considerations with regard to animal welfare issues. Because they are considered more clinically relevant, the advantages and disadvantages of these models must be considered carefully with regard for scientific goals.

Implantation models. Although several infected materials have been used, models that use a bacteria-impregnated fibrin clot have received the most attention.[11,22,32] This model requires a major surgery to implant a fibrin clot into the peritoneal cavity to serve as a deep-seated focus of infection. Implantation of a microbe-laden clot has been used to produce sepsis in a variety of species, including the rat,[2] dog,[33,61] pig,[38] and baboon.[49] In general, this type of model replicates features of sepsis in humans, such as the hyperdynamic cardiovascular state, leukocytosis, extent of myocardial depression, magnitude of the cytokine response, and delayed mortality.[49,60,61] This model offers further advantages because the type and dose of infecting organism can be manipulated.[49] The use of a single organism in the fibrin clot elicits the same criticisms about clinical relevance as does IV injection of pure bacterial cultures. Still, the fibrin clot model, particularly when used with fluid resuscitation and supportive care, has been deemed clinically relevant.

Cecal ligation and puncture. The cecal ligation and puncture (CLP) model is considered the cornerstone of sepsis research. The model received initial favor because it reproduces the dynamic changes in cardiovascular function seen in humans with sepsis. In addition, CLP recreates the progressive release of proinflammatory mediators.[13,25,26] Therefore, CLP is considered to be one of the most clinically relevant models of sepsis.[11,46]

Although it models a complex series of pathologic events, the CLP technique is fairly simple and yields a reliable outcome. Early models of sepsis induced by cecal ligation without puncture in dogs[14] and pigs[47] have been described. However, in rodents, ligation alone resulted in an intrabdominal abscess without consistent systemic signs.[95] Consequently, a model of cecal ligation and puncture was proposed for use in rats[95] and later was adapted to mice.[6] In brief, the cecum is exteriorized through a small abdominal incision, a suture ligature is placed distal to the ileocolic junction, and a needle is used to perforate the viscus. If the cecum is perforated without ligation, the small holes may seal, and peritonitis does not occur reliably. For experimental studies, controls are generated by sham surgery in which the cecum is exteriorized and then replaced without ligation or puncture. Subcutaneous fluids are given after surgery. In mice and rats, the course of disease is accelerated (a few days) compared with that seen in humans (days to weeks). Initially, animals develop peritonitis secondary to infection. A mixed population of enteric bacteria (*Proteus mirabilis, E. coli, Bacteriodes fragilis, Enterococcus*) occurs in rats.[95] Blood cultures may be positive for several bacterial species.[95] Within a few hours after CLP, the animals demonstrate overt signs of illness, including piloerection, hunched posture, diarrhea, weight loss, and disruption of diurnal activity patterns.[25,26,64] Fever is not usually detected in mice, but postoperative hypothermia does occur and usually is progressive.[25] As described for the rat model, mice demonstrate features of early-stage sepsis (increased blood flow to organs, hyperglycemia and hyperinsulinemia), followed by characteristics of late-stage sepsis (decreased blood flow, hypoglycemia, increased serum lactate).[13,94] Without intervention, CLP results in progressive morbidity. However, the eventual outcome of CLP can be controlled experimentally. In acute models, severe sepsis leads to a moribund state, usually within the first 3 d after CLP.[25] With chronic models, the signs of sepsis are less severe, and animals can recover after several days.[26]

A number of technical variations can influence the intensity of inflammation and severity of outcome produced by the CLP model. The needle size and number of cecal punctures will modulate outcome. For example, two punctures created with an 18-gauge needle produces 100% mortality in BALB/c mice. Yet the same procedure yields mortality rates of 50% or 0% when performed with 21-gauge or 25-gauge needles, respectively.[25,26] Levels of proinflammatory cytokines in the peritoneum and plasma also increase in direct relationship to needle size.[25,26] The amount of devitalized cecum influences the degree of peritoneal inflammation; therefore, the location of the cecal ligature is a key determinant of inflammation and mortality after CLP.[81] In some experimental protocols, the necrotic cecum is excised, replicating surgical treatments in humans and, if performed early in sepsis, increasing survival rates.[6,75] Because CLP requires a major abdominal surgery, anesthesia and postoperative hypothermia become factors that modulate outcome. Various anesthetic agents can influence the inflammatory response.[37,40,83] In particular, the antiinflammatory effects of ketamine can have a protective effect in the CLP model.[83] Prompt correction of postoperative hypothermia can decrease mortality in the CLP model.[96] Finally, treatments given after surgery, which may include fluids, antibiotics, or both, can affect the syndrome produced by the CLP model. Postoperative fluids generally are recommended and required to produce the hyperdynamic cardiovascular state seen in early sepsis.[13,95] Antibiotics may reduce the dissemination of bacteria,[27] and sepsis severity will vary with the type of antibiotic administered.[65] Although these variables can influence outcome, a standardized technique tends to reliably reproduce a particular state of sepsis. In practice, a flexible model can be advantageous when studying a heterogenic disease process such as the sepsis syndrome.

In addition to surgical variables, laboratory animals have several inherent characteristics that can profoundly influence the resulting disease process in CLP models. Genetic composition

related to mouse strain can greatly influence the outcome after CLP,[19] and the procedure must be adjusted accordingly to create the desired severity of disease. For instance, the mortality rate is higher in C57BL/6 mice than A/J mice at distinct time points after CLP with a 25-gauge needle.[85] Similarly, sex steroids influence sepsis, and male mice may be less resistant to CLP than are female mice.[23,99] Likewise, aged mice appear to be more susceptible to sepsis in the CLP model than are young mice.[80,89] Because age, gender, and genetic background influence sepsis in human patients,[19] the CLP model is used extensively to study the mechanisms behind these effects. However, for individual sepsis experiments, strain, age, and gender must be standardized to produce uniform results with the CLP model.

Despite numerous advantages, the CLP model does have some limitations. With regard to the suitability of the model, the CLP procedure results in a substantial amount of devitalized tissue that eventually is contained in an abscess. The effects of this necrotic tissue are superimposed on the pathophysiology of the infection. Therefore, CLP creates a model of abscess formation and a course of sepsis that may differ from many cases in humans.[11,46] Further, the CLP model is not standardized within the published sepsis literature. Therefore, comparisons between studies must be made carefully, accounting for a number of potential differences in surgical technique and postoperative care. Despite these limitations, CLP is the most frequently used animal model of sepsis.

Colon ascendens stent peritonitis. Colon ascendens stent peritonitis (CASP) is a relatively new model of polymicrobial sepsis with limited exposure in the published literature. First described in mice,[98] CASP recently has been adapted to rats.[54] For both species, a midline laparotomy is used to expose the colon ascendens, and a stent is placed through the antimesenteric wall at a standardized distance aboral to the ileocecal valve. The stent is sutured in place and acts as a conduit for persistent fecal content leakage into the peritoneal cavity. Intraperitoneal fluids are given at the end of surgery.[54,98] Intestinal bacteria disseminate through the bloodstream, and the number of CFUs in whole blood rise exponentially from 3 to 12 h after implantation before reaching a plateau. Infection of internal organs, including liver, lung, and spleen, mirrors the pattern in the blood, with a time lag of several hours.[98] In addition, LPS is present in circulation as early as 2 h after implantation and rises in a similar manner to that of bacteria in the blood.[98] Systemic inflammatory and compensatory antiinflammatory response syndromes develop rapidly and concomitantly in the CASP model.[55,98] The inflammatory response of the host steadily increases over time, as evidenced by increases in serum cytokines from 6 to 18 h after CASP surgery.[55] In rats, IL-6, a potential prognostic indicator of mortality in sepsis models,[75] is the first serum cytokine to rise in the CASP model.[54] After approximately 12 h, rats begin to show signs of lethargy and ruffled hair coat.[54] Cardiovascular parameters reported for rats remain stable until 1 h before death and then drop rapidly.[54] In both rats and mice, deaths generally occur 1 to 2 d after implantation, with almost no deaths seen after 3 d.[54,55,98] Death occurs after multiple organ failure, including lung, liver, and kidney.[31]

As with the CLP model, variations in the surgical procedure can alter outcome in the CASP model. The severity of sepsis can be modulated reproducibly by varying the diameter of the stents.[54,55,98] This variation appears to affect the mortality rate, but not the time course, associated with CASP-induced sepsis. In addition, serum cytokine levels vary in magnitude with stent size.[55] The stent can be removed after CASP to create a more clinically relevant model, and the timing of this intervention will affect outcome. Removal of 14-gauge stents at 3 h after implantation led to 100% survivability, whereas removal after 9 h did not prevent death.[98]

Although currently limited in popularity, CASP appears to be a well-received model of acute polymicrobial septic peritonitis[11] with several advantages. CASP is reproducible and replicates several important features of sepsis in humans. In particular, similarities in some cytokine profiles (TNF-α and INF-γ) between CASP-operated mice and septic human patients are evident,[98] although IL-6 and IL-10 levels generally are greater in mice than in human patients.[30,94] In addition, LPS levels in the CASP model are similar to those in patients with sepsis.[98] Contributing to the clinical relevance of the model, the prolonged phase of hemodynamic stability seen after CASP potentially may allow its application to more complex and invasive experiments.[54] CASP may offer advantages over CLP because CASP does not impair cecal blood flow with resultant necrosis and abscess formation.[54] However, the leading drawback of CASP is that it is a more challenging procedure to perform than is CLP. Improper surgical technique during placement of the stent can lead to lack of patency or abscess formation.[11] In addition, the hemodynamics associated with CASP, primarily cardiac output and systemic vascular resistance, are not yet well defined.[11] Because cardiovascular parameters have been important determinants of model applicability,[95] CASP may require further investigation before it is widely accepted in sepsis research.

Animal Welfare Concerns in Sepsis Studies

Several factors appear to put the field of sepsis research at odds with key tenets of animal welfare. The primary animal welfare issues are the product of the severity of the disease and the ultimate goals within the field of sepsis research. The following paragraphs describe the goals of sepsis research that influence investigators' decisions in the use of animal models of sepsis. In addition, we present ways in which these issues that conflict with the most humane care for laboratory animals can be overcome.

Scientific considerations and constraints. Because the cause of multiple organ failure and death in sepsis is unknown,[43] researchers focus on late-stage sepsis and factors that lead to death. In addition, success in clinical sepsis trials is judged by 28-d all-cause mortality,[73] and many investigators judge animal studies by the same standard. These issues drive investigators toward the use of severe, endstage models. From an ethics standpoint, this use contradicts views that death as an endpoint is objectionable and should be replaced by alternatives. However, the use of alternative endpoints can cause scientific concerns. Because slight improvements in mortality rates are considered significant advances in sepsis treatment, imprecise endpoints could lead to premature euthanasia and skewed data. In addition, various investigators suspect that some publishers will not accept data from sepsis studies that use alternative endpoints[12] because in that event, no benefit would be derived from the animal studies. The determination of when to terminate sepsis studies will be a continued source of debate until alternatives that address these concerns can be offered to investigators and accepted by reviewers and publishers.

Concerns about animal welfare also arise because sepsis models are often combined with other models of disease. Clinical sepsis

may develop as a result of risk factors, such as advanced age or diabetes.[4] In addition, sepsis can develop concurrently with other inflammatory insults, and many research studies are devoted to understanding the effects of combined injuries.[5,62,67] The 'two-hit' theory of inflammation suggests that an initial event will prime the host for an exacerbated inflammatory response to a second insult.[21] However, the outcomes appear even more complicated, depending on the strength, timing, and compartmentalization of the insults. To study this phenomenon, sepsis models are often paired with other major insults, such as hemorrhage–CLP,[99] CLP–pulmonary aspiration,[62] burn–CLP,[67] and laparotomy–endotoxemia models.[5] These studies often increase animal welfare concerns exponentially. However, given the clear clinical relevance of these situations, investigators are compelled to pursue these studies, and animal care committees will be asked to evaluate them.

To compound the concerns about the severity of sepsis models, sepsis studies often do not use analgesics. Investigators opt not to provide analgesics for a number of reasons. Analgesics are not the first line of defense in cases of infection, which may be a justification for excluding the use of analgesics from some sepsis protocols. However, this issue is particularly difficult because the most clinically relevant models require surgery. In addition, sepsis often occurs secondary to conditions that are treated with analgesics, such as trauma, burns, and surgery,[4,58,92] thereby suggesting that modeling sepsis without analgesics provides an inaccurate clinical picture in some situations. Whether animals with sepsis actually experience or are aware of pain is another question, partly because human patients develop sepsis-associated encephalopathy, a diffuse cerebral dysfunction induced by a systemic response to infection.[15] This encephalopathy has been identified at all stages of sepsis as a result of multiple organ failure or other, as yet unidentified, causes.[15] Humans with sepsis-associated encephalopathy have attenuated or even absent pain responses, and this characteristic may be a consideration in animal models. However, whether an unresponsive animal is completely unaware of pain is unknown,[88] and the role of sepsis-associated encephalopathy has not formally been investigated as a determinant for analgesic use in animal models. Of greatest concern, the use of analgesics may alter the course of the disease syndrome. Because sepsis is caused by aberrant inflammatory responses, the use of steroids and nonsteroidal antiinflammatory agents is problematic. Exogenous opioid administration also has immunomodulatory effects, including inhibition of antibody and cellular responses, natural killer cell activity, cytokine expression, chemokine-induced chemotaxis, and phagocytic acitivity.[58,90] Although the exact mechanisms are unknown, the effects likely are due to opioid receptor binding on immune cells[90] and indirect activation of the hypothalamic–pituitary–adrenal axis and sympathetic nervous system.[58,79,90] As a result of these mechanisms, morphine administration can increase susceptibility to numerous pathogens including viruses, bacteria, and parasites.[41] Likewise, morphine-treated mice appear to be more susceptible to LPS, because of a combination of immunosuppression, decreased gastrointestinal transit time, and increased bacterial translocation.[41] These points suggest that, even if analgesics were required, finding an agent that would not interfere with the established sepsis model might be difficult. Further research is needed to determine the effects of analgesics on the sepsis syndrome in both humans and animal models. Until then, the justification for or against analgesic use must be addressed on an individual basis.

Clearly, several welfare issues in sepsis research are the subjects of recurrent debate. Considering the number of questions that arise, a search for ways to improve animal welfare seems warranted. These issues can be addressed through the concepts of replacement, reduction, and refinement.[78]

Finding solutions that incorporate the 3Rs. Complete replacement of animal models of sepsis is difficult. The conglomerate of immune responses associated with sepsis is too complicated to model in cell culture systems. However, cell culture certainly can model specific aspects of the biological response and may be used in the initial steps toward understanding mechanisms. More complex biological interactions may potentially be modeled in whole blood. Whole-blood assays offer the possibility of studying serial samples from a single subject.[63] However, the volumes necessary for these assays may limit their use to larger animals. In addition, the results of whole-blood assays may not always reflect the responses generated in the whole animal.[23] Mathematical models have been developed to examine acute inflammatory responses in sepsis and someday may allow replacement of some animal models.[93] A recent proposal suggests the value of spontaneous models of sepsis. This concept uses a comparative, 'one medicine' approach, suggesting that information from clinical veterinary medicine can be used to advance sepsis research. Studying the pathophysiology and treatment of clinical diseases, such as parvovirus in dogs, offers advantages over rodent models, particularly with regard to availability of intensive monitoring and more invasive treatments.[70] Previous successes in the fields of cancer and orthopedic research suggest that spontaneous disease may offer answers to questions once studied only in laboratory models.

Reduction of animal numbers in sepsis research can be accomplished through several means. The use of appropriate study design is paramount. In addition, standardization of sepsis models between laboratories would decrease the need to repeat experiments for comparison. High-throughput technologies also offer the opportunity to reduce animal numbers. When small samples can be used to obtain large amounts of information, trends can be followed by serial sampling of subjects, thereby markedly reducing animal numbers and improving the quality of results.

Refinement is the greatest challenge with the highest potential gain in regard to animal welfare issues in sepsis studies. Perhaps the most valuable refinement would be to create a model that truly mimics the clinical scenario in humans, complete with vasopressors, artificial ventilation, blood transfusions, and dozens of treatment options.[11,22,28] Successful treatment modalities in this type of model would translate most readily to the clinical situation, offering solutions rapidly and decreasing the need for animal experimentation. Producing a more clinically relevant model inherently would require better understanding of pain perception and analgesic use during sepsis. For example, some opioids (buprenorphine, hydromorphone, oxycodone, and tramadol) appear to be less immunosuppressive than others (morphine and fentanyl).[39,56,79] In addition, tramadol, unlike fentanyl, does not prolong gastrointestinal transit in the CLP model in rats.[87] These agents may provide options for sepsis models and should be investigated further.

Until the welfare of animals in sepsis studies can be refined further, humane endpoints need to be defined for sepsis models. Several humane endpoints have been recommended for rodents used in infectious disease and other fields of research.[51,64,66,84,88] In

addition, humane endpoints for studies using endotoxins[53,86] and specific microbes[51,84] have been reported. Because these models do not universally replicate the sepsis syndrome, it is no surprise that their endpoints do not directly apply to the polymicrobial sepsis induced with the CLP model. For instance, hypothermia must be more pronounced to predict death after CLP than after intratracheal infection with *Klebsiella pneumoniae*.[51] For a specific murine CLP model, changes in body weight, temperature, and plasma IL-6 levels were identified as potential surrogate endpoints.[64] However, these values will not pinpoint the actual time of death and are of value only if the study can be terminated early in the course of sepsis. The inability to ambulate identifies endstage illness after CLP in mice, but whether late endpoints actually reduce animal suffering is a point of debate.[64] Some of these parameters might also be used as biomarkers to provide alternative refinements to sepsis studies. As a biomarker of sepsis, IL-6 levels have been used to stratify animal groups according to disease severity after CLP.[74] This measure allows evaluation of therapeutic agents for efficacy and toxicity at multiple levels of sepsis severity. Used in this manner, biomarkers address the heterogeneity of immune responses and reduce animal numbers in sepsis studies. When used as biomarkers or humane endpoints, the absolute values for many parameters will change depending on variations in CLP model and the severity of sepsis. Consequently, individual investigators likely should establish unique values for accuracy with endpoints.[64] Once endpoints are established, the use of scoring sheets can provide an objective way to measure multiple clinical signs of illness. This practice facilitates consistent application of human endpoints points for septic animals. Therefore, noteworthy refinements of sepsis models likely are possible but will require further investigation to ensure valid experimental results.

Conclusion

At this time, it is widely accepted that there will never be a 'magic bullet' to treat sepsis—treatment of sepsis likely will remain as complex and variable as the syndrome itself. Therefore, studies probably will remain highly dependent on animal models to discern the causes of multiple organ failure and death in sepsis. These models likely will evolve over time to more accurately portray the clinical pathophysiology, intensive monitoring, and critical care of humans with sepsis. In the meantime, valuable results can be obtained from many models, but the information must be interpreted with respect to the characteristics of the model. The evaluation of promising therapies may require evaluation in a series of preclinical models. Out of scientific necessity, these preclinical studies will focus on severe and late disease. This requirement will continue to prompt concerns for animal well-being and the need to refine sepsis models. Beneficial refinements appear to be possible but must be approached carefully, on a case-by-case basis, to ensure accuracy of the sepsis models in individual laboratories. Armed with a comprehensive understanding of the animal models, we can more readily achieve the goals of sepsis research to the benefit of both humans and animals.

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