MAJOR ARTICLES

What Are the Microbial Components Implicated in the Pathogenesis of Sepsis?
Report on a Symposium

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Despite considerable efforts in the past quarter century to improve therapy for sepsis, mortality rates remain unacceptably high. Microbe-derived constituents can induce the host to produce many mediators that can contribute to immune dysregulation, tissue damage, and death. Although endotoxin-mediated events are clearly important in gram-negative infections, gram-positive bacteria can also play a dominant role. Understanding the interplay of microbial constituents and host immune or inflammatory responses prompted a meeting at Rockefeller University in May 1998. Participants discussed the relative merits of a “2-hit” hypothesis to explain the course of lethal septic shock and a “multihit” synergistic threshold hypothesis. Recommendations include the following: (1) developing animal models that closely mimic human sepsis; (2) further investigating antibiotic effects on bacteria; (3) assessing the relationships between endotoxin, prokaryotic DNA, and peptidoglycan (i.e., independent, additive, or synergistic) in inducing host responses; and (4) developing new strategies to improve outcomes. Studies are needed to better define which and how different microbial constituents lead to sepsis and to provide critical leads for therapeutic intervention.

Mortality rates due to sepsis remain unacceptably high and have not improved substantially over the past 25 years. The induction of a host systemic inflammatory response due to microbial infections is complex and incompletely understood [1]. Components of invading microbes can induce the host to initiate a cascade of events that, if unchecked, can lead to irreversible tissue damage and death. Many of the therapeutic interventions have been directed against endotoxin [2]; however, one of the drawbacks of specific anti-endotoxin strategies for the treatment of septic shock is the observation that it may not benefit patients with gram-positive sepsis. Endotoxin-mediated events are, in many instances, insufficient to explain fully the multitude of potential deleterious effects of polymicrobial bacterial infections, including exaggerated host immune responses, observed in actual clinical practice [3].

Gram-positive bacterial pathogens are increasingly recognized as major contributors to sepsis, as evidenced by recent clinical studies of sepsis [4]. The immunopathogenesis of septic shock related to gram-positive bacteria is increasingly appreciated to differ significantly from that seen in gram-negative sepsis. Exotoxins from many gram-positive bacteria can function as superantigens to promote the release of lymphokines and monokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β, that can then function as proinflammatory mediators of sepsis. Additionally, bacterial peptidoglycan can activate inflammatory mediators observed in sepsis via CD14-dependent and perhaps CD14-independent activation of monocytes and macrophages [5]. Bacterial DNA, moreover, has also been recently recognized to activate inflammatory cells and results in the secretion of inflammatory mediators. Unmethylated CpG motifs found in bacterial DNA serve as the major structural feature of prokaryotic DNA that promotes a proinflammatory response. The most potent motifs include CpG dinucleotides flanked by two 5’ purines and two 3’ pyrimidines. These motifs are less prevalent in vertebrates and are highly methylated [6–9].

Interactions between superantigens and bacterial endotoxins that can induce host inflammatory responses have been the subject of much recent research [10]. Superantigens and endotoxin initiate inflammatory networks that work in an additive or synergistic fashion in many experimental models [11]. Significantly less is known, however, about the potential interplay between peptidoglycan and bacterial DNA with superantigens, endotoxin, or both in systemic bacterial infection. The relative contributions and clinical relevance of these microbial com-
The pathogenesis of septic shock. Endotoxin and peptidoglycan bind to receptors and proteins that activate macrophages to release inflammatory mediators that, under some circumstances, can lead to tissue damage and death. In addition, other microbial constituents may bind to these and other receptors to elicit a host response.

Table 1. Host-derived inflammatory mediators.

<table>
<thead>
<tr>
<th>Proinflammatory cytokine networks</th>
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<tr>
<td>Complement system</td>
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<tr>
<td>Bradykinin</td>
</tr>
<tr>
<td>Prostaglandins and leukotrienes</td>
</tr>
<tr>
<td>Reactive oxygen intermediates</td>
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<tr>
<td>Nitric oxide</td>
</tr>
<tr>
<td>Coagulation and fibrinolytic system</td>
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<tr>
<td>Platelet-activating factor</td>
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<td>Other host-derived mediators [16-18]</td>
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Figure 1. The pathogenesis of septic shock. Endotoxin and peptidoglycan bind to receptors and proteins that activate macrophages to release inflammatory mediators that, under some circumstances, can lead to tissue damage and death. In addition, other microbial constituents may bind to these and other receptors to elicit a host response.

Components to septic shock are unknown at present; however, they may work in concert to produce a septic syndrome in the presence of polymicrobial bacterial infections (i.e., intra-abdominal infection or aspiration pneumonitis).

The recognition of the complexity of the microbial components that cause lethal septic shock led to a day-long symposium at Rockefeller University. The issues on the agenda focused upon 2 overriding questions facing clinical medical practice: Why do patients with sepsis die? And how can we prevent this? Although the discussion raised many more questions than answers, it was concluded that a much more complex understanding of the role of endotoxin, peptidoglycan, exotoxins, and prokaryotic DNA and the interrelationships in effecting an immune or inflammatory response in septic shock would likely lead to better therapeutic interventions.

Contribution of Endotoxin to the Septic Process

Bacterial endotoxin (or lipopolysaccharide [LPS]) is widely considered to be the principal component responsible for the induction of septic shock that often accompanies severe infection with gram-negative bacteria. Despite several recent well-documented failures of anti-endotoxin monoclonal antibodies as treatment for gram-negative septic shock [12, 13], endotoxin remains an active target for improving the treatment of sepsis, and a number of additional clinical trials are currently underway.

Endotoxin is an essential structural component of the outer membrane of all gram-negative bacteria. It is a unique macromolecular structure that is highly anionic and possesses variable hydrophobic and hydrophilic regions. It has been known for almost half a century that systemic injection of highly purified bacterial endotoxin is highly toxic and results in a pathophysiologic state compatible with septic shock from bacteremic gram-
negative bacilli [1, 14]. Endotoxin is thought to function, therefore, as a microbe-derived alarm molecule that indicates the presence of invasive gram-negative bacteria within the body.

Pattern recognition molecules of the innate immune defense system (i.e., CD14, mannose binding protein, toll-like receptors, and alternative complement system) expressed on membrane surfaces or in soluble form detect microbial structures such as endotoxin, peptidoglycan, and prokaryotic DNA [15], all of which are foreign to, and structurally distinct from, host-defined structures. This important recognition system serves to alert the host of the necessity to initiate a series of host clearance mechanisms that eliminate the invading microbial pathogen or pathogens. These physiologic defense mechanisms have appeared to evolve as survival strategies to localize, contain, and eradicate invasive pathogens. Paradoxically, these same immunologic responses that defend the host in localized bacterial infections may be responsible for a generalized and potentially fatal systemic inflammatory response [1, 12–14] (figure 1). For example, uncontrolled activation of host-derived inflammatory mediators (table 1) by bacterial endotoxin and, presumably, a variety of other microbial mediators, is currently accepted as the major pathway to account for the deleterious pathophysiologic state that characterizes gram-negative septic shock. Anti-endotoxin treatments (table 2) have many advantages over anti-mediator therapies (such as anti-TNF antibodies or platelet-activating factor antagonists) that are currently under investigation for sepsis, in that LPS is an unwanted microbial toxin that can be completely eliminated without potential harm to the patient. This may not be true for other treatments directed to essential elements of the host immune system [39, 40].

Key components of inflammation, such as TNF-α and IL-1β, are critical factors in the host defenses against invasive microbial pathogens. Although uncontrolled production of these mediators can lead to adverse pathophysiological consequences and tissue damage, it is also conceivable that inhibitors of these cytokines may place the patient at increased risk for overwhelming infection from the microorganisms that caused the initial septic insult. In some experimental animal systems and in at least 1 controlled clinical trial [41], anti-mediator therapies have actually been shown to be disadvantageous to the host in the presence of an invasive infection [40]. Anti-endotoxin therapies, in contrast, do not appear to impair these intrinsic host defense mechanisms, and this may allow their use as preventative agents (e.g., as vaccines or passive immune therapy in patients at high risk for sepsis) or treatment interventions in the early phases of sepsis, when therapeutic agents are likely to be most effective. However, a rapid host response to the presence of microbial factors such as endotoxin may have precluded otherwise effective therapies [42].

### The Role of Gram-Positive Bacteria in the Pathogenesis of Sepsis

As pointed out above, LPS has been widely studied as the chief causative factor in gram-negative sepsis. It is clear, however, that gram-positive bacteria can also cause sepsis and septic shock. This is not likely to be mediated through LPS directly, as gram-positive bacteria lack endotoxin. However, it has been hypothesized that the mechanism by which endogenous endotoxin and gram-negative microbes are introduced during gram-positive sepsis is the disruption of the permeability barrier of the large bowel by means of inflammation-mediated mechanisms [43]. Nevertheless, it is important to recognize and study other microbial factors capable of inducing the host inflammatory response. Possible candidates—a few of many—are exotoxins, peptidoglycans, and prokaryotic DNA [44, 45].

Although studies that date back >30 years have strongly implicated bacterial exotoxins as potentially capable of perturbing host immune systems, not until recently have these important microbial products been identified as potent inducers of lethal shock. Exotoxins, moreover, have a role in inflammation, interact with Vβ domains in the T lymphocyte antigen receptor to act as superantigens, and can also stimulate macrophages directly [46–48]. The pyrogenic exotoxins of group A streptococci and the staphylococcal enterotoxins are a family of structurally related toxins with similar biological activity [49]. These antigens act like superantigens to cause toxic shock-like syndromes in both streptococcal and staphylococcal infections [47].

The structural similarity among the exotoxins from various organisms has been exploited to create a potential anti-exotoxin therapy. To date, 2 distinct molecular domains have been identified that have a highly conserved amino acid homology common to most members of the known toxin families [46]. A peptide

### Table 2. Proposed anti-endotoxin strategies.


### Table 3. Lethality of purified Escherichia coli DNA in C3H/HeJ mice at 48 h.

<table>
<thead>
<tr>
<th>DNA, μg</th>
<th>Control mice no. dead/no. alive</th>
<th>Mice treated with dexamethasone, no. dead/no. alive</th>
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<tbody>
<tr>
<td>300, double stranded</td>
<td>0/5</td>
<td></td>
</tr>
<tr>
<td>400, single stranded</td>
<td>1/3</td>
<td></td>
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<tr>
<td>400, double stranded</td>
<td>4/5</td>
<td>0/5</td>
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<td>600, single stranded</td>
<td>3/3</td>
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<tr>
<td>600, double stranded</td>
<td>—</td>
<td>0/5</td>
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<tr>
<td>1000, single stranded</td>
<td>3/3</td>
<td>—</td>
</tr>
<tr>
<td>1000, double stranded</td>
<td>—</td>
<td>0/5</td>
</tr>
<tr>
<td>1000, double stranded, + deoxyribonuclease</td>
<td>0/3</td>
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NOTE. Mice were resistant to the lethal effects of lipopolysaccharide (C3H/HeJ) but sensitive to the lethal effects of tumor necrosis factor due to treatment with D-galactosamine [60].

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based on these 2 highly conserved regions of the staphylococcal and streptococcal toxins has been synthesized. This peptide, as in the native toxin molecule, is representative of the 2 consensus regions joined in the proper sequence. Antisera to this peptide have been shown to block blastogenesis and proliferation induced in assays by many superantigens. The antibody, furthermore, showed passive protection in an in vivo model [50].

In addition to exotoxins, peptidoglycans have been implicated as mediators of septic shock [44, 45, 51]. Peptidoglycans are components of the cell wall of gram-positive and gram-negative bacteria that provide structural integrity to the cell wall. The basic repeating unit is an alternating motif of β 1→4 linked neuraminic acid–N-acetyl glucosamine molecules, in which the interpeptide bridges vary from organism to organism. As illustrated in figure 2, part of the peptidoglycan molecule bears a striking 2-dimensional structural similarity to part of the lipid A moiety of gram-negative organisms [5, 44]. In addition, both of these molecules bind to CD14 [5].

Peptidoglycans possess a range of biological activities in mammalian hosts, including cytotoxicity and pyrogenicity. Purified staphylococcal and group A streptococcal peptidoglycans have demonstrated the capacity to induce in vitro the release of TNF-α from human peripheral blood mononuclear cells [52, 53]. Peptidoglycan also has been shown in rats to cause the release of TNF-α and interferon gamma, which in turn increases expression of inducible nitric oxide in various tissues [54]. Peptidoglycan, moreover, can induce platelet aggregation, which may be an important precursor to disseminated intravascular coagulation—a serious manifestation of septic shock [55]. Streptococcal cell walls and peptidoglycan, furthermore, can induce an inflammatory
arthritis in the rat and stimulate synovial fibroblast plasminogen activator activity by monocytes [56].

There is also evidence of synergy between peptidoglycan fragments and LPS. Mice given iv peptidoglycan in pyrogen-free saline were injected 4 h later with sublethal doses of LPS. The mice exhibited immediate shock and death in response to some forms of peptidoglycan [57].

Antibiotics used in the treatment of microbial sepsis may release increased amounts of microbial constituents such as exotoxins and peptidoglycans, much in the same way that they have been previously shown to affect the release of bacterial endotoxin. Many of the antibiotics affect peptidoglycan synthesis in treated bacteria. Peptidoglycans are released from a number of gram-positive bacterial organisms when they are grown in the presence of penicillin; however, when staphylococcal organisms are grown in the presence of vancomycin, there is no corresponding increase in peptidoglycan levels [58]. Use of β-lactam antibiotics, moreover, greatly enhances the release of peptidoglycan from Staphylococcus aureus in culture, which does not occur with the addition of protein synthesis inhibitor antibiotics [59].

The clinical choice of antibiotics, therefore, can affect the release, the production, or both of endotoxin and peptidoglycan and might affect exotoxin activity. Antibiotics, moreover, may differentially affect the interrelationships between these microbial constituents, either additively or synergistically, in eliciting an exuberant immune response, thereby effecting the eventual outcome for the host—recovery or death [1].

Evidence for Microbial DNA as a Proinflammatory Mediator in Sepsis

Collectively, the data summarized above strongly support the hypothesis that the host immune or inflammatory response pathways that may be initiated after the introduction of microbes depend heavily on recognition of structures not shared between prokaryotic microbes and their eukaryotic hosts. In this respect, endotoxin, peptidoglycans, and exotoxins are all unique to prokaryotic organisms and thus would serve as ideal pattern recognition ligands for signaling host responses. Importantly, however, significant differences are now known to exist between microbial and eukaryotic DNA in terms of methylation patterns, with CpG sequences being routinely methylated in the latter but not in the former. It might not be surprising, therefore, to find that the host immune or inflammation systems have capitalized on these differences and that microbial DNA can indeed serve as a proinflammatory signal.
Table 4. Future actions that will help researchers learn how to better explain why patients with sepsis die or prevent their deaths.

| Creation, standardization, or both of animal models that use actual infections and closely mimic human clinical sepsis, especially for patients in an intensive care unit setting |
| Further investigations of the effect of antibiotics on releasing microbial constituents from bacteria, especially concerning the release of biologically active DNA, peptidoglycan, and/or exotoxins |
| Future studies to determine the role and interrelationships between gram-positive and gram-negative bacterial DNA, lipopolysaccharide, peptidoglycan, and/or exotoxins in potentiating the inflammatory response |
| Development of therapeutic interventions directed singly or in combination against the immune effects of endotoxin, exotoxins, peptidoglycan, and/or microbial DNA |

For example, investigators in several different laboratories have demonstrated that bacterial DNA can function as follows: (1) as an immunologic adjuvant to promote host immune responses to unrelated antigens, primarily within the format of DNA vaccines [7]; (2) as an immunostimulant for the production of TNF from mouse macrophages in vitro [6]; and (3) as an agent capable of eliciting a lethal response in TNF-hypersensitized mice in vivo (table 3) [6, 60]. Collectively, these findings support the hypothesis that microbial DNA itself may be a significant microbial mediator of inflammation in both gram-positive and gram-negative sepsis.

Recent studies suggest that both purified microbial DNA and synthetic DNA oligonucleotides can induce the production of TNF-α and nitric oxide in vitro in cultures of mouse peritoneal exudate macrophages [61]. To begin to explore structure/function studies of various chemical properties that dictate the ability of DNA to induce the production of inflammatory mediators, 18 mers of palindromic DNA oligonucleotides were synthesized and tested in vitro in culture for their ability to activate mouse peritoneal macrophages to produce proinflammatory cytokines. Because previously published results supported the hypothesis that oligonucleotides containing CpG sequences were necessary for optimal activity, this variable was specifically tested by the synthesis of oligonucleotides with these sequences as well as those in which the CpG motif was reversed. Preliminary results suggest that significantly (~100-fold) more immunostimulation occurs with oligonucleotides containing CpG sequences in comparison with sequences in which these 2 bases are reversed. Additional results show that the minimal effective size for such oligomers is at least a 12-mer containing 2 CpG sequences and that the 18-mer is ~10-fold more active than the 12-mer. These preliminary findings support the concept that specific structural features of the DNA may be important for macrophage activation and production of inflammatory mediators such as TNF that can then induce lethality.

Recent studies to assess the in vivo effects of bacterial DNA have also been performed. In one published study in which lethality in the D-galactosamine (TNF hypersensitized) mouse model was assessed, it was shown that DNA, like LPS, could serve as a potent in vivo inducer of TNF production [6]. These initial results have been confirmed, and additional experiments have established that the in vivo biological activity is both sensitive to prior in vitro treatment of DNA with deoxyribonuclease and by treatment of mice with the anti-inflammatory agent dexamethasone. The results of these additional experiments are summarized in table 3 and document that both single- and double-stranded DNA can elicit a lethal response in this mouse model. These data therefore support the concept that microbial DNA in and of itself can induce a lethal inflammatory response.

Discussion

At the completion of the meeting, several future actions and considerations were underscored as being particularly important for future directions of sepsis-related research efforts (table 4). It is now well recognized that many microbial constituents have a profound capacity to induce the host to produce a spectrum of inflammatory mediators that can result in an overwhelming systemic inflammatory response. The resulting destruction of organs and tissues, leading to the syndrome of multiorgan dysfunction, can cause irreversible damage and death. Clearly, antibiotic chemotherapy cannot protect the host against this type of tissue damage and may even, at least under some circumstances, actually contribute to a detrimental host response via proinflammatory stimuli. The optimal effective therapeutic approach therefore might well be expected to include both the elimination of microorganisms as well as control or prevention of an exaggerated host inflammatory response to microbial constituents.

It is reasonable to conclude that the current lack of a full appreciation for the magnitude and multitude of the various microbial elements may have contributed to inconsistent and largely unsuccessful clinical trial results in human sepsis carried out to date [2, 39, 62–64]. Additionally, these trials may have failed because of the preferential emphasis on endotoxin (table 2) without regard to other potential microbe-derived factors. The rapid host responses and establishment of pathology before clinical recognition and therapy, moreover, may have been another factor precluding successful interventions. In contrast to the admittedly very complicated human clinical trials, therapies tested in well-controlled animal models of sepsis are often inherently biased toward a favorable outcome that may not even be reflected by, or achievable in, human investigations. For example, the early signs of sepsis may be subtle, leading to a delay of treatment in humans. Animal models must take into account these anticipated delays in treatment often seen in actual clinical practice.

The observation that antibiotics can induce the release of proinflammatory constituents from both gram-negative and gram-positive microbes offers a potential opportunity to limit the extent of inflammation by selection of antibiotics on the basis of how microbes are killed or inhibited from growth, as well as the extent to which the host mounts an inflammatory response.
response to the antibiotic-microbe interaction. Experimental evidence supports the concept that different antibiotics that manifest similar efficacy in their ability to kill gram-negative microbes may show significant differences in their capacity to cause the release of soluble endotoxin. Multiple experimental in vitro, in vivo, and ex vivo studies have amply documented major differences among antibiotics with respect to endotoxin release, production of endotoxin-induced proinflammatory cytokines, induction of cytokines, and differential levels of survival in experimental animal models of gram-negative sepsis in response to antibiotic chemotherapy [1, 3]. Moreover, additional studies suggest that experimental approaches used to assess antibiotic therapeutic efficacy within the framework of microbe-induced inflammatory mediators also may extend to gram-positive microorganisms as well.

In view of the evidence and concepts presented above, a “2-hit” hypothesis versus a “multihit” synergistic threshold hypothesis of lethal septic shock may provide a convenient conceptual framework from which to consider the complex interrelationships occurring in sepsis. We hypothesize that patients with sequential gram-negative and gram-positive infections are at increased risk for the development of lethal septic shock. For example, one possible scenario (figure 3) supporting the 2-hit hypothesis entails the release of LPS from a gram-negative infection. This would trigger a host inflammatory response with the development of shock. The hypothetical patient receives supportive medical care, including antibiotics and iv fluids. A short period later, the patient has a superinfection (e.g., iv line cellulitis, bacteremia, or both) with gram-positive organisms. The host, already primed by the previous release of LPS, responds further with a fatal exaggerated inflammatory response. In this example, components from gram-negative organisms (e.g., endotoxin, DNA, and peptidoglycan) may well function in concert with pyrogenic exotoxins, prokaryotic DNA, or peptidoglycan from gram-positive organisms to produce lethal septic shock. Alternatively, the closely temporal release of multiple microbe-derived components may synergistically reach a threshold that can induce an undesirable host immune or inflammatory response in any particular individual with a previously genetically programmed predisposition to an exaggerated immune response.

Components from gram-positive organisms may potentiate a deleterious immune response initiated by gram-negative organisms, or vice versa. The clinical choice of antibiotics raises other potential concerns. Antibiotics may differentially affect the release of various microbial constituents, and it is quite possible that each of these microbial components may have additive or even synergistic effects in promoting a deleterious host immune or inflammatory response.

A better fundamental understanding of the microbial factors that produce sepsis and how they can induce a detrimental immune response may lead to new therapies for improving survival in septic patients, a goal that has been elusive for 25 years.

References


