SPLANCHNIC PRODUCTION OF CYTOKINES IN PORCINE MODELS OF SEPTIC SHOCK AND MESENTERIC ISCHEMIA-REPERFUSION

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ABSTRACT

Multiple organ dysfunction syndrome (MODS) is a lethal sequela of septic shock and may occur in association with intestinal ischemia and/or reperfusion. Its pathogenesis may be mediated by endogenous endotoxin and cytokines. It is postulated that, during septic shock and intestinal ischemia, translocation of endotoxin from the gut lumen to the portal circulation occurs and the gut and the liver are major sources of cytokines. Two experiments were performed to test these hypotheses.

In the first experiment, differences in fluxes of endotoxin, tumor necrosis factor (TNF) and interleukin-6 (IL-6) across the gut and liver were determined over 4 h in animals given endotoxin (50 μg/kg; N=6) and in control animals (N=6). At no time did gut efflux of cytokines or endotoxin exceed gut influx of these substances, in either control or septic animals. Moreover, at no time did hepatic influx and efflux of TNF and IL-6 differ in either group. Therefore, net gut production of LPS, TNF or IL-6 in this porcine model of septic shock was not demonstrated. Further, net production of TNF or IL-6 by the liver was not observed.

In the second experiment, endotoxin, TNF and IL-6 levels were measured from the carotid artery, portal vein and hepatic vein every 30 minutes over 330 min in pigs, following occlusion of the superior mesenteric artery (SMA; N = 7) and following sham surgery (N = 7). In animals subjected to mesenteric ischemia, the SMA clamp was released twice: once at 240 min (for a duration of 40 s), and once at 300 min (for the remainder of the experiment). Gut efflux of TNF and IL-6 did not exceed gut influx, and hepatic influx of TNF and IL-6 was the same as hepatic efflux in both groups throughout the experiment. The temporal relationships of the appearances of TNF and IL-6 at the various vascular sites suggested TNF is produced in a partially perfused splanchnic bed (eg: pancreas, duodenum, liver, left colon) and IL-6 is produced in ischemic gut. There was no apparent splanchnic release of endotoxin secondary to mesenteric ischemia-reperfusion.
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Chapter 1
INTRODUCTION

1.1 OVERVIEW

Multiple organ dysfunction syndrome (MODS) is a frequent sequela of septic shock and numerous other critical illnesses. Its mortality rate is greater than 30% and increases progressively with the number of organs involved (1)(2)(3). Since its description in 1975 (4), its pathogenesis is still poorly understood, despite extensive investigation.

While MODS is frequently associated with sepsis, it is not known whether infection (including transient or occult bacteremia) is a prerequisite to its occurrence. Clinical manifestations are strikingly similar to those seen with infection, but a focus of infection is often not found. Investigators have been striving to provide an explanation for this paradox. One hypothesis is that, during critical illness, the gut becomes ischemic. As a result of the ischemia, the gut mucosa loses its barrier function and leaks bacteria and bacterial products into the circulation, stimulating a systemic inflammatory response that ultimately results in progressive, widespread organ dysfunction. Alternatively, as a result of local injury, the ischemic gut may be the site of activation of the systemic inflammatory response. The presence of gut ischemia as a prerequisite of MODS is not well established and more study of the consequences of gut ischemia are required. The role of bacteria and bacterial products in the pathogenesis of MODS also requires further study. Investigation on these lines will lead to a better understanding of this syndrome that mimics infection.

The systemic inflammatory response accompanying MODS and sepsis involves both cellular and humoral mediators. Cytokines, peptide inflammatory mediators, appear to be particularly important in the pathogenesis of septic shock and, possibly, MODS. When administered to experimental animals and to humans, some cytokines are known to reproduce the physiologic aberrations seen in septic shock. Further, some of the pathologic and pathophysiologic changes characteristic of MODS have been observed following
administration of these cytokines. While numerous studies have documented the presence of cytokines during septic shock, MODS and gut ischemia, little is known of the source of the cytokines. What is known about cytokine production and secretion is based mainly on \textit{in vitro} studies, which merely document the \textit{ability} of a particular cell line or organ to elicit cytokines in the presence of various stimuli. There is some evidence of "compartmentalization" of cytokine production: the release of cytokines from a focus of injury. With the release of large amounts of cytokines from a focus of injury, some cytokines may spill over into the systemic circulation, further activating the systemic inflammatory response or causing distant organ damage. Thus, MODS is characterized by a systemic inflammatory response in which cytokines that may originate from foci of injury appear to play a central role.

In this thesis, an attempt was made at documenting the presence of gut ischemia in a porcine model of septic shock. Under these same conditions, an attempt at demonstrating the translocation of bacterial products from the gut was made. The release of cytokines from the gut and a distant focus, the liver, was also studied. To determine if ischemia \textit{per se} was the cause of translocation of bacterial products or gut and liver cytokine production, these same factors were studied in a porcine model of mesenteric ischemia/reperfusion.

The literature pertaining to these topics has been reviewed and is discussed in detail below.

1.2 \textbf{MULTIPLE ORGAN DYSFUNCTION SYNDROME (MODS)}

Surgeons have faced the problems associated with organ failure for most of this century. During World Wars I and II, surgeons had to learn to manage cardiovascular failure (shock). Studies establishing the role of acute blood loss in the development of shock led to the liberal use of blood to prevent and treat shock, thereby eliminating the previously common syndrome of irreversible "wound shock". During the Korean conflict, renal failure was a major contributor to the mortality associated with trauma in successfully
resuscitated patients. By the time of the Vietnam war, with the realization that injury-induced renal failure could be largely prevented by resuscitating these patients with sufficiently large amounts of crystalloids in addition to blood, the incidence of renal failure had decreased markedly. However, as more severely injured patients survived for longer periods, acute respiratory failure (adult respiratory distress syndrome; ARDS) was increasingly more frequently observed (5)(6). Finally, in the 1970's, with improvements in the management of patients with ARDS, multiple system organ failure - the progressive dysfunction of physiologic systems in the presence of a clinical picture of sepsis - became recognized (4). Currently, more than 75% of the patients dying with ARDS now die of MODS and systemic hemodynamic instability rather than of hypoxia (7).

1.2.1 Terminology

Although much has been written about MODS in the past two decades, definitions and terminology are inconsistent. In order to better diagnose, study, and report on these processes, a consensus conference was held in 1991 to bring some consistency to the literature, as well as in the clinical and laboratory settings (8). It was thought that the term "organ failure" should be largely avoided, since "failure" describes a dichotomous event (ie: present or absent organ function). Multiple organ dysfunction syndrome was thought to better describe the continuum of changes that occurs in more than one organ system following a significant injury. Dysfunction can include the complete failure of an organ (eg: oliguric renal failure) or the biochemical failure of an organ (eg: an elevated serum creatinine). Deitch (6) attempted to define organ dysfunction more precisely and his criteria for organ dysfunction/failure are summarized in Table 1.

As will be discussed below, MODS is a complication of any one of a number of serious insults and, at its early stages, it is clinically indistinguishable from severe infection. Systemic inflammatory response syndrome (SIRS) characterizes the clinical manifestations of hypermetabolism often seen after a serious insult. This term replaces
"sepsis syndrome". It is defined as the presence of two or more of the following: (a) temperature > 38°C or < 36°C; (b) heart rate > 90 bpm; (c) respiratory rate > 20/min or \( P_aCO_2 < 32 \text{ mm Hg} \); (d) white blood cell count > 12 x 10\(^9\)/L or < 4 x 10\(^9\)/L; and (e) more than 10% band forms. SIRS is present in most patients admitted to a critical care unit and it is a nonspecific response to tissue injury. Finally, sepsis means that SIRS is caused by an infection. Sepsis is further classified as severe sepsis, sepsis with hypotension, and septic shock, depending on the presence of lactic acidosis and the degree of hemodynamic derangement.

Table 1. Criteria for organ dysfunction/failure*

<table>
<thead>
<tr>
<th>Organ or System</th>
<th>Dysfunction</th>
<th>Advanced Failure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pulmonary</td>
<td>Hypoxia requiring ventilator assistance for at least 3 - 5 d</td>
<td>Progressive ARDS requiring PEEP &gt; 10 cm H₂O and ( F_{1}O_2 &gt; 0.50 )</td>
</tr>
<tr>
<td>Hepatic</td>
<td>Serum bilirubin ≥ 2 - 3 mg/dL or liver function tests ≥ twice normal</td>
<td>Clinical jaundice with bilirubin ≥ 8 - 10 mg/dL</td>
</tr>
<tr>
<td>Renal</td>
<td>Oliguria ≤ 479 mL/24h or rising creatinine</td>
<td>Renal dialysis</td>
</tr>
<tr>
<td>Intestinal</td>
<td>Ileus with intolerance to enteral feeding &gt; 5 d</td>
<td>Stress ulcers requiring transfusion, acalculous cholecystitis</td>
</tr>
<tr>
<td>Hematologic</td>
<td>PT and PTT increased &gt; 25% or platelets &lt; 50 - 80 000</td>
<td>Disseminated intravascular coagulation</td>
</tr>
<tr>
<td>Central Nervous System</td>
<td>Confusion, mild disorientation</td>
<td>Progressive coma</td>
</tr>
<tr>
<td>Cardiovascular</td>
<td>Decreased ejection fraction or capillary leak syndrome</td>
<td>Hypodynamic response refractory to inotropic support</td>
</tr>
</tbody>
</table>

*Adapted from Deitch, 1992 (6).

1.2.2 The Clinical Syndrome

"Sequential system failure" was initially described by Tilney et al. in 1973 (9). A group of patients with ruptured aortic aneurysms, following massive acute blood loss and shock, suffered postoperative failure of initially uninvolved organs. The concept that a severe physiologic insult could result in damage to distant organs was formalized in a classic report by Baue (4). MODS was initially thought to be a sign of occult or
uncontrolled infection (4)(10)(11), but the syndrome has now been documented to occur after a number of diverse clinical conditions, including trauma (1), burns (12), pancreatitis (13), aspiration, massive blood transfusions, pulmonary contusion (14), and shock (15). Thus, although infection and shock are the two most common predisposing factors, other processes in which severe or extensive tissue injury occurs may induce a major inflammatory response capable of culminating in MODS.

The normal response to stress and injury has been well described. The response includes cardiovascular changes (eg: tachycardia, increased contractility, increased cardiac output) and an increase in oxygen consumption. Neuroendocrine responses include increased release of catecholamines, cortisol, antidiuretic hormone, growth hormone, glucagon, and insulin. The coagulation cascade, complement cascade and fibrinolytic systems become activated. These normal responses to stress peak within 3 - 5 days after the initial insult and abate by 7 - 10 days (16). A progressive decrease in third space fluid losses and normalization of temperature and cardiovascular changes herald an uncomplicated course; however, some patients maintain their hypermetabolic state and the systemic inflammatory response syndrome ensues.

SIRS is a prolongation and exacerbation of the hypermetabolic state associated with acute injury. The major metabolic change that occurs in SIRS is an initial increase in oxygen consumption (17)(18). This must be met by an increase in oxygen supply or anaerobic conditions and tissue ischemia will result. Heart rate and cardiac output are increased, as in the normal stress response. Concomitantly, there is a fall in systemic vascular resistance due to widespread vasodilation (17). In the early stages of SIRS, the arterial-venous oxygen content difference is normal if oxygen delivery has been maintained (17)(18). When sepsis is present or when MODS begins, there is a further drop in systemic vascular resistance and a failure of cellular oxygen utilization occurs (17)(19). In addition, noticeable changes occur in carbohydrate, lipid and protein metabolism in SIRS/MODS. There is a reduction in the use of glucose as an energy source and gluconeogenesis is
stimulated, resulting in hyperglycemia that is relatively unresponsive to exogenous insulin. Lipolysis is stimulated and lipogenesis is decreased, but ketone body levels in blood are low compared with starvation (17). Amino acids derived from skeletal muscle, connective tissue and intestinal viscera become an important energy source, leading to a dramatic loss of lean body mass, "autocannibalism" (17)(20). The metabolic changes occurring in SIRS and MODS are strikingly similar to those seen in sepsis; these clinicopathologic entities may therefore be clinically indistinguishable from sepsis.

Frequently, the inflammatory response is associated with acute respiratory insufficiency or failure. The pathologic pulmonary changes vary in severity; adult respiratory distress syndrome (ARDS) describes the most severe changes. Acute lung injury begins with an alteration in the pulmonary capillary endothelium. The endothelial injury allows fluid and inflammatory cells to enter the interstitium. Subsequent and progressive alveolar epithelial injury by inflammatory cells and their mediators lead to alveolar flooding, inactivation of surfactant, and collapse of individual alveoli. These pathological changes give rise to ventilation-perfusion abnormalities, hypoxemia, and diffuse pulmonary infiltrates. Acute lung injury that progresses in this way presages MODS.

After the onset of acute lung injury, two clinical patterns of organ dysfunction are common. The first pattern, most frequently seen after an insult such as trauma, burns or surgery, has been termed a "two-phase pattern" (1). In this pattern, the lung remains the primary dysfunctional organ. A clinical lag phase with signs of SIRS may continue for weeks and the progressive and sequential failure of other organ systems may then ensue. Death is most common 14 - 21 days after the initial insult (21). The second common clinical pattern is seen after trauma and delayed or inadequate resuscitation. Signs of MODS are evident shortly after the injury and tend to progress relatively quickly (16).

Transition from the hypermetabolic state of SIRS to clinically defined MODS does not occur in a clear-cut manner; rather, the two entities represent a continuum.
Nevertheless, regardless of the cause, MODS generally follows a predictable course, beginning with the lungs and followed by intestinal, hepatic, and renal failure, in that order (6)(16). Hematologic and myocardial failure usually occur later, and central nervous system manifestations can occur either early or late (6). The classic sequential pattern of organ failure may be altered, however, by the presence of pre-existent disease or by the nature of the precipitating event. That is, while the sequence of organ failure is generally predictable, it can be influenced by the patient's physiologic reserve or by the acute disease process itself.

Numerous risk factors for the development of SIRS/MODS have been implicated and these are summarized in Table 2. Identification of persons at risk for development of MODS is important for a number of reasons. First, it facilitates the study of very early stages of MODS. Secondly, it helps identify individuals in whom prophylactic measures may be taken. Various techniques have been examined and tested in the hope of predicting who will develop MODS, but results are inconsistent (22)(23). Further, to some extent, risk factors vary with the underlying cause or mechanism of the injury or critical illness. Sauaia et al. (24), recognizing this latter phenomenon, attempted to find a predictive model for post-traumatic MODS and determined that age greater than 55 years, Injury Severity Score (ISS) greater than or equal to 25, and transfusion requirements greater than 6 units/12 h are early predictors of MODS. To date, there is no consensus on what variables are most predictive of the development of MODS and much work has yet to be done in this regard, in various critical illnesses.

The mortality rates of patients with established MODS or ARDS have not appreciably improved since the initial description of these syndromes 20 years ago (6). The two most important prognostic indicators are age and number of dysfunctional organ systems. Mortality rate progressively increases with the number of organs involved: single-organ dysfunction has a mortality of 30 - 40%; two-organ dysfunction increases the mortality rate to greater than 60%; and three-organ dysfunction has a mortality rate in
excess of 90%. If a patient is older than 65 years old, then mortality rises as much as an additional 20% in these groups (1)(2)(3). In a prospective multi-center study on acute organ failure involving 5677 intensive care patients, the length of time the patient was in organ failure also correlated directly with mortality rate (2). No intervention has yet been described that alters the influence of these factors on prognosis.

Table 2. Risk factors for SIRS/MODS*

<table>
<thead>
<tr>
<th>Risk Factor</th>
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<tbody>
<tr>
<td>Inadequate or delayed resuscitation</td>
</tr>
<tr>
<td>Persistent infectious focus</td>
</tr>
<tr>
<td>Persistent focus of inflammation</td>
</tr>
<tr>
<td>Preexisting organ dysfunction (e.g., chronic renal failure)</td>
</tr>
<tr>
<td>Age &gt; 65 y</td>
</tr>
<tr>
<td>Alcohol abuse</td>
</tr>
<tr>
<td>Bowel infarction</td>
</tr>
<tr>
<td>Malnutrition</td>
</tr>
<tr>
<td>Surgical &quot;misadventures&quot;</td>
</tr>
<tr>
<td>Diabetes</td>
</tr>
<tr>
<td>Steroids</td>
</tr>
<tr>
<td>Cancer</td>
</tr>
<tr>
<td>Presence of hematoma</td>
</tr>
</tbody>
</table>

* Adapted from Beal and Cerra, 1994 (16).

1.2.3 Pathogenesis of MODS

Organ failure in MODS is unique in several respects. First, the organs that fail are not necessarily directly injured or involved in the primary disease process. Secondly, there is a delay of days to weeks between the initial or subsequent inciting events and the development of distant organ failure. These observations suggest that MODS is a systemic process mediated by circulating factors which produce effects not immediately apparent following the initiating insult. It is not clear, however, whether these circulating factors are exogenous in origin or whether MODS is a consequence of the host's own endogenously produced mediators. The mechanism of activation of the various factors found to be elicited
in this "mediator disease" and their influence on distant organs is where the mystery currently lies.

Deitch, in his review, recounted the various theories on the pathogenesis of MODS (6). These will be summarized below.

**The Role of Infection in MODS**

Because patients with MODS appear clinically septic and because several groups have documented an association between an untreated septic focus and the development of MODS, the syndrome was initially thought to be a sign of occult or uncontrolled infection (4)(10)(11). However, not all patients who appear septic who develop MODS have untreated infections (25). Although uncontrolled infection is the initiating cause of MODS in about one half of patients, in the other half, the syndrome occurs either in the absence of a clinically identifiable infection or the development of infection is a preterminal event of no apparent prognostic significance (6). Further, no septic focus can be found clinically or at autopsy in more than 30% of bacteremic patients, including those dying with clinical sepsis and MODS (25)(26). In experimental animals, it has been reliably induced by an intraperitoneal sterile inflammatory stimulus (27). Finally, sepsis, SIRS and MODS all share the same clinical manifestations because they represent a stereotyped response to a severe insult. The generalized inflammatory response characterizing these syndromes is likely identical, involving the same mediators. This assumption is supported by the observation that a classic septic response can be induced in normal human volunteers by the injection of inflammatory agents (28), endotoxin (29), or cytokines such as tumor necrosis factor (30). Infection therefore does not appear to be a prerequisite to MODS.

**The Macrophage Hypothesis**

The macrophage hypothesis states that excessive or prolonged activation or stimulation of macrophages ultimately results in excessive production, surface expression and liberation of cytokines and other products which, through a cascade effect involving additional humoral and cellular effector systems, exert deleterious local and systemic
effects. The clinical correlate of this macrophage hyperactivation is the uncontrolled inflammatory response. Inflammatory processes, while generally beneficial to the host, are intrinsically destructive to surrounding tissues. When activated to a greater extent, the inflammatory response can "spill over" from the local environment to induce a generalized systemic response, resulting in the activation of other inflammatory effector cells, including fixed tissue macrophages, neutrophils and lymphocytes, as well as activation of the coagulation and complement systems. Ultimately, the systemic inflammatory state becomes self-perpetuating because of the continued local and systemic production of inflammatory mediators and because of inadequate regulation of the inflammatory response by the host. This hypothesis is consistent with the autopsy findings of Nuytinck et al. (31), who found an association between the presence of ARDS and MODS and histologic evidence of organ inflammation. Further, administration of some inflammatory mediators, such as the cytokine tumor necrosis factor, produces pathologic changes characteristic of ARDS and MODS in some animal models (32)(33). The inflammatory response of stimulated macrophages is thus thought to result in the clinical manifestations of infection and, ultimately, distant organ dysfunction.

The role of cytokines in the pathogenesis of MODS will be further discussed in section 1.6.5: "The Role of TNF and IL-6 in the Pathogenesis of MODS".

The Microcirculatory Hypothesis

The microcirculatory hypothesis proposes that organ injury is related to ischemia or vascular endothelial injury. Several overlapping potential mechanisms of organ injury are related to this: inadequate tissue and cellular oxygen delivery, the ischemia-reperfusion phenomenon, and tissue injury due to endothelial-leukocyte interactions.

Since many patients who eventually develop SIRS and MODS have been subject to hemodynamic derangements and variable degrees of fluid resuscitation, it is not difficult to imagine that organ ischemia and reperfusion injury may play a role in organ injury. The first mechanism of organ injury, ischemia, is well characterized; in the absence of adequate
oxygen, energy stores are depleted because of the continuing cellular energy demands and because of a reduced capacity to regenerate adenosine triphosphate (ATP) by oxidative phosphorylation. Cellular dysfunction, injury and death occur, resulting in organ dysfunction. This may occur due to decreased tissue perfusion, as in circulatory shock, and it is perpetuated by microcirculatory changes such as vascular congestion, the formation of microthrombi composed of leukocyte and platelet aggregates, interstitial edema, and increased capillary permeability (34). While ischemia is a well-recognized factor contributing to the pathogenesis of organ injury, the role of reperfusion in this process has only recently been appreciated (35)(36). Frequently, much of the tissue damage occurs after oxygenation is restored rather than during the period of ischemia and this presumably occurs because of the formation of oxygen free radicals (36). Thus, following severe injury, tissue ischemia and subsequent reperfusion injury with resuscitation may cause organ injury, stimulating an inflammatory response.

It has become apparent that endothelial cells actively contribute to tissue ischemia and injury. Their roles in the regulation of blood flow (37), coagulation and inflammation (38)(39), as well as their interactions with circulating neutrophils, appear to contribute to tissue ischemia and injury. Endothelial-leukocyte interactions may be a common pathway by which diverse initiating factors such as bacteria, endotoxin, cytokines and ischemia can lead to organ failure and MODS. For example, endotoxin, TNF and IL-1 induce a change in endothelial cell phenotype from a noninflammatory to a proinflammatory, procoagulant phenotype. That is, they lose their anticoagulant properties and express tissue factor, activating the extrinsic clotting pathway; express surface receptors that promote leukocyte adherence; and secrete numerous cytokines that accelerate the inflammatory process. Neutrophil adherence to endothelial cells appears to be a prerequisite for endothelial and subsequent tissue injury. This is supported by the observation that shock or ischemia-reperfusion-mediated endothelial cell and organ injury can be ameliorated by preventing neutrophil adhesion to endothelial cells (40)(41). Thus, stimulated endothelial cells may
contribute to organ injury by their interactions with circulating leukocytes, as well as by activating the coagulation cascade and various inflammatory mediator systems.

It is not difficult to see that the microcirculatory hypothesis of organ failure overlaps with the macrophage hypothesis in a number of ways. Clinical and experimental observations clearly document that systemic inflammation adversely affects the microcirculation (38)(39), and ischemia-reperfusion can exaggerate the host's inflammatory response to subsequent stimuli by activating neutrophils and priming macrophages (35)(42). Cytokines affect endothelial function and changes in endothelial function may result in the release of more cytokines (39), perpetuating the uncontrolled inflammatory response and affecting local and distant organs.

There are a number of clinical observations supporting the microcirculatory hypothesis. Firstly, circulatory shock with resultant tissue hypoxia is one of the most common clinical events preceding MODS (6). Secondly, neutrophils, platelets and fibrin are characteristically found in the pulmonary microcirculation in ARDS (43). Thirdly, there is autopsy evidence of diffuse microvascular injury in patients with MODS (31). Lastly, the results of hemodynamic studies in patients with ARDS and MODS indicate that oxygen delivery is not sufficient to meet oxygen demands (17)(19). The microcirculatory hypothesis further explains why identification and treatment of the precipitating factor (e.g.: infection) does not help some patients: once the microcirculatory inflammatory/injury process is established, removal of the initiating or perpetuating stimuli will not rapidly reverse or prevent further tissue injury and organ failure.

**The Gut Hypothesis of MODS**

This hypothesis states that intestinally-derived bacteria or endotoxin serve as triggers to initiate, perpetuate or exacerbate the septic state and thereby promote the development of MODS. Again, this hypothesis clearly overlaps with the macrophage and the microcirculatory hypotheses, as bacteria and endotoxin are powerful stimuli for cytokine secretion by tissue macrophages, induce a proinflammatory endothelial cell
phenotype, stimulate neutrophil protease and oxidant production, and activate the complement and coagulation cascades. Once this process is initiated, it can become self-sustaining. For example, activation of the cytokine cascade can stimulate endothelial cells, promoting microcirculatory events that further impair oxygen delivery to the gut. As a result of the ischemic insult, the gut loses its mucosal barrier function and bacterial translocation or endotoxin transmigration is exacerbated. Such bacterial translocation may therefore provide the stimulus for the activation or release of inflammatory mediators responsible for the clinical and pathologic manifestations of sepsis syndrome and MODS.

The gut hypothesis is attractive because it would explain the apparent paradox of why no septic focus can be identified clinically or at autopsy in more than 30% of bacteremic MODS patients dying with clinical sepsis. In the presence of an occult source of bacteria or bacterial products, patients can become clinically septic with or without bacteremia.

The gut hypothesis is supported by several lines of evidence. Firstly, there is experimental evidence from many laboratories documenting that enteric bacteria can escape from the gut and cause systemic or peritoneal infections (6). This has also been documented under certain clinical circumstances: life-threatening infections with gut-associated bacteria have been observed in the absence of an infectious focus in burn patients (44), trauma patients (26)(45), and patients developing MODS (25). In addition, nosocomial infections such as pneumonia are common in the critically ill and the majority of these result are from autoinfection with gut organisms (46). Secondly, the administration of oral nonabsorbable antibiotics in critically ill patients has been shown by some investigators to reduce the incidence of pneumonia, primary bacteremia, and other infectious complications, although mortality has not been improved by selective gut decontamination (47). Lastly, in vitro and in vivo studies have demonstrated an important relationship between the state of the intestinal barrier function, Kupffer cell function, the hypermetabolic response, and distant organ injury (17)(48)(49). That is, gut derived
endotoxin may stimulate Kupffer cell activity and the subsequent release of endogenous mediators that modulate hepatocyte function. The gut hypothesis is therefore supported by experimental and clinical evidence of bacterial translocation following a diverse number of insults, the ability to prevent infectious complications by selective gut decontamination, and evidence of changes in function of an organ distant to the focus of infection.

The Two-hit Phenomenon in MODS

The phrase "two-hit phenomenon" describes the theoretical biological phenomenon in which an initial insult primes the host such that, with a second or subsequent insults, the host's response is greatly amplified (6). Faist et al. (1) described the "two-phase pattern" of MODS in trauma patients and the same clinical pattern of MODS has been observed following burns and surgery (16). For example, in the polytrauma patient, an episode of hypotension due to blood loss could produce a clinically occult focal or global ischemia-reperfusion injury, priming the host's inflammatory response. Any subsequent insult, such as infection or further trauma, could then lead to an amplified tissue response manifested as increased cytokine response, endothelial-leukocyte dysfunction, and microcirculatory disturbances. The host's risk of ultimately developing MODS would thus be higher in patients with multiple injurious stimuli. The "two-hit" model of MODS has been substantiated in basic laboratory work (50)(51).

Despite intensive investigation since its description, the etiology and pathogenesis of MODS is not well understood. MODS was originally thought to occur as a result of infection (10). Indeed, many patients who develop MODS do so as a consequence of a primary infection. However, it has become increasingly more obvious that overt bacterial infection is not always present in patients with a septic clinical picture and progressive organ failure. MODS has been described following a number of insults, with and without evidence of infection, and it has been reliably induced in experimental animals by an intraperitoneal sterile inflammatory stimulus (27). The mystery lies in why such a diverse
set of circumstances would lead to the development of the syndrome of MODS. Perhaps the physiologic responses of the body to injury are limited and any injury of sufficient severity will invoke a stereotyped (uncontrolled) systemic inflammatory reaction; the chemical mediators of this response provoke organ and tissue responses characteristic of SIRS or MODS. The reason it is stereotyped, one might postulate, is because there is a common mechanism by which MODS occurs. There are a number of possible pathophysiologies described that may explain the phenomenon but, by no means should they be considered mutually exclusive. It is entirely possible that each of these mechanisms plays an important role in the pathogenesis of this complex syndrome.

1.3 BACTERIAL ENDOTOXIN

It has long been recognized that the injection of aqueous solutions of gram negative bacteria into patients results in a febrile response, "injection fever". The principle causative agent of this pyrogenic response was found to be a component of the Gram negative bacterial cell wall, endotoxin, which is shed into the environment during cell growth. Because of the chemical and physical stability of endotoxin, it may persist in conditions that kill bacteria. Endotoxin has been purified and the pyrogenic component is now known to be lipopolysaccharide (LPS). Endotoxin consists of LPS plus other closely associated substances also found on the Gram negative cell wall. These other substances influence the degree of biological activity demonstrated by the LPS.

1.3.1 Physical Properties

Endotoxins (LPS) are an extremely heterogeneous group of similar structures that vary between species as well as between strains of Gram negative bacteria (52). In general, they have two major parts: a hydrophilic polysaccharide chain and a hydrophobic lipid group. The polysaccharide region may be subdivided into a relatively conserved core
region and a variable antigenic region; longer polysaccharide chains result in greater solubility of the endotoxin in water. The lipid portion of the of LPS is called lipid A and, because of its hydrophobic nature, endotoxins tend to aggregate in aqueous solutions to form vesicles consisting of variable numbers of LPS molecules (depending on the pH, salt concentration, presence of surfactants, etc. in solution). The molecular weight of LPS varies with the size of the polysaccharide component, ranging from 3 to 25 kilodaltons.

1.3.2 Distribution and Elimination

The fate of endotoxins after they are injected into the blood or tissues or after release from bacteria during infection is not completely understood. The variety of methods used to measure endotoxin in studies attempting to define the pharmacokinetics of LPS and the considerable interspecies variation are some of the obstacles.

Absorption

After enteral administration of radiolabelled E. coli endotoxin, no radioactivity is absorbed in normal or shocked dogs (53). However, when $^{32}$P-labelled E. coli endotoxin is administered enterally to coliform-free rabbits, $^{32}$P is detected in the liver of normal animals and in the blood, liver, spleen, and kidney of animals subjected to hemorrhagic shock (54). The difference between the two animal models may be attributed to invasion and infection of the bowel wall by E. coli, which is an abnormal bacterial species in the bowel of most rabbits (55). Infection may increase gut permeability, especially in the presence of the ischemic effects of shock on the intestine.

Absorption of endotoxins from tissue sites other than the bowel varies between tissues. The amount of E. coli LPS required to kill mice is the same when injected intracranially as when administered intravenously (IV). Twice that quantity is required when injected intraperitoneally (IP) and four times as much is required when injected intramuscularly (IM) (56). Upon intracerebral injection of radiolabelled LPS, radioactivity leaves the cranium rapidly and appears in the liver 3 hours later. Following IM inoculation,
radioactivity in the blood and liver increases very slowly; it continues to rise even after 24 h. The skin and knee also appear to retain endotoxin, for injection into these tissues causes less pyrogenicity and hematological effects and yields lower levels in other tissues than after IV injection of a similar dose (55).

**Distribution**

Numerous studies in various animal models, with different endotoxins and labels indicate that LPS passes from the blood into the liver shortly after IV injection (55)(57). Following IV injection of lethal doses of radiolabelled *E. coli* endotoxin into rabbits, 20% of the radioactivity is found in the liver after 15 min (58). Proportionally more radioactivity is found in the liver after administration of sublethal doses of LPS. Similar differences in distribution of radioactivity of lethal and sublethal doses has been noted in mice (59). Fluorescent antibody studies in rabbits demonstrate that LPS localizes in the Kupffer cells (60). In rats, LPS first becomes detectable in Kupffer cells 2 - 7 h after IV injection, although there is some evidence of direct uptake of LPS by hepatocytes as well (57). Three days after the injection, LPS redistributes from the Kupffer cells to hepatocytes. Pulmonary localization of LPS does not occur consistently and may depend on the solubility of the endotoxin preparation. Relatively insoluble endotoxins are filtered out in the lung, while soluble endotoxins are not (55). Splenic localization of LPS also does not occur consistently, although this may be a function of animal species. *E. coli* endotoxins localize in the spleens of rabbits (58)(59) and rats (57), but not in mice (56)(59). Attempts to detect endotoxin in the brain following IV administration have been uniformly negative (55). Finally, LPS has been found in polymorphonuclear leukocytes in the circulation and in tissues 10 min to 13 h after IV injection in dogs (57). In contrast, LPS has not been detected in erythrocytes (55)(57).

**Degradation and Excretion**

After IV injection of lethal doses of radiolabelled *E. coli* LPS into rabbits, 30 - 50% of radioactivity is removed from the blood during the first 15 min, 50 - 70% is removed at
2 h, and 20% still circulates at 5 h (58). Freudenberg et al. (57) showed that the half-life of IV administered endotoxin from *Salmonella minnesota* in rats is 30 min., but that from *S. abortus-equii* is 7.5 h. The clearance of LPS from blood is therefore dependent not only on the animal species, but on the biophysical properties of the LPS molecule as well. Most of the endotoxin that reaches the liver remains there for long periods. 60% of the radioactivity from radiolabelled *E. coli* LPS is found in the liver of mice 60 days after injection (56) and *S. abortus-equii* LPS also persists in the liver of rats for periods as long as weeks (57). Once taken up by Kupffer cells, LPS undergoes degradation of its lipid A component. It is mostly excreted through the gut, although small amounts of degraded endotoxins are eliminated in the urine (55)(57).

### 1.3.3 Biological Activity

The biological activity of endotoxin varies with its molecular configuration. The major part of the polysaccharide chain, the O antigen, is responsible for producing host immunity to Gram negative bacterial infections. The lipid A portion is primarily responsible for the rest of the biological effects of endotoxin. Because of the variability in the relative sizes of the polysaccharide and lipid components, endotoxins from different species of bacteria exhibit markedly different potencies as measured in terms of concentration required to induce a pyrogenic response. Endotoxins with shorter polysaccharide chain lengths (ie: more lipid A per unit weight) are more potent. In light of this variable potency, a unit of potency, the Endotoxin Unit (EU), was devised by the USFDA; comparison of a sample to a USP reference endotoxin standard allows one to assign an EU value. Thus, where biologic activity is a factor under study, it is preferable to report endotoxin concentrations in terms of biologic activity, or EU.

Animal species vary widely in their sensitivity to LPS and the target organs susceptible to endotoxin-induced injury are species- and dose-dependent. Among the most sensitive species are humans, sheep and rabbits (61). Pigs and ruminants exhibit marked
cardiopulmonary effects with administration of relatively low doses (< 5 µg/kg) of endotoxin and pulmonary hypertension and lung injury are the most common sequelae of endotoxin infusion in these models (62)(63)(64). In contrast, dogs and rodents appear to be less sensitive to endotoxin and the doses required to cause lethality in murine species are typically several orders of magnitude higher than doses required in rabbits; doses of ≥ 1 mg/kg are commonly used in experiments involving these animals (55)(61)(65). Infusion of endotoxin primarily affects the gastrointestinal tract and is typically associated with a loss of plasma volume and cardiovascular collapse, in the absence of marked pulmonary hypertension (66)(67). Administration of endotoxin to healthy human volunteers produces a febrile response accompanied by chills, malaise, headache and nausea (68) and hemodynamic changes resembling septic shock have also been reported (69)(70). The biologic effects, while variable in different species, are summarized in Table 3.

**Hemodynamic Effects of Endotoxin**

Clinically, advanced septic shock in an unresuscitated patient usually manifests as systemic hypotension associated with a variable degree of peripheral vasodilation. Following resuscitation, the majority of cases are characterized by a high cardiac index and a low systemic vascular resistance (71)(72)(73). Despite this hyperdynamic state, myocardial function is abnormal, for a decrease in left ventricular ejection fraction and an increase in end-diastolic volume index occur several days after the onset of septic shock (74)(75)(76). Pulmonary hypertension is a variable finding.

It is unclear whether endotoxin alone is capable of causing all of the manifestations of the septic shock syndrome. In animals, endotoxemia is often associated with a hypodynamic form of shock (ie: low cardiac output) (77)(78). On the other hand, animal models of endotoxemia in which large volumes of fluid are administered do demonstrate hemodynamic changes more consistent with the hyperdynamic cardiovascular profile of septic shock observed in humans (79)(80). In addition, hemodynamic derangements indistinguishable from septic shock have been reported following accidental injection of a
Table 3. Biological effects of endotoxin. The biological effects are species- and dose-dependent. Many of the listed effects are mediated by other factors.

<table>
<thead>
<tr>
<th>Biological Effects</th>
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<tbody>
<tr>
<td><strong>Flu-like Syndrome</strong></td>
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<tr>
<td>Fever, Headache</td>
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<tr>
<td>Malaise, Nausea</td>
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<tr>
<td><strong>Hemodynamic Effects similar to septic shock</strong></td>
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<tr>
<td><strong>Pulmonary</strong></td>
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<tr>
<td>Increased pulmonary arterial pressure</td>
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<tr>
<td>Pulmonary capillary hyperpermeability</td>
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<tr>
<td>Pulmonary edema, ARDS</td>
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<tr>
<td><strong>Myocardium</strong></td>
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<tr>
<td>Myocardial depression</td>
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<tr>
<td>Increased myocardial compliance</td>
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<td><strong>Splanchnic Effects</strong></td>
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<tr>
<td>Splanchnic hypoperfusion</td>
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<tr>
<td>Intestinal hyperpermeability</td>
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<tr>
<td>Liver</td>
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<tr>
<td>Decreased hepatocyte protein synthesis</td>
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<tr>
<td>Hepatic uptake of FFA</td>
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<tr>
<td>Decreased total hepatic blood flow</td>
</tr>
<tr>
<td>? activation of Kupffer cells</td>
</tr>
<tr>
<td>Pancreas</td>
</tr>
<tr>
<td>Decreased perfusion</td>
</tr>
<tr>
<td>? release of myocardial depressant factor</td>
</tr>
<tr>
<td>Renal vasospasm</td>
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<tr>
<td><strong>CNS</strong></td>
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<tr>
<td>Decreased cerebral blood flow</td>
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<tr>
<td>Alterations in brain metabolism of amino acids</td>
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<tr>
<td><strong>Skeletal Muscle</strong></td>
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<tr>
<td>Inhibition of neuromuscular transmission</td>
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<tr>
<td>Alterations in blood flow</td>
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<tr>
<td><strong>Vascular Endothelium</strong></td>
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<tr>
<td>Activation of nitric oxide synthase</td>
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<td>Leukocyte adhesion/margination</td>
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<td>Production/release of cytokines</td>
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<tr>
<td>Platelet aggregation</td>
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<tr>
<td>Increased capillary permeability</td>
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<tr>
<td>Procoagulant activity</td>
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<tr>
<td><strong>Metabolic</strong></td>
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<td>Hypermetabolic state</td>
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<tr>
<td>Hyperglycemia</td>
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<tr>
<td>Increased cortisol</td>
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<tr>
<td>Increased epinephrine, norepinephrine</td>
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<tr>
<td>Increased glucagon</td>
</tr>
<tr>
<td>Mobilization of FFA, TG</td>
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<tr>
<td>Hypoaaminoacidemia</td>
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<tr>
<td><strong>Systemic inflammatory response</strong></td>
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<tr>
<td>Cytokines</td>
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<tr>
<td>Eicosanoids (prostaglandins, thromboxanes)</td>
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<td>Complement</td>
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<td>Coagulation factors</td>
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<td>Disseminated intravascular coagulopathy</td>
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high dose of endotoxin, in humans (69). The changes in myocardial function seen in septic shock have also been demonstrated in human volunteers following administration of endotoxin (70). It therefore appears that endotoxin causes hemodynamic manifestations that are variable but similar to septic shock.

Endotoxin has variable effects on different vascular beds and these effects are often species- and model-specific. These effects have been summarized by Bond (77). Briefly, cerebral blood flow and vascular resistance are decreased in primates and dogs. Myocardial blood flow is maintained in most animal models of endotoxemia, although it is increased in human septic shock (81). In the kidneys, following endotoxin administration in dogs, transient vasoconstriction followed by a progressive loss of vascular tone occurs; a similar phenomenon has been reported in nonhuman primates. A reduction in vascular resistance and blood flow to skeletal muscles has been observed in these same models. Immediately following endotoxin administration in monkeys, vascular resistance in the cutaneous vasculature increases, resulting in decreased cutaneous blood flow. After 15 minutes, vascular resistance decreases, but cutaneous blood flow continues to decrease. In humans, within one hour of IV administration of endotoxin, splanchnic blood flow increases and this occurs in the absence of changes in extremity blood flow (82). However, changes in splanchnic blood flow vary considerably between animal models and this will be discussed in more detail below.

**Metabolic Responses to Endotoxin**

In general, endotoxemia is associated with a hypermetabolic state. Administration of endotoxin to human volunteers results in increased whole body oxygen consumption. Splanchnic oxygen consumption increases to a significantly greater degree than the increase in whole body oxygen consumption (82) and this mimics the disproportionate increase in splanchnic O₂ consumption compared with total body O₂ consumption seen in septic humans (83). Progressive hyperglycemia associated with elevated blood cortisol and epinephrine levels is seen (68)(82). Revhaug et al. reported increased glucagon levels in
endotoxic humans (68), but this was not observed by Fong's group (82). Insulin levels are unchanged (82). Hypoaminoacidemia associated splanchnic amino acid uptake occurs. Free fatty acid (FFA) levels and triglyceride levels increase and this is associated with efflux of FFA from the extremities, hepatic uptake of FFA, and splanchnic efflux of triglycerides (82). Thus, endotoxicosis is associated with a hypermetabolic state that is not unlike that seen during sepsis.

**Endotoxin-induced Organ Injury**

A single large dose of endotoxin has been reported to cause hemodynamic derangements qualitatively similar to septic shock (69). In addition, multiple organ dysfunction including disseminated intravascular coagulopathy, abnormalities of hepatic and renal function, and noncardiogenic pulmonary edema have been reported. Numerous animal models of endotoxemia have similarly demonstrated various degrees of dysfunction of various organ systems. However, there is little evidence that endotoxin itself is directly responsible for organ injury. Rather, endotoxin may exert its effects via one or more mediators. Even low serum endotoxin concentrations (ie: 5 - 10 pg/mL) induce the production of cytokines such as tumor necrosis factor (TNF) and interleukin-1 (IL-1), as well as eicosanoids such as prostaglandin E2 (84). In addition, endotoxin is a potent activator of the complement cascade (85)(86) and the coagulation cascade (86)(87). These and other inflammatory mediators may be the ultimate mediators of endotoxin-induced organ injury. Endotoxin is therefore more accurately referred to as an initiator of a number of proinflammatory events which ultimately cause organ injury.

**Pulmonary Dysfunction**

The clinical features of ARDS include elevated pulmonary arterial pressure, pulmonary vascular resistance, and pulmonary capillary permeability in addition to decreased lung compliance and functional residual capacity. These features, as well as the ultimate manifestations of ARDS - pulmonary edema, ventilation/perfusion mismatch and
hypoxia - are easily reproduced during experimental endotoxemia, especially in sheep and pigs (62)(88).

The pulmonary response to endotoxemia can be seen as a two-phase insult. In the early phase, within approximately two hours of endotoxin infusion in sheep and porcine models, significant increases in the mean pulmonary arterial pressure and pulmonary resistance are seen (64)(89). Following the early response, a less marked increase in mean pulmonary arterial pressure is seen and the alveolar-endothelial permeability is increased.

Thromboxane A2 and its metabolites are thought to be the principal mediators of the early phase of the pulmonary response to endotoxin, for increases in thromboxane concentrations parallel the pulmonary hemodynamic changes. Further, inhibition of thromboxane synthesis attenuates the characteristic response (90)(91). In contrast, the pulmonary hemodynamic changes typifying the late phase can not be prevented by inhibition of the cyclooxygenase or leukotriene pathways and levels of prostaglandins and thromboxanes do not appear to be elevated (92)(93). Thus, while eicosanoids do not play a pivotal role in the pulmonary changes seen later, they appear to be important mediators of the early phase of endotoxin-induced pulmonary injury.

The most prominent pathophysiologic event of the late phase of the pulmonary response to endotoxin is an increase in pulmonary microvascular permeability (62)(89). The mechanisms responsible for the disruption of the endothelial (and epithelial) barriers are unclear. It has been suggested that complement activation and the resultant generation of the chemotactic factors C3a and C5a cause margination of polymorphonuclear leukocytes (PMNs) and a subsequent release of oxygen free radicals and proteases, damaging intercellular integrity (88). This hypothesis is supported by the observation that the microvascular hyperpermeability is diminished with depletion of PMNs with hydroxyurea (94) and by scavenging peroxides with catalase (95). However, depletion of PMNs using other means has no such protective effect (96)(97). Further, complement depletion has no effect on the response (98). Other cellular or humoral factors are therefore probably
involved in the pathogenesis of the pulmonary permeability changes. The factors responsible for the late changes in endotoxin-induced lung injury remain poorly understood.

It has been suggested that TNF is the major mediator of the endotoxin response, for a single infusion of recombinant human TNF in sheep results in a biphasic pulmonary response similar to the response seen following endotoxin administration (99). Furthermore, TNF is capable of initiating endothelial injury and increased microvascular permeability in vitro as well as in vivo (33). Thus, while numerous mediators have been found to contribute to the pathogenesis of either the early or the late pulmonary changes seen during endotoxicosis, it appears that TNF is the primary mediator that stimulates the factors that produce the overall pulmonary response.

**Myocardial Effects**

Septic shock in adequately resuscitated humans is associated with a high cardiac index and a low systemic vascular resistance (71)(72)(73). Despite this hyperdynamic circulatory state in the experimental and clinical settings, myocardial dysfunction has been noted: ejection fraction is decreased and ventricular dilation in the presence of normal filling pressures occurs, suggesting increased myocardial compliance (74)(75)(76). Administration of endotoxin in humans results in qualitatively similar hemodynamic changes (70).

The mechanism responsible for the impairment of myocardial performance seen during sepsis and endotoxicosis not fully understood. Endotoxin itself has no direct depressant effect on the myocardium in vitro (100). Further, myocardial depression is not due to ischemia, for coronary blood flow is disproportionately increased in relation to the observed increase in myocardial oxygen consumption (81). It is possible that TNF is a major mediator of the response, since TNF administration to dogs has the same hemodynamic effects as endotoxin infusion (101). Nitric oxide has been postulated to be a mediator of both TNF- and LPS-associated myocardial dysfunction, for nitric oxide
synthase in the myocardium is activated by endotoxin and is activated in other sites by TNF. The generation of myocardial depressant factor, which appears to be released from pancreatic acinar cells following splanchnic ischemia, has also been postulated to cause the myocardial effects seen during endotoxemia (102). Thus, while endotoxemia is associated with myocardial depression, it is likely that mediators stimulated by endotoxin are responsible for the changes in myocardial performance.

**Renal Sequelae**

In primate models of endotoxemia, the greatest decreases in organ blood flow are seen in the kidneys and in the pancreas (103). Renal blood flow rates in endotoxic baboons fall to about 20% of control values. This paucity of blood flow, the associated renal vasospasm, and the presence of intravascular coagulation all contribute to the renal failure accompanying severe septic shock.

**Liver and Pancreatic Dysfunction**

The magnitude of the decrease in blood flow to the pancreas is of the same order as that seen in renal blood flow (103); endotoxemic baboons display decreases in pancreatic blood flow to approximately 30% of control values. Pancreatic ischemia results in the release of proteolytic enzymes as well as other mediators of systemic events, such as myocardial depressant factor (102). More study is required to better characterize the entire spectrum of systemic events attributable to pancreatic ischemia.

Endotoxemia causes an overall reduction in hepatic blood flow (103),(104). This appears mainly due to a reduction in portal venous flow, for the proportion of blood flowing through the hepatic artery is increased. Hepatic ischemia may have yet undefined systemic consequences in view of the liver's central role in metabolism and as a component of the reticuloendothelial system. Further, endotoxin has direct effects on Kupffer cells: Kupffer cells in culture are activated by endotoxin to inhibit hepatocyte protein synthesis (49).
Cerebral Effects

Septic shock is associated with alterations in mentation. While endotoxin is prevented from exerting direct cerebral effects by exclusion by the blood-brain barrier, the metabolic effects accompanying endotoxemia may induce alterations in mental status (65). For example, as appears to be the case in the pathogenesis of hepatic encephalopathy, an increase in circulating aromatic amino acids relative to branch-chain amino acids in the circulation may be responsible for septic encephalopathy (105).

Skeletal Muscle Dysfunction

Endotoxemia predisposes to diaphragmatic failure as well as respiratory failure (65), but the mechanisms responsible for this are unclear. Work by Hopkins et al. suggests that endotoxin affects neuromuscular transmission in a manner similar to low doses of the nondepolarizing muscle relaxant tubocurarine (106).

Changes in Mesenteric Perfusion

Changes in splanchnic hemodynamics during endotoxemia differ between species and also between different models using the same species. Dogs given lethal doses of endotoxin have decreased mesenteric blood flow due to increased mesenteric vascular resistance. These physiologic changes are associated with intestinal microvascular morphologic changes as well as the appearance of subepithelial congestion and mucosal and submucosal hemorrhage. (107). Pigs treated with endotoxin have decreased splanchnic blood flow (108). However, with aggressive fluid resuscitation, mesenteric flow can be maintained in endotoxemic pigs, since mesenteric vascular resistance is decreased (79)(80). Baboons given lethal doses of endotoxin have decreased mesenteric vascular resistance and maintenance of mesenteric blood flow, with none of the morphological changes in the gut microvasculature seen in dogs (107). Finally, following a single IV bolus of endotoxin in human volunteers, splanchnic blood flow is increased, peaking 3 hours after the infusion (82). It is therefore apparent that changes in mesenteric hemodynamics during endotoxemia differ between species and also vary according to the resuscitation protocol.
**Gut Mucosal Hyperpermeability**

Endotoxin induces increased gut mucosal permeability in humans, as well as in animals (109)(110)(111)(112). This topic will be discussed in more detail below (Section 1.5.2: "Intestinal Mucosal Hyperpermeability During Septic Shock and Endotoxicosis").

### 1.4 Splanchnic Ischemia in Critical Illness

Shock may be defined as a condition in which oxygen delivery \( (\text{DO}_2) \) to the body fails to meet the metabolic needs of the tissues (113). That is, \( \text{DO}_2 \) is inadequate for the generation of the adenosine triphosphate (ATP) necessary to maintain the function and structural integrity of tissues; tissues become dependent on anaerobic mechanisms for energy production - a condition known as ischemia. Oxygen delivery is a product of blood flow and arterial oxygen content. Hence, there are 3 ways by which oxygen supply may be limited: by a decreased blood flow (stagnant hypoxia), by a reduced arterial hemoglobin saturation (hypoxic hypoxia), and by a decreased blood hemoglobin concentration (anemic hypoxia).

Under normal resting conditions, tissue oxygen uptake \( (\text{VO}_2) \) is independent of oxygen supply (Figure 1). Rather, oxygen uptake is fixed by metabolic demand. To compensate, as oxygen delivery falls along the "plateau phase", tissues extract larger and larger proportions of the oxygen supplied (represented numerically as an increased extraction ratio). Both active processes (eg: compensatory redistribution of blood flow) and passive processes (eg: convection and diffusion of gases) are responsible for this increased extraction ratio. However, the ability of tissues to extract oxygen is limited. When tissues meet this limit (ie: an extraction ratio of approximately 65 - 75%, depending on tissue type and species), in the face of an additional fall in oxygen supply, oxygen consumption begins to fall. The phenomenon by which oxygen utilization progressively falls with reductions in oxygen delivery beyond the critical level is referred to as "supply dependence".
Figure 1. Relationship between oxygen delivery and consumption in septic and nonseptic states. While oxygen supply is greater than the critical \( \text{DO}_2 \), oxygen consumption is maintained by the tissues' ability to extract a greater proportion of the oxygen supplied. When oxygen supply is lower than the critical \( \text{DO}_2 \), oxygen consumption begins to decrease and tissues become reliant on anaerobic metabolism. In septic individuals, baseline oxygen demand is increased, while the critical \( \text{DO}_2 \), where oxygen uptake becomes supply-dependent, is significantly increased (crit. \( \text{DO}_2' \)).
Sepsis may alter the normal relationship between tissue oxygen uptake and delivery, impairing tissues' ability to extract oxygen from blood. Tissue oxygen availability can thus be impaired, even when total oxygen delivery is in its normal range. Oxygen consumption falls with higher levels of oxygen delivery than in normal individuals. While pathological supply-dependence of oxygen uptake has been frequently observed in septic animals (114)(115)(116) as well as in a number of human studies (117)(118)(119)(120), it is not a consistent finding (121). Further, if it does occur, its significance is not clear: is it a normal response to critical illness or is it a pathologic response that contributes to the overall morbidity of the individual? Gilbert et al. reported that pathologic supply-dependence occurs in septic patients with lactic acidosis, but not in those without lactic acidosis (119). This suggests that pathologic supply-dependence really is an indicator of tissue hypoxia. This has not been a universal finding either (120). Besides, elevated lactate levels alone must be interpreted with caution, since elevated rates of glycolysis can cause lactate levels to rise, even in the absence of anaerobic metabolism. In sum, the literature would suggest that pathologic supply dependence is probably a real phenomenon in some septic patients, but its significance is not yet known.

Several mechanisms that might contribute to pathologic oxygen supply dependence have been suggested. First, there may be an impairment of normal autoregulatory control in tissues exhibiting pathologic supply dependence of oxygen transport. Vascular catechol responsiveness is abnormal in experimental sepsis (122) and vessels fail to relax normally to topically applied vasodilators (123). In addition, endothelial cells, which play a central role in regulation of vascular tone, exhibit structural and functional changes during sepsis (123)(124). Secondly, peripheral oxygen extraction deficits may be due to microembolization, for sepsis is associated with an increase in neutrophil margination (40)(41), platelet aggregation and activation of the coagulation cascade (86)(87). Thirdly, there may be an impairment of utilization of oxygen at the cellular level: an "uncoupling" of
mitochondrial oxidative phosphorylation (125)(126). Any of these mechanisms may play a role in the impairment of oxygen utilization by susceptible tissues.

Pathologic oxygen supply dependence is important because it may predispose certain tissues to ischemia, despite achieving normal levels of oxygen delivery. This has several clinical implications. If tissues are more susceptible to ischemia during sepsis, then tissue beds at risk must be carefully monitored for occult ischemic injury. Ischemia that has not been detected and that has been allowed to continue may result in focal tissue injury, which may stimulate the inflammatory response responsible for SIRS and MODS. In particular, ischemic injury to the gut may promote the escape of its intraluminal contents, including Gram negative bacteria and their products. It is therefore important to establish the susceptibility of various tissue beds to ischemia and to document the consequences of this vulnerability.

1.4.1 Gut Ischemia During Septic Shock

As previously discussed, endotoxin-induced changes in gut perfusion vary considerably between animal species and also between different experimental models using the same species. Similarly, changes in gut perfusion during experimental and clinical Gram negative sepsis vary considerably between studies. For instance, pigs with fecal peritonitis have decreased splanchnic blood flow (127). In humans with hyperdynamic septic shock, however, splanchnic blood flow is increased (128). Thus, while mesenteric hypoperfusion has been demonstrated in some models of septic shock and endotoxemia, this is not consistently observed.

The lack of consistent changes in mesenteric perfusion does not exclude the possibility that the gut is ischemic during septic shock. The gut may be particularly susceptible to ischemia compared to other organs, especially during septic shock, making it vulnerable even in the absence of grossly detectable changes in perfusion. In the normal intestine, $DO_2$ far exceeds the tissue requirements and blood flow must be reduced to about
40% of control to affect $\text{VO}_2$ (129). While in hemorrhagic shock splanchnic $\text{VO}_2$ is unchanged or slightly reduced in parallel with reduced $\text{DO}_2$, sepsis causes the gut and liver $\text{VO}_2$ to increase markedly (83)(108). The tissue requirements quickly augment to approximately double the normal requirements, so a normal blood flow and $\text{DO}_2$ become barely sufficient to supply enough oxygen. There is therefore no reserve for further increases in oxygen extraction to maintain $\text{VO}_2$ if $\text{DO}_2$ declines further, if oxygen utilization becomes impaired, or if the tissue requirements are further increased. In a canine model of septic shock, Nelson et al. (130) showed that oxygen supply dependency occurs in the gut at a point when systemic $\text{DO}_2$ still exceeds the minimum $\text{DO}_2$ needed to maintain nongut tissues independent of $\text{DO}_2$. This is reflected by lower maximal extraction fractions in the gut than in nongut tissues. The authors suggested that differences in the critical $\text{DO}_2$ are the result of diffusional arteriovenous shunting in the intestinal villi or a smaller capillary density in the gut than in other tissues. In humans, following a single IV bolus of endotoxin, an increase in splanchnic oxygen consumption was observed and the increase was greater than the elevation in total body $\text{VO}_2$ (82). This was not unlike the disproportionate increase in splanchnic $\text{VO}_2$ relative to total body $\text{VO}_2$ seen in septic humans (83). Ruokonen et al (128) also showed data consistent with impaired splanchnic oxygen extraction: increases in oxygen demand in excess of the enhanced oxygen delivery (ie: increased splanchnic blood flow). The gut, due either to its anatomic or its physiologic properties, may therefore be particularly susceptible to ischemia during sepsis.

Gut ischemia as detected by tonometry has been reported in some animal models of sepsis, as well as in humans. Administration of endotoxin or viable bacteria and fecal peritonitis in pigs result in intestinal mucosal acidosis (79)(109)(127), which presumably reflects the presence of ischemia. In humans, tonometrically measured gastric pH has been used as a measure of splanchnic perfusion and oxygenation. Gastric mucosal acidosis is a strong predictor of mortality in critically ill patients (131)(132) and improved survival has been demonstrated in those patients in whom the gastric mucosal acidosis can be reversed.
(133)(134). However, it remains unclear whether this is the case for patients whose physiologic derangements are due mainly to Gram negative sepsis. Tonometrically determined gastrointestinal mucosal acidosis has thus been observed experimentally and clinically, in sepsis and endotoxemia, but the physiologic derangements responsible for the changes are not fully understood.

1.4.2 Intestinal Mucosal Injury Following Ischemia-Reperfusion

Splanchnic ischemia may produce a variable degree of injury, ranging from increased capillary permeability and mucosal hyperpermeability to frank necrosis of the superficial layer of the mucosa (135). Partial ischemia, or a reduction in blood flow to approximately one-third of the normal resting level, causes increased mucosal permeability to macromolecules within one hour. Morphologically apparent injury to the small intestinal villi is detectable via light microscopy within two hours. The colonic mucosa appears to be more resistant to these changes, for ischemia must be more prolonged and/or more severe to induce similar changes in colonic mucosa (136). During a sufficiently prolonged period of ischemia, irreversible tissue injury may occur. Gut ischemia therefore produces a continuum of changes that are first manifested as changes in function and progress to morphologically apparent lesions.

While ischemia is a well-recognized factor contributing to the pathogenesis of organ injury, the role of reperfusion in this process has only recently been appreciated (35)(36). Reperfusion of a previously ischemic bowel intensifies the increased capillary and mucosal permeability that occurs during ischemia (137)(138) and aggravates the resultant mucosal injury (139)(140). The tissue damage occurring after oxygenation is restored is thought to result from the formation of oxygen free radicals (137)(141). There are several important biologic sources of oxygen free radicals, including xanthine oxidase, activated leukocytes, mitochondria, prostaglandin synthetase, and catecholamine auto-oxidation; xanthine oxidase and leukocytes appear to be the major sources in clinical disease states.
radicals and nonoxidative toxins.

Activated neutrophils contribute to the tissue injury by producing further oxygen and reperfusion. These activated neutrophils become activated during ischemia by the enzymatic action of xanthine oxidase. In addition, neutrophils become activated during ischemia xanthine dehydrogenase to xanthine oxidase. On reperfusion, oxygen radicals are formed from oxygen xanthine dehydrogenase to xanthine oxidase. On reperfusion, hypoxanthine results in the production of hypoxanthine from ATP and conversion of tissue injury. Tissue ischemia results in the production of hypoxanthine phenomonon results in microvascular and...

Figure 2. The pathway by which the ischemia-reperfusion phenomenon results in microvascular...
The fact that the conversion of xanthine dehydrogenase to xanthine oxidase takes only 10 s in intestinal tissue, 8 min in cardiac muscle and about 30 min in the liver, spleen, kidney and lung may help explain the differential relative susceptibilities of these organs to ischemia-reperfusion-mediated injury (6)(142). The pathway by which ischemia-reperfusion results in microvascular and tissue injury is depicted in Figure 2.

Ischemia-reperfusion produces a profound inflammatory response with activation of macrophages and neutrophils and the release of proinflammatory cytokines, oxygen metabolites and other cytotoxic substances (42). The endothelium of the splanchnic vasculature has been implicated as a "trigger" of a cascade of humoral and cellular events. Endothelial dysfunction is evident within less than 3 minutes of reperfusion following a period of ischemia and it is manifested as intractable vasoconstriction (143). Following onset of endothelial dysfunction, neutrophil activation occurs, amplifying the magnitude of the reperfusion injury. Much work has been done on determining the mediators of the observed endothelial dysfunction following ischemia-reperfusion. Postreperfusion endothelial dysfunction appears to be primarily due to the early formation and release of oxygen-derived free radicals and superoxide radicals generated from the endothelial cells themselves and, subsequently, from neutrophils attracted to and adherent to the endothelium. In addition, cytokines such as TNF, IL-1 and IL-2 are thought to act as mediators of ischemia-reperfusion injury (32). For example, TNF exerts potent antiendothelium-derived relaxing factor effects in blood vessels (144). It is the inflammatory response that is triggered by endothelial dysfunction secondary to ischemia-reperfusion that may ultimately result in SIRS and MODS.

1.4.3 Gut Ischemia-Reperfusion as a Cause of MODS

MODS appears to be a systemic response to a variety of severe insults, but the pathogenesis is unclear. Intestinal ischemia and/or reperfusion may be important precursor events. In animals, reperfusion of intestine following a period of ischemia causes
functional and pathological changes of organs distant to the primary site of injury, including the heart, lungs, and liver (145)(146)(147)(148). Intestinal ischemia-reperfusion-associated lung injury is characterized by pulmonary vascular hyperpermeability and pulmonary edema (146)(149). Liver injury induced by mesenteric ischemia-reperfusion is manifested by neutrophil sequestration within the hepatic parenchyma, fatty degeneration, focal necrosis, hepatocellular enzyme release, reduced bile flow rates, and impaired hepatocyte metabolism (145)(146)(147). Surgical revascularization in patients with chronic mesenteric ischemia has been reported to be associated with pulmonary dysfunction consistent with adult respiratory distress syndrome, hepatic dysfunction, renal failure and coagulopathy (150). According to this report, the mortality rate after operation was 14% and all deaths resulted from MODS. Distant organ dysfunction has thus been shown to occur following mesenteric ischemia-reperfusion in animal models as well as in humans.

The mechanism by which distant organ dysfunction occurs following reperfusion of ischemic intestine is not well understood. One hypothesis is that mesenteric ischemia-reperfusion incites a stereotyped systemic response by the release of gut-derived bacteria or endotoxin. Dogs treated with partial occlusion of the SMA have a high incidence of Gram negative bacteremia and this is associated with functional and morphological evidence of injury of distant organs (145). Prophylactic administration of amikacin provides some protection from this distant organ injury, suggesting distant organ injury may be a function of Gram negative bacterial products. On the other hand, inhibition of endotoxin activity does not prevent post-reperfusion liver or lung injury in rats (146)(149). Therefore, the role of endotoxin (or other products of Gram negative bacteria) in the pathogenesis of distant organ injury during mesenteric ischemia-reperfusion is yet to be determined. Inflammatory mediators released by the injured tissue have also been thought to be important proximal mediators of distant organ injury under these conditions. For example, blockade of tumor necrosis factor, a cytokine inflammatory mediator, prevents some of the pulmonary changes associated with intestinal ischemia-reperfusion (148). Finally, oxygen
radicals generated by reperfusion may be responsible for the distant organ injury, for administration of antioxidants provides some protection from distant organ injury in dogs submitted to mesenteric ischemia-reperfusion (145). However, other studies do not support this theory, at least not with respect to liver injury, for significant hepatic exposure to oxidant stress could not be demonstrated in rats following intestinal ischemia-reperfusion (151). Thus, while multiple organ dysfunction secondary to intestinal ischemia-reperfusion has been reported in a number of animal models as well as in humans, the mechanism for the phenomenon must still be elucidated.

1.5 BACTERIAL TRANSLOCATION

The loss of the intestinal barrier function leading to the systemic spread of bacteria has been termed bacterial translocation (6). In 1950, Fine et al. described a canine model of chemical peritonitis in which peritoneal seeding with gut-derived $^{131}$I-tagged *E. coli* was observed (152). Subsequently, bacterial translocation across the intestinal wall was described in rats (153). Fine, in his review article in 1965, concluded that absorption of endotoxin from the gastrointestinal tract in conjunction with reduced hepatic detoxification was the fundamental basis for irreversible postinjury shock (154). Since then, bacterial translocation has been described in association with various insults in various animal models, including burn injuries (155), endotoxemia (156), hemorrhagic shock (157)(158), malnutrition (159), immunosuppression (160), skeletal trauma (161), bacterial overgrowth (160)((162), radiation (163) and malignancy (164). In humans, previous studies have shown marked changes in intestinal permeability in the presence of diseases causing morphological disruption of the intestinal mucosa, such as Crohn's disease, celiac disease, and following chemotherapy or radiotherapy. In addition, administration of small doses of endotoxin to healthy human volunteers has been shown to cause an increase in intestinal permeability (112). While increases in intestinal permeability have been documented in
sepsis (165) and following thermal injury (166), as well as in patients with prolonged starvation reliant on parenteral nutrition (167), conflicting data are seen in trauma patients (168)(169). Further, while increases in intestinal permeability have been observed in humans in a number of clinical conditions, more direct evidence of bacterial translocation - positive cultures of gut organisms in the blood, mesenteric lymph nodes, and/or peritoneum - is sparse. One recent study reported translocation of bacteria in 16% of patients undergoing abdominal surgery (170). Bacterial translocation has thus been demonstrated in animals and, less frequently, in humans, in various stressful conditions.

Although bacterial translocation can be induced in a variety of animal models and by a variety of insults, experimental studies on the pathophysiology of bacterial translocation and gut barrier failure indicate that one or more of three basic pathophysiologic conditions is necessary for bacterial translocation to occur (171). These are: (a) disruption of the ecological balance of the normal indigenous microflora, resulting in bacterial overgrowth with Gram negative enteric bacilli; (b) impaired host immune defenses; and (c) physical or functional loss of the mucosal barrier. These conditions are commonly present in the critically ill patient at risk for developing MODS. That is, they have often experienced major blood loss or a hypotensive episode, which may injure the gut mucosal barrier; they are frequently immunocompromised; and antibiotics administered may disrupt the normal ecology of the gut flora (172). In addition, gastric acid neutralization for stress ulcer prophylaxis or hyperosmolar enteral feeds may result in the colonization of the stomach and proximal intestine (173)(174). Lastly, prolonged total parenteral feeding may result in mucosal atrophy and altered intestinal mechanical defenses (167)(175). It is conceivable that the presence of these risk factors for bacterial translocation in critically ill patients results in the susceptibility of this population of patients to the development of MODS.

Despite the abundance of information available regarding the factors that promote bacterial translocation, the anatomic route(s) by which bacteria spread remains unclear. Sori et al. (158) showed that radiolabelled E. coli fed to rats appeared in the blood stream during
hemorrhagic shock. Mainous et al. (176), using a rodent model of systemic inflammation, showed that the route of translocation varies with the severity of the inflammatory stimulus. With a mild inflammatory stimulus, bacterial translocation is mainly via the mesenteric lymph nodes; a severe inflammatory state is accompanied by bacterial dissemination via portal blood. Brooks et al. reported that, in surgical patients, positive cultures are seen most frequently in the mesenteric lymph nodes, followed by intestinal serosa; blood cultures are least frequently positive (170). Translocation has therefore been documented on numerous occasions by a hematogenous route and by a lymphatic route, but the conditions favouring one route over the other have not yet been defined.

1.5.1 Intestinal Mucosal Hyperpermeability During Ischemia

The mechanisms responsible for alterations in the barrier function of the GI tract in sepsis and trauma are incompletely understood, although recent data suggest that one important factor may be mesenteric ischemia. After a period of partial intestinal ischemia, an increased passage of bacteria and endotoxin has been demonstrated (177). When mucosal injury is microscopically visible, significantly increased portal bacteremia or endotoxemia has been observed (178). In addition, high concentrations of bacteria and endotoxin are seen in the intestinal lymph nodes (179) and the mesenteric lymph nodes (180). In sheep subjected to combined cutaneous thermal and smoke inhalation injury, mesenteric hypoperfusion is seen; both mucosal ischemia and bacterial translocation are prevented by pharmacologic blockade of mesenteric vasoconstriction with nitroprusside (181). It therefore appears that mesenteric ischemia may be a cause of bacterial translocation and mesenteric hyperpermeability.
1.5.2 Intestinal Mucosal Hyperpermeability During Septic Shock and Endotoxicosis

Intestinal hyperpermeability to various hydrophilic solutes has been observed in various models of sepsis including pigs (109)(182) and rats (156). In addition, administration of endotoxin leads to gut mucosal hyperpermeability in humans (112). The mechanism(s) by which endotoxin enhances intestinal permeability are unknown. There are a number of possible explanations. There may be a direct effect of endotoxin on enterocytes, producing functional and/or morphologic impairment of their normal barrier function by mechanisms such as ischemia. Alternatively, endotoxin may have an indirect effect on enterocytes which is mediated by cytokines (eg: TNF) or other inflammatory mediators.

Because endotoxin also causes mesenteric hypoperfusion and a decrease in mucosal pH, gut barrier dysfunction associated with endotoxicosis has been attributed to functional impairment of the epithelium secondary to hypoxia or to ischemic damage to the splanchnic epithelium (79)(183). Fink et al. (109) addressed this question in a euvolemic pig model in which LPS was infused to produce a reduction in mesenteric blood flow to approximately 50% of the baseline value. This was associated with an almost six-fold increase in $^{51}$Cr-EDTA clearance, indicating a markedly impaired mucosal barrier function. When mesenteric perfusion was reduced to 50% of baseline values by mechanical occlusion, however, mucosal permeability was not affected. Results obtained by Bulkley et al. on a canine model were similar (129). Further, it was shown that intestinal permeability was not affected by ischemia-reperfusion unless oxygen extraction was reduced by more than 50% during the period of hypoperfusion. It therefore appears, from these two studies, that the effect of endotoxin on gut mucosal permeability is mediated by events other than ischemia, although a reduction of perfusion may be a potentiator of the phenomenon in the presence of some other factor(s). However, in contrast, Fink's group also demonstrated that endotoxin-induced ileal mucosal acidosis - presumably indicative of gut ischemia - is a
causal factor in the pathogenesis of endotoxin-induced ileal mucosal permeability (182). While the association between mesenteric ischemia and gut mucosal hyperpermeability has been made, it is unclear whether mesenteric ischemia plays an etiologic role during sepsis and endotoxemia.

1.5.3 The Gut as a Source of LPS in Critical Illness

Presumably, the same factors that favour bacterial translocation promote escape of their toxic cell membrane from the gut lumen. Numerous animal models of shock have been studied to determine what conditions are associated with endotoxemia and, if endotoxemia does occur, whether the gut is the origin of the endotoxin. Human studies have endeavoured to achieve the same objectives. Data from animal and human studies are confusing and contradictory. While animal models of a given condition differ considerably from each other, perhaps explaining some of the disparity of results seen in the literature, even very similar models do not seem to yield consistent findings. Further, in humans with a given clinical condition, investigators have failed to demonstrate any consistency in their findings.

Animal models of shock and intestinal ischemia have perhaps been best studied, since these models are used to investigate the hypothesis that intestinal ischemic injury is a cause of endotoxemia. During hemorrhagic shock, systemic endotoxemia has been reported and this was thought to be secondary to intestinal ischemia (184). In addition, portal and systemic endotoxemia have been observed during occlusive ischemia of the colon and small bowel in rodents (148)(185), rabbits (186), cats (187), dogs (188), and primates (189), but not all have been able to demonstrate such a phenomenon (149)(190). Reperfusion following a period of ischemia is associated with an even more pronounced endotoxemia, according to some investigators (146)(148)(186)(187)(189). However, a number of other authors have failed to reproduce this finding (149)(190). The finding that intestinal injection of antibiotics (186) or administration of oral nonabsorbable antibiotics (189)
attenuates the endotoxemia associated with mesenteric ischemia-reperfusion suggests that
the endotoxin detected originates from the gut. On the other hand, the failure to demonstrate
a difference between systemic and portal venous levels of endotoxin is not consistent with
this hypothesis (146). Thus, in animal models of mesenteric ischemia-reperfusion,
endotoxemia has been inconsistently observed. Endotoxemia may be most evident during
ischemia or following reperfusion and its origin is not well established, even in these well
controlled experimental conditions.

Evidence supporting the occurrence of endotoxemia in the human portal circulation
is sparse and contradictory. Jacob et al. demonstrated LPS in the portal circulation in 97%
of patients undergoing elective abdominal surgery (191). LPS was seen less frequently in
the systemic circulation. Others have failed to show portal endotoxemia with any degree of
consistency in patients during elective abdominal surgery (192)(193). High levels of
endotoxin have been detected in patients with ulcerative colitis and Crohn's disease (194),
as well as in patients following colonoscopy (195). In patients with liver cirrhosis, LPS is
found in the portal vein more frequently than in peripheral veins (196) and portal venous
LPS levels are higher than LPS levels in the systemic circulation (197). Incidentally, higher
portal venous LPS levels in cirrhotics are associated with more severe liver dysfunction and
with a worse prognosis (198). Gut decontamination with oral nonabsorbable antibiotics
decreases the incidence of endotoxemia, suggesting the gut is a source of endotoxin under
these conditions, but the clinical consequence of this is unknown (199). In patients
undergoing abdominal aortic surgery, during which the colon may be ischemic following
aortic cross-clamping, portal endotoxemia occurs infrequently and endotoxin levels are low
(200)(201). Finally, endotoxemia was not detected in patients after major accidental injury
(169).

Lack of consistent data supporting the presence of portal endotoxemia may be due
to a number of factors other than the disparity of clinical and experimental conditions under
study. Endotoxin may contaminate catheters and instruments and such contamination may
produce spuriously high measurements. This is particularly a problem in animal studies, where the precautions of sterility are not as strictly upheld as in a hospital operating room. Further, any spillage of gastrointestinal contents is sure to skew results. On the other hand, assays for endotoxin, until recently, were relatively insensitive and there were numerous factors that could interfere with these assays, producing false-negative results. Endotoxin assays currently available are extremely sensitive and it has thus become even more important to avoid contamination. Further study under sterile and endotoxin-free conditions, using sensitive assays for endotoxin, is clearly required to resolve the controversy.

1.6 **CYTOKINES**

The first humoral mediators of the response to injury and infection identified were hormones such as catecholamines and glucocorticoids; they are produced in relatively large amounts by specialized tissues and they exert their biologic effects mostly through endocrine mechanisms. Recently, a number of additional protein mediators produced in small amounts at the site of injury and by immune cells throughout the body have also been identified. These proteins are collectively called cytokines and they have an important influence on the response to injury and infection.

Cytokines differ from classic endocrine hormones in several ways. Firstly, they are produced by many cell types, rather than by a distinct gland. Secondly, cytokines are able to elicit significant tissue responses at very low concentrations by stimulating the release of other cytokines, producing a cascade effect which amplifies the response to injury. Finally, cytokines are able to exert their influence locally, in a paracrine fashion, or when produced in excess, they may spill into the circulation to produce systemic effects, like an endocrine hormone. The metabolic and hemodynamic instability and fever characteristic of septic shock are the most clinically evident manifestations of acute cytokine-mediated injury. In addition, chronic excessive production of cytokines may be responsible for the tissue
Figure 3. Schematic representation of the cytokine cascade. TNF production appears to be a primary event, followed by IL-1 production by the same cells, as well as endothelial cells. IL-6, IL-8, colony stimulating factors (CSFs), and various other cytokine chemotaxins are produced by a large variety of cells. IL-6 appears to be able to exert a negative feedback effect on TNF production. Most of the cytokines have both local and distant effects, some of which are represented in the diagram. Adapted from Billiau and Vandekerckhove, 1991.
Figure 4. The interaction of various groups of inflammatory mediators. Each group of mediators serves numerous functions. Activation of one pathway can result in activation of various other cytokine and non-cytokine pathways. The cytokine cascade is a central component of the inflammatory response.
wasting, anorexia and anemia seen in cachexia (202). These systemic manifestations may be due to a diffuse cytokine-secreting response by numerous circulating inflammatory cells or they may be secondary to production of large amounts of cytokines at a focus of injury, where excessive accumulation of cytokines results in their overflow into the circulation.

Numerous cytokines including tumor necrosis factor, interleukins, interferons and colony-stimulating factors, have now been characterized and are described elsewhere (202)(203). Endotoxin is a very potent stimulus for the production of cytokines in vitro and in vivo and other stimuli of an inflammatory response similarly are associated with release of cytokines, which appears to occur by a cascade-type effect. This cascade-type effect may cause an amplification of this inflammatory response or, alternatively, release of certain cytokines may serve to control production of cytokines released earlier on in the cascade (Figure 3). Tumor necrosis factor and interleukin-6, the cytokines most thoroughly studied to date, will be described below.

Non-cytokine mediators of inflammation such as complement, the clotting cascade, platelet activating factor, eicosanoids, and the kallikrein-kinin system can be activated under the same circumstances as cytokines (203). The interaction of cytokines with non-cytokine mediators during a systemic inflammatory response such as sepsis or a localized inflammatory response such as that seen during focal injury is the subject of intensive investigation at present. While these inflammatory events are in no way completely understood, one postulated way in which these various mediators might interact is shown in Figure 4.

1.6.1 Tumor Necrosis Factor (TNF)

TNFα, also known as cachectin, and TNFβ, also known as lymphotoxin, are two closely related proteins that bind to the same cell surface receptors and produce similar, but not identical, biological effects. The TNFs show approximately 30% homology at the amino acid residue level. TNFα is a polypeptide with a molecular weight of 17 kilodaltons
(kD). It is translated as a precursor with 233 amino acids and it then undergoes proteolytic cleavage to be secreted as a protein of 157 amino acids (204). Increased transcription and translation of the protein is stimulated by numerous infectious and inflammatory stimuli, including bacteria, endotoxin, bacterial exotoxins, fungi and viral particles (205)(206); the release of the mature protein occurs within minutes of stimulation. TNFβ is a somewhat larger protein of 171 amino acids, with a molecular weight of about 45 kD (207). TNFα is secreted from many cell sources, including peritoneal macrophages, Kupffer cells, pulmonary macrophages, endothelial cells, mast cells and circulating monocytes, natural killer cells and lymphocytes (202)(208). The circulating half-life is relatively short: 5 - 30 minutes (209). TNFβ is produced by lymphocytes and the circulating half-life is 14 - 18 minutes in humans (210). TNF is degraded in many organ systems, including the liver, the skin, the kidney and the gastrointestinal tract (211).

**Biological Activities**

TNF has numerous effects on virtually every organ system in the body and this is attributable to the ubiquity of its receptors, to its ability to activate multiple signal transduction pathways, and to its ability to induce or suppress the expression of a vast number of genes, including those for growth factors, cytokines, inflammatory mediators, and receptors. One of its more prominent roles is as a stimulator or initiator of other inflammatory responses. For example, TNF invokes the release of neutrophils from the bone marrow and stimulates their activation, margination and transendothelial passage (212)(213). It similarly activates monocytes against viruses and parasites and activates eosinophils against parasites (214)(215). In addition to activating immune cells, TNF is chemotactic for neutrophils and macrophages and stimulates phagocytosis of pathogens by these cells (209). Finally, TNF stimulates the production and release of other cytokines, including IL-1 and IL-6 (209), thus activating the proinflammatory "cytokine cascade".

TNF appears to be important in the maintenance of homeostasis during episodes of acute stress. Its administration results in the secretion of counterregulatory hormones (216);
blockade with antibodies to TNF attenuates this response (217). Further, TNF promotes mobilization of nitrogenous and carbonaceous substrates from peripheral muscle and adipocytes to splanchnic tissue. It induces a release of amino acids from skeletal muscle (218) and simultaneously promotes increased uptake of amino acids by the liver and enhances hepatic protein synthesis (218). This results in preservation of the liver mass at the expense of skeletal muscle and it allows continued synthesis of proteins that may be central to immune, oncotic and coagulation functions. TNF also increases glucose uptake, glycogen utilization and cellular efflux of lactate in skeletal muscle (219), consistent with stimulation of anaerobic glycolysis. The changes in fat metabolism characteristic of sepsis are similarly induced by TNF. Triglycerides are mobilized from adipocytes (211) fatty acid synthesis is inhibited, and lipolysis is enhanced (82). All of these changes in metabolic homeostasis are acutely beneficial to the host. However, it is thought that chronic hypersecretion of TNF may lead to cachexia (220), as seen in patients with chronic inflammatory conditions and malignancies.

TNF may be important in wound healing and remodeling. It stimulates the coagulation cascade and increases vascular permeability at the site of injury. Furthermore, it acts as a growth factor, stimulating microvascular angiogenesis and fibroblast proliferation (202)(209).

In addition to the effects mentioned above, most of which are beneficial to the host in the event of acute illness or infection, a number of detrimental effects of TNF have been described. These have generally been described in animal models in which large doses of TNF have been administered, but may theoretically occur with acute hypersecretion of the cytokine. Acute hypersecretion may occur in septic shock and the potential role of TNF in this regard will be discussed below. Some of the biological effects of TNF are summarized in Table 4.
<table>
<thead>
<tr>
<th>Cell/Tissue Type</th>
<th>Biological Effects</th>
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| Endothelium     | Enhances procoagulant activity  
|                 | Suppresses cofactor activity for anticoagulant protein C  
|                 | Decreases thrombomodulin expression  
|                 | Increases expression of ICAM, ELAM, MHC antigens  
|                 | Increases extravasation of macromolecules and plasma  
|                 | Induces IL-1 and PAF biosynthesis and release  
| Leukocytes      | Stimulates neutrophil degranulation  
|                 | Induces respiratory burst with release of superoxides  
|                 | Chemotactic for macrophages and neutrophils  
|                 | Increases phagocytic capacity against pathogens  
|                 | Induces further TNF release  
|                 | Stimulates IL-1 production  
|                 | Induces PGE2 release  
| Lung            | Increases capillary permeability  
|                 | Induces PGE2 and leukotriene synthesis  
|                 | Mediates leukocyte margination and activation  
|                 | Induces alveolar wall thickening  
|                 | Increases lung water and edema  
| Liver           | Increases acute phase protein synthesis  
|                 | Promotes glucagon-mediated amino acid uptake  
|                 | Increases liver size and DNA content  
|                 | Stimulates lipogenesis  
| Adipocytes      | Suppresses lipoprotein lipase  
|                 | Stimulates free fatty acid and triglyceride release  
|                 | Net insulin resistance  
|                 | Suppresses lipogenesis  
| Muscle          | Increases protein catabolism  
|                 | Decreases resting transmembrane potential  
|                 | Suppresses structural protein synthesis  
|                 | Increases amino acid release  
|                 | Decreases glycogen content and increases lactate efflux  
| CNS             | Fever  
|                 | Anorexia  
|                 | Sympathetic discharge  
|                 | Stimulates ACTH and other pituitary hormones  

Table 4. Biological effects of tumor necrosis factor on some tissues and cells. (Note: ICAM = intracellular adhesion molecule; ELAM = endothelium-leukocyte adhesion molecule; PAF = platelet-activating factor; PGE2 = prostaglandin E2; ACTH = adrenocorticotropic hormone). Adapted from Tracey and Cerami, 1993 (209).
1.6.2 Interleukin-6 (IL-6)

IL-6 is a phosphoglycoprotein that exists in various forms in different animal species and is expressed by many different cell lines (221)(222). Its molecular weight ranges from 23 to 45 kD (223). Endotoxin, viruses and other cytokines (eg: TNF, IL-1) are potent stimulators for transcription and translation (224). Transcription of mRNA of IL-6 by fibroblasts can be detected within 30 minutes of endotoxin stimulation and it is sustained for at least 20 hours (225). IL-6 is produced by endothelial cells, fibroblasts and epithelial cells, as well as circulating monocytes, macrophages and T-lymphocytes (202).

**Biological Activities**

IL-6 serves predominantly as an immunostimulant. It enhances differentiation of lymphocytes (226), stimulates proliferation of B-lymphocytes, invokes the production of immunoglobins by B-cells (227), and activates T-lymphocytes (228)(229). Further, the cytokine has colony-stimulating activity on hematopoietic stem cells (230)(231). IL-6 is a known pyrogen (232).

In addition to the above immunostimulatory effects, IL-6 may exert antiinflammatory effects. It inhibits production of other cytokines, such as TNF. This effect has been observed *in vitro* and *in vivo* (233) and its role as an immunomodulant is further supported by the observation that, in severe infections, peak IL-6 levels occur after TNF and IL-1 peak (202). In addition, IL-6 stimulates the release of adrenocorticotropin in the blood stream which ultimately results in immunosuppression through the action of steroids (203).

IL-6 enhances the hepatic synthesis of acute-phase proteins during injury (234). Further metabolic effects are currently being elucidated. There is no evidence that IL-6 influences cardiovascular stability or cellular integrity.
1.6.3 Compartmentalization of Cytokine Production

It is thought by some investigators that cytokine production and release is "compartmentalized". That is, these peptide inflammatory mediators are produced at sites in which tissue injury occurs, acting in a paracrine fashion to effect local responses to injury. With sufficient stimulation, local tissue levels of cytokines increase to such a magnitude that the cytokines begin to spill into the circulation, potentially allowing them to exert their effects on distant organs. Since sepsis is thought to cause occult injury to the splanchnic organs, resulting in an inflammatory response that assumes systemic proportions, such a theory is quite attractive, as it may help explain the pathogenesis of MODS.

Several studies of focal injuries support the presence of differential rates of cytokine production by various tissues, or "compartmentalization". For example, in meningitis, levels of TNF (235) and IL-6 (236) are higher in the CSF than in the systemic circulation. The intratracheal administration of endotoxin in experimental models is associated with the appearance of TNF in bronchoalveolar lavage fluid while, in the same model, administration of IV endotoxin increases circulating levels of TNF without producing an increase in levels in the bronchoalveolar lavage fluid (237). Hypersecretion of IL-6 in injured skin in burn patients has been reported (238). It therefore appears that injured tissues are most likely to be the predominant source of cytokines.

Evidence of compartmentalization of cytokine production in splanchnic organs, particularly during sepsis, is scant. The splanchnic organs contain a large number of tissue macrophages (e.g. Kupffer cells, in the liver) capable of producing TNF as well as other inflammatory proteins (239). Gene transcription for TNF and IL-6 is increased in the liver, kidney, spleen and lung in rodents treated with endotoxin (240)(241). Fong et al. (82) measured arterial and hepatic venous cytokines following endotoxin infusion in human volunteers. Hepatic venous TNF levels were significantly higher than arterial levels whenever the protein was detectable in the circulation, but this was not the case with IL-6. Splanchnic production of TNF has therefore been demonstrated in animals and humans,
but in each instance there was no documentation of splanchnic organ injury. On the other hand, there have been reports of gut production of cytokines in instances of gut injury in the absence of sepsis. Baigrie et al. demonstrated elevated levels of plasma IL-6 originating from the colon during abdominal aortic surgery, where colon ischemia was thought to be present (200). In addition, TNF-secreting cells have been demonstrated in the human intestinal mucosa. The number of these cells is increased in the presence of mucosal inflammation, regardless of its etiology, and is proportional to the severity of inflammation (242). These studies suggest that injury to the gut may cause splanchnic production of cytokines.

While there is evidence of differential cytokine production by various organs when these organs are injured, it is not yet clear whether the same phenomenon occurs during sepsis. Further, if differential rates of cytokine production do occur during sepsis, the stimulus for this response is as yet undefined: is it a result of organ injury, stimulation by endotoxin circulating throughout the body, or some other unidentified stimulus?

1.6.4 The Role of TNF and IL-6 in the Pathogenesis of Septic and Endotoxic Shock

While sepsis and endotoxemia are associated with elevated levels of cytokines, among other numerous inflammatory mediators, the exact role of cytokines in the pathogenesis of Gram negative septic shock is not known. Evidence is accumulating that at least some cytokines are important proximal mediators of the many pathophysiologic derangements seen during septic and endotoxic shock.

**Tumor Necrosis Factor**

TNF is detected in the serum during various infectious conditions, including bacterial infections (243), parasitic infections (244), and septic purpura (245). In patients with meningococcal infection, circulating levels greater than 100 pg/mL are associated with increased mortality (243). TNF is seen in the circulation of experimental animals and
human subjects within minutes of bacterial or endotoxin infusion and peak serum levels occur within 90 - 120 minutes (29)(82)(217)(246). Circulating levels return to baseline (ie: undetectable) within 4 hours.

Acute hypersecretion of TNF may be a contributing factor in the cardiovascular collapse characteristic of septic shock. In acute models of septic shock, increased serum TNF levels occur at approximately the same time hemodynamic changes characteristic of septic shock appear. Intravenous administration of recombinant TNFα in various animal models duplicates many of the hemodynamic changes of septic shock, including systemic hypotension, decreased systemic vascular resistance, pulmonary hypertension, and hemoconcentration (30)(32)(216)(247)(248). Animals resistant to the effects of endotoxin appear to have protection conferred by a genetic inability to synthesize native TNF (249). However, hemodynamic changes in septic and endotoxic shock persist despite the relatively short time TNF is seen in the bloodstream. Therefore, if TNF produces the hemodynamic effects seen during septic shock, the hemodynamic effects appear to be sustained by other mediators which are perhaps activated by the presence of TNF.

TNF may be responsible for a number of other pathophysiologic changes in septic and endotoxic shock as well. TNF infusion causes pyrexia (30)(248). The metabolic derangements induced by infusion of TNF in animals are similar to those seen in human septic shock and these include lactic acidosis, hyperkalemia, hemoconcentration, and hyperglycemia followed by hypoglycemia (30)(32)(248). TNF-induced shock is also associated with marked elevations of circulating glucose counter-regulatory hormone levels, including epinephrine, norepinephrine, glucagon and cortisol. Neutropenia is also sometimes seen with infusion of TNFα (247). This characteristic "stress" response is typically seen in septic humans.

Prophylactic administration of monoclonal antibodies to TNF prevent many of the physiologic changes in experimental sepsis, including some of the hemodynamic changes, the metabolic acidosis, and the neutropenia. Further, they decrease mortality under these
conditions (250)(251)(252). These antibodies confer some protection even when administered after the onset of septic shock (253). The protective effects of anti-TNF occur even in the face of persistent bacteremia (250). This latter observation suggests that the hemodynamic effects, as well as the lethality, of septic shock is independent of the presence of circulating bacteria. Rather, they are dependent on the effects of an endogenous mediator: either TNF or another mediator stimulated by the action of TNF.

**Interleukin-6**

Unlike TNF, there is relatively little evidence that IL-6 is an important proximal mediator of septic shock. Elevated levels of IL-6 are seen in patients with acute bacterial infections (236)(254). In humans following endotoxin challenge, IL-6 levels increase rapidly, peaking at 2 - 4 h (ie: after TNF) (82). While IL-6 causes pyrexia and induces hepatic synthesis of acute-phase proteins (234), it does not have any known hemodynamic effects. In addition, IL-6 generally appears in the circulation after the onset of sepsis and after TNF has appeared. The temporal relationship of the appearance of septic shock and the appearance of IL-6 in the circulation and the lack of known hemodynamic effects of IL-6 therefore do not support the hypothesis that it is an important proximal mediator of septic shock.

There is evidence that IL-6 persists in the circulation for many hours or even days in some individuals with severe septic or endotoxic shock (69)(217)(255). This suggests that it may be an important mediator in sustaining the pathophysiologic derangements seen in septic shock. The observation that circulating levels correlate with mortality in patients with meningococcal sepsis (255) supports this view. It is further supported by the findings of a unique study by Starnes and coworkers (256). They showed that administration of anti-IL-6 antibody to septic mice causes higher levels of TNF. Although this observation suggests that IL-6 acts as a counter-regulator of the cytokine cascade, mortality in the antibody-treated mice was lower. In addition, mortality caused by administration of exogenous TNF is also prevented by administration of anti-IL-6 antibody. Thus, while
there is very little evidence that IL-6 is an important proximal mediator of septic and endotoxic shock, it may play a role in sustaining the various derangements seen.

1.6.5 The Role of TNF and IL-6 in the Pathogenesis of MODS

Because in MODS the organs that dysfunction are not necessarily those that are directly injured and because it has become apparent that MODS and sepsis syndrome are somehow caused by endogenous factors, these syndromes have been termed "mediator disease" by some investigators (6). While cytokines produced in splanchnic organs may be involved in the local response to injury, they may also exert effects in distant organs when spilled into the circulation. There is some evidence that cytokines, especially TNF, are important proximal mediators in the pathogenesis of MODS.

The most compelling evidence that activation of the cytokine cascade is an important pathogenic factor is that TNF, perhaps the primary cytokine in the cascade, causes dysfunction of numerous different organs in a number of animal models. In rodents treated with a TNF infusion, grossly visible hemorrhagic necrosis in lungs, kidneys, adrenal glands, and the GI tract has been reported (32). The lungs invariably exhibit severe peribronchiolar pneumonitis, thickened alveolar membranes, and pulmonary edema (32)(247). Administration of TNF has also been shown to cause acute lung injury, characterized by pulmonary hypertension and pulmonary capillary hyperpermeability, in sheep (99). Blockade of TNF activity protects septic pigs from lung injury (253). Examination of the kidneys in rodents following TNF administration reveals evidence of acute tubular necrosis (32). In the gastrointestinal tract, the cecum appears particularly sensitive to TNF-induced hemorrhagic necrosis and histologic evidence of ischemia is noted in animals exposed to doses too low to produce shock. Histopathological analysis of each of the organs injured in rodents treated with TNF infusion shows evidence of diffuse capillary thrombosis and acute inflammation. Since TNF is capable of initiating endothelial injury and increased microvascular permeability \textit{in vitro} as well as \textit{in vivo} (33), it is
therefore possible that TNF causes dysfunction in various organs by means of microvascular events.

There is a paucity of clinical studies that support the role of cytokines in the pathogenesis of MODS and some studies even suggest that activation of the cytokine cascade may not be an important factor. For example, Moore et al. addressed the issue in patients with severe trauma and neither TNF nor IL-6 levels correlated with the eventual development of MODS (169). The failure to consistently and reproducibly identify elevated levels of cytokine levels in patients with sepsis, SIRS and MODS has been one of the major factors limiting acceptance of the role of cytokines in the pathogenesis of MODS (6). There are several potential reasons for these inconsistent clinical results. Firstly, because the half-lives of cytokines are very short (ie: minutes), random blood sampling may miss the peaks of activity. Secondly, because some cytokines (eg: TNF) are present in the circulation only briefly during the earliest phase of the critical illness or infection, samples taken once the disease process is established may be too late. Thirdly, circulating levels of cytokines may not reflect their tissue levels or biologic activity, since their major function is as paracrine mediators. Thus, although most clinical studies investigating the role of cytokines in injury and infection have measured circulating cytokine levels, the concentrations of these proteins in the tissues may be of greater importance.
Chapter 2

OBJECTIVES OF THE THESIS

The pathogenesis of MODS appears to be linked with cytokine production, but the source of the cytokines and the exact stimulus for their release are poorly defined. In this thesis, an attempt at demonstrating the compartmentalization of cytokine release during septic shock, a risk factor for the development of MODS, will be made. In addition, the roles of gut ischemia-reperfusion and transmigration of endotoxin from gut to the portal circulation in the production of cytokines will be determined. Specifically, the following questions are addressed, using porcine models of septic shock and mesenteric ischemia/reperfusion:

1) Is the gut ischemic during septic shock?

Septic shock, in the absence of adequate resuscitation, undoubtedly results in mesenteric ischemia. In the clinical situation, it is more common to encounter septic shock that has been treated by fluid resuscitation. Under these circumstances, it is less clear whether septic shock causes ischemia of the gut. Using a porcine model of Gram negative septic shock treated by aggressive fluid resuscitation, changes suggestive of mesenteric ischemia will be examined.

2) Does gut leak endotoxin into the portal circulation during septic shock?

During endotoxemia, there is evidence that the gut becomes hyperpermeable to various macromolecules. Whether this results in the transmigration of endotoxin into the portal circulation is not known. While the primary infection may be the initial cause of hemodynamic instability and a systemic inflammatory response during Gram negative sepsis, it is possible that transmigration of endotoxin into the circulation sustains this response, even after the main source of infection has been controlled.

If the gut does significantly add to the pool of circulating endotoxin, the presence of gut ischemia must be noted. The presence of gut ischemia and transmigration of endotoxin
might suggest that ischemia of the gut, which is potentially reversible, is the underlying cause of gut hyperpermeability. On the other hand, if the gut is not ischemic, then this would suggest that leakage of endotoxin into the portal circulation is secondary to a direct effect of endotoxin or another mediator stimulated by endotoxin.

3) **Does the gut or the liver produce TNF and IL-6 during septic shock?**

TNF and IL-6 are found at increased levels in the circulation during septic shock and endotoxemia. Numerous sources have been determined *in vitro*, but few data document sources *in vivo*. The gut and liver may be important sources of cytokines for two reasons: they contain cells that are known to have the ability to produce cytokines *in vitro* and they may be foci of injury during septic shock.

The gut and the liver contain numerous macrophages, which are known to produce TNF and IL-6 in response to endotoxin *in vitro*. Since there are numerous other cell interactions and humoral factors present in a living system, it is of interest whether these organs are indeed important sources of these cytokines *in vivo*.

Data from previous studies suggests that cytokines may emanate from a focus of injury. In addition to being subjected to the effects of endotoxin, the gut and the liver may be injured to some degree during septic shock. One possible mechanism for the injury is ischemia, although it has not been determined whether this does in fact occur.

4) **Does gut submitted to ischemia or reperfusion injury leak endotoxin into the portal circulation?**

It is possible that ischemia causes the gut to become hyperpermeable, perhaps leading to transmigration of endotoxin into the portal circulation. Reperfusion injury may further injure the gut, exacerbating any effect that ischemia has on the mucosal barrier function. Determination of whether mesenteric ischemia causes endotoxin transmigration is central to proof of the theory that septic shock causes endotoxin transmigration by causing gut ischemia.
5) Does the gut or the liver produce TNF or IL-6 during mesenteric ischemia-reperfusion?

If the gut is shown to be an important source of TNF and/or IL-6 during septic shock, then it is important to determine whether this occurs as a result of exposure to endotoxin, due to ischemic injury, or because of reperfusion injury secondary to successful resuscitation. Comparison of results in a porcine model of mesenteric ischemia-reperfusion to the results obtained in experiments on the resuscitated septic shock model will aid in clarifying this.

If the liver is shown to be an important source of TNF and/or IL-6 during septic shock, then it must be determined whether this occurs as a result of exposure to endotoxin or direct injury from another insult. The latter possibility will not be addressed in this thesis. However, another potential cause for the production of inflammatory mediators by the liver is an effect by mediators originating from an injured (ischemic) gut. Endotoxin itself may be one of those mediators. Alternatively, inflammatory mediators such as TNF or IL-6 originating from the gut may stimulate the cytokine cascade in the liver. This last possibility will be addressed superficially in this thesis.
Figure 5. General hypothesis. Septic shock causes gut ischemia. As a result of ischemic injury, the gut produces inflammatory mediators, including cytokines. In addition, endotoxin transmigrating from the gut lumen to the portal circulation may stimulate distant organs to produce cytokines. Cytokines are among the mediators that cause multiple organ dysfunction syndrome (MODS). In MODS, gut ischemia is exacerbated, perpetuating the systemic inflammatory response.
Chapter 3

HYPOTHESIS

The following is a description of the author's hypothesis, based solely on data presented in the literature. The hypothesis is summarized diagramatically in Figure 5.

1) **The bowel is ischemic during septic shock.**

The whole of the bowel, especially the terminal ileum, is susceptible to ischemia during Gram negative septic shock and endotoxemia. The mechanism for bowel ischemia during septic shock or endotoxemia is not known and will not be addressed in the investigations accompanying this thesis. However, there are a number of reasons why the bowel may become ischemic. Septic shock implies various hemodynamic derangements that result in impaired tissue perfusion. Even with resuscitation that is judged adequate (ie: good urine output, normal cardiac output and systemic blood pressure), the splanchnic circulation may continue to be affected, by a persistent vasoconstrictive response, for instance. Numerous other microcirculatory events associated with endotoxemia may potentiate the derangements in the splanchnic circulation. Finally, alterations in metabolism secondary to endotoxemia may impair the gut's ability to utilize oxygen and this may result in occult ischemia, even in the presence of acceptable parameters of adequate resuscitation.

2) **The bowel is a source of endotoxin, TNF and IL-6 during septic shock.**

As a result of injury secondary to ischemia, the bowel loses its normal mucosal barrier function. This results in mucosal hyperpermeability, allowing some of the intestinal contents to escape the confines of the gut lumen. The degree of hyperpermeability is at least sufficient to allow transmigration of endotoxin. Transmigration may occur via hematogenous, lymphatic or transcelomic routes; the hematogenous route (ie: gut lumen to portal vein) is an important pathway by which endotoxin reaches the circulation during septic shock.
Cytokine production is stimulated not only by chemical stimulants such as endotoxin, but by injury as well. Since the gut is a focus of injury, the cells of the gut are stimulated to produce cytokines. TNF is the primary cytokine produced, followed by other mediators of the cytokine cascade, such as IL-6.

3) The liver is a source of TNF and IL-6 during septic shock.

The liver is laden with tissue macrophages, Kupffer cells, which respond to the presence of endotoxin by producing cytokines.

4) The bowel is a source of endotoxin, TNF and IL-6 during mesenteric ischemia-reperfusion.

During gut ischemia, endotoxin transmigration and gut cytokine production occurs. Because reperfusion may exacerbate the associated injury and, hence, potentiate the effects on mucosal permeability, reperfusion causes even greater endotoxin transmigration and cytokine production.

5) The liver is a source of TNF and IL-6 during mesenteric ischemia-reperfusion.

The endotoxin that is introduced into the circulation from the bowel secondary to ischemia-reperfusion injury stimulates the Kupffer cells of the liver to produce cytokines.

6) The release of endotoxin, TNF and IL-6 by the bowel during septic shock is secondary to ischemic injury of the gut.

If, during endotoxemia, the bowel does prove to be a source of endotoxin and cytokines, is it due mainly to the stimulus from endotoxin or due to ischemic injury? It is hypothesized that ischemia is the main stimulus for endotoxin transmigration and gut cytokine production. This will be evident by demonstration of similar responses during septic shock and mesenteric ischemia-reperfusion.
4.1 THE PORCINE MODEL OF SEPTIC SHOCK

Clinically, septic shock is treated by fluid resuscitation and, if hemodynamic instability persists, inotropic support is administered. A porcine model of septic shock in which hemodynamic stability is achieved by an aggressive fluid resuscitation protocol has been previously described (79)(80). The hemodynamic effects reportedly assimilate those changes seen in septic humans following fluid resuscitation. This model will be used to address the questions described above. Most of the phenomena of interest (ie: hemodynamic effects, cytokine production) are likely best seen during the onset of septic shock and in the period immediately following. For this reason, experimental parameters will be frequently monitored prior to induction of septic shock and in the four hours following this stimulus.

The hemodynamic effects of endotoxemia will be monitored over the experimental period to confirm that this model is indeed comparable to changes seen in septic humans. Using tonometry, the terminal ileum will be monitored for changes in intramucosal pH and $\text{PCO}_2$ which suggest the presence of ischemia. To determine whether the gut is a source of endotoxin, TNF and IL-6, fluxes of these substances into and out of the gut will be monitored. Similarly, hepatic influx and efflux of TNF and IL-6 will be monitored to determine if the liver is a source of these cytokines. Net production of TNF and IL-6 by the gut and liver is suggested when efflux of these substances from the gut or liver exceeds influx. Conversely, net catabolism of TNF and IL-6 is suggested when influx exceeds efflux.
4.2 The Porcine Model of Mesenteric Ischemia-Reperfusion

It is hypothesized that gut ischemia is responsible for transmigration of endotoxin and production of cytokines by the gut during septic shock. Reperfusion injury may exacerbate any of the effects attributable to ischemia and this may occur following resuscitation. To address these possibilities, a porcine model of ischemia-reperfusion was developed. The model is unique in that a period of ischemia is followed by two episodes of reperfusion: one brief, the other prolonged. Occlusion of the superior mesenteric artery (SMA) causes segmental ischemia, especially to that region of the gut thought most susceptible to ischemia during sepsis (32). During this period, there will be a complete lack of blood flow in a segment of gut and any events observed at this time will be attributable to mediators originating from splanchnic beds that are better perfused. To detect mediators originating from the ischemic segment, a brief period of reperfusion, washing out those mediators that have accumulated during the stagnant period, will be used. A one hour period of stagnation will then follow to see if there are any secondary effects from the substances released during the brief reperfusion. Finally, the SMA will be permanently reperfused. Again, mediators detected immediately following reperfusion originate from the "washed out" segment of gut; mediators appearing later may be from anywhere and are more likely elicited secondary to the effects of reperfusion.

In addition to studying the temporal relationships of the appearances of endotoxin, TNF and IL-6 in the circulation with periods of gut circulatory stagnation and reperfusion, gut and liver production of these substances will be demonstrated in the same way as in the septic shock model. That is, gut efflux of endotoxin, TNF and IL-6 will be compared to gut influx of these substances to see if the gut is a source; differences in hepatic efflux and influx of TNF and IL-6 will be studied to see if these substances originate from the liver. Net production of TNF and IL-6 by the gut and liver is suggested when efflux of these substances from the gut or liver exceeds influx. Conversely, net consumption of TNF and
IL-6 is suggested when influx exceeds efflux. Because differences between fluxes across a tissue bed may be transient after an event (i.e.: clamping and unclamping the SMA), measurements will be taken with more frequency immediately following these events.

These studies were approved by the University of British Columbia Animal Care Committee and conform with the NIH standards for animal care.

4.3 DEVELOPMENT OF ENDOTOXIN, TNF AND IL-6 ASSAYS FOR USE IN PORCINE PLASMA

The most recently introduced assay for the measurement of endotoxin is the Limulus amebocyte lysate (LAL) kinetic turbidimetric assay. Its utility in the measurement of endotoxin in aqueous solutions is well described. However, there are a number of potential obstacles if the assay is to be used in measuring endotoxin in a biological medium such as plasma. That is, because of the presence of numerous factors in plasma that may interact with endotoxin or the biochemical reactions on which the assay depends, the reliability of the assay is unpredictable in such conditions. While modifications of similar assays have been described to enable measurement of endotoxin in human plasma, these modifications have not been described for use in the LAL kinetic turbidimetric assay. Further, these modifications have only been described for use in human plasma, not porcine plasma. The assay will be evaluated for use in measurement of endotoxin in pig plasma and modified as required.

Bioassays for measurement of TNF and IL-6 are well characterized, but they are expensive and work-intensive. These assays may require some modifications for measurement of TNF and IL-6 in porcine plasma and this will require evaluation. Enzyme-linked immunosorbent assays (ELISA) have been developed for measurement of numerous cytokines in humans and murine species, but none have been developed for use in pigs. Because an ELISA is faster and easier to perform than a bioassay, it is a more desirable method for measurement of TNF and IL-6, particularly when a large number of
measurements is required. If the homology between murine or human TNF or IL-6 and porcine TNF or IL-6 is sufficient, it may be possible to measure porcine TNF and/or IL-6 with the currently available ELISAs. The utility of the currently available ELISAs for measurement of porcine TNF and/or IL-6 will be evaluated. If the readily available ELISAs do not prove feasible for use in pigs, bioassays will be used.

In light of the lack of information on use of various assays for endotoxin, TNF and IL-6 in pigs, the use of these assays will first be evaluated and modified appropriately.
Chapter 5
EVALUATION AND DEVELOPMENT OF ASSAYS FOR ENDOXOTIN, TNF AND IL-6

5.1 BLOOD SAMPLE COLLECTION

Blood samples for measurement of endotoxin and cytokines were collected under sterile conditions from the carotid artery (a), the portal vein (pv), and the hepatic vein (hv). These samples were immediately heparinized and centrifuged (1000*g, for 5 min). The plasma was placed in sterile cryotubes (NUNC) and immediately frozen in liquid nitrogen. The frozen plasma was stored at -72°C until required for measurement of endotoxin and cytokines, when it was filtered with a 0.45 μm-pore sterile syringe filter (Nucleopore) prior to analysis.

Pooled pig plasma used as diluent and negative controls for endotoxin and cytokine assays was collected from normal sedated pigs prior to any surgical manipulation. The blood was drawn under sterile conditions from the external jugular vein or the brachiocephalic vein; it was heparinized, centrifuged and frozen in the same manner as samples.

5.2 MEASUREMENT OF PLASMA ENDOXOTIN

5.2.1 General Description of Assay

The most sensitive and specific assay currently available for endotoxin is the Limulus amebocyte lysate (LAL) assay. LAL, an extract made from the blood cells (amebocytes) of the horseshoe crab (Limulus polyphemus), becomes turbid and clots in the presence of bacterial endotoxins. Previous methodologies using this assay were semiquantitative and accurate endotoxin concentrations were difficult to determine. In the
studies described in this thesis, plasma endotoxin levels were measured with the LAL 5000, an automated endotoxin detection system utilizing the Limulus amebocyte lysate kinetic turbidimetric assay. The test is performed by adding LAL to the liquid sample. Prior to clot formation, a change in turbidity (optical density) can be detected in the mixture. The LAL 5000 measures and records the optical density of the mixture at regular intervals and determines the time for the optical density to reach a fixed threshold level (Figure 6). This "onset time" decreases with higher endotoxin concentrations in the sample.

5.2.2 Specific Methodology

The assay was performed under sterile conditions, using endotoxin-free borosilicate glassware (Associates of Cape Cod, Inc.). *E. coli* endotoxin (Sigma), which has a potency of 5.0 EU/ng, was used as the standard. Standards were prepared in a dilution of pooled pig plasma. The diluent used for the pooled pig plasma and for samples was endotoxin-free water; dilution of the plasma ranged from 1:10 to 1:100. Aliquots of 1 mL of standard or sample were heated in a water bath at 90°C for 15 min and allowed to cool for 10 min. Samples and standards (0.4 mL) were then mixed with 0.1 mL of lyophilised LAL reagent (Associates of Cape Cod, Inc.) reconstituted with tris[tris-(hydroxymethyl)aminomethane]HCl buffer (pH 7.4; Pyrosol, Associates of Cape Cod, Inc.) in a 10 x 75 mm borosilicate glass reaction tube. The reaction tubes were inserted into assigned wells in the LAL 5000, immediately following addition of the LAL reagent to the sample/standard. Onset times to the threshold optical density were automatically recorded by the machine (Figure 6). All samples/standards were tested in duplicate. The standard curve was constructed by the LAL 5000 software by plotting the log of the endotoxin concentration vs. the log of the onset time (Figure 7). New standard curves were constructed every day tests were run. Standard curves in which the correlation coefficient was <0.985 and in which the slope did not fall in the range of 0.1 - 0.2 were not used.
Figure 6. Optical density (turbidity) plotted against time for eleven standard endotoxin concentrations in porcine plasma (1/100), each measured in duplicate. Optical density is recorded by the LAL 5000 at regular intervals. The software analyzes the data and determines the time taken for the optical density to reach a fixed threshold level. This time ("onset time") decreases with higher endotoxin concentrations in the sample. The set of points plotted at the bottom (approximating a flat line) represents the changes in optical density in a water negative control.
Figure 7. Standard curve constructed by regression of the log_{10} of the corrected onset times against the log_{10} of the endotoxin concentrations of the standards tested in Figure 6. Baseline correction of the raw onset times is performed by the LAL 5000 software to ensure that the average optical density (OD) for each sample is zero prior to any increase in turbidity resulting from the LAL-endotoxin reaction. Correction for subtle increases in the baseline OD (as reflected by a steady increase in the OD of the negative control) is also performed by the system software. The corrected onset time is thus an estimate of the time at which the measured OD of the sample reaches the threshold OD after any baseline or drift correction.
5.2.3 Evaluation and Modification of the LAL Kinetic Turbidimetric Assay

The presence of endotoxin was first determined using the rabbit pyrogen test. The in vivo test was positive if injection of the substance tested produced a fever in the rabbit. Obviously, this was not quantitative, it had limited sensitivity, and it was in no way specific (52). In 1958, Bang noticed that the horseshoe crab, Limulus polyphemus, underwent a type of disseminated intravascular coagulopathy when infected with marine Gram negative bacteria (257). The horseshoe crab has an open circulatory system containing hemolymph, in which the only formed element is a cell called the amebocyte. The clotting reaction of the hemolymph was later found to be a result of the presence of endotoxin and the amebocyte was found to be the source of all of the factors necessary for hemolymph coagulation. Tissue damage that occurs in the crab causes aggregation and degranulation of the amebocytes, activating a coagulation cascade (258). Physical disruption of amebocytes which have been harvested under suitable conditions yields a suspension (the Limulus amebocyte lysate, LAL) containing the coagulation components which may be activated only by bacterial endotoxin. Besides Limulus polyphemus, related horseshoe crabs -- Tachypleus tridentatis, Tachypleus gigas, and Carcinoscorpius rotunda cauda -- have identical coagulation systems with similar reactivity to endotoxin which yield amebocyte lysate with the properties of LAL (259).

The lipid A component of the endotoxin molecule, which is responsible for its biological activity, reacts with the LAL in two stages. Firstly, an inactive proenzyme present in the lysate is activated by LPS. Activation of the proenzyme is dependent on the presence of divalent cofactors, such as Ca$^{2+}$, Mg$^{2+}$ and Mn$^{2+}$ (259). The presence of substances that chelate these ions, such as EDTA, would therefore interfere with the reaction. Secondly, the activated proenzyme cleaves peptide bonds in a coagulant protein. The coagulin molecules thus formed interact with each other to form an aggregated secondary structure, resulting in the visible gelation.
LAL assays are the most sensitive tests for LPS available. LAL has been shown to
detect either bound (ie: cell-associated) or free endotoxin (259). However, different
endotoxins prepared by different extraction procedures, from different species of bacteria,
may vary widely in reactivity. For this reason, a reference standard endotoxin has been
prepared by the U.S. Food and Drug Administration as a means of standardization of LAL
tests. One endotoxin unit (EU) was defined as 0.2 ng of an E. coli endotoxin designated
EC-2. Results in this thesis are expressed in EU/mL, to circumvent problems associated
with using different lots of endotoxin throughout the study and to allow comparison with
other similar studies.

The visible end result of the cascade of reactions causing hemolymph coagulation is
seen as an opaque gel or clot. However, the completion of the reaction requires a long
incubation period, especially when small amounts of LPS are present. This has led to the
development of different LAL methods with variable end-points reliant on either
chromogenic substrates or the changes in optical density preceding development of the clot.
The chromogenic methods have a sensitivity of 0.1 - 0.5 EU/mL (260) and the gelation
method is less sensitive (259). The kinetic turbidimetric LAL method, used in the present
study, measures the time taken to reach a threshold level of turbidity in the sample tested.
The greater the amount of LPS present, the faster the reaction proceeds. The assay is
extremely sensitive, detecting as little as 0.001 EU/mL, and it is accurate. In addition, the
method is technically relatively simple, especially with the aid of the LAL 5000.

A potential obstacle to measurement of LPS in mammalian blood is coagulation in
the blood sample. In the process of clotting in the sample, endotoxin appears to become
trapped in the clots (259)(260). Serum levels of endotoxin are thus invariably lower than
those seen in plasma, since the coagulation system in plasma has not been activated.
Virtually all clinical studies of endotoxemia are therefore performed on plasma, generally
using heparin as the anticoagulant which least interferes with the LAL reaction (260).
Similarly, the present study utilized plasma samples prepared in endotoxin-free heparin.
The presence of "inhibitors" of the LAL reaction in blood has also made it difficult to reliably detect LPS in blood. The exact identity of the inhibitor(s) has not been conclusively determined, but it appears to be an alpha globulin which reversibly binds free endotoxin or IgG and IgM antibodies directed against the lipid A region of the LPS molecule (261). Numerous methods of treating plasma to rid it of its inhibitory activity have been attempted, including chloroform extraction (to denature the inhibitors) and a pH-shift technique (to precipitate inhibitors by acid, then restore neutral pH for optimum LAL reactivity). A number of methods relying on dilution of the inhibitors followed by destruction of the inhibitors by heat have also been described. In a comparison of these methods in the measurement of LPS in human plasma by the chromogenic LAL assay, the heating-dilution method was found to be 10 times more sensitive than the pH shift method and approximately 100 times more sensitive than treatment of plasma by chloroform extraction (260).

Several of the dilution/heating methods previously described were attempted prior to embarking on the present study. Porcine plasma had to be diluted to at least a 1:10 dilution to minimize the intrinsic inhibitory activity in the plasma, regardless of the heating method employed. A lesser dilution also precluded heating of the sample, since undiluted plasma clots upon heating, immediately making the sample too turbid to test. This was consistent with the experience of Roth et al. (260). Insufficient heat or too short a duration of heating resulted in enhancement of the LAL reaction. That is, the reaction was made considerably faster, making it difficult to differentiate onset times in standards containing different concentrations of endotoxin and therefore limiting the utility of the kinetic turbidimetric assay. The presence of enhanced activity in plasma treated by the dilution-heating method has also been described in human plasma (260), but the identity of these "enhancers" is not known. To avoid the enhanced activity seen with underheating the plasma, diluted samples were heated for 15 min at a relatively high temperature, a more
severe treatment of the sample than described in the literature for testing of human plasma with the chromogenic LAL method.

Plasma was stored at -72°C until use. Endotoxin is completely stable under these conditions and no loss of activity should be expected, even following prolonged storage. There is no difference in stability of LPS in whole blood or plasma (261). However, (porcine) plasma stored in the freezer over a long period of time becomes quite turbid, limiting the utility of the kinetic turbidimetric test. This was ameliorated by filtering all samples prior to testing; results were more consistent when plasma was treated in this way.

The LAL reaction is optimally sensitive at a pH range of 6.0 - 8.0. While the pH of the heated and diluted plasma tested ranged from 7.1 - 7.4, it was found that more consistent results and better standard curves were obtained when the lyophilised LAL reagent was reconstituted with a buffer, as opposed to LPS-free water. Sensitivity was also improved with the use of the buffer. It is possible that, during the course of the LAL reaction, previously undetected pH changes occurred, causing some samples/standards to slip out of the optimal pH range. In addition, since plasma samples from the carotid artery, portal vein and hepatic vein have a different pH, different degrees of reactivity can be expected in samples collected from different sites. In an effort to avoid these phenomena, all tests were performed with LAL freshly-reconstituted with buffer.

The nature of the tubes used to make dilutions and to monitor the LAL reaction is an important detail to note. Roth et al. (260) observed a lesser sensitivity by a magnitude of several logs when polystyrene or polypropylene tubes were used instead of borosilicate tubes. Because of the hydrophobicity of the lipid A portion of the LPS molecule, LPS in an aqueous medium (eg: plasma) will readily adhere to certain surfaces with a high affinity to endotoxin, especially if the endotoxin has a relatively short (hydrophilic) polysaccharide component (52). Such high-affinity surfaces therefore limit the sensitivity of the LAL test, since the amount of LPS in solution is decreased. For this reason, regular glass tubes and plastic tubes were not used for measurement of endotoxin. Similarly, new borosilicate
glass has a high affinity for LPS, which is corrected by subjecting the tubes to a number of washings (52). Borosilicate glass that has been treated with an appropriate washing procedure by the manufacturer was therefore used in this experiment.

The specificity of the LAL reaction for LPS has been questioned. Thrombin, thromboplastin and ribonucleases were claimed to cause gelation of LAL (262), but later studies suggested that the reaction observed could have been due to inadvertent contamination of the preparations tested (263). Exotoxins of some streptococci, at high concentrations, have been shown to react with LAL (263); but it is unlikely that exotoxins in the high concentrations reported to produce such a reaction were present in the samples tested in the present study.

5.3 MEASUREMENT OF PLASMA TNF

5.3.1 General Description of Assay

TNF levels were quantified using an *in vitro* cytotoxicity assay employing MTT staining of L929 tumorigenic murine fibroblasts incubated in an effector with known or unknown amounts of TNF. The colorimetric assay is based on the ability of live, but not dead, tumor cells to reduce a tetrazolium-based compound [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MTT] to a blue formazan product.

5.3.2 Culture of L929 Cells

L929 cells were grown and maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells in growth medium were incubated at 37°C in 5% CO₂. Nonviable cells were removed from the culture stock at least every 3 days by pouring out the old growth medium and replacing it with fresh medium; viable cells remained attached to the floor of the culture flask. Cells were removed from the culture flask for subculture and for use in the bioassay by adding
10 mL of trypsin-EDTA in RPMI 1640 and incubating for 3 - 5 minutes, until the cells detached from the bottom of the flask. Following centrifugation at 400*g for 5 minutes, cells were washed once with growth medium and resuspended in growth medium. All manipulations of the culture were performed under sterile conditions.

5.3.3 Specific Methodology

Prior to each run of tests, L929 cells were subcultured in growth medium at 37°C, 5% CO₂ until a confluent monolayer of cells formed on the floor of the culture flask. Cells were then removed from the flask as described above, washed with growth medium, and resuspended in growth medium. The cells were diluted with growth medium containing 6 μg/mL of actinomycin D (Sigma; reconstituted in 99% ethanol) to a final concentration of 5 x 10⁵ cells/mL and 100 μL (5 x 10⁴ cells) were pipetted into each well of a 96-well flat-bottom tissue culture plate (Falcon Labware). Plasma samples were diluted 1:2 with growth medium. Standards consisted of recombinant human TNF (rh-TNFα; R & D Systems) in pooled pig plasma diluted in the same way as test samples. Effector (50 μL) was then added to each well and the plates were incubated for 20 h. The positive control (ie: "0% lysis control") consisted of cells incubated in growth medium only. The negative control (ie: "100% lysis control") consisted of cells incubated in 2% sodium dodecylsulfate (SDS) after the 19th hour of incubation. All samples and standards were tested in triplicate and standards were tested on every run of tests. Following the 20 h incubation period, the effector was removed and the cells were incubated in 100 μL of MTT (0.5 mg/mL MTT in RPMI 1640 without phenol red dye or FBS) for an additional 3 h. After 3 h, fluid was again removed and dimethyl sulfoxide (DMSO; 100 μL) was added to each well, taking care to dissolve any formazan dye crystals by mixing the DMSO/cell solution thoroughly while minimizing any air bubble formation. Major air bubbles that formed during the mixing procedure were burst with a needle prior to colorimetric analysis.
Figure 8. An example of a standard curve used for calculation of TNF concentrations. The proportion of cells lysed was measured in standards prepared with rh-TNF in porcine plasma diluted 1:2 in growth medium. The standard curve was constructed by plotting cytotoxicity (%) vs. Log[TNF]. The smallest concentration of TNF consistently on the linear part of the standard curve was 9.75 pU/mL, giving the assay a sensitivity of 19.5 pU/mL.
The optical density of each well was measured with the Dynatech MR 5000 microplate reader (Dynatech Laboratories, Inc., Chantilly, VA) at a wavelength of 540 nm. The TNF concentration was calculated by comparison with the standard curve, which was constructed by plotting % cytotoxicity vs. log[TNF] (Figure 8). Sensitivity of the assay was 19.5 pU/mL, where 1 pU is defined as the amount of porcine TNF that has the same bioactivity as 1 pg of rh-TNFα.

5.3.4 Derivation of Methods of the L929 Assay for Measurement of TNF in Porcine Plasma

TNF activity was originally measured using an in vivo assay (264) whereby a methylcholanthrene induced BALB/c tumor was implanted in the subcutaneous tissue of syngeneic mice. Activity was measured by the ability of TNF-containing serum to cause hemorrhagic necrosis of the malignant implant. Since then, numerous in vitro cytotoxicity assays for TNF have been described; these are considerably simpler and much more sensitive (265). Ruff and Gifford (266) described an in vitro assay employing crystal violet staining of TNF-treated L cell cultures. It is from this assay that the assay utilized in the above experiment was derived. The crystal violet assay has been compared to several other previously published in vitro cytotoxicity assays, including another colorimetric assay using neutral red and a [3H]-thymidine release assay, each utilizing TNF-treated L cell lines (265). The crystal violet assay is the most sensitive assay. Further, the sensitivity of each of these assays is increased substantially by the addition of actinomycin D. Addition of actinomycin D to the cell cultures greatly enhances killing by an unknown mechanism, shortening the necessary incubation time from 48 h to 20 h.

Because fibroblasts live and grow by adhering to a solid surface (eg: the bottom of the culture flask), removal of the cells for manipulation of the culture requires some method of interfering with the ability of the cells to adhere. Trypsin enzymatically cleaves those proteins responsible for adherence to the culture flask. Further, the addition of EDTA,
which chelates calcium and magnesium, promotes the adhesion-inhibiting effects of the trypsin, for these ions are required for normal function of the fibroblast adhesion mechanism. The presence of significant amounts of calcium or magnesium in the growth medium interferes with the effects of trypsin, limiting the number of cells available for the bioassay. It is important, when exposing cells to be used for bioassay to a substance such as trypsin-EDTA, which inhibits normal growth in the culture flask, to thoroughly wash the culture prior to resuspending it in growth medium. Residual trypsin-EDTA results in excessive cell death or limited cell growth.

The L929 cytotoxicity assay is dependent on the cellular reduction of (yellow) MTT by the mitochondrial dehydrogenase of viable cells to a blue formazan product which can be measured spectrophotometrically. DMSO dissolves the formazan dye crystals (ie: crystals formed upon reduction of the MTT), producing a homogeneous colour. Other solvents have been tested, but the most complete solubilization of the formazan crystals is achieved with DMSO and mineral oil, each yielding relatively narrow, distinct absorbance peaks (267). To completely solubilize the crystals, it is imperative to thoroughly mix the DMSO with the MTT-treated cells. Inadequate solubilization of the dye leads to spurious optical density readings, much the same as large or numerous air bubbles would. Further, MTT is light sensitive and a large delay in obtaining absorbance measurements with overexposure to light will yield spurious results.

Since plasma may interfere with the assay by inhibiting or enhancing survival of L929 cells, different dilutions of plasma with known concentrations of rh-TNFα required preliminary testing. That is, standard curves were constructed with different dilutions of pooled pig plasma to determine which dilution would yield the best standard curve, minimizing background activity without sacrificing too much of the sensitivity of the assay. The following dilutions of pooled pig plasma were tested: 1:2, 1:10, and 1:100. The diluent used for the plasma and for making standard concentrations of rh-TNFα was L929 growth medium. Standard concentrations of rh-TNFα were then tested in each dilution of plasma.
and standard curves were constructed in the usual fashion. Linear standard curves were easily achieved using each of the plasma concentrations tested, so a 1:2 dilution was used in all subsequent test samples, in order to preserve the sensitivity of the test (Figure 8).

Because some of the samples were filtered and previously thawed to perform other assays, an experiment was done to see if filtering or repeated freezing/thawing would alter the results of the assay. This was important because either of these processes may potentially cause a loss or degradation of cytokines. Samples filtered and thawed several times before were compared to samples that were unfiltered and that had never been thawed. Samples compared were from the same experimental subject, collected at the same time. In addition, standard curves of known amounts of rh-TNFα before and after filtering were constructed. Samples that had been filtered and thawed did not have significantly decreased cytotoxic activity. Moreover, the sensitivity of the assay was enhanced, as there was a more intense chromogenic reaction in negative controls and in standards containing a low concentration of TNFα treated by filtration. These results suggest that there was a minimal degree of degradation of TNF secondary to the freeze/thaw cycles and that filtering enhances the chromogenic reaction in the presence of lesser degrees of cytotoxicity. The likely explanation for the enhanced activity following filtration is that filtering the samples somehow reduced the levels of biological or chromogenic inhibitors present in the samples.

Some samples from the experimental subjects produced cytotoxicity and this was attributed to the presence of TNF. To document the specificity of the assay and to determine whether the cytotoxic effects seen in the samples of porcine plasma were secondary to TNF, several attempts at constructing neutralization curves with various antibodies to cytokines were made. If cytotoxicity was blocked by antibodies to TNF but not by other antibodies, this would suggest that the assay is specific to TNF. Unfortunately, no antibodies to porcine cytokines are available. Recombinant antibodies to human TNFα (anti-rhTNFα; R & D Systems) and IL-1β (anti-rhIL-1β; R & D Systems) were combined in a number of concentrations (ie: 10, 1.0, 0.1, 0.01 and 0.001 μg/mL).
with samples known to produce a high degree of cytotoxicity, at a 1:2 dilution. Neither of
the antibodies resulted in neutralization of cytotoxicity. The fact that anti-rhTNFα did not
neutralize the cytotoxic activity suggests that there is inadequate homology between human
and porcine TNF. Indeed, studies by Pauli et al. confirm that there is only 85% homology
at the gene level; eighteen of the amino acids of the deduced mature peptide sequence of
porcine TNFα are different from those in the sequence of human TNFα (268). Therefore,
the measurements of TNF reported in this thesis are more accurately referred to as "TNF-
like activity". In light of the apparent lack of homology between porcine TNF and human
TNF, and since this assay was relatively easy to perform, an ELISA was not attempted.

5.4 MEASUREMENT OF PLASMA IL-6

5.4.1 Use of an ELISA for Measurement of IL-6 in Porcine Plasma

Because the bioassay for IL-6 is expensive and time consuming, a heterogeneous
enzyme-linked immunosorbent assay (ELISA) directed at human IL-6 was evaluated for
use in the measurement of porcine IL-6. As with other heterogeneous enzyme
immunoassays, antigen (ie: IL-6) in plasma (the liquid phase) is measured as it binds to a
monoclonal antibody adsorbed to the vessel surface (the solid phase). A general description
of the assay is as follows: Polyclonal anti-IL-6 is adsorbed to the vessel surface by coating
the surface of the vessel with the antibody and incubating overnight. The sample,
presumably containing IL-6, is then added to the vessel coated with antibody and the
antigen in solution is allowed to bind. A labelled antibody is then added. Following another
incubation period, any unbound labelled antibody is washed away, leaving the antigen (IL-
6) "sandwiched" between the labelled and unlabelled antibodies in the solid phase. Enzyme
substrate is then added, yielding a colorimetric reaction that is proportional in intensity to
the amount of IL-6 present.
More specifically, the methods of the human IL-6 ELISA as utilized in the porcine plasma samples were as follows:

1) Each well of a 96-well flat-bottomed microtiter plate (Immulon I) was coated with 100 μL of goat anti-human IL-6 in carbonate-bicarbonate buffer, pH 9.6 (at a final concentration of 2 μg/mL). This was incubated overnight at room temperature.

2) Plates were washed for 2 minutes, three times, with phosphate buffered saline (PBS)/0.1% Tween, pH 7.4; then rewashed once for 2 minutes with PBS, pH 7.4.

3) Human IL-6 standards (R & D Systems) ranging from 1.25 to 5000 pg/mL were prepared, using pooled pig plasma diluted in PBS/3% bovine serum albumin (BSA) as the diluent. Similar dilutions of sample were prepared. Dilutions prepared included 1:5, 1:10 and 1:20. Samples tested were from septic animals, over several time periods.

4) A number of background controls were prepared. Three consisted of pooled pig plasma diluted in the same way as standards and samples. The other two background controls consisted of PBS/3% BSA and carbonate buffer.

5) Prepared samples, standards and background controls were pre-incubated at 37°C for 2 hours. Samples, standards and background controls (100 μL) were added to assigned wells of the microtiter plate. All samples, standards and controls were tested in triplicate.

5) Once all samples, standards and background controls were plated, the plate was incubated at 37°C for 90 minutes.

6) The contents of the plate were emptied and the plate was thrice washed for 2 minutes, with PBS/0.1% Tween.

7) Biotinylated goat anti-human IL-6 (R & D Systems) diluted 1:8000 in PBS/3% BSA (100 μL) was added to each well. The plate was incubated for 90 minutes at 37°C.

8) The contents of the plate were emptied and the plate was washed three times, for 2 minutes each time, with PBS/0.1% Tween.
9) Streptavidin alkaline phosphatase conjugate (Life Technologies, Inc.) diluted 1:8000 in PBS/3% BSA (100 μL) was added to each well. The plate was incubated for 20 minutes at 37°C.

10) The contents of the plate were emptied and the plate was washed five times, for 1 minute each time, with 50 mM Tris-(hydroxymethyl)aminomethane, 0.15 M NaCl, pH 7.5 (200 μL/well).

11) The remaining steps were performed in a dark room at room temperature:
   a) BRL substrate (50 μL; Life Technologies, Inc.) was added to each well and incubated for exactly 15 minutes.
   b) BRL amplifier (50 μL; Life Technologies, Inc.) was added to each well and incubated for exactly 15 minutes.
   c) 0.3 M H₂SO₄ (50 μL) was added to each well to stop the reaction.

12) The plate was read with a Dynatech MR 5000 microplate reader (Dynatech Laboratories, Inc., Chantilly, VA) at a wavelength of 490 nm. The IL-6 concentration was calculated by comparison with the standard curve, which was constructed by plotting optical density vs. log[IL-6]. Sensitivity of the assay was 31 pg/mL.

While a linear standard curve was easily achieved over a wide range of concentrations, no IL-6 activity was seen in samples tested. The following possible conclusions could be made from the inability of the ELISA to detect the presence of IL-6 in the samples tested: a) There was no IL-6 present; b) the presence of soluble receptors for IL-6 or anti-pIL-6 in samples inhibited detection; or c) the ELISA is specific to human IL-6 and there is inadequate homology between porcine IL-6 and human IL-6. In view of the high levels of IL-6 in the samples tested when the B9 proliferation assay was used (below), the latter explanation is the most likely reason for the inability of the ELISA tested to detect IL-6 in plasma from septic pigs.
5.4.2 B9 Proliferation Assay for IL-6

General Description of Assay

Quantification of IL-6 levels was determined by a $[^3]H$-thymidine uptake assay with the IL-6-dependent murine hybridoma subclone B9 as described previously (269). The protocol was refined for measurement of IL-6 in humans by Dr. W.W.S Kum of the Division of Infectious Diseases, Department of Microbiology, Vancouver General Hospital (270). The rate of proliferation of B9 cells is proportional to the rate of incorporation of thymidine in nucleic acid synthesis.

Culture of B9 Cells

B9 cells were grown and maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 25 mM HEPES, 1% penicillin/streptomycin, and 8 pM human recombinant IL-6 (rh-IL-6; R & D Systems). Cells were incubated at 37°C in 5% CO$_2$. Nonviable cells were removed from the culture stock at least every 3 days by washing with growth medium without IL-6 and the remaining viable cells were resuspended in the enriched growth medium described above. All manipulations of the culture were performed under sterile conditions.

Specific Methodology

Cells were subcultured in growth medium without rh-IL-6 for 72 h at 37°C in 5% CO$_2$, washed and diluted with plain RPMI 1640 to a cell density of $5 \times 10^4$ cells/mL. Cells (100 µL, 5000 cells) were pipetted into each well of a 96-well round-bottom tissue culture plate (Falcon Labware). Plasma samples were diluted with growth medium not containing IL-6. Standards consisted of recombinant human IL-6 (rh-IL-6; R & D Systems) prepared in pooled pig plasma diluted in the same way as test samples. The effectors (100 µL) were then added to each well and the plates were incubated for 68 h. All samples and standards were tested in triplicate and standards were tested on every run of tests. Following the incubation of the B9 cells in the effector, cells were pulsed with 0.5 µCi of $[^3]H$-thymidine (1 mCi/mL; Amersham) in 20 µL of plain RPMI and incubated for another 6 h. Cells were
Figure 9. An example of a standard curve used to calculate IL-6 concentrations. The degree of proliferation of B9 cells (as reflected by radioactivity following incubation in [3H]-thymidine) was measured in standards prepared with rh-IL-6 in porcine plasma diluted 1:100 with growth medium. The standard curve was constructed by plotting counts per minute vs. Log[IL-6]. The linear part of the curve was used for calculation of IL-6 concentrations. The smallest concentration of IL-6 on the linear part of the standard curve is 2.5 pg/mL. Because porcine plasma had to be diluted to at least 1:100, the sensitivity of the assay was therefore limited to 250 pg/mL.
then harvested onto glass-fiber filter paper using an automatic harvester (Skatron, Lierbyen, Norway) and air-dried. Scintillation fluid (2 mL) was added to each dried filter and radioactivity was counted on a liquid scintillation counter (Beckman LS 1800, Beckman Instruments, Inc., Irvine, CA). IL-6 concentrations were calculated using a standard curve constructed by plotting counts per minute vs. Log[IL-6] (Figure 9). Sensitivity of the assay was 250 pU/mL, where 1 pU is defined as the amount of porcine IL-6 that has the same bioactivity as 1 pg of rh-IL-6.

**Derivation of Methods of the B9 Proliferation Assay for Measurement of IL-6 in Porcine Plasma**

Since plasma may interfere with the assay by inhibiting or enhancing growth of B9 cells, different dilutions of plasma with known concentrations of rh-IL-6 required preliminary testing. That is, standard curves were constructed with different dilutions of pooled pig plasma to determine which dilution would yield the best standard curve, minimizing background activity without sacrificing too much of the sensitivity of the assay. The following dilutions of pooled pig plasma were tested: undiluted plasma, 1:10, 1:100, 1:1000, and 1:10000. The diluent used for the plasma and for making standard concentrations of rh-IL-6 was plain RPMI 1640. Standard concentrations of rh-IL-6 were then tested in each dilution of plasma and standard curves were constructed by plotting IL-6 concentration vs. counts/minute.

During this preliminary testing, it was determined that samples must be diluted to at least 1:100 to construct a reliable and linear standard curve with minimal background activity. Since the smallest concentration of IL-6 on the linear part of the standard curve was 2.5 pg/mL, this limited the sensitivity of the assay to 250 pU/mL (Figure 9). In addition, because of the very narrow range of valid values in the standard curve, at least 3 standard curves (eg: 1:100, 1:1000, 1:10000) must be constructed with every run of the experiment; samples must also be tested in the corresponding dilutions. The reasons for the unusually narrow range have not specifically been investigated. Possible explanations may
be as follows: a) the concentration of tritiated thymidine may have been inadequate; b) the suspension of cells placed into each wells may have been too dilute or the viability of the cells may have been inadequate; c) the incubation time following the tritium pulse may have been too long; or d) the subclones of cells used may have been relatively resistant to the proliferative effects of the IL-6. Each of these factors would have resulted in an early plateau of proliferative activity with higher concentrations of IL-6 in the sample/standard. That is, maximal thymidine uptake would occur with relatively low concentrations of IL-6.

B9 cell proliferation is reported to occur specifically in response to the presence of IL-6. Ulich et al. (241) investigated the effects of a number of cytokines, including IL-1α, IL-1β, IL-2, IL-4, IL-8, TNF, interferon-γ, and several colony stimulating factors; none elicited a proliferative response. In addition, LPS at doses less than 1μg/mL did not produce the proliferative response. While previous work has indicated that B9 proliferation is dependent on the presence of IL-6 and that other substances do not invoke the proliferative response, it was necessary to confirm that the proliferative activity effected by the samples from experimental animals in this study was due to the presence of IL-6. To prove the specificity of the proliferative response, various concentrations of polyclonal anti-hIL-6 were combined with a sample from an animal treated by LPS infusion which was known to induce B9 cell proliferation in effort to "neutralize" this response. In this way, a dose-response curve (or neutralization curve) could be constructed. Further, the addition of a high dose of an "irrelevant" antibody, which presumably lacks the ability to neutralize porcine IL-6, was combined with the same sample. A lack of inhibition of the proliferative response with the addition of the irrelevant antibody would ensure that the neutralizing activity seen with the addition of anti-hIL-6 was not secondary to cytotoxic effects of antibodies. The irrelevant antibody used for this experiment was anti-hIL-1β.

Undiluted pig plasma from normal pigs (ie: plasma collected from animals prior to any surgery) was seen to produce some proliferation of B9 cells. The proliferative activity was neutralizable with the addition of anti-hIL-6. However, the proliferative activity was
not neutralizable with addition of anti-hIL-1β (ie: irrelevant antibody), suggesting that the animals are eliciting some IL-6 prior to any experimental manipulation. A sample from an animal treated with endotoxin infusion in which the calculated IL-6 activity in the test well was 14.6 pU/mL was combined with several concentrations of anti-hIL-6 (Figure 10).

![Neutralization of Cell Proliferation With Anti-hIL-6](image)

**Figure 10.** Neutralization curve of porcine IL-6-dependent B9 cell proliferation with anti-human IL-6 antibody.

An exponential increase in neutralization was observed with increasing concentrations of anti-hIL-6. The degree of neutralization is only a fraction of the degree of neutralization one would expect with the same concentration of hIL-6; 10 µg/mL of anti-hIL-6 completely neutralizes the activity seen with 3 ng/mL of hIL-6. This explains why the ELISA utilizing antibodies to human IL-6 did not work.

In summary, the B9 proliferation assay using the methods described in this thesis is a sensitive test for the detection of IL-6. Further, it appears to be specific to the detection of IL-6. However, the assay is extremely work-intensive, owing to the narrow range of
concentrations within the standard curve and to the use of radionuclides, which require special precautions. A chromogenic derivative of the B9 proliferation assay may prove to be more useful for the measurement of large numbers of samples with extremely variable levels of IL-6.
6.1 INTRODUCTION

Multiple organ dysfunction syndrome (MODS) is associated with a mortality rate of 30 - 100% (2)(3). It is a frequent sequela of septic shock, but also occurs following trauma (169), thermal injury (238), pancreatitis (13), and a host of other severe injuries (6). An uncontrolled inflammatory response - manifested, in part, by the release of cytokines - has been implicated as an important pathogenic component of MODS (6). If cytokine mediators are indeed important in the pathogenesis of MODS, then it is important to identify the source of these cytokines and the stimulus for their release. While numerous sources of cytokines have been identified in vitro, relatively few studies have attempted to identify the origin of cytokines in vivo. Investigations of localized injuries suggest that cytokines, in particular tumor necrosis factor (TNF) and interleukin-6 (IL-6), originate from injured tissues, spilling into the circulation when large amounts have accumulated (235)(237)(238)(242). During sepsis, the gut is thought to be such an injurious focus as a result of occult ischemia (6)(109)(271). The role of gut ischemia in the pathogenesis of MODS is supported by the improved survival of those shock patients in whom gut ischemia, as determined by gastric tonometry, can be reversed (133). Perhaps it is this focus of injury that leads to a sustained spillage of inflammatory mediators into the systemic circulation, resulting in MODS.

Endotoxin is perhaps the most powerful stimulus for cytokine synthesis and release. It is this property which suggests that its presence, even in very small quantities, may be important in the perpetuation of the inflammatory response that leads to MODS. Fink et al. have demonstrated increased intestinal mucosal permeability in a porcine model
of septic shock (109). Presumably, transmigration of endotoxin from the gut lumen to the portal circulation occurs under the same conditions. Portal endotoxemia could then result in the release of cytokines from the gut. Thus, the gut has been postulated to be the "motor" by which endotoxemia and the resulting inflammatory response is perpetuated (271).

While the gut has been postulated to be a major source of cytokines during septic shock, the liver may also play an important role by contributing to the systemic inflammatory response. Endotoxin is known to rapidly and preferentially accumulate in Kupffer cells in vivo (57). Further, the liver is a potential focus of ischemic injury. The liver contains numerous tissue macrophages, known to have the ability to elicit TNF and IL-6 in vitro in the presence of endotoxin (272). The liver is therefore a potential major source of cytokines during septic shock.

Fink and coworkers have investigated the pathogenesis of septic shock using a porcine model (109). This model has been well characterized and demonstrates many of the same hemodynamic characteristics as septic shock in humans following fluid resuscitation. Using a similar porcine model of septic shock, the following questions were addressed: 1) Does the gut leak endotoxin into the portal circulation during septic shock? 2) Does the gut produce TNF and IL-6 during septic shock? and 3) Does the liver produce TNF and IL-6 during septic shock?

6.2 METHODS

6.2.1 Experimental Design and Protocols

Juvenile pigs weighing 22-35 kg were prepared as described below. During surgery, instrumentation and stabilization, normal saline was infused intravenously (IV) at 25 mL/kg/h. Animals were allowed to stabilize over 1 hour and were then allocated to one of two groups. The septic group (N = 6) received 50 μg/kg of purified E. coli lipopolysaccharide (Strain 0111:B4, Sigma, MO) infused into the external jugular vein over
1 hour. The control group (N = 6) received sham saline infused over 1 hour. Once the endotoxin or sham infusion was initiated, the rate of IV saline infusion was increased to 48 mL/kg/h for the remainder of the experiment. Measurements and blood samples were taken as described below prior to the endotoxin or sham infusion (time '0'), immediately after the infusion, and every 30 minutes thereafter for 4 hours. The experimental protocol is depicted in the time-line diagram below (Figure 11).

**Control (N = 6)**

```
0  60  90  120  150  180  210  240  270  300
NS | Infusion
```

**Septic (N = 6)**

```
0  60  90  120  150  180  210  240  270  300
LPS | Infusion
```

Figure 11. Schematic time-line representation of experimental protocol: control vs. septic animals.

### 6.2.2 Surgery and Instrumentation

The pigs were fasted overnight and premedicated with lorazepam 0.5 mg given intramuscular (IM) on the morning of the experiment. Ketamine 500 mg IM was administered, followed by sodium pentothal IV until sleep was induced (3 - 5 mg/kg). The animals were ventilated via a tracheostomy with an \( F_{1O_2} \) of 0.5, maintaining a \( PCO_2 \) of 35 to 45 mm Hg. Anesthesia was maintained with 0.75% isoflurane and a ketamine infusion...
(1.5mg/kg/h). A sterile carotid arterial catheter and bilateral external jugular catheters were established by cutdown.

A midline suprapubic incision was made and a catheter was inserted directly into the bladder for urine collection. An upper abdominal midline incision was then made. Following ligation of the splenic artery and the short gastric vessels, a sterile polyethylene catheter (PE 240) was placed in the portal vein via a splenic vein cutdown. Its position in the portal vein was confirmed by palpation. A sterile catheter was placed in the hepatic vein via the right external jugular cutdown, under fluoroscopic guidance. Its position in the hepatic vein was confirmed by injection of contrast. Ultrasonic flow probes (Transonic Systems Inc., Ithaca, NY) were placed on the portal vein and the hepatic artery. A tonometer (Tonometries Inc., Bethesda, MD) was placed in the terminal ileum through an enterotomy situated approximately 90 cm from the ileocecal junction.

### 6.2.3 Hemodynamic Measurements

Mean arterial pressure was measured via the carotid arterial catheter. Temperature, central venous pressure, pulmonary arterial pressure and pulmonary artery occlusion pressure were measured with a Swan-Ganz catheter inserted into one of the external jugular veins. Cardiac output was measured by the thermodilution method (Edwards Model 9250, Baxter Health Care, Irvine, CA) and recorded as the mean of three measurements. Portal venous flow ($Q_{pv}$) and hepatic arterial flow ($Q_{ha}$) were measured directly from the flow probes.

### 6.2.4 Ileal Tonometry

The gastrointestinal tonometer consists of a saline-filled silicone balloon that is placed in the gut lumen. The silicone balloon is highly permeable to oxygen and carbon dioxide. Normal saline (5.0 mL, at room temperature) was injected into the ileal tonometer balloon and was left there for at least 25 min. At the end of the equilibration period, the
saline was removed under anaerobic conditions. The first milliliter of saline removed was discarded, as this was not in direct contact with the balloon (since the tonometer tube has a residual volume of one milliliter). The CO₂ content of the subsequent volume of saline removed, which reflects the mucosal CO₂, was measured using the ABL Blood Gas Analyzer. Simultaneously, the arterial bicarbonate - assumed to be equal to the bicarbonate content of the gut mucosa - was measured. Using a conversion table supplied by the manufacturer, measured PCO₂ was transposed to a steady state PCO₂ (PCO₂ss), depending on the exact duration of equilibration. Gut mucosal pH was calculated by substituting the PCO₂ss and the simultaneously measured arterial bicarbonate in the Henderson-Hasselbalch equation (ie: pHᵢ = 6.1 + Log([HCO₃]/PCO₂)). The use of tonometry to measure changes in ileal intramucosal pH induced by endotoxin infusion in pigs has previously been validated (273).

6.2.5 Blood Sample Analysis

Blood was collected from the carotid artery and portal vein in heparinized 1 mL tuberculin syringes. The samples were immediately put on ice and blood gases were measured within 20 minutes, using the ABL 30 Blood Gas Analyser (Radiometer, Copenhagen).

Blood samples for measurement of endotoxin and cytokines were collected under sterile conditions from the carotid artery (a), the portal vein (pv), and the hepatic vein (hv), and treated as described in Chapter 5. Plasma endotoxin levels were measured from the carotid artery and portal vein with the LAL 5000, an automated endotoxin detection system utilizing the Limulus amebocyte lysate kinetic turbidimetric assay. Plasma TNF and IL-6 levels in the carotid artery, portal vein and hepatic vein were determined using the L929 cytotoxicity assay and the B9 proliferation assay, respectively. These assays were described previously.
6.2.6 Data Analysis

To determine whether gut and/or liver were a source of cytokines, plasma levels over time between sites (carotid artery, portal vein and hepatic vein) and between groups (Control vs. Septic) were compared. In addition, gut influx was compared to gut efflux of endotoxin, TNF and IL-6; hepatic influx of TNF and IL-6 were compared to hepatic efflux of these cytokines. Gut fluxes and hepatic fluxes of each substance of interest (Z) were calculated using the following equations:

\[
\text{Gut Influx}(Z) = Q_{pv} \cdot [Z]_a
\]
\[
\text{Gut Efflux}(Z) = Q_{pv} \cdot [Z]_{pv}
\]
\[
\text{Hepatic Influx}(Z) = Q_{pv} \cdot [Z]_{pv} + Q_{ha} \cdot [Z]_a
\]
\[
\text{Hepatic Efflux}(Z) = (Q_{pv} + Q_{ha}) \cdot [Z]_{hv}
\]

where \([Z]\) is the concentration of endotoxin, IL-6 or TNF at each vascular site.

Statistical significance of changes of each parameter over time and of differences between groups was tested by a one-way repeated measures ANOVA with one or two repeating factors as calculated by BMDP/Dynamic Version 7 (BMDP Statistical Software, Inc., Los Angeles, CA). LPS, TNF and IL-6 levels and fluxes were log transformed to normalize the data. Multiple comparisons were corrected for by using the sequential rejective Bonferroni procedure. A corrected p-value < 0.05 was considered significant.

The power of the study was calculated using NCalculator version 0.9 (by Mark L. Mitchell). The desired power (\(\beta - 1\)) was 0.9. With 6 animals/group and given a coefficient of variation (\(\sigma\)) of 80\% in plasma endotoxin levels, a difference of 190\% of the mean would be detectable. Similarly, given a \(\sigma = 75\%\) in gut and liver fluxes of TNF and IL-6, a difference of 175\% can be detected.
6.3 RESULTS

6.3.1 Hemodynamic Changes

Hemodynamic changes during the experiment are graphically summarized in Figures 12 and 13. The endotoxin infusion did not significantly alter the cardiac output. There was a significant decrease in mean arterial pressure associated with a significant drop in the systemic vascular resistance in the septic group, beginning 90 minutes after initiation of the LPS infusion and persisting for the remainder of the experiment. In septic animals, pulmonary arterial pressure (PAP) increased following the LPS infusion. The increase was only statistically different from baseline at 150 and 180 minutes and, when septic and control animals were compared, there was no significant difference in PAP at 150 and 180 min. Portal venous flow did not change during the experiment in either group, corresponding to the absence of change in cardiac output.

6.3.2 Tonometric Measurements

Changes in ileal mucosal pH and PCO₂, as measured by tonometry, are summarized in Figure 14. In septic animals, ileal intramucosal pH decreased, tonometer PCO₂ increased, and arterial bicarbonate decreased following the LPS infusion; the difference between septic and control animals became statistically significant 90 minutes after initiation of the LPS infusion. Thereafter, the ileal intramucosal pH remained significantly depressed in the septic group. Ileal intramucosal pH and tonometer PCO₂ were unchanged during the entire experiment in nonseptic control animals. This data is consistent with the presence of gut ischemia in the septic group, but not in the control group.
Figure 12. Hemodynamic changes in controls (solid circles) and septic animals (open squares). Top: cardiac index (CI). Middle: Portal venous (PV) flow index. Bottom: Hepatic arterial (HA) flow index. All values in this and subsequent graphs are expressed as mean +/- SEM.
Figure 13. Hemodynamic changes in controls (solid circles) and septic animals (open squares). Top: systemic mean arterial pressure (MAP). Middle: pulmonary arterial pressure (PAP). Bottom: systemic vascular resistance (SVR).
Figure 14. Tonometric parameters in controls (solid circles) and septic animals (open squares). Top: calculated ileal mucosal pH. Middle: tonometer PCO₂. Bottom: arterial bicarbonate concentration.
6.3.3 Endotoxin Levels

Figure 15 demonstrates arterial and portal venous LPS levels in septic and control animals throughout the experiment. In controls, plasma endotoxin was detectable at minimal levels throughout the experiment, but no significant changes over time were observed. In addition, no difference between carotid arterial and portal venous levels were seen. Thus, in the control group, endotoxin of unknown origin was present throughout the experiment; it did not appear to originate from the gut.

Due to technical difficulties, baseline (pre-LPS infusion) samples from the septic group were contaminated such that endotoxin data was not obtained at baseline in the septic group, but presumably baseline was similar to baseline control data which indicated minimal plasma endotoxin levels. In the septic group, plasma endotoxin was highest at the conclusion of the LPS infusion. Endotoxin levels then decreased with a half-life of approximately 30 minutes for the following 60 minutes and remained elevated over baseline at a steady level for the remainder of the experiment. At no time was there a difference between carotid arterial and portal venous endotoxin levels or between gut influx and efflux of endotoxin in septic animals (Table 5). The similar endotoxin levels in the portal vein and the systemic circulation suggests the elevated endotoxin levels are secondary to the exogenously administered endotoxin and that the gut did not significantly contribute to this large pool of circulating endotoxin.
Figure 15. Arterial and portal venous endotoxin (LPS) levels in controls and septic animals.
6.3.4 TNF and IL-6 Levels

In the control group, plasma TNF was undetectable in all subjects throughout the majority of the experiment (Figure 16). In the septic group, plasma TNF levels peaked at the conclusion of the LPS infusion, then decreased to baseline levels within 90 minutes (Figure 16). There was no significant difference between carotid arterial, portal venous and hepatic venous TNF levels during this time. There was no difference between gut influx and efflux of TNF (Figure 17, Table 5). Hepatic efflux of TNF tended to exceed influx of TNF to the liver immediately following the endotoxin infusion (uncorrected  P < 0.04 at 60 min, NS after correction). Overall, however, there was no significant difference in hepatic influx and efflux of TNF in either group (Figure 18, Table 6). There was therefore no net production (or destruction) of TNF by the gut or the liver and the elevated levels of TNF seen in the septic group were largely of extrasplanchnic origin.

Plasma IL-6 levels did not change from the baseline level throughout the experiment, in control animals (Figure 19). In the septic group, IL-6 levels increased significantly at the conclusion of the LPS infusion, peaked 180 minutes after initiation of the infusion, and remained elevated for the remainder of the experiment (Figure 19). In the control group and in the septic group there was no significant difference between IL-6 levels in the carotid artery, portal vein and hepatic vein. There was no difference between gut influx and efflux of IL-6 in either group (Figure 20, Table 5), nor was there a difference between hepatic influx and efflux of IL-6 (Figure 21, Table 6). Thus, neither the gut nor the liver were sites of net production (or destruction) of IL-6 and neither of these sites significantly contributed to the elevated levels of IL-6 seen in the septic group.
<table>
<thead>
<tr>
<th>TIME (min)</th>
<th>0</th>
<th>60</th>
<th>120</th>
<th>240</th>
<th>300</th>
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<tr>
<td><strong>LPS (EU/mL)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Influx, Septic</td>
<td>22253 ± 8363</td>
<td>5432 ± 1748</td>
<td>3461 ± 1649</td>
<td>2213 ± 717</td>
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<td>Efflux, Septic</td>
<td>15804 ± 6322</td>
<td>4328 ± 1584</td>
<td>3845 ± 1353</td>
<td>3291 ± 710</td>
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<tr>
<td>Influx, Control</td>
<td>9.7 ± 2.8</td>
<td>19.6 ± 9.5</td>
<td>14.1 ± 7.2</td>
<td>16.5 ± 8.9</td>
<td>7.0 ± 2.5</td>
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<tr>
<td>Efflux, Control</td>
<td>25.5 ± 7.7</td>
<td>14.7 ± 6.2</td>
<td>12.0 ± 7.4</td>
<td>31.4 ± 21.5</td>
<td>4.0 ± 2.5</td>
</tr>
<tr>
<td><strong>TNF (pU/mL)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Influx, Septic</td>
<td>528 ± 212</td>
<td>3312 ± 2207</td>
<td>1931 ± 897</td>
<td>302 ± 45</td>
<td>272 ± 32</td>
</tr>
<tr>
<td>Efflux, Septic</td>
<td>660 ± 246</td>
<td>3274 ± 2020</td>
<td>1838 ± 826</td>
<td>295 ± 36</td>
<td>284 ± 36</td>
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<tr>
<td>Influx, Control</td>
<td>423 ± 59</td>
<td>354 ± 43</td>
<td>395 ± 79</td>
<td>334 ± 56</td>
<td>315 ± 42</td>
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<tr>
<td>Efflux, Control</td>
<td>448 ± 36</td>
<td>344 ± 44</td>
<td>376 ± 79</td>
<td>334 ± 56</td>
<td>423 ± 162</td>
</tr>
<tr>
<td><strong>IL-6 (nU/mL)</strong></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Influx, Septic</td>
<td>38.4 ± 9.4</td>
<td>110.9 ± 52.4</td>
<td>1782 ± 588</td>
<td>1956 ± 575</td>
<td>1061 ± 324</td>
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<tr>
<td>Efflux, Septic</td>
<td>49.9 ± 6.7</td>
<td>111.5 ± 61.3</td>
<td>1887 ± 601</td>
<td>1816 ± 525</td>
<td>1258 ± 318</td>
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<tr>
<td>Influx, Control</td>
<td>19.7 ± 5.4</td>
<td>16.7 ± 2.9</td>
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<td>12.5 ± 4.9</td>
<td>11.1 ± 3.2</td>
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<tr>
<td>Efflux, Control</td>
<td>23.5 ± 6.5</td>
<td>20.3 ± 4.1</td>
<td>18.9 ± 3.4</td>
<td>13.5 ± 4.0</td>
<td>10.3 ± 2.8</td>
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Table 5. Gut influx and efflux of endotoxin (LPS), TNF and IL-6 in septic and control animals.

Data missing is due to experimental protocol violation. Data is expressed as mean ± SEM.
<table>
<thead>
<tr>
<th>TIME (min)</th>
<th>0</th>
<th>60</th>
<th>120</th>
<th>240</th>
<th>300</th>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Influx, Septic</td>
<td>743 ± 251</td>
<td>3987 ± 1748</td>
<td>2224 ± 968</td>
<td>357 ± 43</td>
<td>380 ± 42</td>
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<tr>
<td>Efflux, Septic</td>
<td>560 ± 111</td>
<td>7545 ± 3560</td>
<td>2705 ± 1520</td>
<td>375 ± 57</td>
<td>373 ± 39</td>
</tr>
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<td>Influx, Control</td>
<td>550 ± 91</td>
<td>444 ± 55</td>
<td>548 ± 89</td>
<td>544 ± 92</td>
<td>687 ± 167</td>
</tr>
<tr>
<td>Efflux, Control</td>
<td>898 ± 216</td>
<td>523 ± 111</td>
<td>800 ± 174</td>
<td>895 ± 297</td>
<td>527 ± 53</td>
</tr>
<tr>
<td><strong>IL-6 (nU/mL)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Influx, Septic</td>
<td>56.6 ± 6.9</td>
<td>192.8 ± 68.6</td>
<td>2277 ± 700</td>
<td>2223 ± 644</td>
<td>1626 ± 421</td>
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<tr>
<td>Efflux, Septic</td>
<td>44.7 ± 8.3</td>
<td>237.3 ± 118.3</td>
<td>1986 ± 562</td>
<td>2591 ± 718</td>
<td>2381 ± 584</td>
</tr>
<tr>
<td>Influx, Control</td>
<td>30.1 ± 7.7</td>
<td>27.2 ± 5.8</td>
<td>28.3 ± 6.3</td>
<td>23.6 ± 9.1</td>
<td>17.7 ± 5.1</td>
</tr>
<tr>
<td>Efflux, Control</td>
<td>24.5 ± 6.8</td>
<td>26.4 ± 6.0</td>
<td>27.3 ± 7.1</td>
<td>27.4 ± 11.2</td>
<td>15.2 ± 4.1</td>
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</table>

Table 6. Hepatic influx and efflux of TNF and IL-6 in septic and control animals.

Data is expressed as mean ± SEM.
Figure 16. Arterial, portal venous and hepatic venous levels of TNF in controls and septic animals.
Figure 17. Gut influx and efflux of TNF in controls and septic animals.
Figure 18. Hepatic influx and efflux of TNF in controls and septic animals.
Figure 19. Arterial, portal venous and hepatic venous IL-6 levels in controls and septic animals.
Figure 20. Gut influx and efflux of IL-6 in controls and septic animals.
Figure 21. Hepatic influx and efflux of IL-6 in controls and septic animals.
6.4 DISCUSSION

Cytokinemia may be important in the pathogenesis of MODS in various critical illnesses, including septic shock. However, the source of the cytokines and the mechanism by which their release is stimulated is poorly understood. It was postulated that gut becomes ischemic during septic shock and that, as a result of ischemia, a localized inflammatory response marked by gut production of cytokines occurs. It was further hypothesized that the ischemic injury to the gut would result in the release of endotoxin from the gut lumen to the portal circulation. Finally, it was postulated that the liver would be stimulated to produce cytokines. By measuring the changes in plasma LPS, TNF and IL-6 across the gut and liver, the author attempted to demonstrate their origin from these organs in a porcine model of septic shock. While the tonometric data suggested that the gut was ischemic in our model, the gut was not shown to be a source of LPS, TNF or IL-6. Net production or metabolism of TNF or IL-6 in the liver was also not demonstrated.

The porcine model of septic shock utilized in the present study is analogous to the clinical situation. It is characterized by normodynamic septic shock. The aggressive fluid resuscitation replicates standard practice for the management of septic shock in the clinical setting, ensuring that hypovolemia is not a factor contributing to the hemodynamic derangements resulting from infusion of endotoxin. This model reproduces the hemodynamic features of septic shock in humans (71)(274). That is, septic animals demonstrate systemic arterial hypotension due to decreased systemic vascular resistance and an increase in pulmonary arterial pressure. In addition to the similarity in hemodynamic response to humans in septic shock, the porcine intestinal anatomy and gut flora are similar to that found in humans and other primates. This is in direct contradistinction to ruminants, which possess greater bacterial populations in their proximal bowel and which have a gut vasculature which differs anatomically from humans (155). Because of the similar hemodynamic responses to humans in septic shock and because of comparable intestinal
anatomy, the findings obtained with the saline-resuscitated porcine endotoxicosis model are more likely to be applicable to the clinical situation.

Inadequate splanchnic blood flow may have a special role in the pathogenesis of MODS. This postulate is supported by the improved survival of those shock patients in whom gut ischemia, as determined by a decreased gastric mucosal pH, can be reversed (133). A decreased ileal intramucosal pH associated with decreased mesenteric oxygen delivery has been observed in endotoxemic and septic pigs (79)(127). To monitor whether the gut was ischemic in the present study, indirect measurements of the adequacy of gut perfusion were utilized. Portal venous flow, while lower in septic animals, was not significantly different from controls. However, portal venous flow is a measure of total gut blood flow and it gives no information on regional changes in gut perfusion. For example, Fink and coworkers observed a decrease in superior mesenteric artery flow in a similar model of septic shock (79)(109); regions of gut perfused by the celiac artery or the inferior mesenteric artery may be less prone to hypoperfusion. In addition, in the presence of countercurrent arteriovenous shunting of oxygen at the base of intestinal villi, the tips of the villi may become significantly anoxic, even if blood flow to the superficial mucosa remains unchanged compared to the normal resting state (275). The tonometer revealed a decreased ileal intramucosal pH, but this too has its limitations in detecting gut ischemia. Use of tonometry carries with it a number of assumptions which may not hold true under endotoxic conditions; in a porcine model of endotoxicosis, a profound reduction in mucosal pH as calculated by tonometry was observed to occur in the absence of histological evidence of intestinal ischemia (276). Further, VanderMeer et al., using a porcine model of endotoxicosis similar to the one used in the present study, clearly showed that ileal mucosal acidosis occurred in the absence of mucosal hypoxia and ischemia (277). It has been suggested that measurement of mucosal PCO2 might be useful, but certain caveats must be considered during interpretation. Increased mucosal PCO2 as measured by tonometry could represent anaerobic production of metabolic acids such as lactate, in which case it would
indicate anaerobic metabolism. Alternatively, the increased PCO₂ might be secondary to impaired washout of CO₂ produced by oxidative phosphorylation produced aerobically, representing flow stagnation. Schlichtig and Bowles, recognizing this, determined that anaerobic metabolism occurs in the presence of a mucosal PCO₂ > 65 mm Hg in nonseptic dogs and suggested that mucosal PCO₂ be used to detect ischemia, instead of mucosal pH (278). In the present study, there was no evidence of gut flow stagnation (Figure 12) and the mucosal PCO₂ was well above the level that Schlichtig and Bowles considered to represent ischemia (Figure 14). However, the author wonders whether the gut was ischemic in our septic animals, especially in light of the recent findings reported by VanderMeer and associates (277). A more detailed discussion of the limitations of tonometry in the determination of the presence of gut ischemia is contained in Appendix A.

In view of findings from other studies, it came as a surprise that a release of endotoxin from the bowel to the circulation was not demonstrated. Fink and coworkers demonstrated intestinal mucosal hyperpermeability in a similar porcine model of septic shock. In that study, the increase in permeability became apparent 30 minutes after the onset of the endotoxin infusion, but did not become significant until 2h after the endotoxin infusion (109). Portal endotoxemia in excess of systemic endotoxemia has been demonstrated during abdominal surgery (191) intestinal ischemia (148) and cirrhosis (197), in animals and in humans. In the present experiment, a measurable difference in endotoxin levels in the portal vein and the carotid artery was not observed and this could be due to any of several reasons. First, it is possible that the duration of the experiment (and, hence, the duration of gut ischemia) was too short and that portal endotoxemia would become evident after a longer period of observation. However, mesenteric hyperpermeability, as shown in Fink's porcine model of endotoxicalosis, occurs well within the study period in this experiment (109). Secondly, it may be that in this model, the degree of ischemia observed was not an adequate stimulus for transmigration of endotoxin; perhaps reperfusion injury would produce the insult required to cause the requisite breakdown in the mucosal barrier.
While most studies of the gut ischemia-reperfusion phenomenon report endotoxemia during the ischemic phase (148)(149), some do not demonstrate significant increases in plasma endotoxin until after reperfusion (146). Thirdly, it is possible that the plasma endotoxin levels secondary to the endotoxin infusion were much too high and too variable between subjects to see a small, significant difference in portal venous and arterial levels. Finally, there may not be a difference between portal venous levels and systemic levels because the major route by which endotoxin leaks into the circulation is via the lymphatic route. Mainous et al. showed that the route of bacterial translocation varied with severity of a systemic inflammatory stimulus in a rodent model. With a mild inflammatory stimulus, bacterial translocation was mainly via the mesenteric lymph nodes; a severe inflammatory state was accompanied by bacterial dissemination via portal blood (176). Further, Brooks et al. reported that, in surgical patients, positive cultures are seen most frequently in the mesenteric lymph nodes, followed by intestinal serosa; blood cultures were least frequently positive (170). Therefore, the inability to detect endotoxin in the portal circulation may be because, under these experimental conditions, the major route of transmigration is via the lymphatics.

Tumor necrosis factor is thought to be one of the initial cytokines released in the cytokine cascade and triggers the release of other cytokines in the cascade (217). Injection of TNF in animal models reproduces the hemodynamic effects of endotoxemia in these same models (32)(216) In addition to producing these hemodynamic responses, TNF has been shown in rodents to cause widespread tissue damage, including hemorrhagic necrosis in lungs, kidneys, adrenal glands, and the GI tract; severe peribronchiolar pneumonitis, thickened alveolar membranes, and pulmonary edema; acute tubular necrosis; and diffuse capillary thrombosis (32). This has led investigators to postulate that the presence of high levels of TNF in sepsis, as well as in other critical illnesses, is crucial to the pathogenesis of MODS. In contrast, IL-6 does not have any known hemodynamic effects, but it has important immunostimulatory effects. At the same time, IL-6 inhibits TNF production
High levels of IL-6 are associated with a fatal outcome in Gram negative sepsis (279). Therefore, while IL-6 does not appear to be a causal factor in septic shock, it may be a contributory determinant for MODS.

It was interesting to compare the cytokine response observed in this study to other studies. After bacterial infusions in rats, rabbits and baboons, TNF can be detected within minutes. A monophasic peak is seen at 90 to 120 minutes, and levels decrease until they become undetectable approximately 4 h following infusion (217)(252). Following infusion of bacteria in baboons, IL-6 could be detected in the circulation approximately one hour later; levels continued to rise for up to 8 h after the infusion (217). In human volunteers who received IV endotoxin, the kinetics of circulatory TNF and IL-6 are similar to those seen in baboons who received bacterial infusions (29)(223). Studies by Sugerman's group, in which swine were made septic with a one-hour infusion of live *Pseudomonas aeruginosa*, showed that plasma TNF levels reached a maximum at 120 minutes, remained at this level for an additional 60 min, and began to decline, remaining above baseline for at least 5 hours (253)(280). The author is not aware of studies of the appearance of IL-6 in porcine models of septic shock other than the one presented in this thesis. The experimental design of the present study, in which cytokine levels were analyzed every 30 minutes, prohibited more precise documentation of how soon TNF and IL-6 appeared in the circulation following initiation of the endotoxin infusion. In this study, TNF levels peaked at 60 minutes after initiation of the infusion and IL-6 levels peaked at 180 minutes. Unlike in studies of primates and ruminants, TNF did not persist for hours; it virtually disappeared within 90 minutes. Further, IL-6 levels decreased relatively quickly when compared to other models of endotoxemia. This was a curious finding which may have several possible explanations. First, porcine TNF and IL-6 may have a shorter half-life than TNF and IL-6 of other species. One possible reason for this is that soluble receptors to TNF and IL-6 may be present in particularly high quantities in pigs. Soluble receptors have been described for many cytokines, including TNF and IL-6 (281). TNF receptors are rapidly shed from cell
surfaces into the circulation in response to a variety of inflammatory stimuli, including endotoxemia (282). Further, soluble receptors, because they are naturally occurring antagonists to cytokines, have been reported to interfere with bioassays (283). It is possible that an integral part of the porcine inflammatory response under the experimental conditions in this study consists of the release of relatively large amounts of TNF receptors (as well as receptors for other cytokines) and this phenomenon limited the bioactivity of any TNF present in the latter part of the experiment. However, a particularly short half-life of porcine cytokines or the presence of soluble receptors for cytokines is not the likely explanation, since Sugerman's group reported kinetics of TNF in pigs that closely resembled those in primates and ruminants. Secondly, the porcine cytokine response may have been abbreviated by the aggressive fluid resuscitation instituted in this study. This would explain the differences between the results from this experiment and those of Sugerman's group, for they did not administer large volumes of fluids to their septic animals, which displayed hypodynamic septic shock. More detailed study of the porcine immune response and development of immune markers and assays for use in pigs would be most helpful in clarifying the cytokine response observed in this study.

Much work has been done to identify potential sources of cytokines under various clinical and experimental conditions. In other models of septic shock and endotoxicosis, TNF and IL-6 have been shown in vitro to originate from circulating monocytes, neutrophils, mast cells, natural killer cells and lymphocytes, as well as tissue macrophages and vascular endothelial cells (202)(221)(222). In addition, in rats, TNF mRNA and IL-6 mRNA have been noted in bowel, spleen, liver and lung (240)(241), presumably predominantly in the macrophages residing in these tissues. However, the demonstration of the capability of a tissue or cell to produce cytokines in vitro or the presence of the biochemical machinery necessary to synthesize cytokines is only circumstantial evidence that that tissue is involved in the secretion of cytokines in vivo. The question still remains whether cytokine production is "compartmentalized". That is: do cytokines originate from
sites in which tissue injury is localized? Some evidence of compartmentalization has accumulated. For example, in meningitis, levels of TNF (235) or IL-6 (236) are higher in the CSF than in the systemic circulation. The intratracheal administration of LPS in experimental models is associated with the appearance of TNF in bronchoalveolar lavage fluid while, in the same model, administration of IV LPS increases circulating levels of TNF without producing an increase in levels in the bronchoalveolar lavage fluid (237). Rodriguez and coworkers demonstrated hypersecretion of IL-6 in injured skin in burn patients (238). It therefore appears that tissues injured are most likely to be the predominant source of cytokines.

An attempt was made to demonstrate compartmentalization of cytokine production in gut. Breese et al. previously reported that gut contains increased numbers of TNF-secreting cells in the presence of mucosal inflammation, regardless of its etiology, and the number of such cells is proportional to the severity of inflammation (242). While demonstration of the presence of cytokines in tissue is an adequate method of studying localized tissue injury, it can not be expected to yield useful information in the presence of a systemic inflammatory response, such as that which occurs in endotoxicosis. That is, if one were to demonstrate the presence of cytokines in bowel in septic shock, the origin of the cytokines would still be in question: did they originate within the bowel or did they originate from another site upstream? To circumvent this problem, the differences in plasma TNF and IL-6 entering and leaving the bowel were measured. The failure to show that gut is a source of these cytokines in endotoxicosis, despite the demonstration of ischemic injury by tonometry, may be due to a number of factors. Firstly, endotoxicosis produces a systemic inflammatory response during which a number of tissues may be injured, either secondary to the inflammatory response or as a result of the presence of endotoxin itself. The liver, the spleen or the lungs, with their numerous tissue macrophages, or circulating immune cells may be more important sites of production in the presence of endotoxin. It is possible that, because of the generalized effects of endotoxicosis, where cytokines emanate
from numerous, diffuse sites, gut production of TNF and IL-6 could not be demonstrated. That is, the method of detection may not be sensitive enough under these conditions. Alternatively, while comparison of gut influx and efflux of cytokines demonstrates no net production of TNF or IL-6 in the gut, it is possible that these substances are produced and metabolized at the same rate at this site. Finally, it is possible that the small bowel may not have been ischemic. As mentioned above, only indirect measurements of the presence of gut ischemia were used. Therefore, it may be that the gut was not a significant source of cytokines because it was not sufficiently injured to produce an inflammatory response.

The hepatic Kupffer cell population comprises about 70% of the total population of macrophages in the body and these cells may participate in the initiation of an immune (or inflammatory) response (284). Endotoxin is known to rapidly and preferentially accumulate in Kupffer cells *in vivo* (57). Moreover, in the presence of endotoxin, these tissue macrophages are known to have the ability to elicit TNF and IL-6 *in vitro* (272)(285). Additionally, the liver is a potential focus of ischemic injury during sepsis (108). In rats, following hepatic ischemia-reperfusion, elevated levels of circulating TNF have been demonstrated (286). The role of the liver as a major source of TNF and IL-6 was thus investigated in the present study. By comparison of efflux of TNF and IL-6 from the liver with hepatic influx of these cytokines, hepatic production of TNF and IL-6 could not be demonstrated. One possible reason for this is that hepatic production of these cytokines may have been masked by the diffuse cytokine production by the numerous other cells exposed to endotoxin throughout the body. Another potential explanation is that metabolism of TNF and IL-6 occurred at the same rate as production, resulting in no net production or destruction of these cytokines. On the other hand, Kupffer cells have been shown to have less secretory activity compared with other types of macrophages, suggesting hepatic tissue macrophages may have more of a phagocytic function than a paracrine function (287)(288). Thus, while the macrophages residing in the liver have the
ability to produce cytokines in vitro in the presence of endotoxin, the overall contribution to the pool of circulating cytokines may be minor.

The roles of the gut and liver, endotoxin and cytokines in the pathogenesis of MODS is still unknown. While sepsis clearly differs from trauma, both conditions are often complicated by MODS. It is therefore conceivable that some common pathophysiological event must be present in both conditions. Moore et al., in their investigation of the role of the gut in the pathogenesis of MODS in critically ill trauma patients, reported findings consistent with those presented here (169). They did not detect endotoxin in the portal vein. Further, simultaneously measured TNF and IL-6 levels in the portal and systemic circulations were identical. Finally, neither TNF nor IL-6 levels correlated with the development of MODS.

In summary, transmigration of endotoxin from the gut was not demonstrated, nor was a net production of TNF or IL-6 by the liver or gut detected in a porcine model of fluid-resuscitated septic shock. These results raise a number of questions. Firstly, the presence or absence of gut ischemia during septic shock must be better defined using different indices of ischemia. Secondly, the role of the gut in production of cytokines during a known state of gut ischemia must be explored. This will ultimately result in a better understanding of the role of the gut, other splanchnic organs and cytokines in the pathogenesis of MODS.
Chapter 7

GUT AND LIVER PRODUCTION OF CYTOKINES IN A PORCINE MODEL OF MESENTERIC ISCHEMIA-REPERFUSION

7.1 INTRODUCTION

Loss of intestinal mucosal barrier function secondary to mesenteric ischemia and/or reperfusion, resulting in transmigration of bacteria or endotoxin from the gut, may play an important role in the pathogenesis of MODS (6). Further, injury to the gut may provoke a local inflammatory response leading to a systemic inflammatory state. Mesenteric ischemia occurs commonly in critically ill patients secondary to shock of various etiologies and subsequent resuscitation and reperfusion may result in an even greater injury than that associated with ischemia alone (143)(289). In animals, reperfusion of intestine following a period of ischemia causes functional and pathological changes of organs distant to the primary site of injury, including the heart, lungs, and liver (145)(146)(147)(148). In addition, surgical revascularization in patients with chronic mesenteric ischemia has been reported to be associated with pulmonary dysfunction consistent with adult respiratory distress syndrome, hepatic dysfunction, renal failure and coagulopathy (150). It therefore appears that mesenteric ischemia-reperfusion incites a stereotypical systemic response, perhaps by the release of gut-derived toxins or inflammatory mediators.

Cytokines are peptide inflammatory mediators that are released under various conditions in which tissue injury occurs. Previous animal models of meningitis and pneumonitis suggest that cytokines - in particular, TNF and IL-6 - are primarily released at the site of injury and spill over into the circulation (235)(236)(237). Gut ischemia-reperfusion has not been well studied with respect to localizing the origin of the cytokine release. Since gut ischemia-reperfusion is implicated as a possible inciting factor in the
pathogenesis of MODS, it is important to determine whether the inflammatory response accompanying gut ischemia is a localized phenomenon or whether other organs contribute to the inflammatory response.

Tumor necrosis factor is thought to be one of the initial cytokines released in the cytokine cascade and triggers the release of cytokines in the cascade (6)(202). Injection of TNF in animal models reproduces the hemodynamic effects of endotoxemia in these same models. In addition, TNF has been shown in rodents to cause widespread tissue damage (32). High levels of TNF in critical illnesses may therefore be important in the pathogenesis of MODS. In contrast, while IL-6 does not have any known hemodynamic effects, high levels of IL-6 are associated with a fatal outcome in Gram negative sepsis (279). Therefore, IL-6 may contribute to the pathogenesis of MODS. Previous data from other studies suggest that, during gut ischemia, the gut loses its normal mucosal barrier function, resulting in leakage of endotoxin from the gut lumen into the portal circulation (110)(290).

It is postulated that TNF, IL-6 and endotoxin are released from ischemic gut. Since the liver is the first organ exposed to the effects of any toxins leaking from the gut and because it contains numerous macrophages, the liver could amplify release of TNF and IL-6 into the systemic circulation. The purpose of this experiment was to address the following questions in a porcine model of mesenteric ischemia-reperfusion: 1) Does ischemic gut produce IL-6 and TNF? 2) Does liver, an organ distant to the site of injury, produce IL-6 and TNF during mesenteric ischemia-reperfusion? and 3) Does the gut leak endotoxin into the portal circulation during mesenteric ischemia-reperfusion?

7.2 METHODS

7.2.1 Experimental Design and Protocols

Juvenile pigs weighing 22-35 kg were prepared as described below. During surgery, instrumentation and stabilization, normal saline was infused IV at 25 mL/kg/h.
Animals were allowed to stabilize for 1 hour following surgery. At this time, baseline measurements of hemodynamics and cytokine levels were performed. The rate of IV saline infusion was increased to 48 mL/kg/h following baseline measurements. Animals were allocated to one of two groups. In the ligation group (N = 7), the superior mesenteric artery (SMA) was clamped for 4 h, during which measurements were taken every 30 min. After 240 min, the clamp was released for 30 - 40 s and measurements were taken while the clamp was being released, as well as at 5, 15, 30 and 60 min following reapplication of the clamp. At 300 min, the clamp was completely released and measurements were performed while the SMA clamp was being released, as well as at 5, 15 and 30 min after removal of the clamp. The control group (N = 7) had the same surgery except for dissection of the SMA. In control animals, the retroperitoneum was entered but the pancreas was only partially mobilized and the SMA was not encircled. Measurements were performed every 30 min throughout the entire duration of the experiment (330 min). The experimental protocol is depicted in the time-line diagram below (Figure 22).

Control (N = 7)

SMA Ligation (N = 7)

Figure 22. Schematic time-line representation of experimental protocol: control vs. animals treated by SMA occlusion.
7.2.2 Surgery and Instrumentation

The surgery and instrumentation were performed in the same way as in the previous experiment (ie: as in septic and control animals), with the exception of isolation of the SMA. Isolating the SMA at its origin on the aorta required mobilization of the inferior edge of the pancreas. Once the root of the SMA was isolated, a vessel loop was brought around the SMA. Complete occlusion of the SMA by tightening the vessel loop was confirmed by a major reduction in portal vein flow and a less marked increase in hepatic artery flow. Conversely, re-establishment of flow through the SMA was confirmed by a sudden increase in portal vein flow. During prolonged SMA occlusion, a feculent odor emanated from the abdomen and the bowel was observed to become progressively darker in colour on its serosal surface.

7.2.3 Hemodynamic Measurements and Ileal Tonometry

Methods used to monitor hemodynamic changes and for the collection of tonometric data were exactly as in the previous experiment. With the exception of portal venous flow and hepatic arterial flow, hemodynamic data was collected for each time interval. In addition, because at least 25 min is required for the tonometer balloon contents to equilibrate with the ileal mucosal environment to obtain valid results, tonometric parameters were only recorded every 30 min.

7.2.4 Blood Sample Collection and Analysis

Arterial, portal venous, and hepatic venous blood were collected in heparinized syringes for blood gas analysis as described in the previous experiment, every 30 min. Samples for measurement of endotoxin and cytokines were collected from the carotid artery, portal vein, and hepatic vein on every time period indicated on the experimental
protocol. Plasma endotoxin, TNF and IL-6 levels were determined as described previously.

7.2.5 Data Analysis

All calculations and statistical methods were the same as those used in the first experiment. Net production of TNF and IL-6 by the gut and liver is suggested when efflux of these substances from the gut or liver exceeds influx. Conversely, net catabolism of TNF and IL-6 is suggested when influx exceeds efflux. The temporal relationships of the appearances of TNF and IL-6 at each vascular site following release of the SMA clamp will also aid in determining the source of these cytokines. For instance, immediately following release of the SMA clamp, if a substance has accumulated in the ischemic segment of gut during the period of stagnation, it will first appear in the portal vein, then in the hepatic vein and, lastly, it will appear in the carotid artery. Transmigration of endotoxin from the gut to the portal circulation will be demonstrable by higher endotoxin levels in the portal vein than in the carotid artery (ie: gut efflux > gut influx).

7.3 RESULTS

7.3.1 Characterization of the Model

Hemodynamic changes are summarized in Figures 23 and 24. Systemic blood pressure (MAP) and cardiac index (CI) were the same in both groups and remained unchanged from baseline for most of the experiment. Following complete removal of the SMA clamp at 300 min in the ligation group, the systemic blood pressure and cardiac index suddenly dropped. Systemic vascular resistance (SVR) did not change significantly throughout the experiment. Pulmonary arterial pressure (PAP) remained stable in both groups throughout the experiment, although there was a trend for PAP to decrease upon
Figure 23. Hemodynamic changes in controls (solid circles) and in animals treated by SMA occlusion (open squares). Top: cardiac index. Middle: portal venous (PV) flow index. Bottom: hepatic arterial (HA) flow index. In this graph and subsequent graphs, arrow A is the time at which the SMA was clamped, arrow B indicates the point at which the SMA was unclamped and then reclamped, and arrow C indicates the time of complete removal of the SMA clamp.
Figure 24. Hemodynamic changes in controls (solid circles) and in animals treated by SMA occlusion (open squares). Top: systemic mean arterial pressure (MAP). Middle: pulmonary arterial pressure (PAP). Bottom: systemic vascular resistance (SVR).
Figure 25. Tonometric parameters in controls (solid circles) and in animals treated by SMA occlusion (open squares). Top: calculated ileal mucosal pH. Middle: tonometer PCO₂. Bottom: arterial bicarbonate concentration.
removal of the SMA clamp in the ligation animals. With application of the SMA clamp, portal venous flow decreased. In addition, hepatic arterial flow tended to increase with application of the SMA clamp, but this was not significant. Portal venous flow normalized each time the SMA clamp was removed, confirming re-establishment of flow through the artery.

Figure 25 summarizes changes in terminal ileal intramucosal pH and PCO2 as measured with the tonometer, as well as changes in arterial bicarbonate concentration. Intramucosal pH was significantly lower in the ligation group and the tonometer PCO2 was elevated within 30 min of the application of the SMA clamp, confirming the presence of gut ischemia in this region of gut. No significant change in arterial bicarbonate concentration was seen.

7.3.2 Tumor Necrosis Factor

Figure 26-A shows the changes in TNF levels at each vascular site and Figure 26-B shows changes in TNF levels following each release of the SMA clamp in the ligation group. In control animals, TNF levels remained stable throughout the experiment. In the ligation group, TNF levels at all sites were higher than in controls at the beginning of the experiment (uncorrected P < 0.005, NS after correction). TNF levels then decreased to those levels seen in controls, by 180 min. After 180 min, TNF levels began to increase again, peaking just before the SMA clamp was released the first time (ie: 240 min). At this time point, compared to levels in the carotid artery, TNF levels were higher in the portal vein and hepatic vein (uncorrected P < 0.05, NS after correction). With release of the SMA clamp, TNF levels suddenly decreased. With reapplication of the SMA clamp, they began to increase in the hepatic vein, but did not increase in the portal vein or carotid artery. Hepatic venous TNF levels peaked just prior to complete release of the SMA clamp (HV vs. CA, uncorrected P < 0.02, NS after correction). Again, upon reperfusion of the SMA, TNF levels suddenly decreased. During each of these latter two peaks in TNF levels, TNF
was present in the hepatic vein and in the portal vein, but not in the arterial sample. The disappearance of TNF upon reperfusion suggests that this cytokine does not originate in the ischemic segment of bowel; there was no "washout" effect. The appearance of TNF at these sites while the SMA was clamped (ie: while there was no blood flow through the ischemic segment of gut) suggests that the TNF originates from an organ that is at least partially perfused by collaterals to the SMA.

Gut influx of TNF did not exceed gut efflux of TNF in either group (Table 7). Hepatic influx and hepatic efflux of TNF were not significantly different in either group (Table 8). Thus, no net production or catabolism of TNF by the gut or liver was demonstrated.

7.3.3 Interleukin-6

Figure 27-A shows changes in IL-6 levels at each vascular site throughout the experiment; changes following each episode of reperfusion in the ligation group are shown in Figure 27-B. In controls, IL-6 levels remained constant throughout the experiment. In animals treated by SMA ligation, immediately following the first release of the SMA clamp, IL-6 suddenly increased in the portal vein and, to a lesser extent, in the hepatic vein, but not in the carotid artery. At that instant, compared to levels in the carotid artery, IL-6 levels were higher in the portal vein (uncorrected P < 0.007, NS after correction) and hepatic vein (uncorrected P < 0.05, NS after correction). Portal venous IL-6 levels increased at this time in 6 of the 7 animals studied, but this surge in IL-6 levels in the portal vein was not statistically significant with correction for multiple comparisons. In contrast, less marked increases in IL-6 levels in the hepatic vein and carotid artery occurred in only 4 and 3 animals, respectively. Once the SMA clamp was reapplied, IL-6 levels continued to rise; portal venous levels were significantly higher during this time (CA vs. PV, corrected P < 0.04). No significant changes in IL-6 levels were seen after the second release of the SMA clamp. While not statistically significant after correction, the higher levels seen in the portal
vein (and the greater frequency with which IL-6 was detected at this site) following release of the SMA clamp suggest that IL-6 originates in the ischemic segment of gut.

The gut efflux of IL-6 was not significantly higher than the gut influx of IL-6 throughout the experiment, but inadequate flow data was collected immediately after release of the SMA clamp to calculate fluxes at that moment (Table 7). Net production of IL-6 could therefore not be detected at times outside of the immediate moment in which the SMA clamp was released. Hepatic influx of IL-6 was not significantly different from the hepatic efflux of IL-6, negating the role of the liver in production of this cytokine (Table 8).

7.3.4 Endotoxin

While endotoxin was present in most of the samples from all vascular sites at all times, at no time did endotoxin levels change significantly from baseline levels. In addition, at no time did gut efflux of endotoxin exceed gut influx, suggesting that endotoxin did not translocate into the portal circulation (Figure 28, Table 7).
<table>
<thead>
<tr>
<th>TIME (min)</th>
<th>0</th>
<th>60</th>
<th>120</th>
<th>240</th>
<th>245</th>
<th>300</th>
<th>305</th>
<th>330</th>
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<tbody>
<tr>
<td>LPS (EU/min)</td>
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<td></td>
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<tr>
<td>Influx, Ligation</td>
<td>24.5 ± 11.3</td>
<td>5.8 ± 3.0</td>
<td>10.0 ± 4.3</td>
<td>8.1 ± 2.9</td>
<td>7.0 ± 3.0</td>
<td>18.8 ± 7.8</td>
<td>17.2 ± 3.7</td>
<td>10.8 ± 4.5</td>
</tr>
<tr>
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<td>16.2 ± 7.0</td>
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<td>2.1 ± 0.6</td>
<td>3.8 ± 2.0</td>
<td>6.3 ± 3.2</td>
<td>9.5 ± 2.6</td>
<td>7.8 ± 3.3</td>
<td>17.4 ± 8.4</td>
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<td>19.6 ± 9.5</td>
<td>14.1 ± 7.2</td>
<td>11.2 ± 4.6</td>
<td>7.0 ± 2.5</td>
<td>8.1 ± 6.1</td>
<td>5.1 ± 2.3</td>
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<tr>
<td>Efflux, Control</td>
<td>14.7 ± 6.2</td>
<td>12.0 ± 7.4</td>
<td>4.8 ± 1.9</td>
<td>4.0 ± 2.5</td>
<td>2.1 ± 0.5</td>
<td>2.8 ± 1.0</td>
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<tr>
<td>TNF (pU/min)</td>
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<tr>
<td>Influx, Ligation</td>
<td>997 ± 214</td>
<td>232 ± 43</td>
<td>320 ± 80</td>
<td>167 ± 34</td>
<td>242 ± 113</td>
<td>279 ± 66</td>
<td>307 ± 27</td>
<td>315 ± 68</td>
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<td>Efflux, Ligation</td>
<td>1224 ± 200</td>
<td>261 ± 32</td>
<td>301 ± 116</td>
<td>286 ± 67</td>
<td>205 ± 87</td>
<td>390 ± 96</td>
<td>235 ± 35</td>
<td>320 ± 67</td>
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<td>Influx, Control</td>
<td>354 ± 43</td>
<td>395 ± 79</td>
<td>311 ± 50</td>
<td>315 ± 42</td>
<td>301 ± 34</td>
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<td>Efflux, Control</td>
<td>344 ± 44</td>
<td>376 ± 64</td>
<td>330 ± 59</td>
<td>423 ± 162</td>
<td>286 ± 35</td>
<td>313 ± 53</td>
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<tr>
<td>IL-6 (nU/min)</td>
<td></td>
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<tr>
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<td>12.2 ± 7.1</td>
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<tr>
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<td>20.3 ± 4.1</td>
<td>18.9 ± 3.4</td>
<td>14.4 ± 4.1</td>
<td>10.3 ± 2.8</td>
<td>13.7 ± 3.2</td>
<td>14.8 ± 3.1</td>
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Table 7. Gut influx and efflux of endotoxin (LPS), TNF and IL-6 in ligation and control animals.

Data missing is due to experimental protocol violation. Data is expressed as mean ± SEM.
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<thead>
<tr>
<th>TIME (min)</th>
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<th>120</th>
<th>240</th>
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<tr>
<td>Influx, Ligation</td>
<td>1384 ± 202</td>
<td>645 ± 77</td>
<td>648 ± 121</td>
<td>530 ± 86</td>
<td>396 ± 98</td>
<td>547 ± 99</td>
<td>363 ± 46</td>
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<td>Efflux, Ligation</td>
<td>1474 ± 221</td>
<td>1036 ± 377</td>
<td>622 ± 102</td>
<td>674 ± 244</td>
<td>399 ± 91</td>
<td>873 ± 209</td>
<td>555 ± 220</td>
<td>393 ± 81</td>
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<tr>
<td>Influx, Control</td>
<td>444 ± 55</td>
<td>548 ± 89</td>
<td>504 ± 79</td>
<td>687 ± 167</td>
<td>433 ± 25</td>
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<td>Efflux, Control</td>
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<td>800 ± 174</td>
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<td>527 ± 53</td>
<td>426 ± 28</td>
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<td>Influx, Ligation</td>
<td>40.3 ± 9.2</td>
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Table 8. Hepatic influx and efflux of TNF and IL-6 in ligation and control animals.

Data missing is due to experimental protocol violation. Data is expressed as mean ± SEM.
Figure 26-A. Arterial, portal venous and hepatic venous TNF levels in controls (bottom) and in animals treated by SMA occlusion (top).
Figure 26-B. Arterial, portal venous and hepatic venous TNF levels in animals treated by SMA occlusion at the times of each release of the SMA clamp. Error bars have been omitted to better see each point of measurement.
Figure 27-A. Arterial, portal venous and hepatic venous IL-6 levels in controls (bottom) and in animals treated by SMA occlusion (top).
Figure 27-B. Arterial, portal venous and hepatic venous IL-6 levels in animals treated by SMA occlusion at the times of each release of the SMA clamp. Error bars have been omitted to better see each point of measurement.
Figure 28. Arterial and portal venous endotoxin (LPS) levels in controls and in animals treated by SMA occlusion.
7.4 DISCUSSION

MODS appears to be a systemic response to a variety of severe insults, but the pathogenesis is unclear. Intestinal ischemia and/or reperfusion may be an important precursor event. In animals, reperfusion of intestine following a period of ischemia causes functional and pathological changes of organs distant to the primary site of injury, including the heart, lungs, and liver (145)(146)(147)(148). In addition, surgical revascularization in patients with chronic mesenteric ischemia has been reported to be associated with pulmonary dysfunction consistent with adult respiratory distress syndrome, hepatic dysfunction, renal failure and coagulopathy (150). According to this report, the mortality rate after the operation was 14% and all deaths resulted from MODS. The mechanism by which this occurs is not well understood. One hypothesis is that mesenteric ischemia-reperfusion incites a stereotypical systemic response by the release of gut-derived toxins or inflammatory mediators.

In a porcine model of mesenteric ischemia-reperfusion, the author sought to determine whether the gut and/or liver are stimulated to mount an inflammatory response in the form of TNF or IL-6 production. Additionally, he sought to determine whether the intestinal injury was associated with leakage of endotoxin from the gut into the portal circulation. Although it was found that gut efflux did not exceed gut influx of TNF or IL-6 and hepatic efflux did not exceed hepatic influx of these cytokines, TNF and IL-6 were elevated in the portal vein and hepatic vein around the times of each release of the clamp. TNF levels were elevated prior to reperfusion of the SMA, especially in the portal vein and hepatic vein sites. Reperfusion did not result in a further increase in TNF levels. Immediately following reperfusion of the SMA, IL-6 levels increased in the portal vein and there was a less marked increase in hepatic venous IL-6 levels. No changes in endotoxin levels in response to mesenteric ischemia-reperfusion were documented and there was no evidence of endotoxin translocation.
TNF and IL-6 have been shown in vitro to originate from circulating monocytes, neutrophils, mast cells, natural killer cells and lymphocytes, as well as tissue macrophages and vascular endothelial cells (202)(272). However, very few studies document the origin of these cytokines under various conditions, in vivo. Further, it is not known whether cytokine synthesis and release is "compartmentalized", originating from sites in which tissue injury is localized. Previous studies support the concept of compartmentalization. For example, in meningitis, levels of TNF (235) and IL-6 (236) are higher in the CSF than in the systemic circulation. Administration of IV endotoxin increases systemic levels of TNF without producing an increase in the bronchoalveolar lavage fluid; intratracheal administration of endotoxin in the same animal model results in appearance of TNF in bronchoalveolar lavage fluid (237). The injured skin in burn patients secretes high levels of IL-6 (238). It therefore appears that tissues injured are most likely to be the predominant source of cytokines. The author was previously unable to demonstrate TNF and IL-6 production by the bowel and liver in a porcine model of septic shock where the gut was believed, but not proven, to be ischemic (previous experiment). It is therefore important to document whether these organs release these cytokines in a model in which mesenteric ischemia is known to be present.

In humans, gut contains increased numbers of TNF-secreting cells in the presence of mucosal inflammation, regardless of its etiology, and the number of such cells is proportional to the severity of inflammation (242). However, demonstration of the presence of TNF in the gut does not necessarily demonstrate an intestinal origin, whereas if TNF efflux from the gut were greater than influx, one may conclude that gut is a site of TNF production. By using this technique, it could not be demonstrated that the gut is a source of TNF in a model in which bowel injury was known to be present. The assay used to measure TNF was moderately sensitive and there was considerable variability in the responses to ischemia-reperfusion between animals; this may have resulted in β-error. On the other hand, the temporal relationship of the appearance of TNF at the different vascular
sites prior to each episode of reperfusion might suggest an alternative conclusion (Figure 29). Unlike IL-6, TNF did not suddenly appear in the circulation with reperfusion and this indicates that it did not accumulate in the completely unperfused and ischemic region of bowel. Rather, TNF was highest just prior to release of the SMA clamp, suggesting that it was being released by partially perfused splanchnic organs such as the pancreas, liver, duodenum and left colon, allowing the accumulating TNF to overflow into the circulation. Furthermore, since TNF was initially elevated after the surgical preparation in the ligation group but not in the control group, it is likely that the pancreas is a source of TNF production. These results differ from murine models of mesenteric ischemia-reperfusion. With occlusion of the SMA, TNF levels become slightly elevated after 120 minutes of ischemia and increase dramatically 15 minutes after reperfusion, peaking at 30 minutes (148). With occlusion of the SMA and the celiac artery, TNF levels are increased 40 minutes into the ischemic period and increase even more dramatically when measured 10 and 30 min following reperfusion (291). This latter observation is consistent with the hypothesis that an organ perfused by both the celiac artery and the SMA, such as the pancreas and liver, is the origin of TNF under these conditions.

In a murine model of endotoxicosis, transcription for the synthesis of IL-6 has been demonstrated in bowel (241). Baigrie et al. (200) demonstrated elevated levels of plasma IL-6 originating from partially ischemic colon during abdominal aortic surgery and the magnitude of the IL-6 response did not correlate with the presence of portal endotoxemia. If ischemic injury is the cause for IL-6 production by bowel, then it is expected that little change in systemic IL-6 levels is seen when the ischemic segment of bowel is completely unperfused. Similarly, with reperfusion, a "washout" phenomenon, where IL-6 suddenly and sequentially appears in the portal vein, hepatic vein and the systemic circulation, would be observed. The findings in the present study are consistent with the origin of IL-6 in ischemic gut. On the other hand, these results differ somewhat from those of Bitterman and associates who demonstrated increased systemic IL-6 levels 40 min into the ischemic
Figure 29. Schema describing postulated origin of TNF in mesenteric ischemia-reperfusion. TNF appears in the portal circulation while the SMA is completely occluded, yet its appearance seems related to the presence of ischemic injury. This suggests that TNF originates from a "watershed" organ, at least partially perfused by collaterals to the SMA. While this schema depicts a segment of bowel as being the source of TNF, the liver, pancreas, duodenum and left colon too are possible sources.
period and even more dramatic increases when measured 10 and 30 min following reperfusion in rats (291). This might be explained by inter-species differences. Alternatively, because they occluded the SMA and the celiac artery, it is possible that the magnitude of the response they observed was sufficiently large to cause "spill-over" of IL-6 into the systemic circulation during the ischemic period and/or that IL-6 is also released by an organ which has blood supply from both the SMA and the celiac artery, such as the pancreas and liver.

Intestinal reperfusion induces an acute liver injury manifested by neutrophil sequestration within the hepatic parenchyma, fatty degeneration, focal necrosis, hepatocellular enzyme release, reduced bile flow rates, and impaired hepatocyte metabolism (145)(146)(147). The mechanism is poorly understood. Studies on rats suggest that hepatic exposure from oxidant stress secondary to intestinal reperfusion is not likely to be the cause (151). In addition to being a potential site of injury during mesenteric ischemia-reperfusion, the liver may play a more active role in the pathogenesis of MODS following intestinal ischemia-reperfusion. One hypothesis is that the liver normally removes toxic mediators released by the gut in response to ischemia-reperfusion, decreasing the subsequent distant organ injury. Alternatively, gut-origin mediators or the liver injury itself could cause the release of other mediators by liver reticuloendothelial cells, leading to increased injury to other organs. Demonstration of hepatic production of inflammatory mediators such as IL-6 and TNF would support the latter hypothesis. The biosynthetic machinery appears to be present, for transcription for the synthesis of IL-6 has been noted in liver following endotoxicosis (241). In addition, Kupffer cells - like other tissue macrophages - are able to produce IL-6 and TNF \textit{in vitro} (202)(272). Hepatic production of IL-6 or TNF in this porcine model of mesenteric ischemia-reperfusion was not demonstrated using comparison of hepatic efflux to influx in the present study. However, TNF was slightly more elevated in the hepatic vein than in the portal vein prior to release of the SMA clamp, which may indicate that the liver was the source of some TNF. Alternatively, these results are
consistent with the in vivo observations reported by Billiar and coworkers, who demonstrated production of IL-6 and TNF by liver nonparenchymal cells during endotoxemia, but not in response to a peripheral inflammatory stimulus, despite the activation of a hepatic acute-phase response (285). Further, since administration of TNF induces lung injury (247), these results are consistent with the finding that diversion of portal blood around the liver does not affect the incidence or severity of post-reperfusion lung injury (190). Therefore, at this time, there is no conclusive evidence that the liver does or does not release TNF or IL-6 in response to gut ischemia.

The control group was unfortunately not a perfect control, since dissection of the SMA around the pancreas was not as complete as in the ligation group. Since elevations in systemic levels of IL-6 and TNF can be exacerbated with worse trauma (291), comparison of our two experimental groups may be of limited value. IL-6 levels at the beginning of the experiment (when the effects of trauma would be expected to appear) were not significantly affected by this difference in extent of surgery. In contrast, TNF levels were significantly higher in those animals treated by SMA occlusion at the beginning of the experiment, normalizing 120 minutes into the experiment. In the view of the author, since the major difference is the degree of trauma to the pancreas, this observation is consistent with TNF being of pancreatic origin.

Endotoxin is perhaps the most powerful stimulant for cytokine release known and translocation of endotoxin, perhaps as a result of intestinal ischemia, has been implicated as an important factor in the pathogenesis of MODS (6). Dogs treated with partial occlusion of the SMA and followed for up to 72 hours have a high incidence of Gram negative bacteremia and this is associated with functional and morphological evidence of injury of distant organs (145). Administration of antioxidants provided some protection from these changes, but so did prophylactic administration of amikacin, suggesting distant organ injury may be a function of Gram negative bacterial products. On the other hand, inhibition of endotoxin activity does not prevent post-reperfusion liver or lung injury in rats.
Therefore, the role of endotoxin as a proximal mediator of distant organ injury during mesenteric ischemia-reperfusion is yet to be determined.

Appearance of endotoxin in the circulation varies considerably between species as well as between studies using the same species. Increased venous levels of endotoxin has been reported during intestinal ischemia due to hemorrhage, in dogs (184). Papa et al. also demonstrated the presence of endotoxin in peritoneal washings and in portal and systemic blood samples within 30 min of onset of colon ischemia in dogs (188). In rabbits, systemic endotoxemia is seen 15 min after application of a ligature around the SMA and the endotoxemia is even more pronounced 10 min following reperfusion of the SMA (186). In cats subjected to 60 min of SMA occlusion and 120 min of reperfusion, arterial endotoxin levels begin to rise after 20 min of occlusion, increasing until the end of the ischemic period. Following reperfusion, LPS levels increase further, peaking 20 min after reperfusion (187). In primates subjected to the same treatment, systemic endotoxin levels do not increase during the ischemic period but increase following reperfusion, peaking within 20 min of reperfusion (189). Studies on rats have yielded extremely variable results. Caty et al. reported increased portal venous endotoxin levels after 30 min of SMA occlusion and endotoxin levels were proportional to the duration of ischemia. Reperfusion was associated with even higher endotoxin levels (148). In contrast, Turnage and associates did not demonstrate elevated portal venous or systemic endotoxin levels during a 120 minute period of SMA occlusion; endotoxin levels finally became significantly elevated 60 minutes after reperfusion (146). Koike and coworkers also did not observe elevated systemic endotoxin levels following 45 min of SMA occlusion and 120 min of reperfusion (149). Finally, during a study by Johnstone et al., virtually no endotoxin was detected in the systemic circulation following 60 minutes of ischemia and 60 minutes of reperfusion, even when the liver was bypassed with the presence of a shunt (190).

While endotoxemia has been demonstrated in a number of models of intestinal ischemia-reperfusion, it is not known with any certainty by which route it appears in the
circulation. Gathiram et al. showed that the prophylactic oral administration of nonabsorbable antibiotics to primates prevents the appearance of LPS in the circulation following mesenteric ischemia-reperfusion (189). Similar findings have been reported in rabbits treated with intestinal injection of antibiotics (186), suggesting the LPS is of gut origin. In rats, intestinal permeability as measured by plasma-to-luminal clearance of $^{51}$Cr-EDTA is increased after 20 min of small bowel ischemia and remains elevated following reperfusion (290). This supports the role of a hematogenous route of transmigration of endotoxin, although data from other studies suggest other routes of translocation may also be important. In dogs with ischemic colon, radioactive endotoxin administered per rectum can be detected in the peritoneal fluid and in the portal and systemic circulation within 30 min of the onset of colon ischemia (188). Olofsson et al. (185), using a partial gut ischemia model in rats, detected endotoxin in thoracic duct lymph and in the systemic circulation before significant portal endotoxemia was seen, suggesting systemic endotoxemia mainly reflects lymphatic transport. This is consistent with the observations of Turnage and associates who reported no difference between portal venous and systemic levels of endotoxin, even when endotoxin levels became elevated during reperfusion (146).

In the present study, pigs submitted to a relatively long period of ischemia followed by two periods of reperfusion did not have higher portal venous or systemic levels of endotoxin than controls. Further, there was no difference between portal venous and systemic levels of endotoxin. This came as a surprise, since it was expected that the insult was of such a magnitude to cause necrosis, providing bacteria and endotoxin an unchallenged pathway to the circulation. Peritoneal washings may well have been laced with endotoxin. Alternatively, translocation of endotoxin via a lymphatic route may be of particular importance in the pig. On the other hand, it is conceivable that pigs are not as susceptible as some of the other animal models studied to changes in intestinal permeability. Fink et al. assessed intestinal mucosal permeability in pigs submitted to progressive occlusion of the SMA over 210 minutes followed by reperfusion. While
mucosal permeability tended to increase during the period of ischemia, the increase did not become significant until after 60 minutes of reperfusion (109). While the degree of ischemia was surely greater than that seen by Fink and coworkers, the animals were not observed for 60 minutes following reperfusion in the present study.

Post-reperfusion circulatory collapse is often observed following a period of mesenteric ischemia and mortality is 90 - 100% if not treated aggressively (143). In this study, extremely variable but frequently severe hemodynamic derangements were observed following complete release of the SMA clamp. These changes were characterized by sudden systemic hypotension, occasional pulmonary hypotension, decreased systemic vascular resistance and a drop in cardiac output. During preliminary (unreported) studies, many of the animals died shortly after reperfusion. In the present study, animals were given relatively large volumes of fluid and death infrequently occurred within the study period in animals treated in this way. The cause of the hemodynamic derangements and death are poorly understood and therefore deserve comment. Post-reperfusion circulatory collapse has been attributed to sudden and severe endotoxemia, since prevention of endotoxemia prevents post-reperfusion shock and death in rabbits (186). The data from the present experiment do not support this hypothesis, for no significant endotoxemia was seen in association with reperfusion shock. Further, the hemodynamic changes observed were unlike those seen in endotoxic pigs given the same amount of fluids, who had significant pulmonary hypertension (previous experiment). Similarly, TNF could not be responsible for the hemodynamic derangements. Studies in rats suggest the primary cause of death is loss of plasma into the lumen of the gastrointestinal tract. Volume resuscitation with plasma prolongs life and hypoglycemia becomes the secondary cause of death. Finally, if glucose is administered, the tertiary cause of death in survivors is hyperkalemia which can be obviated to a degree by gastrointestinal lavage (292). Cardiotoxic factors released by the gut or pancreas may be a contributing factor (293), as well as acids, electrolytes and other unnamed toxins resulting from intestinal ischemia-reperfusion injury.
In summary, the data presented are consistent with TNF originating from an organ at least partially perfused by collaterals to the SMA, such as the duodenum, pancreas, liver and left colon. The author suggests that the pancreas and liver are likely sources of TNF. IL-6 appears to originate from the ischemic gut. However, net production of TNF and IL-6 by the splanchnic organs was not apparent from comparison of gut and liver efflux and influx. No evidence of transmigration of endotoxin from the gut to the portal circulation was found in this porcine model of intestinal ischemia-reperfusion injury.
Chapter 8
CONCLUSIONS

In the porcine model of septic shock described in this thesis, data obtained by tonometry suggested that the gut was ischemic, but the validity of tonometry as a measure of gut ischemia under these conditions is not known. Transmigration of endotoxin from the gut lumen to the portal circulation could not be demonstrated, but this does not preclude the existence of endotoxin transmigration via other routes, such as the transcelomic and lymphatic routes. While a dramatic cytokine response as reflected by elevated levels of TNF and IL-6 was observed, net production of TNF or IL-6 by the gut or liver was not demonstrated.

In the porcine model of mesenteric ischemia-reperfusion, transmigration of endotoxin from the gut lumen to the portal circulation was not demonstrated. This suggests that absence of ischemia is not an adequate explanation for the inability to demonstrate this phenomenon in the septic shock model.

In the porcine model of mesenteric ischemia-reperfusion, TNF appeared to originate in a splanchnic organ that was partially perfused by collaterals to the SMA, such as the liver, duodenum, pancreas or left colon. Net production or metabolism of TNF by the liver was not apparent when hepatic influx and efflux of TNF were compared, but observations of the appearance of TNF at different vascular sites prior to release of the SMA clamp suggested that the liver is a potential source. There was no evidence that ischemic gut is a source of TNF. IL-6 appeared to originate from the ischemic segment of gut. There was no evidence of hepatic production of IL-6.

The cytokine response seen with mesenteric ischemia-reperfusion is distinctly different from that seen during septic shock. While it is tempting to interpret this as evidence that gut ischemia was not present in the septic shock model, it is possible that splanchnic production of TNF and IL-6 during septic shock was masked by the production
of cytokines by numerous other cells in the body, including circulating granulocytes, lymphocytes and macrophages. This systemic cytokine response may be of considerable magnitude.
Chapter 9

SUMMARY: CLINICAL RELEVANCE, FUTURE DIRECTIONS

The role of the gut, endotoxin and cytokines in the pathogenesis of MODS is still unknown. If the gut is indeed the "motor" by which the inflammatory response to critical illness is perpetuated because of ischemic injury and/or release of endotoxin and various noxious inflammatory mediators, then therapy directed at the gut would hopefully prevent the pivotal events leading to MODS. Even if the gut is not an important factor in the pathogenesis of MODS, if endotoxin, TNF and/or IL-6 are important proximal mediators, their inhibition might potentially prevent the subsequent events leading to MODS.

It is crucial to determine whether the gut is ischemic during septic shock, for this has direct implications on potential treatment modalities. While irreversible decreases in gastric pH (as calculated by tonometry) are associated with a worse prognosis in critically ill patients (133), should our efforts be directed at preventing or treating gut ischemia? It is possible that changes in gut pH as calculated by tonometry do not necessarily reflect the presence of ischemia. Certainly, gut mucosal acidosis has been demonstrated in septic animals in the absence of mucosal hypoxia and ischemia (277). If the changes in tonometric parameters seen in septic shock and various other critical illnesses actually reflect derangements in physiologic processes other than ischemia, then it is clear that therapy must be directed at preventing or treating those pathophysiologically derangements. Because of therapeutic implications, it must therefore be determined with more clarity and with methods other than tonometry whether gut ischemia occurs during septic shock and whether gut ischemia contributes to the pathogenesis of MODS.

If gut ischemia proves to be an important event in the pathogenesis of septic shock, then effective therapies directed at this phenomenon must be developed. Intestinal mucosal ischemia persisting despite adequate resuscitation may be amenable to local measures of improving mucosal oxygenation. Encouraging results have been reported with direct
supply of oxygen to the mucosa via the lumen in experimental models of intestinal ischemia, but no clinical data is currently available. Intraluminal perfusion with oxygenated saline prevents histologically apparent mucosal injury in animals subjected to mesenteric occlusion (294). Delivery of oxygen by this method also prevents the reduction in intramucosal pH as measured by tonometry (295). Further, intraluminal oxygenation by oxygenated saline prevents the reduction in systemic blood pressure in the early postischemic (reperfusion) period (296). Intraluminal oxygenation with gaseous oxygen has been used in experiments on rats and has been shown to prevent the microscopic villous damage induced by occlusion of the superior mesenteric artery. Following release of the occlusion, the 48h mortality rate is also decreased in those animals treated by intraluminal treatment with gaseous oxygen (39% vs. 89%). Finally, histologic evidence of mucosal injury associated with septic shock is reportedly prevented by intraluminal perfusion of oxygenated saline (297). When this intervention is better characterized in the laboratory and when the indications in critically ill patients are better defined, this may be a potentially exciting prospect for the future, in the clinical arena.

The porcine model of septic shock described in this thesis simulates the physiological derangements seen in septic humans. Endotoxemia in other models of septic shock, as well as in humans, also emulate the pathophysiologic changes seen in septic shock. This demonstrates the importance of endotoxin, of exogenous or endogenous origin, in the pathogenesis of septic shock. Thus, therapeutic maneuvers inhibiting endotoxin activity may be of benefit in septic patients. Immunologic neutralization of endotoxin has been attempted by several investigators. To obviate the problem of narrow specificity with antibodies directed against O-antigenic determinants, research has focused on passive immunization using antibodies directed at highly conserved epitopes in the core structures of the LPS molecule (ie: lipid A and the core polysaccharides). Murine anti-core monoclonal antibodies have been shown to be protective against heterologous Gram negative infections in animal models. Human monoclonal anti-core HA-1A has been shown
to be protective in some animal sepsis models, but does not appear effective in murine models of septic shock (61). It is possible that the variable success seen with monoclonal anti-core antibodies in animal models of septic shock may not be observed in humans, since the efficacy may be determined by mechanisms that are not applicable to murine (or other) species. This has prompted some clinical trials of HA-1A. Ziegler et al. (298) conducted a multi-center, randomized, placebo-controlled, double-blinded trial of HA-1A. In the entire study population, consisting of critically ill patients, no treatment effect was observed. However, in a subset of 200 patients with culture-proven Gram negative bacteremia (analyzed retrospectively), the 28-day mortality decreased significantly, from 49% in the control group to 30% in the treatment group. A murine anti-core antibody, E5, has also been subjected to two prospective clinical trials. In the first trial (299), among 316 patients with Gram negative sepsis, there was no difference in the 30-day survival in placebo- and antibody-treated groups. However, a significantly improved mortality rate (30% vs. 43%) was seen in a subgroup of patients designated as "Gram negative sepsis without refractory shock". Refractory shock was defined as hypotension that did not respond to IV fluids or vasopressors. The second trial (61), designed to verify the treatment effect observed in the first trial in patients with Gram negative sepsis without refractory shock, failed to demonstrate a significantly improved survival following treatment with E5. While animal experiments and clinical trials of antibodies directed against LPS have not yet yielded consistent evidence of their efficacy, further studies are currently being done. Theoretically, it is possible that patients with Gram negative sepsis, by the time they present, are already subject to the cascade of events initiated by the presence of endotoxin. This may be the factor limiting the efficacy of treatments designed to neutralize the effects of LPS and therapeutic strategies targeting later events (eg: activation of components of the cytokine cascade) may therefore be more efficacious.

In this thesis, hematogenous transmigration of endotoxin during septic shock and mesenteric ischemia-reperfusion could not be demonstrated. While measurement of LPS
levels across tissue beds is a good initial approach to determining whether endogenous endotoxin plays a role in perpetuating the systemic inflammatory response, the negative results obtained do not completely rule out the importance of endogenous endotoxin in the pathogenesis of MODS. Several other avenues of investigation require examination. Firstly, other methods of measuring hematogenous transmigration of endotoxin in septic shock must be developed. In the experiment described in this thesis, the biological activity of LPS as measured by an LAL assay was used to determine endotoxin levels; there was no way to distinguish whether the endotoxin measured was of endogenous or exogenous origin. To circumvent this problem, infusion of radiolabelled LPS and measurement of radioactivity as a proportion of total bioactive LPS in all 3 vascular sites over time may be of interest. Secondly, the model described in this thesis was only observed during the acute phase of septic shock; transmigration may not occur until later. Observation for a longer duration following infusion of LPS may be required to detect the onset of endogenous LPS release. Finally, other routes of endotoxin transmigration must be investigated, for lymphatic or transcelomic migration of endotoxin may be the primary mechanisms by which endogenous endotoxin reaches the circulation.

Determination of the importance of endogenous endotoxin in the pathogenesis of MODS in the critically ill is crucial not only to the understanding of the pathogenesis of MODS, but also to planning new therapeutic strategies. Selective gut decontamination, eliminating Gram negative organisms, has been suggested for prevention of MODS. Prophylactic oral administration of nonabsorbable antibiotics prevents appearance of circulating LPS in primates during mesenteric ischemia-reperfusion (189) and prophylactic administration of antibiotics prevents the organ dysfunction associated with intestinal ischemia-reperfusion in dogs (145). Neither of the experiments described in this thesis support a role for selective gut decontamination, as endotoxemia of endogenous origin was not demonstrated. In critically ill patients, numerous studies, including prospective, randomized, controlled trials have attempted to determine the utility of selective gut
decontamination. In sum, while this therapeutic maneuver may reduce the risk of nosocomial pneumonia, mortality rate is not improved (47). Thus, unless experimental evidence strongly (and more consistently) suggests a potential benefit from gut decontamination or unless a subgroup of patients is identified that does benefit, the risks of selective gut decontamination will likely preclude further consideration of this therapeutic strategy.

TNF was elevated for a short time following acute administration of endotoxin and this finding was consequent with numerous hemodynamic derangements. Others have found administration of TNF to produce the same pathophysiological changes as those seen during septic shock (30)(32)(216)(247)(248). The fact that high serum levels of TNF confer a hemodynamic response not unlike that seen in endotoxemia demonstrates the pivotal role of TNF in the pathogenesis of septic shock. Its role in the pathogenesis of MODS has also been implied. Blockade of TNF is therefore a theoretically viable strategy in treating septic shock. Prophylactic administration of monoclonal antibodies to TNF prevent some of the physiologic changes in experimental sepsis and decrease mortality under these conditions (250)(251)(252). If administered after the onset of sepsis, these antibodies normalize some of the hemodynamic changes, reverse the metabolic acidosis, and ameliorate the profound neutropenia characteristic of septic shock in pigs (253). Clinical trials of anti-TNF antibodies are in progress. Preliminary results from one Phase I trial found the antibody to be safe and without acute side effects. In addition, treatment with anti-TNF is associated with increases in mean arterial blood pressure in patients with shock (300). In the model of sepsis described in this thesis, TNF was elevated for only a short time, quite soon after the administration of LPS. Perhaps more prolonged endotoxemia, as may be present in the clinical situation of Gram negative septicemia, would result in a more prolonged elevation in TNF and so the results presented in this thesis do not preclude a potential benefit from this intervention. Moreover, absence of elevated levels of TNF gives
little information on tissue levels of TNF, where antibodies to TNF may exert their most important protective effects.

Results from the ischemia-reperfusion experiment demonstrate that systemic levels of cytokines are not usually markedly elevated. Rather, cytokines are released in small amounts in injured organs, potentially activating a cascade of more directly detrimental events that may result in the manifestations of MODS. It will be important to document with more clarity whether this occurs in septic shock, for if prolonged cytokinemia, even at low levels, can activate subsequent events that lead to MODS, then perhaps this can be prevented by therapy directed at the source. Future experiments may address this issue using different methods than those used in the experiments described in this thesis. For example, the measurement of tissue levels of cytokines (ie: in the bowel, liver, spleen, lung, etc.) may help define the role of these organs in the production of these substances. There are several potential problems with this approach, however. The first is technical: the presence of proteases in the tissues results in the rapid degradation of cytokines in tissue, so great care would be required in the handling and analysis of tissues. In addition, the degree of cytokine production may have regional variations; such an experiment would thus be susceptible to sampling errors. Finally, and most importantly, the results would be very difficult to interpret. That is, the presence of cytokines in a tissue bed does not shed light on the origin of the peptides. Not only may cytokines be detected if produced locally; they would also be present if the organ is perfused by blood containing high concentrations of these substances, which would occur if the source of these cytokines were produced by an organ upstream. Thus, alternative and perhaps more sensitive methods for determining whether the gut is the source of cytokines will have to be developed.

Finally, while current developments of new therapeutic strategies appear to be directed toward prevention of the gut ischemia postulated to occur and to inhibiting mediators thought to be important in the pathogenesis of MODS, perhaps it is time to re-examine the hypotheses on which these new therapies are based. The role of endotoxin as
an important proximal mediator of remote organ injury during intestinal ischemia-
reperfusion was not directly addressed in this study, but the author's inability to
demonstrate significant endotoxemia during gut ischemia-reperfusion supported the view
that the presence of endotoxin in the circulation is not as important as previously thought.
Turnage et al. (146) and Koike et al. (149) addressed this question specifically and each of
these groups found that distant organ injury occurred following intestinal ischemia-
reperfusion by a mechanism independent of endotoxin. The results of Moore and associates
(169), in their study of critically ill trauma patients further shed doubt on the hypothesis
investigated in this set of experiments. Endotoxin was not detected in the portal vein and
simultaneously measured TNF and IL-6 levels in the portal and systemic circulations were
identical. Moreover, neither TNF nor IL-6 levels correlated with the development of
MODS. The results in our septic model are consistent with these findings. Thus, while
results from the experiments described in this thesis do not rule out the role of the gut as the
"motor" - a source of endogenous endotoxin and cytokine mediators perpetuating the
inflammatory response and ultimately resulting in MODS - they do not support this
hypothesis either.
Appendix A

TONOMETRY: ASSUMPTIONS AND LIMITATIONS

Gastrointestinal (GI) ischemia is associated with a high morbidity and mortality. Its symptomatology in the clinical arena is insensitive and nonspecific, often leading to a delayed diagnosis. Early recognition of mesenteric ischemia may improve prognosis and so the recent introduction of GI tonometry as a diagnostic tool is of great interest to clinicians.

Tonometry is based on the premise that ischemic tissues tend to become acidic. This may be due to a number of factors, including increased $H^+$ production from accelerated ATP hydrolysis, increased lactic acid production, and accumulation of $CO_2$. Measurement of mucosal pH (pHi) could therefore provide an indirect means of assessing the state of GI oxygenation. Gut mucosal pH is calculated by substituting the $PCO_2$ measured in the saline in the tonometer balloon (luminal $PCO_2$) and the simultaneously measured arterial bicarbonate in the Henderson-Hasselbalch equation (ie: $pHi = 6.1 + \log([HCO_3]/PCO_2)$).

For tonometry to reliably predict the presence of GI ischemia, a number of assumptions must be verified and these will each be discussed briefly.

1) Arterial [HCO3] closely approximates GI mucosal cell [HCO3].

Intramucosal bicarbonate concentration is assumed to be approximately equal to arterial bicarbonate concentration. While this may be true under conditions of normal splanchnic perfusion, the relationship may not hold when the gut is hypoperfused. Significant disparity between arterial [HCO3] and intramucosal [HCO3] calculated by direct measurement of intramucosal $PCO_2$ and pH have been observed by some investigators (301)(302). In these experiments, intramucosal [HCO3] was higher than arterial [HCO3]. In addition, arterial [HCO3] may decrease during metabolic acidosis secondary to any one of numerous causes that may be totally unrelated to the status of gut perfusion.
2) Increased luminal \( PCO_2 \) is due to increased \( CO_2 \) production and is therefore due to intestinal hypoxia.

Gut \( PCO_2 \) may increase because of increased \( CO_2 \) production or due to impaired clearance of \( CO_2 \). Increased gut \( CO_2 \) production implies gut ischemia. That is, under hypoxic conditions, there is decreased ATP synthesis and accelerated ATP breakdown, leading to increased \( H^+ \) production. In addition, organic anions such as lactate accumulate during anaerobic metabolism. These two factors result in intracellular accumulation of acids; buffering by accumulation of acids with intracellular \( HCO_3^- \) generates \( CO_2 \). Anaerobic decarboxylation will generate additional \( CO_2 \). Impaired clearance of \( CO_2 \), the other possible mechanism for increases in gut luminal \( PCO_2 \), may result from a reduction in splanchnic blood flow, even in the absence of tissue hypoxia. The increase in \( PCO_2 \) and, consequently, the decrease in calculated \( pHi \) may thus occur secondary to impaired perfusion even in the absence of an oxygen deficit.

3) Luminal \( PCO_2 \) is not influenced by other factors.

If tonometry is a specific indicator of GI ischemia, the luminal \( PCO_2 \) that it measures should not be affected by luminal or extraluminal factors. Experimental data suggest that this is not the case. Intraluminal contents affect \( PCO_2 \) by several mechanisms. The intraluminal contents may act as a diffusion barrier between the intestinal wall and the silicone balloon, for measured \( PCO_2 \) was reported to be significantly lower in a canine hemorrhagic shock model if adequate bowel cleansing was not achieved (303). In addition, intraluminal microorganisms may produce varying amounts of \( CO_2 \) and buffering of acids in the GI tract may also yield \( CO_2 \) (304). Extraluminal factors such as respiratory acidosis and alkalosis have also been observed to exert an influence over GI luminal \( PCO_2 \). Hyperventilation decreases intestinal \( PCO_2 \) (305). GI luminal \( PCO_2 \) therefore must be interpreted in the context of the systemic respiratory acid-base status and other GI influences.
4) All bowel segments have similar luminal PCO2.

In the clinical situation, tonometers are generally inserted into the stomach or the rectum, the most accessible sites in a patient. The assumption that the acid-base status of all segments of the GI tract are similar allows the clinician to extrapolate from measurements made in the stomach or rectum, making conclusions on the state of oxygenation of the entire GI tract. During occlusive mesenteric ischemia (e.g., during SMA occlusion), this assumption is clearly not correct. In addition, this may not necessarily be the case in all instances of nonocclusive mesenteric ischemia. For example, during hemorrhagic shock in dogs, there is considerable variability of luminal PCO2 in the stomach, ileum and sigmoid colon (303). On the other hand, in a porcine model of septic shock similar to the one used in the investigations in this thesis, the changes in pH in the stomach, ileum and rectum were similar (P.T. Phang, personal communication).

A.1 Is the Gut Ischemic During Septic Shock?

Ischemia is a condition of profound cellular energy crisis that is caused by a relative or absolute hypoperfusion. It is a dual defect: cellular hypoxia and cellular hypercarbia are both important components of this disorder (306). The assumptions on which conclusions are based on tonometric measurements have been discussed above. In addition, the possibility that tonometric changes observed in the first experiment of this thesis did not reflect gut ischemia were briefly addressed in the discussion accompanying the results of that experiment. This will be addressed in more detail in the context of the underlying assumptions of tonometric measurements.

In the first experiment presented in this thesis, intestinal mucosal acidosis was demonstrated in a porcine model of septic shock; this has also been reported by others in porcine models of sepsis (79)(127)(182). Several mechanisms for this phenomenon are possible. First, regional hypoperfusion may lead to impaired clearance of CO2 produced by normal aerobic metabolism, even in the absence of tissue dysoxia (306). Schlichtig and
Bowles, recognizing this, determined that anaerobic metabolism occurs in the presence of a mucosal PCO₂ > 65 mm Hg in nonseptic dogs and suggested that mucosal PCO₂ be used to detect ischemia, instead of mucosal pH (278). A second possibility is that sepsis leads to a large enough decrement in mucosal perfusion to cause cellular hypoxia. That is, gut mucosal acidosis results from excessive hydrolysis of adenosine triphosphate (ATP), since the rate of ATP synthesis supported by anaerobic metabolism is inadequate to meet the metabolic demands of the tissue (307). The notion that intestinal mucosal acidosis is secondary to ischemia is supported by the fact that sepsis is associated with decreases in gut perfusion at both the macrovascular (110)(127) and microvascular (182) levels. However, studies from a number of laboratories have been unable to document a decrement in transmesenteric oxygen uptake in various animal models of sepsis (79)(108)(110)(308). A third possibility is that intestinal mucosal acidosis during sepsis is caused by alterations in intermediary metabolism that are unrelated to changes in blood flow or tissue oxygenation, but nevertheless are associated with excess production of protons.

Normalization of mesenteric blood flow using volume expansion or administration of dobutamine ameliorates but does not prevent the development of mucosal acidosis in endotoxic pigs (110). This suggests that mechanisms other than global mesenteric hypoperfusion may be responsible for mucosal acidosis in this model. One such mechanism may be dysregulation of perfusion at the microvascular level. That is, intestinal villi may be particularly sensitive to small perturbations in flow because the anatomic architecture of the villous vasculature can lead to diffusional arteriovenous shunting of oxygen (275). However, Vander Meer et al. (277) clearly showed that ileal mucosal acidosis occurred in the absence of mucosal hypoxia and ischemia in septic pigs. These results are consistent with others who have shown disturbances in cellular metabolism in the absence of hypoxia during sepsis. For instance, Hurtado and coworkers found that lactic acidosis develops in the skeletal muscle of septic rabbits despite normal tissue oxygenation (309). Uncoupling of oxidative phosphorylation (126), inhibition of
mitochondrial respiration (310) and reduced availability of substrates for oxidative metabolism (311) are some of the derangements in cellular energy metabolism that have been demonstrated in various models of sepsis and these may help explain how acidosis may occur in the intestinal mucosa even in the absence of ischemia. On the other hand, the findings of Vander Meer et al. are not consistent with those of Vallet and coworkers (312) who demonstrated significantly decreased intestinal PO$_2$ in dogs given a large dose of endotoxin. This may be due to differences in species, for acute endotoxemia in dogs leads to hemorrhagic necrosis of the intestinal mucosa (312). In pigs, endotoxemia results in only minimal histologic changes in intestinal histology (182). Further, in humans, hemorrhagic necrosis rarely, if ever, occurs during septic shock (277).

Use of tonometry requires acceptance of a number of assumptions. While ileal mucosal acidosis is a consistent finding by many investigators using a porcine model of sepsis, there is very little evidence that intestinal ischemia is present under these conditions. Determination of the oxygen delivery and oxygen consumption when the gut becomes reliant on anaerobic metabolism, or defining the critical point, will undoubtedly aid in defining whether ischemia is indeed an important factor during sepsis.
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