ENTERAL FEEDING DECREASES GUT APOPTOSIS, GUT PERMEABILITY, AND PULMONARY INFLAMMATION DURING MURINE ENDOTOXEMIA

by

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ABSTRACT

Background: The systemic response to sepsis is associated with gut mucosal damage, increased permeability, and subsequently increased inflammation in distant organs. Enteral feeding may reduce mortality from multiple organ failure by maintaining the gut mucosal barrier, however the mechanism of this is unclear. We tested the hypothesis that endotoxemia is associated with increased gut apoptosis, increased gut permeability, and increased pulmonary inflammation. Furthermore, we postulate that enteral feeding ameliorates endotoxin's effect on these parameters.

Methods: Four groups of 10 male CD-1 mice were studied: fed/sham, fasted/sham, fed/endotoxemic, fasted/endotoxemic. Fasted mice were denied food for 16 hours prior to injection of either saline or E. coli endotoxin. Fed animals received rodent chow throughout. Six hours post injection the gut and lungs were removed and frozen. We assessed gut apoptosis by measuring gut caspase 3, caspase 6 and PARP enzymatic activity. Gut permeability was quantified by measuring the transfer of fluorescein labeled dextran (FD-4) across the gut wall, and pulmonary inflammation was quantified by lung interleukin 6 (IL-6) and macrophage inflammatory protein-2 (MIP-2) ELISA.

Results: 1) Apoptosis - After LPS injection, gut caspase 3 and 6 activity increased by 4.9 fold and 4.5 fold respectively ($p < 0.001$); fed mice given endotoxin had 40% less caspase 3 activity and 20% less caspase 6 activity than fasted mice given endotoxin. Gut PARP activity was 15% higher in endotoxemic mice than controls ($p < 0.05$) 2) Gut permeability - Endotoxemic mice had 44% ($p < 0.05$) more FD-4 present in their serum than the sham mice;

fed mice given endotoxin had 36% (p<0.05) less FD-4 than the fasted animals given endotoxin. 3) Pulmonary inflammation - After LPS injection, pulmonary IL-6 increased by 7.4 fold (p<0.001); fed animals had 40% less IL-6 than the fasted (p<0.05) mice given endotoxin. Lung MIP-2 increased by 292.1 fold (p<0.001) after LPS injection; fed animals had 35% less MIP-2 than their fasted counterparts within the endotoxemic group (p<0.05).

Conclusions: Gut apoptosis, gut permeability, and pulmonary inflammation are increased during endotoxemia. Enteral feeding decreases gut apoptosis, and this is associated with improved gut barrier function and decreased pulmonary inflammation. Enteral feeding may decrease the systemic inflammatory response by maintaining gut mucosal function during endotoxemia.

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INTRODUCTION

1.1   OVERVIEW

The cost to society of the systemic inflammatory response syndrome (SIRS), sepsis, and multiple organ failure is staggering. Current estimates indicate that there are over 500,000 cases of sepsis in North America annually, with an estimated crude mortality of 35% (21, 240). Fully 25% is mortality directly attributable to sepsis, with 10% of patients dying as a result of underlying disease (239). Septicemia, the now clinically defunct term that is still being used for recording vital statistics, is the 13th leading cause of death in the United States, and imparts a financial cost of $5 to $10 billion US per year (194). Further, multiple organ failure is the number one cause of death in surgical intensive care units, representing 50% to 80% of all such deaths, at an average cost of over $150,000 US per patient (43).

The incidence of sepsis and SIRS has been rising for the last 20 years, yet despite the efforts of thousands of dedicated researchers, and several innovative clinical trials of novel therapies, the mortality rates of patients with established multiple organ failure have not appreciably improved (43). Thus, as spectacular advances are made in the field of organ transplantation in the therapy of patients with end-stage single organ failure, our inability to successfully treat patients with acute multiple organ failure remains a serious hurdle in the care of critically ill postoperative or posttrauma patients.

When multiple organ failure was recognized to be a discrete entity almost 30 years ago, physicians began a quest to find the "magic bullet" that would allow for the directed treatment of sepsis and subsequent end organ failure. Unfortunately, further investigation into the mechanisms of sepsis and SIRS revealed that such processes were incredibly complex, and thus it was unlikely that a single therapy could or would suffice in the treatment of such a heterogeneous group of patients. This has been borne out by the failure of numerous recent clinical trials of innovative therapies intended to reduce the mortality of sepsis (180).

By its very nature, sepsis is a systemic illness, which involves the triggering of a systemic inflammatory response, the magnitude of which is a balance of pro and anti inflammatory processes. A complex interaction exists between cellular and humoral immunity, with the goal being to elicit a systemic response sufficient to rid the body of the triggering factor (be it bacterial, viral or otherwise) without damaging vital organs in the process. Clearly, this is an imperfect mechanism, and thus the progression from sepsis to multiple organ failure is not an uncommon occurrence. This generally occurs as a result of excessive stimulation of host cells, leading to excessive cytokine production. In order to elicit a systemic inflammatory response in a previously healthy patient, an inciting factor or event must be present. Half of all cases of sepsis are caused by gram-negative rods (194). In fact, 50% to 60% of gram-negative bacteremias and 5% to 10% of gram positive or fungal bloodstream infections result in septic shock (180). One of the most fascinating aspects of SIRS is the fact that it can and does exist in the absence of any documented infection. Clinically, blunt trauma is a well known cause of SIRS, however the mechanism by which this occurs is poorly understood.

Although infection is not a prerequisite for SIRS, the most widely described trigger of the systemic inflammatory response is the bacterial endotoxin that exists as a lipopolysaccharide moiety in the outermost membrane of gram-negative bacteria (167). It is often referred to simply as endotoxin, or by the acronym for lipopolysaccharide, LPS. Host monocytes and macrophages bind the lipid A moiety of LPS by way of cell surface receptors including CD-14, and the $\beta2$ (CD11/CD18) leukocyte integrins (102). This specific binding leads to the synthesis and release of proinflammatory cytokines, arachodonic acid metabolites, and various other mediators, which in turn mediate the systemic toxicity. Activation of the septic cascade can result in the liberation of mediators that directly alter vasomotor tone. Nitric oxide (NO), bradykinin, histamine, and prostacyclin are all capable of decreasing vascular tone and producing hypotension (171, 223).

While it was once thought that the hypotension seen in septic shock was the primary cause of multiple organ failure, it is now recognized that even if kept normotensive, many patients with SIRS progress on to multiple organ failure. It is now believed that end organ failure occurs as a result of complex mechanisms affecting the organs at the microvascular and cellular level. Such mechanisms include capillary endothelial cell damage, "sludging" of red blood cells, platelets, and leucocytes, and adhesion and aggregation of leukocytes to specific endothelial cell receptors. These events are believed to adversely affect end organs by causing hypoxia and thus anaerobic metabolism, and acting as triggers of cellular dysfunction, and programmed cellular death, which is known as apoptosis.

While the host response to endotoxin as a trigger of systemic inflammation is well characterized, factors that allow for the propagation of such a response are not. In the mid 1980's, studies done on rodents showed that during sepsis, viable bacteria translocated from the gut lumen to the mesenteric lymph node (47, 49). This was thought to be due to sepsis induced gut mucosal damage, and led to the theory that the gut may act as an "engine" that drives and propagates the systemic inflammatory response. In addition, it was becoming increasingly recognized that feeding critically ill patients enterally, as opposed to parenterally, may act to maintain the barrier and immune functions of the gut mucosa (44, 45, 120). Subsequent clinical studies have shown that enteral feeding is indeed a powerful intervention that has been shown to reduce septic complications in critically ill trauma patients (118, 163, 164), and thus enteral feeding has supplanted parenteral feeding as the nutrition route of choice in most intensive care units worldwide.

Despite the fact that enteral feeding has been so widely accepted as being beneficial in septic and critically ill patients, the exact mechanism by which enteral feeding improves outcomes is not well understood. While significant discoveries have been made regarding the molecular mechanisms of the systemic inflammatory response syndrome, and targeted therapies developed and studied as a result of such research, a relatively small body of work exists which investigates the mechanism of gut dysfunction during sepsis, and how enteral feeding affects this. Given that enteral feeding has been shown to be a more useful and important intervention than any of the targeted sepsis therapies studied to date, it seems intuitive that further study into exactly how this inexpensive, readily available treatment works is warranted.

In this thesis, the effect of endotoxemia and enteral feeding on gut apoptosis, gut function, and distant organ inflammation is studied in a murine model of endotoxemia. The literature pertaining to this field of endeavor is discussed in detail below.

1.2 SEPSIS AND THE SYSTEMIC INFLAMMATORY RESPONSE SYNDROME

1.2.1 Definitions

In order to aid in bedside diagnosis and standardize research protocols, the following definitions were established at the American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference in August 1991 (19) (Figure 1).

Systemic inflammatory response syndrome (SIRS) - Term developed to describe systemic inflammation independent of cause. SIRS can be seen following a wide variety of insults, and included but is not limited to, more than one of the following clinical manifestations: (1) a body temperature greater than 38°C or less than 36 °C; (2) a heart rate greater than 90 beats per minute; (3) tachypnea, manifested by a respiratory rate greater than 20 breaths per minute, or hyperventilation, as indicated by a $PaCO_2$ of less than 32 mmHg; and (4) an alteration in the white blood cell count, such as a count greater than 12,000/cu mm, a count less than 4,000/cu mm, or the presence of more than 10% immature neutrophils ("bands"). Furthermore, such physiologic changes should represent an acute alteration from baseline in the absence of other known causes for such abnormalities. When the inflammatory process is due to infection, sepsis and SIRS are synonymous terms.
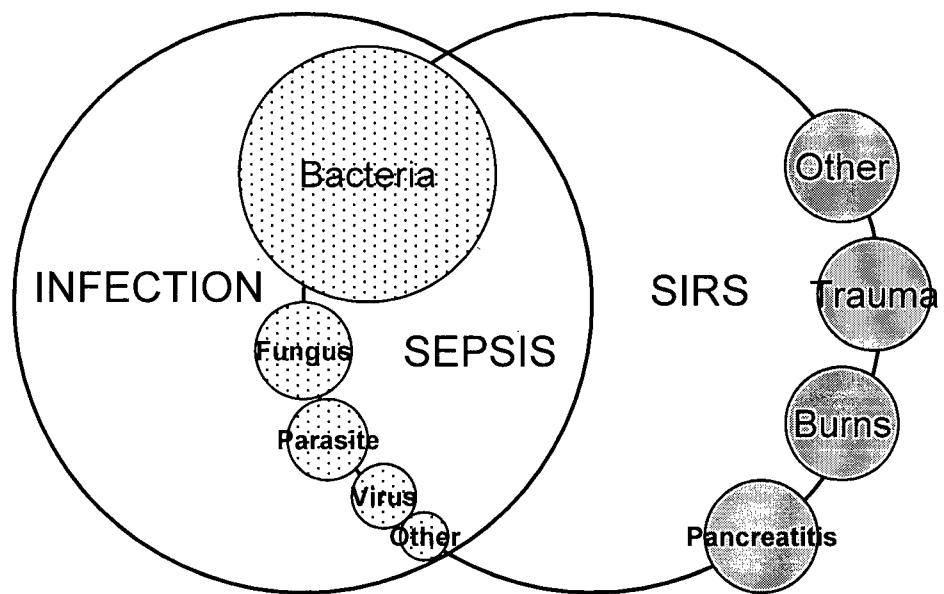
Figure 1.   Sepsis and SIRS

This pictogram illustrates the differences between the terms described by the American College of

Chest Physicians/Society of Critical Care Medicine Consensus Conference (ACCP/SCCM)

Sepsis – The term used when SIRS is the result of a confirmed or suspected infectious process. The term *sepsis* therefore, represents the systemic inflammatory response to the presence of infection.

Infection – a microbial phenomenon characterized by an inflammatory response to the presence of microorganisms or the invasion of normally sterile host tissue by those organisms.

Bacteremia – the presence of viable bacteria in the blood. The presence of viruses, fungi, parasites, and other pathogens in the blood should be described in a similar manner.

Septicemia – was a term used in the past to define the presence of microorganisms or their toxins in the blood. However, this term has been used clinically and in the medical literature in a variety of ways, which had added to confusion in data collection and interpretation. This term, therefore, has been abandoned.

Severe sepsis – sepsis associated with organ dysfunction, hypo perfusion abnormality, or sepsis induced hypotension.

Sepsis-induced hypotension – the presence of a systolic blood pressure of less than 90 mm Hg or its reduction by 40 mm Hg or more from baseline in the absence of other causes for hypotension.

Septic shock – a subset of severe sepsis. Defined as sepsis-induced hypotension persisting despite adequate fluid resuscitation, along with the presence of hypoperfusion abnormalities or organ dysfunction.

Multiple organ dysfunction syndrome (MODS) – this term replaces the previously used term *multiple organ failure,* with the reasoning being that "dysfunction" identifies a process, which is a continuum, in which organ function is not capable of maintaining homeostasis. It is intended to identify relative organ dysfunction, which can be qualified, rather than simply observing organs as either functioning or failing. The term *syndrome* was introduced to refer to a pattern of multiple and progressive symptoms and signs that are thought to be progressively related. MODS may be described as either primary or secondary, as MODS develops by two relatively distinct, but not mutually exclusive pathways. Primary MODS is a direct result of a well-defined insult in which organ dysfunction occurs early, and can be attributable to the insult itself. Secondary MODS is more common, and develops as the consequence of a host response. It usually evolves after a latent period following the triggering injury or event.

1.3   FEEDING IN CRITICAL ILLNESS

1.3.1   Overview

There is an emerging consensus that early nutrition support benefits critically ill patients by decreasing septic morbidity, maintaining immunocompetence, and improving wound healing (4, 22, 29, 125, 161, 184, 242). The indications for providing nutrients by enteral or

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parenteral (intravenous) route are not well defined, and the efficacy of nutritional support in many circumstances is unproven (210). Nonetheless, nutritional support is widely used for several reasons: malnutrition is common in hospitalized patients (17, 18), there is an association between malnutrition and increased morbidity (30, 243) and mortality (94), it seems intuitively likely that well-nourished patients will respond most favorably to treatment, nutritional support can be administered safely to most patients, and clinical trials indicate that it is beneficial in selected patients (7, 163, 168). The optimal route of substrate delivery (enteral *versus* parenteral), however, continues to be the subject of debate (1, 24, 117, 121, 147, 164, 186, 201).

1.3.2 Enteral Feeding

Enteral feeding refers to the act of feeding directly into the gastrointestinal (GI) tract, either orally or by feeding tube placed in the stomach or proximal small intestine. This involves the placement of either a nasoenteric feeding tube, or an operatively placed feeding tube, such as a gastrostomy or jeujenostomy. In contrast to enteral feeding, total pareneral nutrition (TPN) is given entirely intravenously, bypassing the GI tract. The term "enteral" refers only to the route of nutrition, and not to the specific type of food and/or nutritional supplement that is ingested.

Generally speaking, if a patient is capable of tolerating oral intake, that is the preferred route of nutrition. If a patient is incapable of tolerating adequate volumes of oral intake, as is common in critical illness, a decision must be made as to how best to provide nutrition. Traditional arguments favoring the enteral route have been that it provides a safe, convenient,

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and cost effective means of providing nutrition, but fear of GI intolerance has discouraged it's use in the postoperative and critically ill patient (108). Recently, however, basic and clinical research has provided compelling physiologic benefits of enteral feeding. A discussion of the benefits of enteral feeding over parenteral feeding follows shortly.

1.3.3 Parenteral Feeding

Parenteral, or intravenous feeding is a method of nutritional support in which substrate is administered directly into the circulation by way of central venous catheter. This route of nutrition is not reliant on the GI tract for absorption, and is thus very useful in selected cases where enteral feeding cannot be tolerated. Examples of such circumstances include severe ileus, short gut syndrome, high volume diarrhea, enterocutaneous fistula, pancreatitis, and severe inflammatory bowel disease.

Total parenteral nutrition (TPN) has a number of drawbacks that made it less desirable than enteral nutrition for those patients that can tolerate being fed enterally. Due to the expertise required in mixing, prescribing and delivering TPN, its availability is limited to large and medium sized centers. As a result of this, and the fact that frequent bloodwork need be done, TPN is a very expensive way to provide nutrition. In order to administer TPN, a central venous catheter must be placed. Complications related to placement and maintenance of such intravenous access are common, and can be life threatening. These include bleeding, pneumothorax, and line sepsis. Further, it is well recognized that prolonged TPN can lead to severe metabolic derangements and liver failure, which can result in death. In addition to the obvious primary drawbacks of parenteral nutrition, it is now recognized that the route of

nutrition carries an importance far beyond that of which was previously recognized. TPN leads to decreased microvillus height (227), decreases in brush-border hydrolase (83) and nutrient transporter activity (103), and an increase in mucosal permeability (103). It has also been suggested that TPN may amplify the metabolic derangements that develop during sepsis (75).

1.3.4 Enteral vs. Parenteral Feeding

The recommendation that the enteral route be used preferentially for nutritional support is based on cost information (24) and on studies of injured patients that demonstrate the superiority of enteral over parenteral nutrition (118, 160, 163, 164). Compared to TPN, enteral feeding has been shown to decrease the incidence of postoperative pneumonia, intraabdominal abscesses, and catheter related sepsis in blunt and penetrating abdominal trauma patients (118). Similar results have been found in patients with burns (4) and head injuries (80). These clinical findings are supported by basic science research in animals, which demonstate the importance of luminal feedings in supporting the body's mucosal immune system (119, 129).

It is well recognized that TPN and/or lack of enteral feeding leads to gut mucosal atrophy (227). With the resumption of oral alimentation, brush border enzyme activities and microvillus height return to normal values within one week (83). Interestingly, The splanchnic response to endotoxin seems to be exaggerated in normal subjects fed parenterally as compared with those fed enterally (75). The route of feeding appears to be more important than the amount of nutrition provided (118), and outcomes correlate with enteral

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protein intake in injured patients (22). This suggests that even if a patient's calorie and nitrogen requirements cannot be met with enteral nutrition, the enteral route of feeding should be used, unless it is specifically contraindicated.

1.4 THE GUT IN SEPSIS

1.4.1 Normal gut mucosal barrier function

The gastrointestinal (GI) tract functions to absorb nutrients and support important immunologic defenses during health and illness. Under both experimental and clinical conditions in animals and humans, gut stimulation abrogates the body's response to stress by reducing gut mucosal atrophy and permeability (60). In addition, gut stimulation prevents atrophy of the gut-associated lymphoid tissue (GALT), which acts as a primary defense system. GALT consists of Peyer's patches and lymphocytes, and is a major producer of IgA. This antibody prevents the adherence of microbes to mucosal surfaces, and combats an essential first step for systemic microbial invasion.

The gut mucosa acts as the physical and physiological barrier between the systemic circulation and the external environment of the gut lumen (Figure 2). The mucosal barrier consists of both intrinsic and extrinsic defenses. The intrinsic component is made up of the physical and structural properties of the small intestine, while the extrinsic mechanism consists of mucus, humoral factors, resident bacteria, and mucosal immune barrier itself.
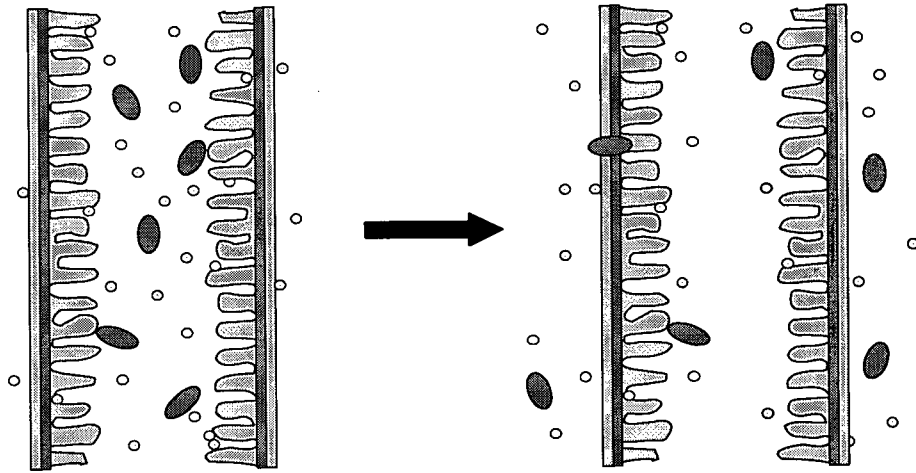
Figure 2.   **Gut Mucosal Barrier Dysfunction**

This figure illustrates the loss of selectivity seen in the gut mucosal barrier during SIRS and sepsis. A longitudinal cross section of small intestine is depicted. The small circles represent macromolecules such as endotoxin, and the larger ovals represent whole bacteria translocating from the gut lumen to the systemic circulation.

Intrinsic Defense

Due to the presence of microvilli, the human GI mucosal surface has a surface area of 300 to 400 square meters. One cell layer thick, the mucosal surface is composed of several cell types, including columnar cells, goblet cells, Paneth cells, enteroendocrine cells, and intraepithelial lymphocytes. This cell layer is protected by a coating of mucus, which prevents direct contact between the cells and the external environment. Lying beneath the mucosal cell layer, the lamina propria contains lymphocytes, macrophages, and dendritic cells, which seem to be crucial to the GI immune response (60).

Enterocytes are the absorptive epithelial cells that make up the vast majority of the mucosal epithelium. These cells form a continuous columnar monolayer, and line the crypts and villi. The enterocytes are attached at their apices by tight and gap junctions as well as desmosomes, which form a selective barrier between the internal and external environments (84).

Enteroendocrine cells are scattered as individual cells throughout the mucosal epithelium. Such cells contain secretory granules in their basal cytoplasm, and release their products into the lamina propria as well as the gut lumen. In addition to effecting gut barrier function by increasing the production of mucin and IgA, these cells also play a neuroendocrine and enteroendocrine role by releasing such substances as serotonin, glucagon, cholecystokinin, gastrin, secretin, motilin, neurotensin, substance-P and endorphins (60).

Intraepithelial lymphocytes are T cells found throughout the mucosal epithelium. These cells are about one third as numerous as epithelial cells, and 80% of them express the CD8+ cytotoxic/suppressor phenotype (25, 68). The precise function of intraepithelial cells is a topic of some debate, but they have been implicated in the modulation of enterocyte apoptosis, as well as playing a role in cytokine secretion, cytotoxicity, and the alteration of mucosal permeability (60).

Paneth cells are generally found in the crypts of the small intestine, and contain various antibacterial secretory granules of lysozymes, defensins, and phospholipase A2. This, combined with their ability to phagocytose immune-coated bacteria, suggest that Paneth cells play a defensive role in the gut barrier (87, 188, 198).

Extrinsic Defense

The luminal surface layer of the gut contains glycocalyx, which is composed of a number of enzyme systems and proteins that are involved in digestion and absorption of nutrients. The glycocalyx is a continuous glycoprotein coat that is adherent to the outer surface of the microvillus membrane. This coat aids in the protection of the apical membrane, as it is resistant to mucolytic and proteolytic agents. The glycocalyx is an integral part of the plasma membrane.

Goblet cells are the mucus secreting cells of the intestinal epithelium. This mucous layer is 30 to 50 microns in thickness, and is believed to prevent mechanical abrasions and adherence

of bacteria to epithelial cells (111, 200). The viscosity of the mucus prevents pathogenic penetration by undesirable microbes (215), and disruption of this layer leads to bacterial overgrowth and increased adherence to the mucosal epithelium (10). As an additional defense, several of the glycoproteins and glycolipids found in the mucus layer are closely related to glycoproteins and glycolipid receptors of the mucosal surface. This is believed to allow for competitive binding of pathogenic bacterial within the mucus layer, preventing attachment to the mucosal epithelium (139, 241).

Resident intestinal microflora play a protective role in gut defenses by preventing the overgrowth and bacterial translocation of pathogenic organisms, and this is diminished with antibiotic administration (230-232). In the small intestine, obligate anaerobes greatly outnumber enteric gram-negative and aerobic gram-positive bacteria, and appear to be responsible for the prevention of overgrowth in the GI tract. These obligate anaerobes form a barrier limiting direct contact between pathogenic bacteria and intestinal epithelium, and may inhibit the growth of coliforms via production of volatile fatty acids (127). Normal intestinal microflora also release low molecular weight substances (LMW) that prime the host immune response (192). Disruption of the anaerobic barrier by the use of broad spectrum antibiotics facilitates overgrowth and attachment of pathogenic bacteria to the intestinal epithelium due to the loss of these LMW substances.

GALT

The gut associated lymphatic tissue (GALT) comprises a large volume of lymphoid tissue that lines the moist mucosal surfaces of the body (124). The GI tract is one of the richest

lymphoid tissues in the body, containing 75% of all the immunoglobulin producing cells. The GALT consists of three components: Peyer's patches, the lamina propria lymphoid cells, and the intraepithelial lymphocytes. CD4+ cells and B cells are predominant in the lamina propria, whereas intraepithelial lymphocytes are mostly CD3+ T cells that express the CD8+ phenotype (cytotoxic/suppressor) (25).

The GALT is comprised of two functional arms: the effector and the affector arms (182, 183). The effector arm consists of Peyer's patches, where naïve B and T cells are sensitized to luminal antigens presented by antigen presenting cells. M cells, which are located on the luminal surface of Peyer's patches, endocytose, transport, and release antigens to dendritic cells, B cells and macrophages, which function as antigen presenting cells. The antigen presenting cells process and present these antigens to CD4+ T helper lymphocytes, and once sensitized, these T helper cells induce the development and isotype switching of IgM plasma cells to become IgA secreting plasma cells (209). Cytokines play a crucial role in this process. Once activated, the B and T cells migrate to the mesenteric lymph nodes, proliferate, and enter the systemic circulation by way of the thoracic duct. Once in the circulation, these cells migrate to the lamina propria and intraepithelial spaces of the small intestine where they serve as the effector arm by producing secretory IgA against the specific antigen. IgA prevents the adherence of microbes to the mucosal epithelium to defend against systemic invasion (172, 195).

The GALT is sensitive to the route and type of nutrition, and lack of enteral feeding during TPN results in a significant reduction in GALT cells within the Peyer's patches, lamina

propria, and intraepithelial spaces (129). This reduction in GALT cell mass is paralleled by reductions of intestinal and respiratory IgA levels (114, 130). There is a functional relationship between the GALT and other mucosal surfaces, and reduction of GALT cell numbers within the GI tract leads to a loss of established IgA mediated defenses within the respiratory tract (114). The same IgA mediated defenses are maintained with enteral feeding, or by the administration of the neuropeptide, bombeisin (105).

1.4.2 Gut Dysfunction in Sepsis

1.4.2.1 The gut as an "Engine" in sepsis and SIRS

In a 1965 review article, Jacob Fine concluded that refractory traumatic shock was caused by the absorption of bacteria and endotoxin from the gut, in conjunction with shock-induced impairment of the ability of the hepatic reticuloendothelial systems to detoxify the portal venous effluent, which potentiated the systemic spread of bacteria and LPS (70). Due to lack of collaborating experimental and clinical data, this theory faded from the literature until the mid 1980's, when experimental work revived this concept. Dietch and Berg performed a series of experiments that elucidated the mechanisms that regulate "bacterial translocation" (53), and popularized that term in the literature. Subsequent studies by them demonstrated that hemorrhagic shock caused bacterial translocation to mesenteric lymph nodes in rodent models. With increasing severity of shock, bacteria were cultured from the liver and spleen, and ultimately bacteria and LPS were recovered from the systemic blood. This early work led to the concept that gut dysfunction acts as an "engine" that drives the post injury systemic inflammatory response (Figure 3).
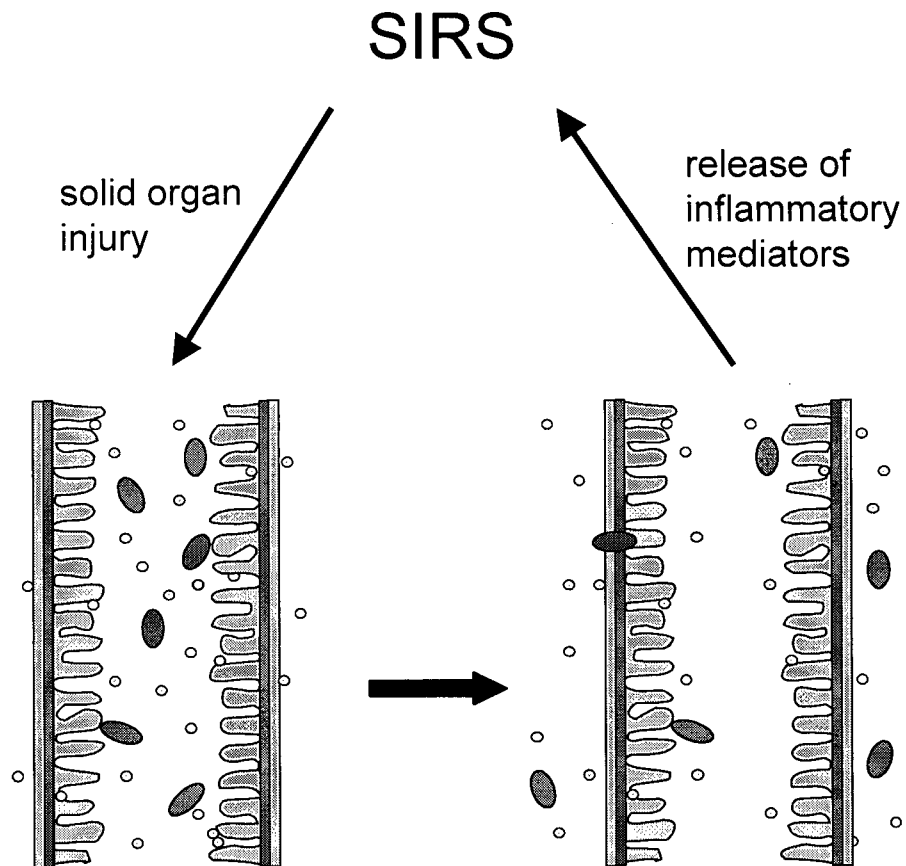
Figure 3.   Gut Dysfunction as the "Engine" of SIRS

This figure illustrates the role of the gut in propagating the systemic inflammatory response. Gut damage during sepsis leads to the release of inflammatory mediators, which fuel the systemic inflammatory response, which leads to solid organ injury.

It is now generally accepted that bacterial translocation itself is not the sole, or even a major effector in post-injury multiple organ failure. However, laboratory and clinical research strongly suggest that the gut plays a pivotal pathogenic role in the propagation of SIRS and MODS. On the basis of their own observations, and those of others, Moore's group reported this year (88) that they believe the following with respect to the gut driving post-injury multiple organ failure: 1) shock with resulting gut hypoperfusion is an important inciting event, 2) the reperfused gut is a source of proinflammatory mediators that can amplify the early systemic inflammatory response and thus contribute to early multiple organ failure, 3) early gut hypoperfusion causes an ileus in both stomach and small bowel that sets the stage for progressive gut dysfunction so that the proximal gut becomes a reservoir for pathogens and toxins that contribute to late sepsis-associated multiple organ failure, and 4) late infections cause further worsening of this gut dysfunction. Thus, he believes the gut can be both an instigator and a victim of multiple organ failure. While the precise mechanisms and sequence of events is debatable, it is clear that gut dysfunction does play an important role in the propagation of the post-injury systemic inflammatory response.

1.4.2.2 Bacterial Translocation

In addition to it's role in nutrient absorption, the intestinal mucosa functions as a major barrier that prevents bacteria colonizing the gut from invading systemic organs and tissues. Based on epidemiologic studies, the mucosal barrier to bacteria appears to be lost under certain circumstances, resulting in systemic sepsis and multiple organ failure (22, 27, 51, 88). Using rodent models of hemorrhagic shock, burn injury, endotoxemia, and obstructive jaundice, it has been reproducibly demonstrated that bacteria normally contained within the

gut lumen can cross the mucosal barrier and survive in the mesenteric lymph nodes and other organs (9, 47, 54, 136). Subsequent work in humans has shown that bacterial translocation occurs as a result of intestinal obstruction as well (46).

Factors that promote bacterial translocation from the gut include disruption of the indigenous gastrointestinal microflora leading to bacterial overgrowth, impaired host immune defenses, and physical disruption of the gut mucosal barrier (53). The precise mechanisms of bacterial translocation have yet to be elucidated. Most of the research performed to this end has focused on the gut's response to endotoxin with or without various dietary and/or stress modifications. The combination of a nonlethal dose of endotoxin plus a protein-malnourished state or a nonlethal thermal injury results in lethal sepsis (48, 57). It is known that endotoxin-induced bacterial translocation requires the presence of the terminal core lipopolysaccharide moiety, and that xanthine oxidase-generated oxidants are important in the pathogenesis of endotoxin-induced mucosal injury and bacterial translocation (52). Furthermore, it has been shown that bacterial translocation is inhibited in inducible nitric oxide synthase (iNOS) knockout mice after endotoxin challenge (157), and that endotoxin-induced mucosal injury and bacterial translocation are associated with iNOS activity and nitric oxide production (158). These findings suggests that physical disruption of the mucosal barrier by highly reactive oxidant species may play a key role in allowing for bacterial translocation.

In order to determine the effect of diet on bacterial translocation, Deitch performed a series of experiments on stressed and nonstressed mice. Knowing that starvation and protein malnutrition disrupts the normal indigenous GI tract microflora and impairs host antibacterial

defenses, Deitch set out to study the effect of these interventions on bacterial translocation. He found, somewhat surprisingly, that neither starvation nor protein malnutrition promoted bacterial translocation, despite the fact that the ecology of the gut microflora was disrupted (56). In a follow up study comparing mice with 25% total body surface area burns to unburned mice, in the presence and absence of endotoxin, it was found that protein malnutrition did not promote bacterial translocation from the gut in either burned or unburned animals (48). However, a nonlethal dose of endotoxin promoted bacterial translocation to the mesenteric lymph nodes in burned and unburned mice, but only in burn mice did the bacteria translocate from the gut to other systemic organs. More recent work has shown that the synergistic effect of endotoxin plus protein malnutrition on bacterial translocation is not primarily related to failure of the gut mucosal barrier, however it is clear that protein-malnourished mice are less able to clear translocating bacteria than normally nourished mice (131)

Experimental work on mice suggests that oral intake of nondigestable fiber or bulk forming agents prevents bacterial translocation induced by oral administration of total parenteral nutrition solution (212). Although the mechanism of this is not clear, this finding has been reproduced. In an experiment comparing three groups of rats (regular food, IV total parenteral nutrition, and oral total parenteral nutrition), Spaeth demonstrated that food without fiber promoted bacterial translocation from the gut (211). A separate arm of that same experiment compared the IV/PO TPN groups with or without oral cellulose powder. The results showed that oral administration of cellulose fiber helps to maintain intestinal barrier function and prevent bacterial translocation even in the absence of oral nutrients. The

presumed mechanism of this is that the cellulose acted to prevent IV-TPN or PO-TPN induced alterations in mucosal structure, although the reason for this has yet to be described. Interestingly, the addition of oral glutamine to the diet of animals receiving elemental PO-TPN does not prevent the observed bacterial translocation or immunosuppression, which supports the idea that lack of nutrients alone is not enough to cause bacterial translocation under normal conditions (246).

1.4.2.3 Macromolecular Permeability

The intestinal epithelium normally functions as a highly selective barrier, permitting the absorption of nutrients, electrolytes, and water, but restricting the passage from the lumen of larger, potentially toxic compounds and/or microbes. In addition to bacterial translocation, administration of LPS to experimental animals causes an increase in gut permeability to various hydrophilic solutes (34, 55, 73, 203). Intestinal permeability is also increased in human volunteers injected with LPS and in septic patients (176, 252). Although it is postulated that increased gut macromolecular permeability plays a role in propagating the systemic inflammatory response, the clinical significance of this in the absence of bacterial translocation is unclear. It is apparent that even relatively minor insults lead to increased gut macromolecular permeability in rodents and humans, thus making its measurement a more sensitive and quantifiable method of judging gut dysfunction than culturing mesenteric lymph nodes for evidence of bacterial translocation.

Controversy exists as to what role inflammatory mediators released from the damaged gut following a traumatic or septic insult play in the systemic inflammatory response. In a rodent

model of gut ischemia/reperfusion injury plasma levels of LPS were elevated after the ischemic insult, but were no higher than that of the control group, which received a sham laparotomy (162). This is important because only the ischemia/reperfusion animals manifested acute lung injury. Thus, the reperfused gut appears to be a source of early proinflammatory mediators that cause acute lung injury independent of circulating LPS. The identity and function of those early mediators remain to be fully elucidated, however it is known that human trauma patients manifest an increase in compliment 3a and interleukin-6 in their portal blood, but not LPS or tumor necrosis factor (96, 165). One explanation for this may be that proinflammatory mediators liberated by the damaged gut may be released into the systemic circulation via lymphatic channels as well as, or instead of, the portal venous system. This possibility is supported by the work of Magnotti, who showed that gut-derived mesenteric lymph but not portal blood increases endothelial cell permeability and promotes lung injury after hemorrhagic shock (137, 138).

1.4.2.4 Ileus

The clinical scenario occurring during and after episodes of sepsis is common, but not well understood. Critically ill patients often do not have adequate gastrointestinal function to permit enteral feeding because of gastric and small bowel ileus due to sepsis (41). This reduced gut motility may lead to alteration of the relatively sterile upper GI tract, resulting in bacterial overgrowth (27). Thus, the gut may serve as a reservoir of pathogens that may enter the systemic circulation and aid in the progression of multiple organ failure.

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Ileus during or after sepsis is likely due to several etiologies, with the two most prominent theories being local gut inflammation, and relative gut ischemia. It is known that a single sublethal dose of endotoxin temporarily disrupts fasting and postprandial canine gastrointestinal motility and transit (40). This finding is supported by work performed on an identical animal model that shows that a single dose of endotoxin results in disruption of gastrointestinal myoelectric activity for up to two days, which is similar to what is seen during postoperative ileus (41). Endotoxin induced ileus appears to occur as a result of acute activation of the muscularis macrophage network, which leads to extravasation of leukocytes and circular muscle impairment (64). This sequence of events has been shown to be dependant on macrophage derived iNOS (65, 89).

The theory that ileus during sepsis is caused by relative gut ischemia is based on the results of rodent studies investigating gut ischemia/reperfusion injury. It is now generally recognized that it is not hypoperfusion per se that is the cause of the ileus. Rather, ileus is believed to result from the sequence of local inflammatory events that follow the ischemic insult, as described above.

1.4.3   Mechanisms of gut dysfunction in sepsis

1.4.3.1 Ischemia/hypoxia

Whether the tissue distress seen in sepsis is caused by microcirculatory hypoxia or disturbances in cellular metabolic pathways is a source of much debate. It is believed that tissue oxygen debt is a major determinant of organ failure and mortality in critical illness

(206). Hypoperfusion of the gut mucosa is considered an important factor leading to alterations in gut epithelial damage in endotoxemia and sepsis (72, 176). However, the relationship between peripheral oxygen delivery, extraction, and use during sepsis has yet to be fully understood. In normal tissues, oxygen consumption is maintained if oxygen delivery decreases due to increased oxygen extraction ratio (Figure 4). Only when oxygen delivery is reduced below a critical value does oxygen consumption begin to decrease, and becomes "delivery dependant oxygen consumption". In septic states consumption is dependant upon delivery over a wide range of flows, thus the value of "critical oxygen delivery" and resting oxygen demand increases while oxygen extraction ratio is unchanged with decreased delivery (32).

The gut is an organ that is very sensitive to the effects of systemic cardiovascular and pulmonary perturbations. Normally, gut oxygen delivery and tissue oxygenation are regulated by a balance of local and systemic effectors. Metabolic vasodilation is an important regulator of gut blood flow and oxygen delivery. When systemic blood flow needs to be conserved, sympathetic adrenergic vasoconstriction increases gut vascular resistance, keeping gut blood flow relatively low. Loss of barrier function is directly related to dysregulation of blood flow in the gut (6). Microcirculation disturbances play a crucial role during endotoxemia (100, 148), and in the gut such changes differ between hemorrhagic shock and septic states (170). Blood hemodynamics in intestinal villi is affected even in normotensive sepsis (170). Intravital microscopy has shown that the density of perfused villi, red blood cell velocity, and flux are dramatically decreased in hypotensive sepsis whereas for the same level of hypotension only moderate changes are seen in hemorrhagic shock (170).

Graph axis labels: Oxygen Consumption (y-axis), Oxygen Delivery (x-axis). Curve labels: Flow Dependent, Flow-Independent, Sepsis, Normal, "Critical O$_2$ Delivery".
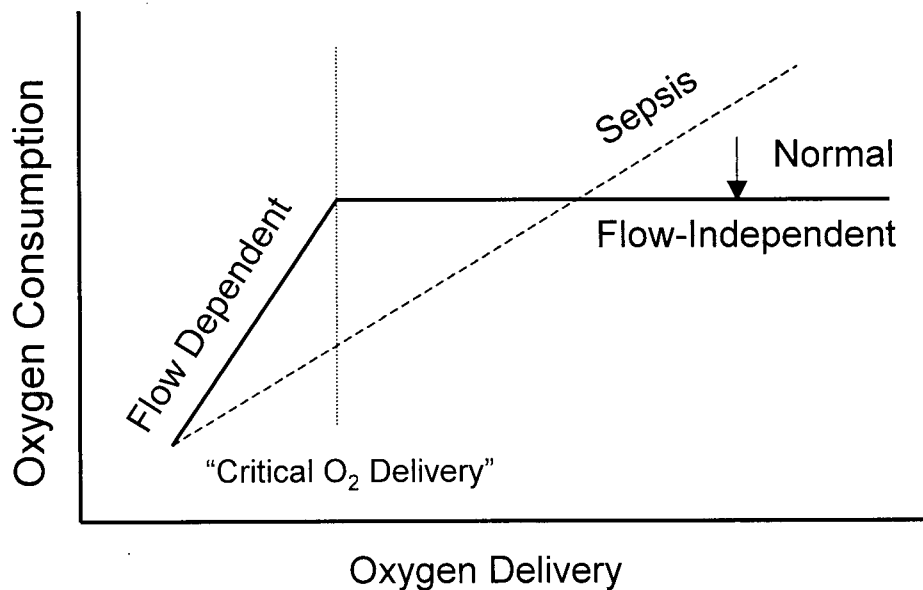
Figure 4.   Oxygen Extraction Ratio

This graph illustrates the relationship between oxygen delivery and oxygen consumption in normal and septic conditions. In normal conditions (arrow), a slight reduction in oxygen delivery is not associated with a decrease in oxygen consumption since extraction increases (flow-independent). At a "critical point", oxygen consumption begins to decrease as flow decreases (flow-dependent) since the extraction ratio is maximal. In sepsis, oxygen consumption is dependent on flow at levels above and below endogenous flow, as shown by the dashed line.

These findings support what has long been suspected with regards to gut dysfunction and tissue oxygenation in sepsis; the situation is much more complex than can be explained by hypotension alone.

Recently, attention has focused on the role of the coagulation pathway during sepsis, and it's role in modulating microvascular changes in SIRS and sepsis. The protein C anticoagulant pathway plays a critical role in controlling thrombin formation, thus preventing microvascular thrombosis. The protein C pathway is initiated by exposure of tissue factor on injured endothelium, causing thrombin generation. Thrombin binds to thrombomodulin on the surface of the endothelial cell. This binding increases the rate of protein C activation by 1000-fold in endothelium and simultaneously blocks the pro-coagulant ability of thrombin. Activated protein C binds protein S, and inactivates factor Va and VIIIa, thus inhibiting coagulation. Targeting the dysregulated coagulation system in septic patients has lead to therapeutic success (13).

1.4.3.2 Local inflammation and immunity

Much of the research focusing on gut damage during sepsis has investigated the role of local inflammation and immunity as a mechanism for the gut mucosal dysfunction seen during endotoxemia and during ischemia/rerperfusion. The general premise is that release of cytokines and proinflammatory proteins from the gut during endotoxemia leads to neutrophil accumulation in the gut mucosa, which leads to physical damage as a result of the liberation of highly reactive oxidative species (78). Although this mechanism is generally accepted,

some investigators have brought into question the role of neutrophils in causing mucosal damage during sepis, favouring mediation by purely enterocyte derived oxidants (112).

Local mucosal production of the proinflammatory cytokines tumor necrosis factor, interleukin (IL)-1 (153), IL-6 (154), and nuclear factor(NF)-kB (191) is increased in endotoxemic and septic mice. In addition, sepsis and endotoxemia are associated with mucosal synthesis of the acute phase proteins compliment component C3 and serum amyloid A (236). Interestingly, activation of NF-kB and production of C3 and amyloid A during sepsis is differentially regulated in different parts of the gut (190, 236). As would be expected, activation of NF-kB and subsequent production of proinflammatory cytokines from the gut mucosa during sepsis and endotoxemia results in neutrophil accumulation in the intestine. This accumulation is dependant on the expression of intercellular adhesion molecule-1 (ICAM-1) in the gut, which is modulated by IL-10 and IL-4, and is influenced by the route of nutrition (77, 78). Regulated adhesion, stimulation, and migration of neutrophils through endothelial cells are thought to be essential steps in acute gut inflammation, with the neutrophil itself contributing to tissue injury by releasing enzymes or toxic oxygen products.

Though many believe neutrophil accumulation plays a pivotal role in modulating gut mucosal damage during sepsis and ischemia/reperfusion, several prominent researchers dispute this. While investigating the role of neutrophils and hydroxyl radicals on hemorrhagic shock-induced bacterial translocation, Deitch found that neutrophil generated oxidants and proteases are not necessary for the initiation of bacterial translocation across the gut mucosal barrier (50). In the same study, Deitch showed that the mucosal injury was a

result of xanthine oxidase-generated oxidants originating from the enterocyte itself, rather than from neutrophils. While not overtly discounting the role of the neutrophil, Salzman is a proponent of the theory that peroxynitrite, a reaction product of nitric oxide (NO) and superoxide, is a final common effector of cytotoxicity and tissue injury (202). The major cell type accounting for mucosal inducible nitric oxide synthase (iNOS) and peroxynitrite formation appears to be the enterocyte (196). Salzman's group has shown that peroxynitrite causes DNA strand breaks in the enterocyte, which activates the nuclear repair enzyme poly(ADP-ribose)polymerase (PARP), leading to the consumption and eventual depletion of adenosine triphosophate (ATP) and oxidized nicotinamide adenine dinucleotide (NAD$^+$) in the enterocyte, damaging the cell (112). A detailed discussion of PARP and it's role in cellular homeostasis follows.

1.4.3.3 Apoptosis

1.4.3.3.1 Definition

Apoptosis, or programmed cellular death, was first described in 1972. It represents the activation of an endogenous cell suicide program in response to a wide variety of diverse exogenous stimuli. Apoptosis is readily distinguishable from the pathological or passive form of cell death known as necrosis. Necrosis usually occurs under conditions of extreme cellular trauma or injury, and is identified by cell swelling, and organelle disruption, followed by membrane rupture and cell lysis. The spillage of intracellular contents into the extracellular space during necrosis can trigger an inflammatory response, which can cause further damage to surrounding tissue. Apoptosis, in contrast, involves chromatin

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condensation, nuclear fragmentation, cell shrinkage, membrane blebbing and the dissociation of the cell into membrane bound vesicles termed apoptotic bodies, which are rapidly phagocytosed. This disassembly and packaging of cellular contents prevents any inflammatory response following cell death. In essence, apoptosis is a mechanism that ensures the safe disposal of unwanted, defective, injured, or infected cells in a manner that poses minimal risk to the organism as a whole.

Apoptosis plays a major role in regulating the constant turnover of the gut mucosa under normal conditions (82), and has recently been implicated as an important mechanism for sepsis related dysfunction of the liver (33, 104) as well as the heart (67, 149, 173). The process of apoptosis begins with the triggering of membrane bound receptors by a wide variety of stimuli, including tumor necrosis factor (TNF) (15). This triggers a complex cascade of pro and anti apoptotic reactions within the cytosol, involving several families of cysteine proteases known as caspases. The caspases then go about dismantling the cell in an orderly fashion, affecting everything from cellular metabolism to cytoskeletal integrity and DNA repair, resulting in death of the cell (Figure 5).

1.4.3.3.2 Caspases

The cloning of the death promoting gene *ced-3* in *C. elegans* revealed significant homology to the mammalian interleukin-1B-converting enzyme (ICE), and provided the first evidence that proteases are important in the apoptotic process (250). Since then, several other homologues have been identified and are now termed caspases (to date, 1-11) to avoid confusing nomenclature. Although apoptosis is a tightly regulated process, it also requires a
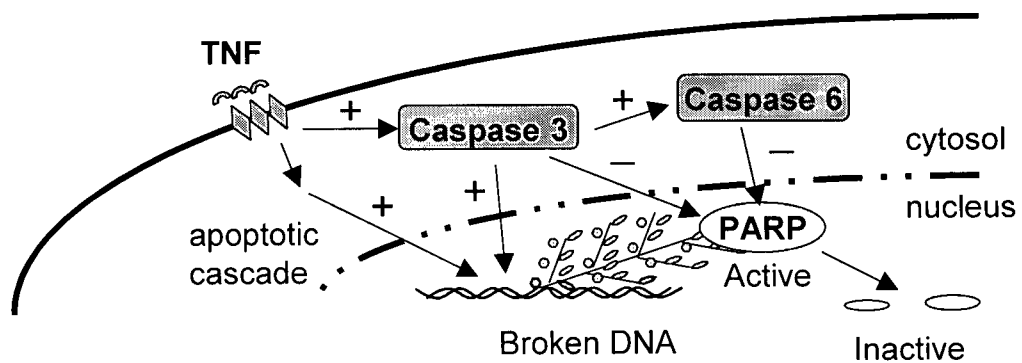
Figure 5.   **Apoptosis**

This figure illustrates some of the basic pathways of apoptosis. A cross sectional view of one quarter

of a cell is depicted. Tumor necrosis factor is acting on a membrane bound receptor to trigger a

complex cascade of proteolytic reactions catalyzed by cytosolic caspases. Caspase 3 activates caspase

6, and both of these enzymes cleave and inactivate the nuclear DNA repair enzyme poly (ADP-

ribose) polymerase (PARP). This effectively shuts off nuclear DNA repair, which, along with other

mechanisms, allows the cell to die.

highly destructive capability in order to dismantle the cell and package it in apoptotic bodies. It is believed that the caspases provide this capability and that they, in essence, represent the effector stage of the apoptotic process. Caspases are divided into three subfamilies based on their homology. Caspase 1(ICE), caspase 4 (ICH-2, ICE-rel-II), caspase 5 (ICE-rel-III), and caspase 11 form the ICE family, whereas caspase 3 (CPP32), caspase 6 (Mch2), caspase 7 (Mch3, ICE-LAP3), caspase 8 (FLICE, Mch5) and caspase 10 (Mch4) form the CPP32 family. Caspase 2 (Ich-1) and caspase 9 (ICE-LAP6, Mch6) constitute the Ich-1 family (76).

After apoptosis is triggered, caspases are activated by the cleavage of proenzymes into distinct subunits that rearrange to form the active cysteine proteases. Caspases transduce and augment the apoptotic signal by activation of other caspases. They are believed to excecute cell death by proteolysis of important functional and structural intracellular proteins, such as PARP and lamin A (126, 221). While lamin A is a caspase 6 specific substrate, PARP is cleaved by members of the CPP32 family of caspases (28). The cleavage of PARP marks a final pathway of apoptosis, and results in PARP inactivation, which impairs DNA repair and facilitates cell death.

1.4.3.3 Poly (ADP-ribose) polymerase

Poly (ADP-ribose) polymerase (PARP - E.C. 2.4.2.30) is a major nuclear protein that plays a role in the cellular response to DNA breakage. The polymerase binds to DNA at single- and double-strand breaks and synthesizes long branched chains of poly (ADP-ribose), which covalently, but transiently modifies itself and numerous other cellular proteins and depletes the cell of NAD^+. PARP is a ubiquitous enzyme found in all mammalian cells and most other

eukaryotes, though lacking in yeast and prokaryotes. The gene for PARP was first cloned in 1987 by Smulson and his colleagues (5). The full length DNA is approximately 3.7 kb in length and the gene is located on chromosome 1. PARP is a 116 kilodalton (kd) protein of 1014 amino acids. Three major domains are recognized by trypsin digestion: a 46-kd N-terminal region responsible for binding to DNA termini, a 22-kd central region that contains an automodification site for polymer attachment, and a 54-kd C-terminal region that is the NAD^+-binding site (109). A shorter region of 29-kd at the N-terminal region contains two potential Zn^{2+}finger-binding motifs that may be responsible for the protein's specificity for binding at DNA termini (146). The protein is found in large concentrations in the nuclei of most eukaryotes, including mammals (69). Approximately 1 enzyme per 10-20 nucleosomes is a typical amount, comparable in importance to DNA topoisomerase II and some histones (98). PARP binds to DNA under normal physiologic conditions, but has a greater avidity for DNA breaks, especially blunt ended double-strand breaks, which act as essential cofactors for enzyme activity (12). On the basis of its location and quantity, PARP is a major nonhistone chromosomal protein which likely exercises an important structural and functional role in chromosome integrity (37).

In terms of enzymatic activity, PARP catalyzes the covalent attachment of ADP-ribose units from NAD^+ to itself and other nuclear proteins to generate long, branched, negatively charged poly (ADP-ribose) chains (37) (Figure 6). When DNA is cleaved by endogenous nucleases, or when cells are exposed to agents that result in DNA breakage, poly (ADP-ribose) is rapidly synthesized from NAD^+, accounting for the majority of NAD turnover (37). This leads to intracellular NAD depletion. The polymer consists of long chains of
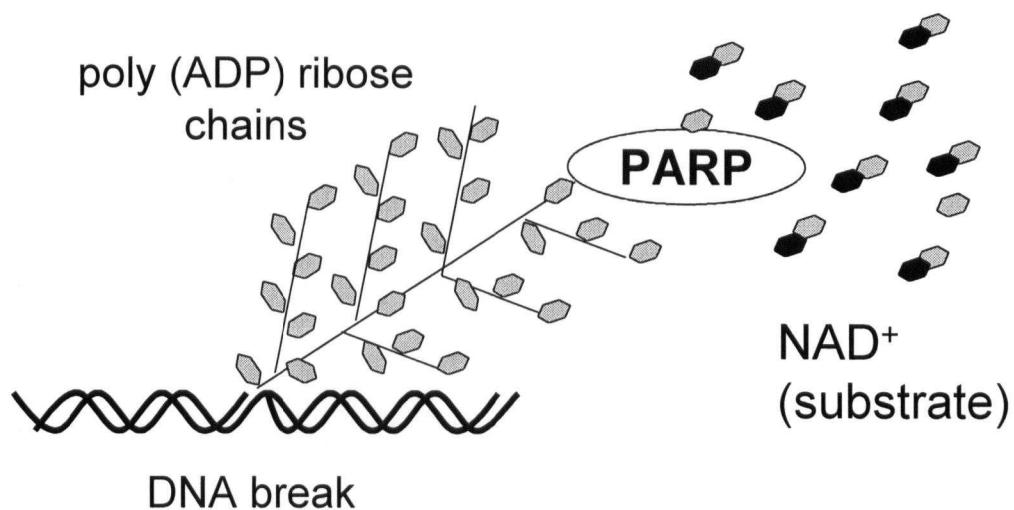
Figure 6.   **Poly (ADP-ribose) polymerase (PARP) Activity**

This figure illustrates the activity of the nuclear enzyme poly (ADP-ribose) polymerase (PARP). Using NAD$^+$ as a substrate, PARP reacts to DNA breaks by placing long, branched chains of poly (ADP) ribose at the site of the DNA break. This is believed to facilitate DNA repair enzymes by creating a physical barrier to prevent nucleases from destroying the DNA while it is being repaired, but it also serves to deplete the cell of much needed energy stores (NAD$^+$).

diphosphoribose units covalently linked at one end to either the polymerase itself (automodification), or other proteins. Up to several hundred residues per minute are added to the chains, which have several branch points (156). The polymer has a short half-life, on the order of minutes, and is hydrolyzed by poly (ADP-ribose) glycohydrolase (37). It is synthesized and broken down rapidly during times of DNA breakage. Once the breaks are sealed, the remaining polymers are completely degraded within minutes (37). Poly (ADP-ribosyl)ation of proteins generally inhibits their function and can dissociate chromatin proteins from DNA (37). Although the precise function of the negatively charged poly (ADP-ribose) chains is unclear, it has been proposed that they function as a physical barrier to prevent the action of DNAses at the site of DNA breaks.

PARP is not considered a damage-inducible gene product, because it is only activated by damage. The levels of the enzyme's gene transcription and mRNA remain unchanged in damaged cells, and the protein is constitutively produced (16). Thus, PARP is best regarded as a damage-responsive element in cellular homeostasis. PARP is specifically cleaved into 116kd and 85kd fragments by the CPP32 family of caspases (caspase 3,6,7,8 & 10) (28), which inactivates the enzyme and impairs the cells ability to repair DNA. This is one of many final pathways of apoptosis.

1.5 MEDIATORS OF SEPTIC TOXICITY

1.5.1 Mediation of septic toxicity

The intensity and duration of the systemic inflammatory response is dependant on a multitude of factors, including the nature and magnitude of the inciting event, propagating forces, and premorbid health and nutrition of the host. The resultant inflammatory response, which is intended as a host defense mechanism, is modulated by a fine balance of pro and anti-inflammatory mediators. In most cases, host defense mechanisms are very effective in protecting the body from invading microbes, and the intensity and duration of the inflammatory response mounted is appropriate. In the case of severe sepsis, which by definition involves host decompensation, one of two scenarios may be present: (1) the systemic inflammatory response is appropriate, however the inciting factor is overwhelming and ongoing (i.e. fecal peritonitis from a perforated viscus). (2) the systemic inflammatory response is inappropriately severe in terms of intensity and/or duration relative to the magnitude and nature of the insult (i.e. inoculation of a single small dose of bacteria into the blood as a result of a contaminated skin laceration). In both scenarios, the inflammatory response is modulated by the balance of the same stimulatory and inhibitory mediators, however in the latter the reason for such a vigorous response is difficult to discern.

1.5.2 Bacterial Endotoxin

For more than a century, endotoxins have been recognized as microbial products capable of eliciting deleterious responses when administered to laboratory animals. More recently, evidence gained from human studies in which purified *Escherichia coli* lipopolysaccharide

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was injected into healthy volunteers supports the concept that endotoxins can serve as proinflammatory mediators in humans (58, 144).

Endotoxin is a complex glycolipid that constitutes the major portion of the outer membrane of gram-negative bacteria, and is often referred to as lipopolysaccharide (LPS), although the two terms are not strictly synonymous (74). The first insights into the detailed structure-function relationship of LPS came in the 1950's when Luderitz and Westphal demonstrated that heating of highly purified LPS with mild acid resulted in the generation of an insoluble lipid-enriched residue that they called lipid A. By the early 1960's animal studies had established that it was indeed the lipid A component of LPS that was responsible for the lethal activity of endotoxin. The final proof of the importance of lipid A came in 1984, when Tetsuo Shiba determined the precise chemical structure of the molecule, and was able to subsequently synthesize it in his laboratory (222). It is now universally recognized that bacterial endotoxins are complex chemical structures composed of two components: the O-antigen and the core (Figure 7). The O-antigen is an oligosaccharide polymer that varies among bacterial strains, accounting for antigenic variability. The core is composed of oligosaccharide, which is covalently bound to the lipid A domain. Lipid A is highly conserved across species and strains of gram-negative bacteria, and constitutes the toxic principle of endotoxin (193, 197). Chemically synthesized polysaccharide-free preparations of lipid A lead to identical manifestations both in vitro and in vivo as highly purified lipid A from natural microbial sources.
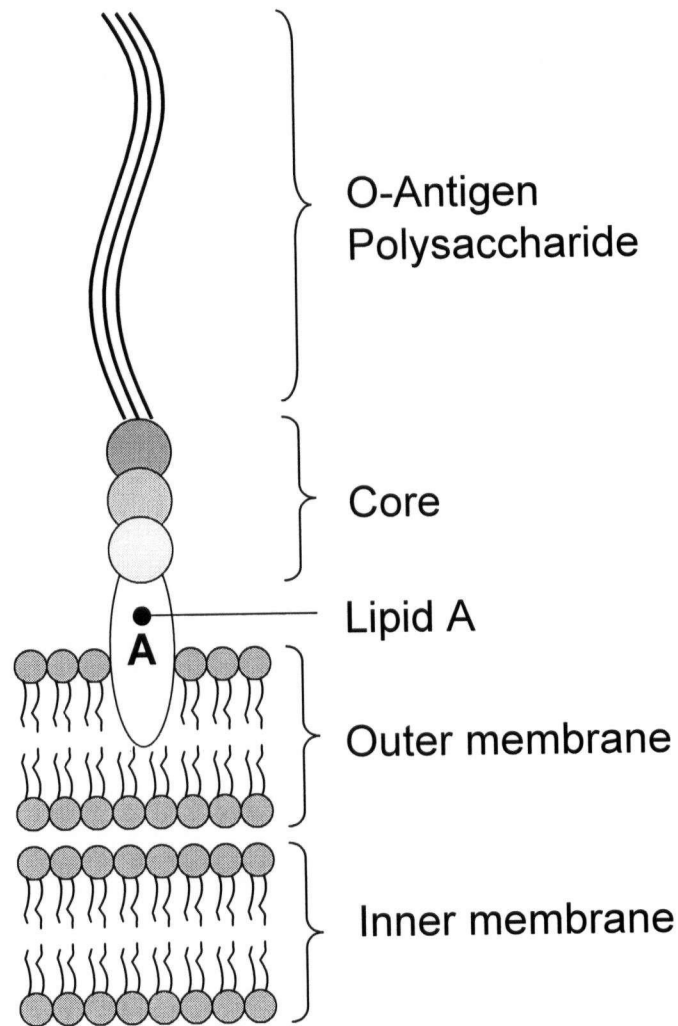
Figure 7.   **Lipopolysaccharide (Endotoxin (LPS))**

This figure shows endotoxin as part of the outer cell wall of a gram negative bacteria. The lipid A moiety is covalently bound to the core oligosaccharide, and accounts for most of the toxic effects of LPS. The O-antigen polysaccharide accounts for the antigenic variability of LPS between different strains of bacteria.

Endotoxin exerts it's effects on the host in a number of ways, including complement activation, procoagulant tissue factor activation, and neutrophil activation (151, 166). The most prominent and well studied effect of endotoxin is in the activation of macrophages, which triggers the synthesis and release of a complex cascade of cytokines, which act to further stimulate and propagate the systemic inflammatory response. Three cloned molecules expressed on the surface of monocytes and macrophages are known to bind lipid A (102): (1) CD14 (2) the macrophage scavenger receptor, and (3) the $\beta2$ (CD11/CD18) leukocyte integrins. Although the scavenger receptor does not function as a signaling receptor for LPS, it does play a role in host-defense (91). Both CD14 (92) and CD11/18 (101) do act to initiate intracellular signals, however, which results in the activation of phagocytes, and stimulates phagocytosis, as well as the synthesis and release of cytokines and highly reactive oxidative species. Additionally, CD14 and CD11/CD18 interact with LPS in concert with serum proteins such as soluble CD14 and LPS binding protein. These soluble LPS receptors enhance the sensitivity of the membranes bound receptors to LPS by 100 to 1000 fold. Recently, in addition to the membrane bound receptors described above, Toll-like receptor (TLR)-2 and TLR-4 have been implicated in LPS signaling, however they are not yet fully characterized (102).

The end result of endotoxin's effect on the host is that a marked systemic inflammatory cascade is triggered, which leads to the release of cytokines, eicosanoids, platelet activating factor, and other vasoactive substances like nitric oxide, as well as numerous other cellular and humoral mediators, both pro and anti inflammatory. This complex cascade eventually leads to tissue and end organ damage by way of hypoxia, tissue necrosis, and apoptosis.

1.5.3 Host Defenses

Host defenses can be divided into specific and nonspecific factors, or local and systemic factors. Non-specific factors include skin barrier, mucous membranes, complement, and random phagocytosis. Specific factors refer to the recognition of specific antigens by T-Cells, and the production of antibodies directed toward those antigens by B-Cells. Such interactions involve the major histocompatibility complex (MHC), which allows the T-cells to differentiate self from non self, and thus, under normal conditions, only mount an immune inflammatory response towards foreign organisms and/or antigens. Like the overall systemic inflammatory response, the specific immune system's response to a challenge is a balance between pro and anti-inflammatory signals. Thus, if conditions favor inflammation, it is possible that the incited inflammatory response could damage uninvolved and distant tissues by way of cytokine amplification, which would act to promote SIRS. A pivotal player in the specific immune system is the antigen presenting cell, or macrophage. Not only do macrophages recognize, phagocytose and present antigen to T-cells as an initial step in specific defense, but they also play a crucial role in immunomodulation by way of the release of inflammatory mediators and toxic substances, as discussed in the sections that follow.

Recently, a great deal of attention has been focused on the role of the neutrophil in systemic inflammation, and in particular, the neutrophil endothelial cell interaction. Endothelial-directed recruitment of neutrophils to extra vascular sites of bacterial infection is essential in host defense (150). A key step in this interaction is the upregulation of adhesion molecules and ligands on neutrophils and endothelial cells by bacterial or host proinflammatory mediators. This results in the localization and adherence of circulating neutrophils to

endothelial cells followed by neutrophil activation and purposeful migration to the site of infection. This is achieved in three discrete steps: rolling, firm adhesion, and transmigration.

As neutrophil products such as enzymes and reactive oxidant species may be harmful to the host, the ability of adhesion molecules to be upregulated in specific regions of need is paramount to avoid unnecessary injury to uninvolved tissues. Unfortunately, under circumstances of systemic inflammation, many of the proinflammatory mediators that stimulate upregulation of adhesion molecules during localized infection are increased in the intravascular space systemically. Recent work has suggested that neutrophil adherence to endothelial cells plays a role in the tissue injury and multiple organ dysfunction seen in sepsis (140, 205). Thus, during sepsis widespread upregulation of adhesion molecules by circulating proinflammatory mediators may result in the injury of non-infected, distant tissues and organs. While neutrophil accumulation and activation likely does play an important role in the development of MODS, it cannot be the sole mechanism of tissue injury during SIRS and/or sepsis, as patients with inherited abnormalities of adhesion molecule function can develop sepsis-associated tissue injury (90).

1.5.4 Inflammatory Mediators

Proinflammatory mediators play a pivotal role in tuning a localized process such as a bacterial infection into a systemic process such as SIRS, and it is through their actions that a host response intended to be beneficial sometimes becomes detrimental, and leads to MODS.

1.5.4.1 Peptide cytokines

Peptide cytokines, which can either be pro or anti inflammatory, facilitate a wide range of inflammatory processes. The most extensively studied proinflammatory cytokines in sepsis are tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1). IL-6, which is both pro and anti inflammatory, IL-12, and interferon-γ (IFN-γ) are also thought to play a role in the pathogenesis of SIRS (229). Peptide cytokines are secreted by phagocytes and blood born monocytes in response to a wide variety of stimuli including endotoxin, other cytokines, compliment, and eicosanoids.

TNF-α and IL-1

Mature secretory TNF-α is composed of three 17-kd polypeptides that form a compact trimer (228). The 26-kd prohormone of TNF-α is transmembrane protein that is processed into a mature secreted form via cleavage by one or more matrix metalloproteinases. Two separate cell surface TNF-α receptors have been found: type I (CD120a); and type II CD120b (11). Triggering of the type I receptor produces a multitude of TNF-α actions, such as expression of adhesion molecules on endothelial cells, frank cytotoxicity, and activation of the transcriptional regulator NF-κB. Activation of the type II receptor may play a role in the signaling effects of membrane bound TNF-α.

IL-1 is the designation for two polypeptides, IL-1α and IL-1β. These two forms, along with their soluble inhibitor IL-1 receptor agonist, recognize the same receptor. Both IL-1s are synthesized in precursor form, but only IL-β is transported out of the cell in any significant quantity, thus IL-1β is the predominant form of IL-1 that is found in circulation during SIRS.

IL-1α is believed to play a role in intracellular and paracrine signaling only. Two IL-1 receptors have been identified: type I is found on virtually all cell types, and is responsible for the transduction of cellular effects of IL-1, while type II receptors have no known biological response (61).

Injection of TNF-α and IL-1 into laboratory animals reproduces many of the hallmarks of SIRS, and both these cytokines have a wide variety of biologic actions (178). Interestingly, simultaneous administration of TNF- α and IL-1 has been shown to cause synergistic effects (178). In humans, both these cytokines are pyrogenic, and cause activation of the cytokine cascade, thrombosis, and fibrinolysis, with TNF-α also causing neutrophil activation.

Interleukin-6

IL-6 has a number of highly diverse biologic activities, which contribute to both the anti and pro inflammatory immune states. The peptide has been found to be involved in acute phase protein synthesis as well as modulating the immune system via it's effects on antibody production, differentiation of T-cells, enhancement of natural killer cell activities, and maturation of megakaryocytes in the bone marrow (115). IL-6 exerts its effects at the cellular level via the cytokine signal transducer gp130, which is triggered after the IL-6 receptor is stimulated. Both membrane bound and soluble forms of the IL-6 receptor exist. The IL-6/IL-6 receptor complex induces formation of a gp130/gp130 homodimer, which leads to IL-6 signal transduction. Cells lacking a cell surface IL-6 receptor can be affected by the peptide through the agonistic effects of the soluble IL-6 protein/receptor complex (229).

IL-6 is considered to be a major regulator of the acute phase response to bacterial infection in animals and humans, with serum IL-6 levels correlating well with mortality in critically ill patients (185, 189). IL-6 is known to stimulate hepatocytes to produce several acute phase reactant proteins, but human studies involving the infusion of IL-6 intravenously have shown that it is not as toxic as TNF-α or IL-1, although it does cause fevers and chills (229). The anti inflammatory effects of IL-6 have been demonstrated both in vitro, where it inhibits endotoxin induced TNF-α and IL-1 production in monocytes, and in vivo, where it inhibits the release of TNF-α in endotoxemic mice (2).

Interleukin-12 and Interferon-γ

IL-12, produced mostly by monocytes and macrophages, acts on T cells and Natural Killer cells to induce the production of interferon-γ (INF-γ), stimulate proliferation, and enhance their cytotoxic activity. INF-γ is mainly produced by natural killer cells, T-helper cells, and cytotoxic T cells. It acts to facilitate cell mediated immunity by inducing class II MHC antigen expression on various cell types, as well as activating macrophages. In addition, INF-γ plays a major role in the production of IgG against bacterial polysaccharides.

INF-γ potentiates many of the in-vivo effects of IL-12, which in turn is believed to be involved in sustaining the inflammatory response to a bacterial insult (225).

Antiinflammatory Cytokines

Anti-inflammatory cytokines act to reduce the production of proinflammatory cytokines, thus inhibiting inflammatory processes. Although IL-6 and IL-10 are the two most closely studied cytokines with anti-inflammatory properties, many other peptide cytokines also possess anti-inflammatory characteristics, including IL-4, IL-11 and IL-13 (229).

IL-6, discussed above, is recognized predominantly for it's pro-inflammatory actions, and the clinical importance of its anti-inflammatory actions are unclear. IL-10, however, is better recognized as a cytokine that reduces the systemic inflammatory response to a degree that may be biologically important. Produced by T cells, B cells, monocytes, and macrophages, IL-10 acts to inhibit pro-inflammatory cytokine production, inhibit MHC class II expression by monocytes and macrophages, and inhibit the killing of intracellular bacteria by macrophages (229).

Activation of the Cytokine Network During Bacterial Infection

Several studies have been published on serum and plasma concentrations of cytokines during sepsis in both animals and humans (3, 8, 213, 234). In human studies results are confounded by the timing at which the samples were taken relative to the onset of sepsis, however, it is accurate to say that certain proinflammatory cytokines can only be found in a subset of patients, while anti-inflammatory cytokines can be found in almost all patients. All published studies looking at IL-6 levels and mortality have found a positive correlation between IL-6 concentration on admission and mortality, however the values found in the

studies were highly variable, and thus it is difficult to set cut-off values for IL-6 for prognostic purposes.

In general, after a bacterial infection occurs, pro-inflammatory cytokines are released very early on in the course. In experimental models of infection, TNF-α is the first cytokine appearing in the circulation (95). The infusion of *Escherichia Coli* endotoxin in healthy humans and rodents results in a transient rise in serum TNF-α, which peaks after 90 minutes. Shortly thereafter, anti-inflammatory cytokines are released during what has become known as the compensatory anti-inflammatory response, or CARS (Figure 8). CARS is followed by a refractory state characterized by endotoxin tolerance, which has been reproduced experimentally in animals and humans by repeated low dose endotoxin infusion. The mechanism of the refractory state has not yet been elucidated.

It is likely that the effect of cytokines is compartmentalized, which is to say that the cytokine profile at the particular site (or organ) of the insult is often different in magnitude and time course than if the insult were genuinely systemic at its inception. For example, patients with unilateral pneumonia have much higher concentrations of proinflammatory cytokines in bronchoalveolar lavage fluid taken from the infected lung than from either the uninvolved lung, or serum (42). This is important to keep in mind when it comes to interpreting cytokine data in the various animal models of sepsis, many of which, unlike the usual human circumstance where a localized process becomes generalized, start off as generalized processes (i.e. infusion of endotoxin systemically).
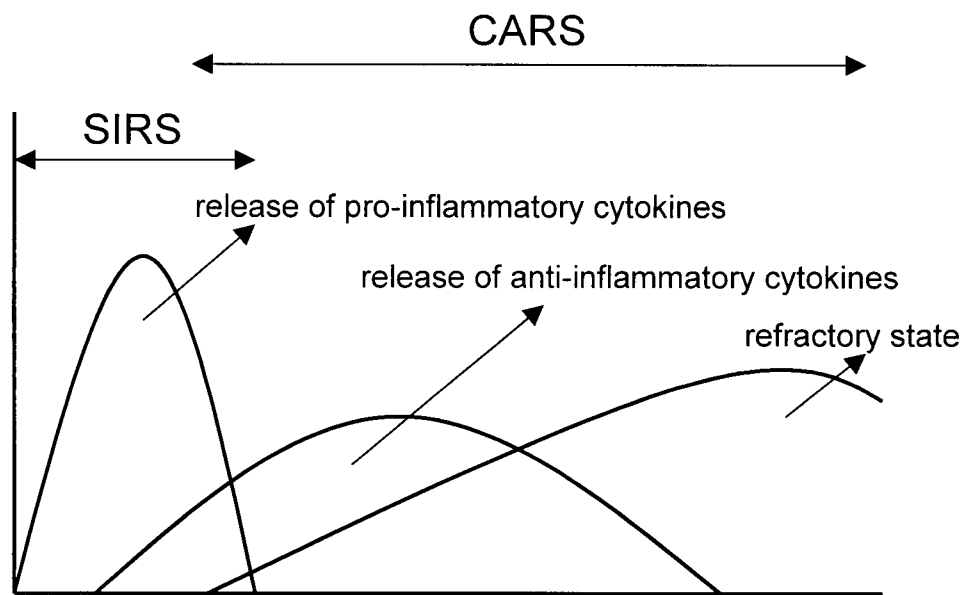
Figure 8. **Pro- and Anti- Inflammatory Events During Sepsis**

Sepsis is associated with an early transient activity of proinflammatory cytokines, corresponding to

the clinical designation SIRS. Subsequent to this, counterregulatory pathways become activated,

which is referred to as the compensatory anti-inflammatory response sydrome (CARS).

1.5.4.2 Platelet Activating Factor

Platelet activating factor (PAF) is a labile lipid first found to be released by platelets in the presence of antigen and leukocytes, and is believed to be an important mediator of SIRS (36). PAF infusion mimics several of the effects of sepsis and endotoxemia, and it is known that PAF is produced during endotoxemia in animals and in severe sepsis (36). Interestingly, several structurally different antagonists of PAF have been found to inhibit endotoxin-induced hypotension, lung injury, and mortality.

1.5.4.3 Eicosanoids

Eicosanoids are derivatives of arachidonic acid, an important cell membrane phospholipid. The term eicosanoids encompasses three major families of mediators: prostaglandins (PG), thromboxanes (Tx), and leukotrienes (LT). A number of inflammatory stimuli can directly or indirectly activate phospholipase leading to release of arachidonic acid from phospholipid aggregations. Once it is released, arachidonic acid is oxidized via two major pathways, leading to the production of the eicosanoids. These derivatives all have numerous functions, a discussion of which is beyond the scope of this thesis.

1.5.4.4 Nitric Oxide

Nitric oxide (NO) has been implicated as the primary agent responsible for the myocardial depression and decreased vasoresponsiveness seen in patients with sepsis (219). It has long been recognized that peripheral vascular tone was modulated by an endothelial derived substance known as endothelium-derived relaxing factor (EDRF). In 1987, NO was identified as the primary constituent of EDRF, thus paving the way for NO related research,

which has subsequently implicated NO as a major modulator of vascular tone during sepsis and SIRS (79, 134, 159). NO is formed by endothelial cells via the action of the enzyme nitric oxide synthase (NOS) on the amino acid L-arginine, which liberates L-citrulline and NO. Three isoforms of NOS exist, with two being constitutive and one being inducible. The constitutive forms eNOS and nNOS are derived from endothelial cells and neuronal cells respectively. NO is generated in response to vasorelaxing mediators such as acetylcholine, bradykinin, or histamine, as well as endothelial mechanical sheer stress. The inducible form iNOS is produced in response to endotoxin, PAF, IL-1 and TNF-α. NO is a highly diffusible substance with a half-life of 6-10 seconds which serves to activate soluble guanylate cyclase in adjacent smooth muscle. The guanylate cyclase acts as a second messenger, which converts GTP to cyclic GMP, which produces vascular smooth muscle relaxation via a cGMP-dependant protein kinase that promotes calcium entry into the sarcoplasmic reticulum. It is recognized that iNOS is capable of producing on the order of ten times the magnitude of NO as is eNOS, and thus iNOS is thought to be the major source of excessive NO production responsible for the hypotension with profound shock and refractory vasodilation that is often observed in severe sepsis and septic shock (38, 79) (169, 171). Of importance, iNOS expression is induced in a number of organs and tissues, including the gut, in rodents challenged with LPS (226).

In addition to it's role in the mechanism of cardiovascular dysfunction during sepsis, NO has also been implicated as an important contributor to the formation of the highly reactant oxidant peroxynitrite (112). Peroxynitrite is formed by the reaction of superoxide with NO, and has been shown in-vitro to contribute to cellular injury by damaging DNA and other vital

intracellular structures, thus triggering apoptosis (253). This will be discussed in greater detail later on.

Modifying the concentration of NO through inhibition of it's enzymatic formation has demonstrated improvement in hemodynamic function and organ damage in animal models of sepsis, but unfortunately, no survival benefit has been seen in human patients suffering from sepsis and/or septic shock (219).

1.6   TREATMENT OF SEPSIS

Despite 30 years of intense clinical and laboratory investigation into the sepsis related inflammatory response, the mortality of sepsis remains essentially unchanged. While great strides have been made towards our understanding of the systemic inflammatory response, the treatment of sepsis remains predominantly supportive, focusing on the treatment of the infectious nidus and support of organ dysfunction. Therapy of sepsis consists of immediate steps to resuscitate and stabilize the patient, followed by definitive therapeutic intervention, if applicable.

1.6.1   Resuscitation and stabilization

The immediate priority in treating patients with sepsis is in the identification and reversal of life-threatening physiologic abnormalities. This process is often referred to as the primary survey, and involves addressing the ABCs: airway, breathing, and circulation, in that order. Decreased level of consciousness may require the patient's airway to be secured emergently

in order to maintain adequate oxygenation in the face of pulmonary capillary leak. This is usually done via insertion of an endotracheal tube, but failing that, a surgical airway may need to be established. Once intubated, the patient may be mechanically ventilated, allowing the use of positive end-expiratory pressure (PEEP). The process of mechanical ventilation allows off loading of respiratory muscles, thus lowering their oxygen consumption and allowing for greater availability of oxygen for other tissues. Due to profound vasodilation, circulation to vital organs may be compromised very early in severe sepsis. Hypotension may necessitate aggressive fluid resuscitation with crystalloid and/or colloid, as well as inotropic and vasopressor support once intravascular volume has been replenished.

1.6.2 Differential diagnosis of sepsis

Once the primary survey has been completed and resuscitation has commenced, the secondary survey is performed. This process involves a detailed examination with the intent of revealing the full extent of a patient's illness, as well as identifying the cause of the physiologic derangements. By definition, sepsis implies an infectious etiology. Hyper/hypothermia, tachycardia and leukocyte abnormalities can result from noninfectious causes, which need to be identified as soon as possible. While these parameters are sensitive findings in sepsis, they are not specific. Hyperthermia can result from drugs, toxins, thyroid storm, hypothalamic injury, and environmental heat injury. Furthermore, multiple organ dysfunction syndrome can result from such noninfectious insults as drug overdose, exposure to toxins, severe trauma, and acute pancreatitis. Although cardiovascular collapse and shock is commonly seen in sepsis, it can also result from noninfectious causes such as cardiogenic

shock (myocardial infarction), pulmonary embolism, hemorrhage, dehydration, anaphylaxis, adrenal insufficiency, and poisoning/overdose.

If the etiology of shock is not immediately apparent and/or sepsis is suspected, blood and other appropriate cultures (sputum, urine, wound, cerebrospinal fluid) should be taken in order to ascertain the location and nature of the suspected infection. Empiric broad-spectrum antibiotics are indicated in such cases, and antimicrobial therapy can be tailored based on the results of the cultures when they are available. Once the patient has been resuscitated and placed on appropriate antibiotics, a combination of serial physical exams, cultures and diagnostic imaging can be used to help identify the infectious nidus.

1.6.3 Monitoring

Monitoring the cardiovascular, pulmonary and metabolic derangements present during sepsis is important as it allows for the selection of an appropriate intervention. This is typically done in the intensive care unit setting, where both invasive and non-invasive monitoring can be carried out. Routine vital signs such as blood pressure, heart rate, respiratory rate, and temperature should be monitored frequently. Oxygen saturation, arterial blood gasses and electrocardiographic monitoring are often routinely performed as well. Ventilated patients can have their airway pressures and lung volumes assessed, and the mode of ventilation can be adjusted accordingly. In order to properly resuscitate the patient in septic shock, a detailed cardiovascular profile need be obtained. In addition to central venous catheter measurement of central venous pressure, a Swan Ganz catheter can be used to measure pulmonary capillary wedge pressure, cardiac output, and systemic vascular resistance. The monitoring of

urine output and mental status allow changes suggestive of impaired end organ perfusion to be detected.

While not routine, monitoring of the gastrointestinal tract can also be done if necessary. Tonometry to measure the gastric intramucosal pH has been advocated as a way of monitoring regional perfusion in critically ill patients. While not difficult to perform, concern has been expressed with regards to its methodology and clinical relevance (179). Similarly, gut permeability can be monitored in critically ill humans by looking at lactulose and mannitol clearance in the urine, but the clinical significance of this parameter is uncertain and it remains a research tool.

1.6.4   Supportive therapy of sepsis

1.6.4.1 Volume resuscitation

In order to increase left ventricular preload and reverse organ hypoperfusion in sepsis the initial therapeutic maneuver is to give intravenous fluids in the form of either crystalloid or colloid. Crystalloids are much less expensive than colloids and are usually used in the initial resuscitation. The low viscosity of crystalloids allows for rapid infusion of volume, although the volume expansion effect of colloids is greater than that of crystalloids. The crystalloid versus colloid debate has been raging for well over three decades, but no clear benefit of either has been demonstrated in the resuscitation of the septic patient (59). Theoretically, the use of crystalloids could lower the oncotic pressure and increase interstitial water with volume loading. The clinical impact of this premise is however, unproven. Conversely,

colloids are known to produce less peripheral edema than crystalloids, but there is no evidence that this is anything more than a cosmetic issue. In addition, there are no data to suggest a benefit of colloid on the acute respiratory distress syndrome (ARDS) (59).

Packed red blood cells should be considered part of volume resuscitation in patients with sepsis if there is evidence of anemia or ongoing bleeding. Transfusion of packed red cells to maintain a normal hemoglobin is believed to be an effective means of increasing arterial oxygen content and systemic oxygen availability. Although this makes empiric sense, clinical studies have not demonstrated that transfusion to normal hemoglobin increases oxygen use or alters outcome in critically ill patients (133, 143). The recently reported TRICC trial randomized patients to maintain hemoglobin levels at a threshold greater than or equal to 70 versus 100, and found that a restrictive strategy of red cell transfusion is at least as effective as a liberal transfusion strategy in critically ill patients, with the possible exception of patients with acute myocardial infarction or unstable angina (93). Transfusion of packed red cells has several drawbacks, including the possibility of viral transmission, immunosuppression, and microcapillary occlusion and subsequent tissue ischemia owing to the poorly deformable transfused cells.

1.6.4.2 Inotropes and vasopressors

If efforts to optimize left ventricular preload with volume resuscitation do not lead to the desired increase in cardiac index and organ perfusion, inotropic agents such as dopamine or dobutamine may be used in an effort to increase cardiac output. Dopamine is an adrenergic agent with dose-dependant pharmacologic effects. Low dose dopamine (less than 5

mcg/kg/min) stimulates dopaminergic receptors in renal, mesentery and coronary tissues, leading to vasodilation. The renal effects include increase in glomerular filtration rate, renal blood flow, and sodium excretion. The clinical impact of the use of low dose dopamine in sepsis remains unclear. At a dose range of 5 to 10 mcg/kg/min, beta-adrenergic effects predominate, and lead to an increase in heart rate and contractility. Above a dose of 10 mcg/kg/min alpha-adrenergic effects predominate, which leads to arterial vasoconstriction. In addition to its cardiac and arterial effects, dopamine may further increase pulmonary capillary wedge pressure by decreasing venous capacitance. Although dopamine increases oxygen delivery, its ability to improve tissue oxygenation is not clear (145). It has been postulated that dopamine may redistribute blood flow within the gut, decreasing mucosal blood flow. Dobutamine has been the focus of several studies of cardiac function in sepsis (85, 106, 199). It has been shown to increase cardiac index and by improving left and right ventricular stroke work, as well as increasing heart rate. Unlike dopamine, dobutamine usually decreases pulmonary capillary wedge pressure, and is an ideal agent for those patients with adequate or increased filling pressures and blood pressure but inappropriately low cardiac index.

Vasopressor therapy has been advocated for subsets of patients with septic shock, but the specific indications remain unclear. Vasopressor therapy is not thought to increase oxygen delivery directly, as the resulting increase in systemic vascular resistance impedes forward flow. Vasopressor agents should be considered when a persistently low mean arterial pressure remains after adequate left ventricular preload has been optimized and inotropic therapy is in place. Increased stroke volume with vasopressor therapy may result from the

inotropic effect of mixed adrenergic agents, or by increased coronary blood flow related to an increase in aortic diastolic pressure. Common agents used as vasopressors include high dose dopamine, norepinephrine, epinephrine, and phenylephrine (near pure vasopressor). Norepinephrine is a mixed adrenergic agent with predominantly alpha effects. It acts to increase blood pressure with little change in heart rate, cardiac index, or pulmonary capillary wedge pressure. A combination of low dose dopamine and norepinephrine has been shown to improve renal function in animals and healthy human volunteers compared to noradrenaline alone (204), but the clinical benefit in severe sepsis is unclear. Epinephrine has been shown to increase mean arterial pressure primarily by increasing stoke volume, however the dose response is unpredictable (132). In addition, epinephrine typically decreases splanchnic blood flow. For this reason, its use should be limited to patients who have failed other therapies. Phenylephrine is a selective alpha-1 adrenergic agonist that results in an increase in mean arterial pressure with no change in heart rate. This agent may lead to a slight decrease in cardiac index, and it remains a useful agent when tachycardia limits therapy with other vasopressors that have concomitant inotropic effects.

1.6.4.3 Vasopressin

A new therapy for septic shock that is currently under investigation is vasopressin. Vasopressin, also known as antidiuretic hormone (ADH) has homeoststic, gastrointestinal, and thermoregulatory effects, and is an adrenocorticotropic secretagogue (97). Released from the axonal terminals of magnocellular neurons in the hypothalamus (23, 128), vasopressin mediates vasoconstriction via V1-receptor activation on vascular smooth muscle and mediates its antidiuretic effect via V2-receptor activation in the renal collecting duct system

(97). At low plasma concentrations, vasopressin also mediates vasodilation in coronary, cerebral, and pulmonary arterial circulations (107, 177).

Septic shock causes first a transient early increase in blood vasopressin concentrations that decreases later in septic shock to very low levels compared to other causes of hypotension (122). Infusion of vasopressin 0.01 to 0.04 U/min in septic shock patients increases plasma vasopressin levels to those observed in patients with hypotension from other causes (122, 123). Increased vasopressin levels are associated with a lesser need for other vasopressors (141). Urinary output may increase, and pulmonary vascular resistance may decrease. Because clinical studies have been relatively small, focused on physiologic end points, and because of potential adverse effects of vasopressin, clinical use of vasopressin should await a randomized controlled trial of its effects on clinical outcomes such as organ failure and mortality (97).

1.6.4.4 Steroid therapy

Steroids in the treatment of septic shock are indicated if adrenal insufficiency is present or suspected. Although systemic steroids have been used empirically for several years in the treatment of severe sepsis n the absence of adrenal insufficiency, well designed prospective studies failed to show a benefit from high dose steroid treatment initiated early in septic shock (20, 214). In fact, one study showed that septic patients with elevated serum creatinine had a worse prognosis when treated with steroids (214). Interestingly, a recent study of steroid therapy in patients with persistent vasopressor requirements treated later in the course of septic shock with lower doses of steroids suggested improved outcome (14).

1.6.4.5 Nutrition

Early enteral nutrition is recommended in patients with severe sepsis and septic shock. A nasogastric feeding tube is ideally paired with a larger nasogastric sump drain such that residuals can be checked and feeding rates adjusted. Post pyloric feeding aids in decreasing gastric distension and subsequent aspiration of gastric contents. In patients that cannot tolerate enteral feeding, total parenteral nutrition (TPN) is recommended. A thorough review of enteral feeding in sepsis is presented in chapter 1.6.

1.6.5 Directed therapy of sepsis

Since 1982, when J5 antisera was trailed in the treatment of septic shock (251), many clinical trials have been undertaken with the purpose of finding a "magic bullet" for the directed therapy of sepsis. Tens of thousands of patients in at least 25 multicenter, double blinded, placebo-controlled, clinical trials have been studied, with generally disappointing results (180). Several trials have shown a hint of efficacy in certain subgroups, but until recently, no clear evidence of overall efficacy has been demonstrated.

While the pro and anti-inflammatory pathways in SIRS and sepsis have been extensively studied, no single agent has been found to effectively inhibit the inflammatory cascade and prevent the onset of the systemic inflammatory response and subsequent multiple organ failure. Part of the difficulty lies in the fact that sepsis is a syndrome, and not a discrete defined, disease. Sepsis results from a multitude of different microorganisms and affects people with an endless array of comorbid conditions. There are multiple determinants of

septic outcome in septic patients in addition to sepsis itself. A variety of genetic (216, 233) , immunologic, microbiologic, and hemodynamic parameters contribute to the outcome of sepsis (180). It is difficult to control for all these variables in multicenter, double-blind clinical trials. Septic populations are profoundly heterogeneous, and the complexity and redundancy of the human humoral and cellular immune system virtually guarantees that a simple, straightforward treatment approach is unlikely to be effective. The likelihood of finding the elusive "magic bullet" that uniformly benefits all septic populations is very small (62, 181).

Clinical trials designed to evaluate drugs for the treatment of sepsis are based one of two unproven assumptions relating to sepsis in humans. The first is that endotoxin contributes to the morbidity and mortality caused by infection with gram-negative bacteria. The second assumption is that a large part of the pathophysiology in septic patients relates to an overproduction of one or many secondarily induced host mediators. These assumptions arise from basic science studies performed on animal models of sepsis. Unfortunately, the situation in human sepsis is much more complicated. Many recent clinical trials in septic shock have used anticytokine strategies such as anti-tumor necrosis factor antibodies or inhibitors if interleukin 1, based on the fact that these are important inflammatory mediators in animal models of sepsis (237). The results of such trials have been disappointing, likely due to the complexity of the human inflammatory response, and our paucity of knowledge regarding the ideal timing and dosage of such anti-inflammatory agents.

Recently, increased attention has been focused on the interaction between coagulation and inflammatory pathways in septic patients, and targeting the dysregulated coagulation system in these patients to therapeutic success. The protein C anticoagulant pathway plays a critical role in controlling thrombin formation, thus preventing microvascular thrombosis. Low levels of protein C are associated with poor outcome in severe sepsis (247). The PROWESS trial, a landmark study designed to demonstrate a reduction in 28-day mortality in patients with severe sepsis who receive recombinant activated protein C, was recently terminated because of efficacy (13). This marks the first real breakthrough in reducing the mortality of severe sepsis by single agent directed therapy since such research began two decades ago, and portends well for the future of directed therapy in sepsis.

Chapter 2

HYPOTHESIS

It has been shown in clinical studies that enteral feeding decreases morbidity in critically ill patients relative to total parenteral nutrition. While it has been know for some time that sepsis leads to gut dysfunction, and that enteral feeding helps to maintain the integrity of the gut mucosa, the precise mechanism of sepsis related gut dysfunction, and the effect of enteral feeding on that has yet to be described. In addition, the mechanistic relationship of distant organ inflammation to gut function during sepsis is not well described. My hypothesis is that endotoxemia is associated with increased gut apoptosis, increased gut permeability, and increased inflammation in a distant organ, the lungs. Furthermore, I hypothesize that enteral feeding ameliorates endotoxin's effect on gut apoptosis and subsequently gut permeability and pulmonary inflammation.

Chapter 3

THESIS OBJECTIVES

It is generally recognized that sepsis and endotoxemia lead to gut dysfunction, which can act to modulate multiple organ failure. Despite the large body of research dealing with sepsis and the systemic inflammatory response, the cause of gut dysfunction in sepsis and the nature of the relationship between sepsis-related gut dysfunction and distant organ inflammation are poorly understood. In a similar vein, the mechanism by which enteral feeding improves morbidity in critically ill patients has not been well defined. In this thesis, an attempt is made to address several important questions regarding the relationship between endotoxin related gut dysfunction and distant organ inflammation, and how enteral feeding affects this.

Specifically, the following questions will be addressed:

1.   What is the effect of endotoxemia on gut apoptosis?

Apoptosis plays a major role in gut mucosal homeostasis, and has been implicated in the sepsis related injury of solid organs such as the liver and heart. Surprisingly, very little work exists which looks at the effect of sepsis and endotoxemia on gut apoptosis, making this an important question to resolve.

2.   What is the effect of endotoxemia on gut function in this model of sepsis?

Others have shown an increase in gut mucosal permeability and bacterial translocation in rodent models of sepsis, but it is important to characterize the exact nature of the gut mucosal

dysfunction in this particular model so that it can be examined in both the fed and fasted states.

3. What is the effect of endotoxemia on pulmonary inflammation in this model?

In order to relate gut dysfunction to distant organ inflammation, it is vital to quantify inflammation in an organ known to be involved in the systemic inflammatory response. Pulmonary inflammation in murine sepsis models similar to the one used here has been well documented.

4. What is the effect of enteral feeding on gut apoptosis, gut function, and pulmonary inflammation?

By studying these three parameters under fed and fasted conditions, one can draw a conclusion as to the relationship between sepsis related gut dysfunction and distant organ inflammation, as well as the mechanism by which enteral feeding affects the systemic inflammatory response.

Chapter 4

RESEARCH PLAN

4.1 THE MOUSE MODEL OF SEPSIS

In considering which model of sepsis to use several points were taken into consideration. Pioneering work in the field of sepsis related gut dysfunction was carried out in the mid 1980's on mouse and rat models of sepsis. This primarily involved culturing the mesenteric lymph nodes of septic mice for the presence of enteric bacteria (47). As one of the purposes of this study is to make an assessment of gut dysfunction, it seemed reasonable to use a mouse model. Another important consideration was the availability of species-specific assays for apoptosis and pulmonary inflammation. Virtually all such assays that are commercially available had been developed for murine applications, making it very easy to adapt them to use in this series of experiments. Finally, a rather large number of animals will be required to complete all of the assays in each of the four experimental groups. The ready availability and low cost of mice makes them an ideal choice in this regard.

Once a suitable animal was chosen, a decision had to be made in regards to which model of sepsis to use. The two most widely studied models in mice are those of endotoxicosis and cecal ligation and puncture (74). Based upon it's reproducibility, ease of use, and ethical suitability, we chose to use intraperitoneal endotoxin. Although not a sine qua non of sepsis, endotoxin is almost certainly of fundamental importance in sepsis (74). Entotoxin is a relatively stable, pure compound, which is convenient to use and store. Unlike other models

of sepsis such as cecal ligation and puncture in which the innoculum cannot be controlled, doses of endotoxin are readily measured and administered. In addition, the dose response of mice to endotoxin is well characterized and predictable, which helps to standardize treatment groups (74).

4.2   ENTERAL FEEDING MODEL

This experiment was designed to investigate the mechanism of sepsis related gut dysfunction, and how that gut dysfunction affects distant organ inflammation in the setting of feeding and fasting. It is of fundamental importance to study the gut in the presence and absence of intraluminal nutrients. This mandated that some of the animals be fasted *prior* to becoming endotoxemic. Although our brief fast is analogous to the preoperative routine of fasting patients prior to surgery, it is different than the usual human circumstance in which the decision whether or not to feed is made after the patient becomes septic. In designing this experiment, it was not our goal to replicate this typical human circumstance. Instead, we were more interested in developing an experiment that would allow us to control for the presence of intraluminal contents, allowing us to study the mechanism by which enteral feeding affects the gut in the presence or absence of endotoxemia.

Several models of enteral feeding exist in the study of rodents. Others have utilized tube gavage feeds or implanted feeding tubes at numerous points along the gastrointestinal tract to quantify the amount of nutrients each animal consumes. These techniques have been used primarily in rats, which are substantially larger than mice. Our experimental design calls for two groups of fed mice (ie. mice with food in their gut lumen at the time of the endotoxemic

insult), and two groups of fasted mice (ie. animals with no luminal contents at the time of endotoxemic insult). For our purposes, the exact amount of food consumed by each animal or group does not matter, as long as those in the fed groups eat *something*, and those in the fasted groups ingest nothing at all. This design allows for a simple model of enteral feeding: the fed mice will be given ad libitum rodent chow, and the fasted mice will be denied anything solid per os for the duration of the experiment. A relatively short 16 hour fast prior to the intraperitoneal injection of endotoxin was chosen because it allows time for the fasted mice to empty their gut contents without them becoming malnourished. This was established by preliminary experiments.

4.3   ASESSMENT OF GUT APOPTOTIC ACTIVITY

One of the primary goals of this experiment is to make an assessment of gut apoptosis in both the fed and fasted state, in the presence and absence of endotoxin. In addition to looking at end stage apoptosis in the enterocyte via in-situ staining for DNA breaks, it is instructive to study the process of apoptosis at an earlier stage. Knowing that the CPP32 family of caspases are sequentially activated during gut apoptosis (82), and that these caspases cleave and inactivate the nuclear DNA repair enzyme poly (ADP-ribose) polymerase (PARP) as a late event in apoptosis (28), we sought assays that would allow us to measure caspase 3 and 6 activity, as well as the activity and cleavage state of PARP. The caspase activity assays we chose are simple in concept. A sample of gut protein is incubated with a labeled caspase-specific tetrapeptide. Cleavage of that peptide by caspases in the gut protein sample leads to a colorimetric reaction proportional to caspase activity, which is easily quantified by spectrophotometry.

PARP is an enzyme that responds to DNA breaks by utilizing NAD$^+$ as a substrate to construct long branched chains of poly (ADP-ribose), which are placed at the site of the DNA break. These short lived, highly charged structures are thought to facilitate DNA repair, and inhibit DNA break down by DNAses. In order to measure the activity of gut PARP, we chose to use a previously described assay in which gut protein samples are incubated with radiolabeled NAD$^+$, forming radiolabeled chains of poly(ADP-ribose). By precipitating out these chains and measuring their radioactivity, the enzymatic activity of PARP will be determined.

It is known that both caspase 3 and 6 cleave the intact PARP molecule into inactive 85 kd and 116 kd fragments. In order to tie together early and late events in apoptosis, we chose to examine the cleavage state of PARP in our gut protein samples. To do this required a technique that was capable of separating proteins based on size, and Western blotting became an ideal candidate for this. Western blotting uses gel electrophoresis to separate proteins on the basis of size and charge. The proteins are then transferred or "blotted" onto a sheet of nitrocellulose, and incubated with labeled protein-specific antibodies to allow for visualization. Although the time frame for the events of gut apoptosis during endotoxemia is unclear, we hope to find a relationship between gut caspase activity, PARP activity, and PARP cleavage state in our model.

4.4 DETERMINATION OF GUT FUNCTION

Previous studies investigating the gut response to sepsis have focused on bacterial translocation to the mesenteric lymph nodes as a measure of gut dysfunction. While this method is validated, it is not very sensitive, and generally requires 24 hours or more of endotoxemia to yield positive results. Measurement of gut macromolecular permeability offers a more sensitive way to measure small changes in gut function. Though difficult to perform on an animal the size of a mouse, macromolecular permeability measurements have been performed on humans and rats, with impressive results. We have adapted a technique that was validated in rats, and involves instilling fluorescein labeled dextran MW 5000 into a closed loop of mouse small bowel. In order to prevent excretion, both renal pedicles are tied off, and the solution is allowed to equilibrate for 45 minutes. Blood is then harvested via cardiac stab and the serum is assayed for fluorescence, which is proportional to gut permeability. The results of this assay will allow us to investigate the relationship between gut apoptosis and gut dysfunction during early endotoxemia, which has not been done before.

4.5 ASSESSMENT OF PULMONARY INFLAMMATION

Distant organ inflammation is a hallmark of the systemic inflammatory response syndrome. Lung inflammation during endotoxemia has been thoroughly studied in mice, and the expected cytokine profile well described (234, 235). We sought to demonstrate lung inflammation in terms of the cytokine response, and confirm this by quantifying the infiltrate of neutrophils into the lung using immunohistochemistry. Cytokine levels of IL-6 and MIP-2 will be determined using enzyme linked immunosorbent assay (ELISA), which is a sensitive antibody-based technique to quantify the amount of a desired protein present in a sample.

IL-6 was chosen because it is a pro-inflammatory cytokine directly related to mortality in human sepsis (185, 189), and MIP-2 because it is the murine functional analogue to IL-8, a key neutrophil chemotactic cytokine in the lungs in human sepsis and ARDS (99, 155). Although lung cytokine levels are important, we felt it was necessary to confirm lung inflammation histologically, and correlate that with the ELISA results. To do this, immunohistochemistry of thin paraffin embedded lung sections will be used to count the number of neutrophils in the lung.

Chapter 5

THE EFFECT OF ENTERAL FEEDING ON GUT APOPTOSIS, GUT PERMEABILITY, AND PULMONARY INFLAMMATION DURING MURINE ENDOTOXEMIA

5.1   RATIONALE FOR EXPERIMENTAL PLAN

Sepsis and the systemic inflammatory response in both animals and humans are associated with gut mucosal damage and dysfunction (51, 176). Gut dysfunction during sepsis is a common problem, resulting in loss of gut mucosal barrier selectivity, increased permeability to various hydrophilic solutes (35), and translocation of bacterial products into the circulation (47, 51), which may then further increase the inflammatory response in distant organs resulting in multiple organ dysfunction and death (175). As a result, the gut is viewed by many as an "engine" that drives sepsis. One possible contributory mechanism to endotoxin-induced gut mucosal damage is increased apoptosis (187). Septic inflammatory mediators enhance apoptosis in a large number of cell lines (86, 116, 152). In intact animals, increased cardiac and hepatic apoptosis during sepsis may contribute to sepsis-related dysfunction of those organs (39, 104, 116). Thus, it is reasonable to postulate that increased gut apoptosis may also occur during sepsis, and that excessive mucosal cell death may contribute to the observed gut mucosal atrophy, damage, and impaired gut barrier function.

In experimental models of sepsis enteral feeding has been shown to reduce mucosal atrophy and improve gut immunity (119) while, clinically, enteral feeding reduces septic morbidity in post operative and post trauma patients (118, 163, 207). If, as we postulate above, sepsis

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induces gut mucosal apoptosis leading to increased gut permeability and translocation with increased inflammation in distant organs, then feeding could conceivably have beneficial effects by impacting the underlying mechanism – feeding may reduce gut apoptosis during sepsis. Indeed, if these steps; 1) increased apoptosis, 2) increased gut permeability, and 3) increased inflammation in distant organs, are causally related then if feeding affects one step it must affect all steps.

Accordingly, we tested the effect of endotoxin in fed versus fasted mice using a 2 by 2 experimental design. We did not wish to study established starvation (>1-3 days in mice (66, 248)). Instead we chose to study a moderate degree of food restriction comparable to moderate limitation of food intake that may frequently be found in critically ill patients. Based on preliminary autopsy studies that showed the fasted mouse gut lumen to be empty, we chose a sixteen hour fast. We first measured caspase 3 activity as an important final common mediator of the intracellular apoptotic cascade and caspase 6 activity because it is activated by caspase 3, and hence provides additional evidence of caspase 3 activity (81, 208, 224). As a further measure of induction of apoptotic pathways we also measured PARP expression and activity since recent investigations have shown that PARP, an enzyme involved in DNA repair, and a final effector in the apoptotic pathway, may play a role in gut dysfunction during sepsis (220). We used TUNEL staining of gut sections to determine which cells were involved. To assess gut dysfunction we measured gut permeability to dextran molecules. Whether increased gut permeability in fasted mice can result in an increase in the inflammatory response in a distant organ is unknown. Therefore, we measured the number of neutrophils in lung sections and the concentration of both IL-6 and

MIP-2 in samples of lung using ELISA. We chose IL-6 because it is a pro-inflammatory cytokine directly related to mortality in human sepsis (185, 189), and chose MIP-2 because it is the murine functional analogue to IL-8, a key neutrophil chemotactic cytokine in the lungs in human sepsis and ARDS (99, 155). We chose endotoxin injection, not as an accurate model of clinical sepsis but, rather, to investigate mechanism in this well studied, reproducible model that results in increased gut permeability (31, 55, 73, 203, 226).

5.2   METHODS

5.2.1   Experimental Design and Protocol

Four groups of 10 mice each were studied: Fed/Sham, Fasted/Sham, Fed/Endotoxin, Fasted/Endotoxin. Those animals in the fed groups received ad libitum rodent chow (Lab Diet Rodent Diet 5001, PMI Intl., Brentwood, MO) throughout the experiment, while the fasted mice were denied food for 16 hours prior to either saline (sham) or endotoxin injection (Figures 9 and 10). We chose a 16 hour fast due to preliminary time course experiments showing that it took 16 hours to clear the mouse gut of solid food after eating, and we wanted to study the gut in the presence and absence of food. All groups had free access to water at all times. The animals were then briefly anaesthetized with 3% inhaled halothane, given a 1 mL subcutaneous injection of sterile normal saline, and 0.25 mL intraperitoneal (I.P.) injection of either sterile normal saline (sham) or endotoxin (E. coli 0111:B4 LPS, 4 mg/kg dissolved in normal saline). Six hours later, the animals were again anaesthetized, and their entire small bowel was rapidly harvested and irrigated with 3 mL ice cold normal saline to
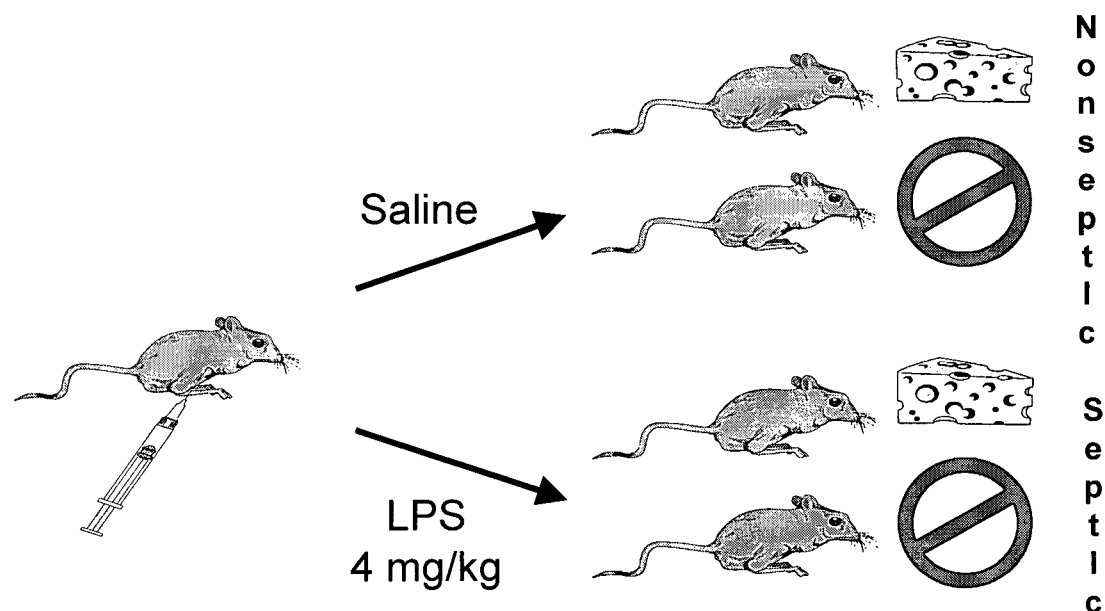
Figure 9. **Experimental Groups**

This figure illustrates the four groups of CD-1 mice used in our experiment: Fed/Sham, Fasted/Sham, Fed/Endotoxin, Fasted/Endotoxin. The fed animals were allowed ad lib rodent chow, while the fasted groups were denied access to any food. Animals in the septic groups were given an intraperitoneal injection of LPS, while their nonseptic counterparts were given a saline injection.

Time (hrs.)

0	16	22

Food
removed
or replaced

LPS or
Sham
Injection

Organs
harvested

Ad lib water throughout

Figure 10.   **Time Line of Experiment**

This figure illustrates a time line of our experimental protocol. At time 0, food was either removed, or replaced into the cages of the mice, depending their group. After 16 hours, the mice were injected with either LPS or saline (sham), and six hour later their organs were harvested. All mice were allowed ad lib access to water throughout the experiment.

clear intestinal contents. The gut and lungs were snap frozen in liquid nitrogen, and stored at

-80 °C. The animals were euthanized by exsanguination while anesthetized.

5.2.2 Caspase Activity

Frozen whole gut tissue was homogenized for 30 seconds in 2 mL of ice cold buffer

containing 50 mM TrisHCl pH 8.0, 25 mM $MgCl_2$, 0.1 mM PMSF. The homogenate was

then placed on ice and sonicated, centrifuged at 5000 rpm for 15 minutes at 4° C, and the

protein concentration of the supernatant quantified using the bicinchoninic acid method

(BCA assay, Pierce, Rockford, IL). The supernatant was then diluted to 2 mg/mL and

assayed for caspase 3 and 6 activity using a colorimetric assay (R&D Systems, Minneapolis,

MN). Gut protein samples were incubated with either the p-nitroanilide (p-NA) conjugated

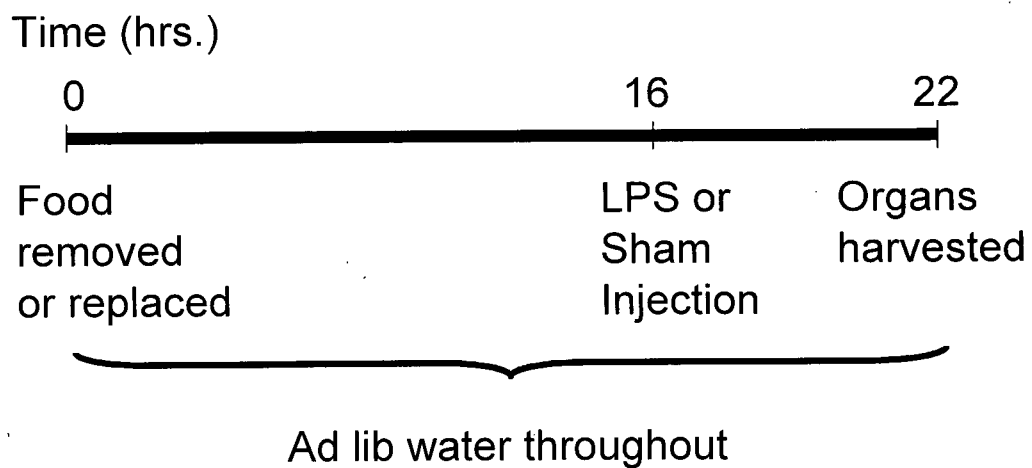caspase 3 specific peptide DEVD-p-NA (Asp-Glu-Val-Asp-p-NA) or the caspase 6 specific

equivalent VEID-p-NA (Val-Glu-Ile-Asp-p-NA) as substrate. The cleavage of the peptide

by the caspase releases the chromphore p-NA, which was quantitated spectrophotometrically

at 504 nm in a microplate reader (Rainbow Reader, SLT Lab Instruments, Germany). The

level of caspase enzymatic activity is directly proportional to the color reaction, and results

were expressed as fold increase in caspase activity over the Fed/Sham control group.

5.2.3 Poly(ADP-ribose) polymerase Activity

In order to determine PARP activity, we used a radiolabeled NAD based enzymatic assay

(R&D Systems, Minneapolis, MN). Ten µL of each sample of gut protein prepared as

described above (2 mg/mL total protein) was incubated with the following: 2 µL of 1 µCi/µL

^{32}P-labeled NAD (New England Nuclear, Boston, MA), 10 µL 10X PARP buffer, 10 µL 1

mM NAD, and 68 µL distilled water. After exactly 1 minute, the reaction was stopped with 900 µL ice cold 20% TCA, passed through a GF/C glass fiber filter pre wetted with 10% TCA, washed four times with 3 mL cold 10% TCA, and twice with cold 85% ethanol, leaving the precipitated chains of radiolabeled poly (ADP-ribose) on the filter. A scintillation counter (Beckman LS8200, Beckman Coulter, California, USA) was then used to quantify the radioactivity on the filter after it was placed in a glass scintillation vial containing 3 mL scintillant (Scintisafe 30%, Fisher Scientific, ON, Canada). The specific activity of NAD was then calculated and used to ascertain the PARP activity in each sample, which was expressed in nmol/min/µL. A positive control used damaged DNA and stock PARP enzyme.

5.2.4 Poly (ADP-ribose) polymerase Protein Expression

In it's active intact form, PARP exists as a 116 kd molecule. During apoptosis, PARP is specifically cleaved by the CPP32 caspases such as Caspase 3 and 6 to form a major and minor fragment, the former of which is 85 kd (185). In order to determine PARP protein expression in the gut, we performed Western analysis using the same gut protein samples as in the PARP activity assay described above. 200 µg of each gut protein sample, 5 µL of molecular weight marker, and 7.5 µL of HeLa cell extract was loaded on a 7.5% SDS-PAGE gel. After separation by gel electrophoresis at 200 volts for 55 minutes, the protein was then transferred onto a nitrocellulose membrane at 300 milliamps for 25 minutes using a transfer buffer of 192 mM glycine, 25 mM Tris, and 20% methanol. Membranes were then washed and blocked for 1 hour with TBS–TWEEN + 5% milk protein while shaking at room temperature. The primary PARP antibody consisting of rabbit anti-mouse polyclonal IgG

(Upstate Biotechnology, Lake Placid, NY) was then prepared in blocking solution to a concentration of 2 μg/mL and added for 1 hour. This antibody was specific for both the intact (116 kd) and cleaved (85 kd) forms of PARP. The next day, the membrane was washed with TBS and incubated for 1 hour with the secondary antibody of HRP-goat anti-rabbit IgG (Upstate Biotechnology, Lake Placid, NY). Blots were developed using ECL (Amersham, England) following the manufacturer's instructions. Membranes were exposed to Hyperfilm™ ECL™ film (Amersham, England) and developed. Quantification of the amount of protein present in each sample was done by measuring the densitometry of each of the visible bands using the Eagle Eye II still video imaging system (Stratagene, La Jolla, CA). Background readings were subtracted for each band, and results were expressed in arbitrary units

5.2.5 Gut TUNEL Staining

TUNEL staining was carried out using TdT-FragELTM DNA Fragmentation Detection Kit (Oncogene, Cambridge MA). Positive and negative controls consisted of gut samples treated with 0.1 mg/mL DNase or incubated without Tdt respectively. TUNEL staining was performed according to manufacture instructions with the following exception. The permeablization step was carried out using 40 μg/mL Proteinase K for 30 minutes at 37°C. A single blinded observer determined the number of apoptotic cells per high power field and whether the cells were mucosal epithelial or other cells.

5.2.6 Intestinal Mucosal Macromolecular Permeability

In separate but identical experiments, we measured the passage of macromolecules from the lumen of the small intestine to the systemic circulation as previously described (71). Four groups were studied: Fed/Sham (n=10), Fasted/Sham (n=10), Fed/Endotoxin (n=10), and Fasted/Endotoxin (n=9). Following the same feeding and injection protocol as described earlier, the mice were anaesthetized with inhaled halothane 5 hours after sham or endotoxin injection. Via a midline laparotomy, a 21 gauge plastic catheter was inserted into the proximal jejunum, just distal to the pylorus. The catheter was secured in place with a 5-0 silk suture which also occluded the bowel just proximal to the opening of the catheter. Intestinal contents were gently expressed manually from this gut segment to clear the gut lumen. Absence of lumen contents in this gut segment was confirmed at autopsy. In order to create a closed segment of small bowel, a 5-0 silk suture was used to tie off the terminal ileum. Both renal pedicles were ligated with 5-0 silk to prevent urinary excretion of the fluorescent probe. Once the surgical procedure was completed, 0.6 mL of freshly mixed 25 mg/mL fluorescein labeled dextran MW 4000 (FD-4; Sigma, Oakville, ON, Canada) in normal saline was slowly injected into the gut lumen, avoiding any distension of the gut wall. The entire procedure took 15 minutes to complete. To keep the animal warm and protect the dye from light, the intestine was covered with a blanket of aluminum foil, during which the animal remained anesthetized. After 45 minutes (6 hours after sham or endotoxin injection), 0.5 mL of blood was collected by cardiac stab, anticoagulated using citrate dextrose, centrifuged at 5000 rpm for 10 minutes, and supernatant diluted 1:2 in phosphate-buffered saline (PBS; pH 7.3). The concentration of FD-4 was determined using a fluorescence spectrophotometer (LS-50; Perkin Elmer, Palo Alto, CA) with the following parameters set: excitation wavelength, 492

nm; excitation slit width, 2.5 nm; emission wavelength, 515 nm; emission slit width, 10 nm; and integration time 10 seconds. A standard curve relating FD-4 concentration to fluorescence intensity was generated for each day's experiment by adding known amounts of FD-4 to PBS.

5.2.7 Measurement of Lung Cytokines

We used enzyme linked immunoassay (ELISA) to measure the concentration of both IL-6 and MIP-2 in lung tissue. Lung tissue, stored at -80 °C, was homogenized for 30 seconds (Tissue-Tearor, BioSpec, Bartlesville, OK) in 1 mL of ice cold 1X PBS pH 7.4. The samples were then centrifuged at 1500 rpm for 10 minutes at 4 °C, and the supernatant assayed for protein concentration (BCA assay, Pierce, Rockford, IL). All samples were then made up to a uniform protein concentration with PBS. The ELISA antibodies for IL-6 were chosen on the basis of their ability to be paired (IL-6: MP5-20F3 and MP5-32C11, PharMingen, San Diego, CA). MIP-2 was measured using a Quantikine M mouse MIP-2 Immunoassay kit (R&D Systems, Minneapolis, MN). ELISA plates were incubated at 4 °C overnight with 50 µL per well of desired capture antibody. Plates were washed four times and non-specific binding was blocked using 200 µL of PBS with 2% bovine serum albumin (BSA) per well for 90 minutes. Plates were washed three times followed by incubation of 75 µL of diluted cell free supernatant for 3 hours at room temperature. The plates were again washed and 50 µL/well (1 µg/mL) of the paired biotinylated antibody was added and incubated for 60 minutes. Avidin-peroxidase conjugate was added followed by chromagen substrate (OPD, Dako, Carpinteria, CA). Plates were read at 490 nm using an ELISA plate reader (Rainbow Reader, SLT Lab Instruments, Germany).

5.2.8 Lung Tissue Neutrophils

Four-micron sections of paraffin-embedded lungs or gut were deparaffinized and rehydrated. Sections were autoclaved in citrate buffer for 20 minutes to allow for antigen retrieval. Cooled sections were stained for neutrophils as previously described (235) using rat anti-mouse neutrophil antibody (Serotec, UK) diluted 1/500 in TBS + 1% BSA (fraction V, Sigma). Biotinylated rabbit anti-rat IgG (Dako) diluted to 1/300 was selected as the secondary antibody. The number of neutrophils per 40X field was determined in 5 random fields per mouse

5.2.9 Statistical Analysis

All results are expressed as mean $\pm$ SE. We used a two factor ($\pm$ feeding and $\pm$ endotoxin) analysis of variance to determine whether these factors had a statistically significant effect, choosing $p < 0.05$ as significant. When a significant difference was found, we used an unpaired Student's t test corrected for multiple comparisons using a sequentially rejective Bonferroni procedure to identify specific differences.

5.3   RESULTS

5.3.1   Gut Apoptotic Activity

5.3.1.1 Gut Apoptosis

Six hours after endotoxin injection caspase 3 activity increased 4.9 ± 0.6 fold $(p<0.001)$ (Endotoxin groups compared to sham groups in Figure 11) and caspase 6 activity increased 4.5 ± 0.5 fold $(p<0.001)$ (Figure 11) relative to sham groups, indicating that endotoxin activates apoptotic pathways in the gut. Enteral feeding decreased caspase 3 activity by 41% in the endotoxin groups $(p<0.05)$ (Figure 11). In sham groups not treated with endotoxin, feeding did not alter caspase 3 activity. Similarly, feeding decreased gut caspase 6 activity in both sham and endotoxin groups although these differences were not statistically significant. TUNEL staining also demonstrated an endotoxin-induced increase in total apoptotic cells in the fasted group that was inhibited by feeding $(p<0.05)$ (Figures 11 and 12). In particular, apoptosis of mucosal epithelial cells was markedly inhibited by feeding $(p<0.05$, Figures 11 and 12). However, cells in all layers of the gut wall contributed to TUNEL staining, including infiltrating leukocytes - although there was no difference in the number of neutrophils infiltrating the gut wall between the 4 groups $(1.0 \pm 0.2$ neutrophils per 40X field). Thus, enteral feeding ameliorates endotoxin-induced apoptosis.

Figure 11. Gut Apoptosis Assay Results (see next page)

Average gut caspase 3 activity (panel A), caspase 6 activity (panel B) and number of TUNEL positive cells per 40X field (panel C, stipled bars are total TUNEL positive cells, shaded bars are TUNEL positive mucosal epithelial cells) are shown for each of the 4 experimental groups (n=10 per group). Endotoxin (black bars, panels A and B) increases caspase-3 and 6 activity compared to sham-treated mice (white bars) ($p < 0.001$). Within the endotoxin-treated groups, feeding reduces caspase-3 activity (* indicates $p < 0.05$) and reduces the total number of TUNEL positive cells (* indicates $p < 0.05$) and the number of TUNEL positive mucosal epithelial cells (* indicates $p < 0.05$). Error bars indicate standard error of the mean.
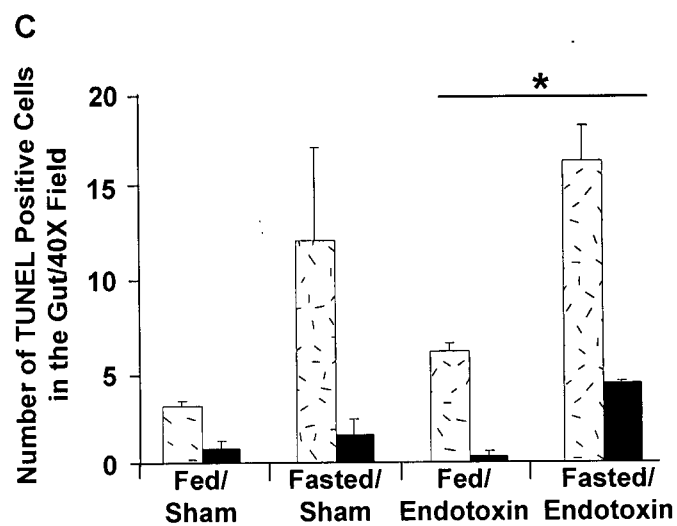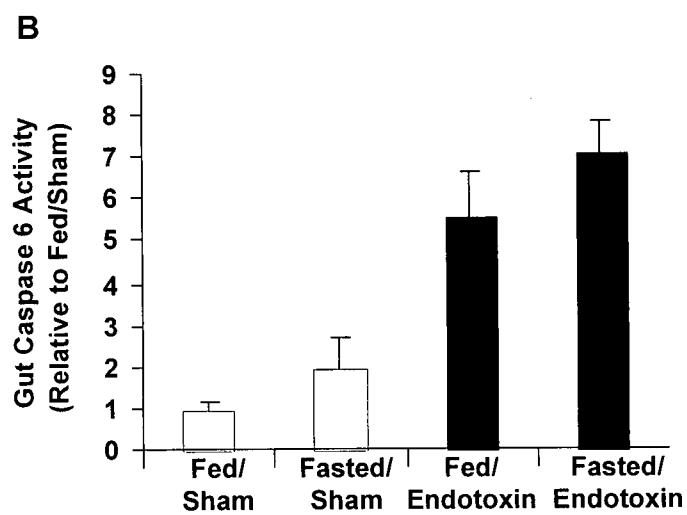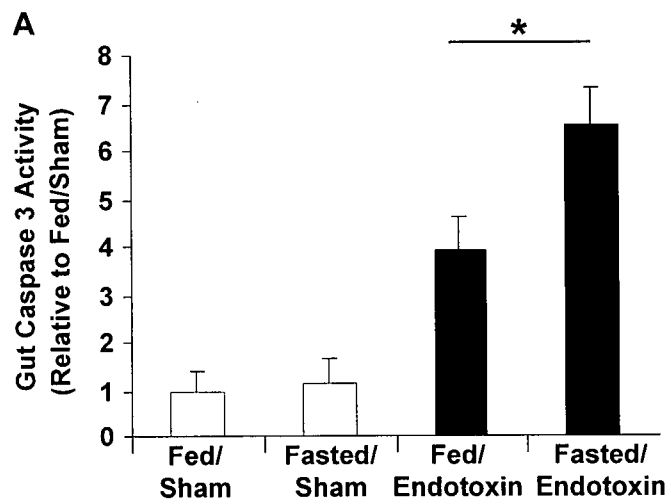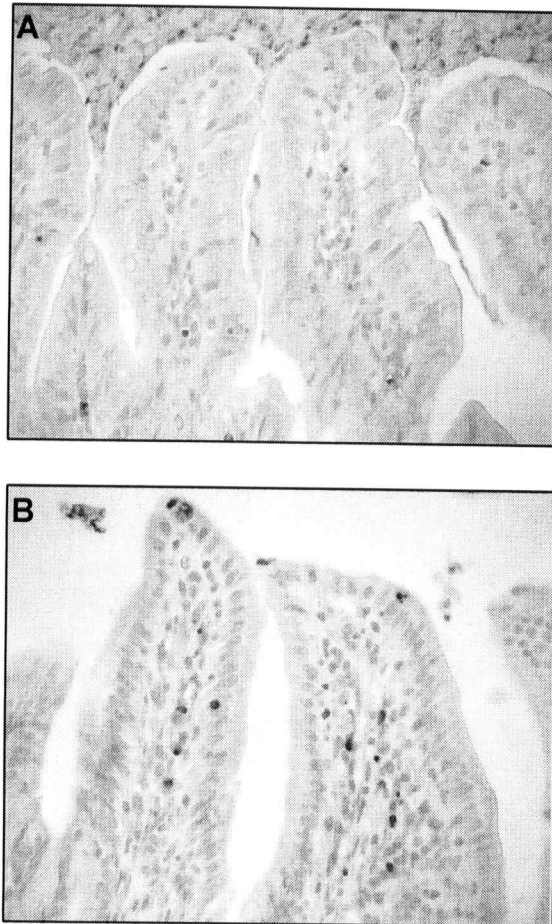
Figure 12.   Gut TUNEL – Representative Sections

TUNEL staining of representative gut sections. Fed/Endotoxin (Panel A) mice have fewer TUNEL positive (dark) cells in total and TUNEL-positive mucosal epithelial cells than Fasted/Endotoxin (Panel B) mice (p<0.05). Total TUNEL positive cells include cells in all layers of the gut wall, including infiltrating leukocytes

5.3.1.2 Gut PARP expression and activity

PARP is cleaved and inactivated by CPP32 caspases, which include caspase 3 and 6. However, PARP activity increases in response to DNA strand breaks. DNA strand breaks increase with caspase 3 and subsequent endonuclease activity (245) and are believed to increase in enterocytes during inflammatory injury (113). Therefore we measured both PARP protein expression and PARP activity in gut tissue to resolve the net effect of increased caspase activity on the competing potential outcomes of decreased PARP expression and increased PARP activity. Gut PARP protein expression at 6 hours mirrored caspase 3 and 6 activity, the proteins that cleave PARP. That is, endotoxin decreased PARP expression by 60% (Figure 13). Feeding resulted in greater PARP expression in both Sham and Endotoxin groups but this difference was not statistically significant. We did not observe 85 kD bands, characteristic of cleaved PARP, at 6 hours but 85 kd bands were detectable at 12 hours (Figure 13). In parallel with the increase in PARP expression, feeding also significantly increased PARP activity at 6 hours (p<0.05) (Figure 14). In contrast, endotoxin, which decreased PARP expression, resulted in an 18% increase in gut PARP activity (p<0.05) (Figure 14). Thus, although PARP expression is reduced by endotoxin administration, possibly due to the observed increased CPP32 caspase activity that cleaves PARP, endotoxin administration increases PARP activity similar to other models of sepsis (220).
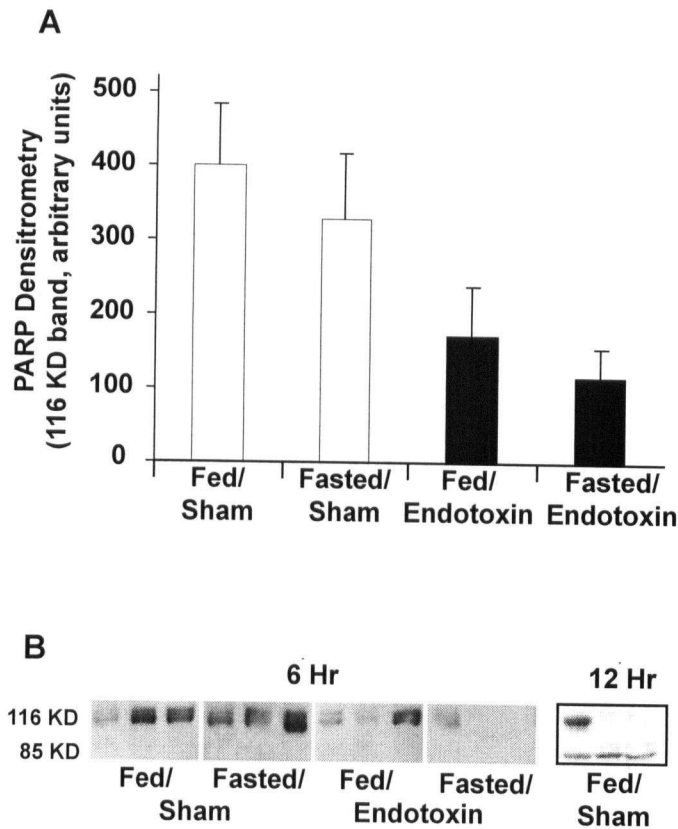
Figure 13. **Gut PARP Expression and Western Analysis**

Average PARP expression (panel A), measured using Western analysis, is shown for each of the 4 experimental groups (n=10 per group). Endotoxin (black bars) decreases PARP expression ($p<0.005$). Feeding results in a trend to increased PARP expression in both sham and endotoxin groups (p = NS). These results mirror caspase 3 and 6 activity (Figure 1). Representative Western analysis images are shown in panel B. Cleavage of 116 kD PARP is not seen at 6 hours but is visible at 12 hours (85 kD product). Error bars indicate standard error of the mean.
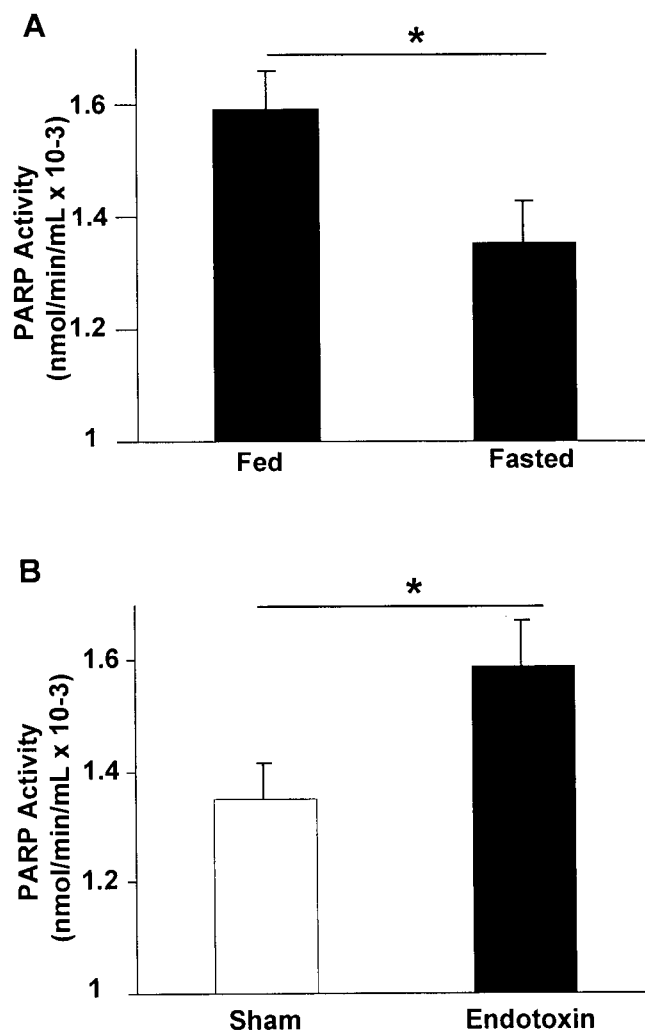
Figure 14.   Gut PARP Activity

Average PARP activity is shown comparing Fed to Fasted groups in the upper panel A and Sham

to Endotoxin groups in the lower panel B. Feeding increased PARP activity ($p<0.05$), consistent

with the observation that feeding decreased caspase activity (Figure 1) and therefore reduced

PARP breakdown (Figure 3). Endotoxin also significantly increased PARP activity ($p<0.05$)

consistent with previous observations (40). Error bars indicate standard error of the mean.

5.3.2   Intestinal Mucosal Macromolecular Permeability

Whether the above evidence of increased gut apoptosis is related to impaired gut barrier function is unknown. Whether reduction in endotoxin-induced apoptosis by feeding then reduces gut permeability during endotoxemia is also unknown. Therefore, we measured gut macromolecular permeability by quantifying the transfer of fluorescein labeled dextran MW 4000 (FD-4) from the gut lumen into the systemic circulation. Gut permeability increased by 44% in endotoxin-groups ($p<0.01$) (Figure 15). Furthermore, fasted mice had 38% higher gut permeability than fed mice ($p<0.01$) in both sham and endotoxin groups (Figure 15). These results indicate that even an overnight fast may be intervention enough to significantly affect gut permeability and, further, that fasting adds to endotoxin-induced increases in gut macromolecular permeability.

5.3.3   Lung Inflammatory Response to Fasting and Endotoxemia

Whether increased gut permeability in fasted mice can result in an increase in the inflammatory response in a distant organ is unknown. Therefore, we measured the concentration of both IL-6 and MIP-2 in samples of lung protein extract from each group using ELISA and measured neutrophils in lung sections. Endotoxin treated mice had a 5.9 ± 0.1 fold increase in lung IL-6 concentration ($p<0.001$) (Figure 16) consistent with the expected systemic inflammatory response (234). Enteral feeding decreased lung IL-6 concentration by 40% in the endotoxin groups ($p<0.05$) (Figure 16). There was no difference in lung IL-6 between the fed and fasted mice that did not receive endotoxin. A similar pattern was seen with lung MIP-2 concentrations (Figure 16). Endotoxin-treated mice had a 290 ± 40 fold increase in lung MIP-2 concentration ($p<0.001$). As with lung IL-6, we found
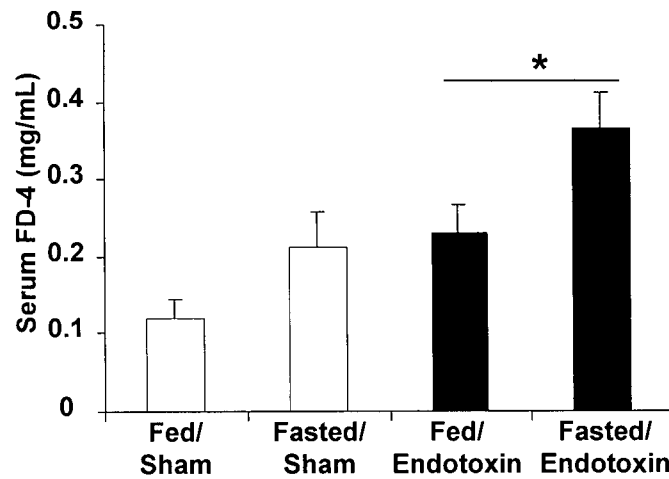
Figure 15. **Gut Macromolecular Permeability**

Average gut permeability, assessed by measuring fluorescein labeled dextran having a molecular

weight of 4000 (FD-4) in the blood after instilling FD-4 within the gut lumen, is shown. Endotoxin

(black bars) increases gut permeability ($p<0.05$). Furthermore, feeding decreases gut permeability (*

indicates $p<0.05$) in both the Sham and Endotoxin groups. Error bars indicate standard error of the

mean

Figure 16.   **Lung Cytokine Assay Results (see next page)**

Average lung IL-6 (panel A) and MIP-2 (panel B) concentration, and number of neutrophils per 40X field (panel C), is shown for each of the 4 experimental groups. We measured IL-6 because it is a pro-inflammatory cytokine closely related to mortality in human sepsis. Endotoxin (black bars) greatly increases IL-6 expression ($p < 0.001$) as expected. Within the endotoxin-treated groups, feeding reduces lung IL-6 expression (* indicates $p < 0.05$). We measured MIP-2 because it is the murine functional analogue to IL-8, a key chemotactic cytokine in the lungs in human sepsis and ARDS. Endotoxin (black bars) greatly increases MIP-2 expression ($p < 0.001$) as expected. Within the endotoxin-treated groups, feeding reduces lung MIP-2 expression (* indicates $p < 0.05$). Similarly, endotoxin increases the number of infiltrating neutrophils ($p < 0.001$), an effect partially reversed by feeding (* indicates $p < 0.05$). Error bars indicate standard error of the mean.

A

Lung IL-6 concentration (pg/mL)

2500 — 2000 — 1500 — 1000 — 500 — 0

*

Fed/Sham Fasted/Sham Fed/Endotoxin Fasted/Endotoxin

B

Lung MIP-2 concentration (pg/mL)

12000 — 10000 — 8000 — 6000 — 4000 — 2000 — 0

*

Fed/Sham Fasted/Sham Fed/Endotoxin Fasted/Endotoxin

C

Number of PMNs in the lung/40X Field

140 — 120 — 100 — 80 — 60 — 40 — 20 — 0

*

Fed/Sham Fasted/Sham Fed/Endotoxin Fasted/Endotoxin
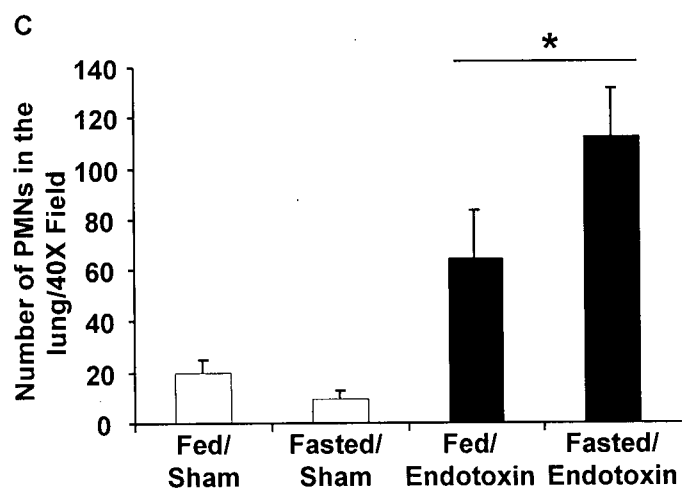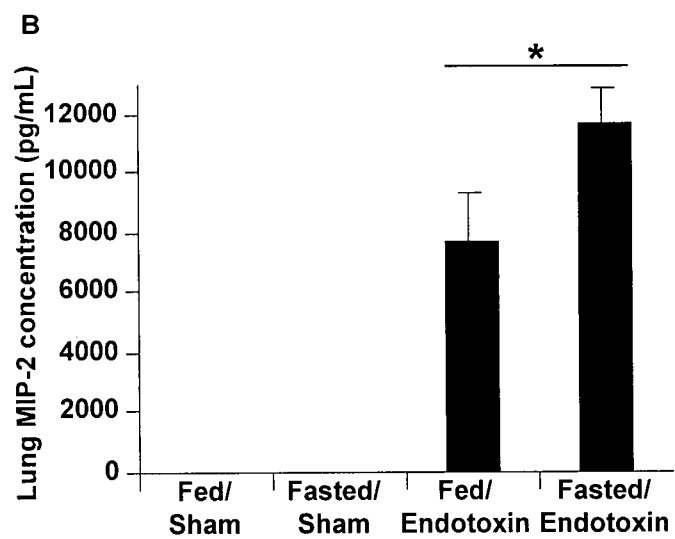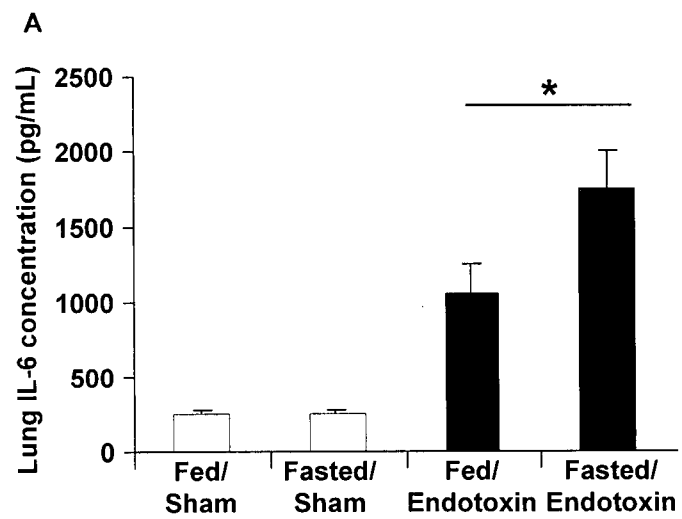
that lung MIP-2 concentration within the endotoxemic group was 35% less in fed mice than in fasted mice (p<0.05). There was no difference in lung MIP-2 between fed and fasted mice within the sham groups. Similar results were found when these whole-lung ELISAs were not corrected for total sample protein. Endotoxin and feeding altered pulmonary neutrophils infiltration (p<0.05) in parallel with the changes observed in pro-inflammatory cytokine expression (Figure 16). These results indicate that enteral feeding reduces pulmonary inflammation during endotoxemia, but has no effect on pulmonary cytokine expression or neutrophils under normal circumstances.

5.4   Discussion

The novel findings in this study are that an increase in gut apoptotic activity occurs following endotoxin infusion, is associated with increased gut macromolecular permeability, and increased pulmonary cytokine expression. In this setting, the co-intervention of feeding decreases the extent of apoptotic activity, gut permeability, and pulmonary cytokine expression. In other experimental settings increased gut apoptosis results in increased gut permeability (217, 218). Further investigations suggest that increased gut permeability increases the systemic inflammatory response and involvement of distant organs (175). Taken together, our new results and previous observations suggest an important association between gut apoptosis, gut barrier function, and pulmonary inflammation during sepsis. Enteral feeding ameliorates increased gut apoptotic activity which, we postulate, may be a key step in improving gut barrier function and decreasing inflammation in distant organs during sepsis.

Gut injury in sepsis and in other models of gut injury results in loss of gut barrier function to bacteria and bacterial products such as endotoxin (47, 51, 72, 110, 176). The cause and mechanism of gut dysfunction in sepsis is unclear. Gut mucosal injury seen in septic rodents is not associated with concurrent mucosal perfusion deficits, suggesting that ischemia may not play a prominent role in normotensive septic gut dysfunction (174). However, gut mucosal atrophy occurs during sepsis (187). We suggest that one possible mechanism for sepsis related gut dysfunction is increased apoptosis.

Recent studies have shown that increased cardiac and hepatic apoptosis during sepsis may contribute to the sepsis-related dysfunction of those organs (104, 116). Apoptosis plays an important role in gut mucosal homeostasis under normal physiologic conditions. After differentiating from stem cells located at the base of intestinal crypts, enterocytes migrate towards the villus tip, where they undergo apoptosis, detach, and are shed to complete their 3-5 day life cycle (82). In inflammatory states TNF induces caspase activation within enterocytes, causing enterocyte detachment in the lumen and resulting in villus atrophy (187). In another model, apoptosis induced by doxorubicin results in increased gut permeability, which can be reduced by inhibitors of apoptosis (218). Here we extend these results to show that endotoxin-induced inflammatory response in mice also induces increased apoptosis in the gut and, similar to previous observations (187, 218), this is associated with gut barrier dysfunction.

The DNA repair enzyme poly (ADP-ribose) polymerase (PARP) is strongly activated in enterocytes by endotoxin both in-vitro and in-vivo due to DNA breaks caused by

peroxynitrite and other reactive oxygen intermediates produced as part of an inflammatory response (113), and by caspase activity (26). Our results are consistent with these findings and show that gut PARP activity is increased by endotoxin administration. Activation of PARP depletes cellular energy stores and may contribute to cell and organ dysfunction, such as the increase in gut macromolecular permeability we observed (113, 220). However, caspase 3 and 6 then result in cleavage and inactivation of PARP (28). Therefore, we measured PARP expression as evidence of caspase 3 and 6 activity. The decrease in PARP expression, measured by Western analysis, that we observed during endotoxemia is consistent with the increase in gut caspase 3 and 6 activity that we observed during endotoxemia.

Our results are consistent with the hypothesis that failure of gut barrier function may play a role in the etiology of the systemic inflammatory response and the acute respiratory distress syndrome (45, 63, 135). As anticipated, endotoxin increased pulmonary IL-6, MIP-2, and neutrophil infiltration. We note that the observed increases in pulmonary pro-inflammatory cytokine expression may represent increased local cytokine production, increased leakage of circulating cytokines into the lung, increased activation of alveolar macrophages, or increased trafficking of inflammatory cells to the lung. Indeed, we observed an increase in pulmonary neutrophil content so that part of the increase in pulmonary pro-inflammatory cytokines may be related to the increased inflammatory cell infiltrate.

Feeding had a number of important effects. We found that enteral feeding partially reversed endotoxin-induced increases in CPP32 caspase activation and reduced the number of TUNEL

stained cells in gut sections. This effect of enteral feeding on gut apoptosis has not previously been elucidated. Our subsequent finding that endotoxemia increases gut macromolecular permeability supports previous observations in animals as well as humans (73, 176). Our results extend these observations by demonstrating that enteral feeding significantly decreases gut macromolecular permeability during endotoxemia. A further novel finding is that feeding reduced the inflammatory response in a distant organ. These results do not prove a causal link between gut apoptosis, gut permeability, and inflammation in a distant organ. That is, the effects of feeding on gut permeability and pulmonary inflammation may be due to effects unrelated to gut apoptosis. However, the concordant effect of endotoxin on apoptosis, gut permeability, and lung inflammation, and the concordant reverse effect of feeding, are consistent with an important mechanistic connection. It is interesting to note that even this mild to moderate degree of fasting has significant physiologic effects.

A number of limitations with these results should be considered. First, while endotoxin infusion is a well established tool for investigating gut barrier dysfunction, it is not a realistic model of human sepsis. CD-1 mice are relatively sensitive to endotoxin. Different effects are possible in other strains of mice or in other species. Therefore, extrapolation of these findings to human sepsis should be limited. Second, while feeding had a beneficial effect in this model, it is important to recognize that this was not a study to assess the clinical efficacy of enteral feeding. Third, the 16 hour fast used here represents mild to moderate fasting and analogous food deprivation is commonplace in both animals and humans when surgery or other invasive procedures are planned. This degree of fasting is not as severe as 1 to 3 day

fasts used in previous experiments (57, 66, 248). Furthermore, we did not find any differences in apoptosis, gut permeability, or pulmonary inflammation between the Fed/Sham and Fasted/Sham groups indicating that this moderate fast by itself was insufficient to cause a significant physiologic effect by these measures. Finally, while we have shown that the pattern of gut permeability in each of the four groups is paralleled by a corresponding change in pulmonary IL-6, MIP-2 and neutrophils, the precise mechanism of how increased gut macromolecular permeability leads to increased pulmonary inflammation has yet to be determined

We conclude that gut apoptotic activity, gut permeability, and pulmonary inflammation are increased during endotoxemia. Enteral feeding decreases gut apoptotic activity, and is associated with improved gut barrier function and decreased inflammation in a distant organ, the lungs. These data support the hypothesis that enteral feeding may decrease the distant organ inflammation by reducing gut apoptosis, thereby maintaining gut mucosal function during endotoxemia.

Chapter 6

CONCLUSIONS

A review of the current literature on the effect of enteral feeding on multiple organ failure led us to our hypothesis that increased gut apoptosis during endotoxemia leads to gut dysfunction and subsequent pulmonary inflammation, and that enteral feeding ameliorates this. By quantifying both caspase 3 and 6 activity and end stage apoptosis we found that experimental endotoxemia led to a significant increase in gut apoptosis. In an effort to tie together these findings relating to the early and late stages of apoptosis respectively, we investigated the activity and expression of an intermediary enzyme of apoptosis, poly (ADP-ribose) polymerase. With the prior knowledge that significant DNA breaks occur during endotoxin infusion in mammals, we expected to find an increase in PARP activity during endotoxemia. Indeed, this was the case in our model. In addition, the decrease in PARP expression that we observed during endotoxemia was consistent with the increase in gut caspase 3 and 6 activity that we observed during endotoxemia. Our next goal was to establish an association between gut apoptosis and gut dysfunction during endotoxemia. This was done by measuring gut macromolecular permeability. We found that gut apoptotic activity correlated remarkably well with gut macromolecular permeability in both endotoxemic and non-endotoxemic animals. Next, in order to demonstrate an association between gut apoptosis, gut function, and distant organ inflammation, we measured the levels of two pulmonary cytokines, IL-6 and MIP-2, and also quantified histologic inflammation by performing immunohistochemistry for neutrophils in the lung. We found that endotoxemia increased

lung IL-6 and MIP-2, and that this increase correlated well with the observed histologic inflammation. In addition, a close correlation between lung inflammation, gut apoptosis, and gut dysfunction was found. Finally, we sought to investigate the effect of enteral feeding on these three parameters. We observed that enteral feeding acts to partially reverse the increased gut apoptotic activity seen during endotoxemia, as well as the increases seen in both gut permeability and pulmonary inflammation. These results do not prove a causal link between gut apoptosis, gut permeability, and inflammation in a distant organ, however the concordant effect of endotoxin on apoptosis, gut permeability, and lung inflammation, and the concordant reverse effect of feeding, are consistent with an important mechanistic connection.

We conclude that gut apoptotic activity, gut permeability, and pulmonary inflammation are increased during endotoxemia. Enteral feeding decreases gut apoptotic activity, and is associated with improved gut barrier function and decreased inflammation in a distant organ, the lungs. These data support the hypothesis that enteral feeding may decrease the distant organ inflammation by reducing gut apoptosis, thereby maintaining gut mucosal function during endotoxemia.

Chapter 7

SUMMARY: CLINICAL RELEVANCE, FUTURE DIRECTIONS

The role of the gut in SIRS and sepsis has yet to be defined. In designing this study, we sought to elucidate a mechanism by which endotoxemia led to gut dysfunction and subsequent distant organ inflammation. Further, we set out to study the effects of enteral feeding on the gut and a distant organ during endotoxemia. We found that endotoxemia led to increased gut apoptosis, gut dysfunction, and subsequent pulmonary inflammation, and that this was partially reversed with enteral feeding. These novel findings provide basic science rationale to the clinical observation that enteral feeding is beneficial in the treatment of critically ill patients. While it is likely that there are several mechanisms involved in gut injury during sepsis, apoptosis appears to play a major role in the modulation of gut function and systemic organ inflammation in that setting. We would anticipate that if our findings held true in humans, therapies could be developed to alter the effect of sepsis on gut apoptosis, which could potentially decrease inflammation in distant organs, effectively shutting down the "engine" that drives sepsis. There are several areas of possible further study. These include extensions of the current project, as well as logical follow-up studies to investigate potential therapeutic agents to ameliorate gut dysfunction during sepsis.

With the results of the current study as groundwork, several extensions to the protocol could be incorporated in order to further our understanding of the nature of gut dysfunction during experimental endotoxemia. While this study demonstrates a very close association between

gut apoptosis, gut dysfunction, and pulmonary inflammation in both the fed and fasted states, it does not prove a causal relationship between these phenomena. The most accepted way to prove mechanistic causation in such circumstances is to deliberately inhibit one or more steps along the pathway being studied. In this case, the earliest and most obvious step to inhibit would be gut apoptosis during endotoxemia. If gut apoptosis truly is causally related to gut dysfunction and pulmonary inflammation, these events should be reduced or ameliorated when gut apoptosis is inhibited. The introduction of global and/or specific caspase inhibitors into the experimental protocol should inhibit gut apoptosis. The effect of endotoxemia on gut function and pulmonary inflammation could then be studied in the absence of excess gut apoptosis, which would allow for further delineation of the mechanism by which the gut effects systemic organ inflammation during sepsis.

Another obvious target for further investigation in our current study is the enzyme poly (ADP-ribose) polymerase (PARP). It is postulated that PARP plays a major role in cellular energy metabolism, especially during times of increased DNA breakage (113). This is believed to result from PARP depleting the cells of NAD^+, and subsequently ATP, as it uses these substances as substrate to form branched chains of poly(ADP-ribose), which acts as an adjunct to enzymes of DNA repair (113). In addition, the cleavage of PARP by CPP32 caspases is one of the final steps in the apoptotic pathway. The incorporation of a specific PARP inhibitor into our experimental protocol would allow for the study of the effects of this enzyme on cellular energy metabolism as well as endotoxin associated apoptosis, which would provide further insight into the mechanism of gut damage during sepis. By inhibiting the CPP32 caspases and PARP in turn, the interaction between the two moieties and their

respective effects on cellular homeostasis, gut function, and distant organ inflammation could be studied.

While it has been established clinically that enteral feeding is beneficial to critically ill patients (161, 163, 164), and that the amino acid glutamine plays an important role in enterocyte metabolism (142, 238, 244, 249), the effect of differing enteral diets or feeds on gut function and systemic organ inflammation is poorly understood. Introducing several specifically tailored diets into out current experiment would allow for the study of individual dietary components on gut apoptosis, gut function, and lung inflammation. Given the recent interest in glutamine, it would seem reasonable to subdivide the "fed" groups into three subgroups based on diet: those fed standard rodent chow, those fed a glutamine-enriched diet, and those fed a diet absent of glutamine. Information gathered from such an experiment could be used to study the effect of glutamine, or any other supplement of interest, on gut apoptosis and energy metabolism, which would provide valuable insight as to how glutamine modulates gut function during sepsis. Finally, our model could be used to study any number of nonspecific therapies in an effort to understand their effect on the gut during sepsis. Examples include free radical scavengers, xanthine oxidase inhibitors, and steroids, to name a few.

Whether apoptosis is a clinically relevant mechanism of sepsis related gut dysfunction and subsequent distant organ injury remains to be seen. It is our sincere hope that the findings of this experiment and the information presented in this thesis will be used by others to further the study of the role of the gut in sepsis.

REFERENCES

1.   Adams, S., E. P. Dellinger, M. J. Wertz, M. R. Oreskovich, D. Simonowitz, and K. Johansen. Enteral versus parenteral nutritional support following laparotomy for trauma: a randomized prospective trial. *Journal of Trauma-Injury Infection & Critical Care* 26: 882-91, 1986.

2.   Aderka, D., J. M. Le, and J. Vilcek. IL-6 inhibits lipopolysaccharide-induced tumor necrosis factor production in cultured human monocytes, U937 cells, and in mice. *Journal of Immunology* 143: 3517-23, 1989.

3.   Adrie, C., and M. R. Pinsky. The inflammatory balance in human sepsis. *Intensive Care Medicine* 26: 364-75, 2000.

4.   Alexander, J. W., B. G. MacMillan, J. D. Stinnett, C. K. Ogle, R. C. Bozian, J. E. Fischer, J. B. Oakes, M. J. Morris, and R. Krummel. Beneficial effects of aggressive protein feeding in severely burned children. *Annals of Surgery* 192: 505-17, 1980.

5.   Alkhatib, H. M., D. F. Chen, B. Cherney, K. Bhatia, V. Notario, C. Giri, G. Stein, E. Slattery, R. G. Roeder, and M. E. Smulson. Cloning and expression of cDNA for human poly(ADP-ribose) polymerase. *Proceedings of the National Academy of Sciences of the United States of America* 84: 1224-8, 1987.

6.   Alteveer, R. J., R. D. Goldfarb, J. Lau, M. Port, and J. J. Spitzer. Effects of acute severe hemorrhage on metabolism of the dog intestine. *American Journal of Physiology* 224: 197-201, 1973.

7. Anonymous. Perioperative total parenteral nutrition in surgical patients. The Veterans Affairs Total Parenteral Nutrition Cooperative Study Group. *New England Journal of Medicine* 325: 525-32, 1991.

8. Arnalich, F., J. Lopez, R. Codoceo, M. Jim nez, R. Madero, and C. Montiel. Relationship of plasma leptin to plasma cytokines and human survivalin sepsis and septic shock. *Journal of Infectious Diseases* 180: 908-11, 1999.

9. Baker, J. W., E. A. Deitch, M. Li, R. D. Berg, and R. D. Specian. Hemorrhagic shock induces bacterial translocation from the gut. *Journal of Trauma-Injury Infection & Critical Care* 28: 896-906, 1988.

10. Banwell, J. G., R. Howard, D. Cooper, and J. W. Costerton. Intestinal microbial flora after feeding phytohemagglutinin lectins (Phaseolus vulgaris) to rats. *Applied & Environmental Microbiology* 50: 68-80, 1985.

11. Bazzoni, F., and B. Beutler. The tumor necrosis factor ligand and receptor families. *New England Journal of Medicine* 334: 1717-25, 1996.

12. Benjamin, R. C., and D. M. Gill. ADP-ribosylation in mammalian cell ghosts. Dependence of poly(ADP-ribose) synthesis on strand breakage in DNA. *Journal of Biological Chemistry* 255: 10493-501, 1980.

13. Bernard, G. R., J. L. Vincent, P. F. Laterre, S. P. LaRosa, J. F. Dhainaut, A. Lopez-Rodriguez, J. S. Steingrub, G. E. Garber, J. D. Helterbrand, E. W. Ely, C. J. Fisher, Jr., and C. W. E. i. S. S. s. g. Recombinant human protein. Efficacy and safety of recombinant human activated protein C for severe sepsis. *New England Journal of Medicine* 344: 699-709, 2001.

14. Bersten, A. D., and A. J. Rutten. Renovascular interaction of epinephrine, dopamine, and intraperitoneal sepsis. *Critical Care Medicine* 23: 537-44, 1995.

15.   Beutler, B., and C. van Huffel. Unraveling function in the TNF ligand and receptor families. *Science* 264: 667-8, 1994.

16.   Bhatia, K., Y. Pommier, C. Giri, A. J. Fornace, M. Imaizumi, T. R. Breitman, B. W. Cherney, and M. E. Smulson. Expression of the poly(ADP-ribose) polymerase gene following natural and induced DNA strand breakage and effect of hyperexpression on DNA repair. *Carcinogeneis* 11: 123-128, 1990.

17.   Bistrian, B. R., G. L. Blackburn, E. Hallowell, and R. Heddle. Protein status of general surgical patients. *Jama* 230: 858-60, 1974.

18.   Bistrian, B. R., G. L. Blackburn, J. Vitale, D. Cochran, and J. Naylor. Prevalence of malnutrition in general medical patients. *Jama* 235: 1567-70, 1976.

19.   Bone, R. C., R. A. Balk, F. B. Cerra, R. P. Dellinger, A. M. Fein, W. A. Knaus, R. M. Schein, and W. J. Sibbald. Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. The ACCP/SCCM Consensus Conference Committee. American College of Chest Physicians/Society of Critical Care Medicine. *Chest* 101: 1644-55, 1992.

20.   Bone, R. C., C. J. Fisher, T. P. Clemmer, G. J. Slotman, and C. A. Metz. Early methylprednisolone treatment for septic syndrome and the adult respiratory distress syndrome. *Chest* 92: 1032-6, 1987.

21.   Bone, R. C., C. J. Fisher, T. P. Clemmer, G. J. Slotman, C. A. Metz, and R. A. Balk. A controlled clinical trial of high-dose methylprednisolone in the treatment of severe sepsis and septic shock. *New England Journal of Medicine* 317: 653-8, 1987.

22.   Border, J. R., J. Hassett, J. LaDuca, R. Seibel, S. Steinberg, B. Mills, P. Losi, and D. Border. The gut origin septic states in blunt multiple trauma (ISS = 40) in the ICU. *Annals of Surgery* 206: 427-48, 1987.

23.   Bourque, C. W. Osmoregulation of vasopressin neurons: a synergy of intrinsic and synaptic processes. *Progress in Brain Research* 119: 59-76, 1998.

24.   Bower, R. H., M. A. Talamini, H. C. Sax, F. Hamilton, and J. E. Fischer. Postoperative enteral vs parenteral nutrition. A randomized controlled trial. *Archives of Surgery* 121: 1040-5, 1986.

25.   Brandtzaeg, P., T. S. Halstensen, K. Kett, P. Krajci, D. Kvale, T. O. Rognum, H. Scott, and L. M. Sollid. Immunobiology and immunopathology of human gut mucosa: humoral immunity and intraepithelial lymphocytes. *Gastroenterology* 97: 1562-84, 1989.

26.   Bursztajn, S., J. J. Feng, S. A. Berman, and A. Nanda. Poly (ADP-ribose) polymerase induction is an early signal of apoptosis in human neuroblastoma. *Brain Research. Molecular Brain Research* 76: 363-76, 2000.

27.   Carrico, C. J., J. L. Meakins, J. C. Marshall, D. Fry, and R. V. Maier. Multiple-organ-failure syndrome. *Archives of Surgery* 121: 196-208, 1986.

28.   Casciola-Rosen, L., D. W. Nicholson, T. Chong, K. R. Rowan, N. A. Thornberry, D. K. Miller, and A. Rosen. Apopain/CPP32 cleaves proteins that are essential for cellular repair: a fundamental principle of apoptotic death [see comments]. *Journal of Experimental Medicine* 183: 1957-64, 1996.

29.   Cerra, F. B. Hypermetabolism, organ failure, and metabolic support. *Surgery* 101: 1-14, 1987.

30.   Chandra, R. K. Nutrition, immunity, and infection: present knowledge and future directions. *Lancet* 1: 688-91, 1983.

31.   Chen, K., M. Inoue, and A. Okada. Expression of Inducible Nitric Oxide Synthase mRNA in Rat Digestive Tissues after Endotoxin and Its Role in Intestinal Mucosal Injury. *Biochemical and Biophysical Research Communications* 224: 703-708, 1996.

32.   Chittock, D. R., and J. A. Russell. Oxygen delivery and consumption during sepsis. *Clinics in Chest Medicine* 17: 263-78, 1996.

33.   Chung, C. S., S. Yang, G. Y. Song, J. Lomas, P. Wang, H. H. Simms, I. H. Chaudry, and A. Ayala. Inhibition of Fas signaling prevents hepatic injury and improves organ blood flow during sepsis. *Surgery* 130: 339-45, 2001.

34.   Ciancio, M. J., L. Vitiritti, A. Dhar, and E. B. Chang. Endotoxin-induced alterations in rat colonic water and electrolyte transport. *Gastroenterology* 103: 1431-1443, 1992.

35.   Ciancio, M. J., L. Vitiritti, A. Dhar, and E. B. Chang. Endotoxin-induced alterations in rat colonic water and electrolyte transport [see comments]. *Gastroenterology* 103: 1437-43, 1992.

36.   Cipolle, M. D., M. D. Pasquale, and F. B. Cerra. Secondary organ dysfunction. From clinical perspectives to molecular mediators. *Critical Care Clinics* 9: 261-98, 1993.

37.   Cleaver, J. E., and W. F. Morgan. Poly(ADP-ribose)polymerase: a perplexing participant in celular responses to DNA breakage. *Mutation Research* 257: 1-18, 1991.

38.   Cobb, J. P., and R. L. Danner. Nitric oxide and septic shock. *Jama* 275: 1192-6, 1996.

39.   Comstock, K. L., K. A. Krown, M. T. Page, D. Martin, P. Ho, M. Pedraza, E. N. Castro, N. Nakajima, C. C. Glembotski, P. J. Quintana, and R. A. Sabbadini. LPS-induced TNF-alpha release from and apoptosis in rat cardiomyocytes: obligatory role for CD14 in

mediating the LPS response. *Journal of Molecular & Cellular Cardiology* 30: 2761-75, 1998.

40.   Cullen, J. J., D. K. Caropreso, and K. S. Ephgrave. Effect of endotoxin on canine gastrointestinal motility and transit. *Journal of Surgical Research* 58: 90-5, 1995.

41.   Cullen, J. J., K. S. Ephgrave, and D. K. Caropreso. Gastrointestinal myoelectric activity during endotoxemia. *American Journal of Surgery* 171: 596-9, 1996.

42.   Dehoux, M. S., A. Boutten, J. Ostinelli, N. Seta, M. C. Dombret, B. Crestani, M. Deschenes, J. L. Trouillet, and M. Aubier. Compartmentalized cytokine production within the human lung in unilateral pneumonia. *American Journal of Respiratory & Critical Care Medicine* 150: 710-6, 1994.

43.   Deitch, E. A. Multiple organ failure. Pathophysiology and potential future therapy. *Annals of Surgery* 216: 117-34, 1992.

44.   Deitch, E. A. Nutrition and the gut mucosal barrier. *Current Opinion in General Surgery* : 85-91, 1993.

45.   Deitch, E. A. The role of intestinal barrier failure and bacterial translocation in the development of systemic infection and multiple organ failure. *Archives of Surgery* 125: 403-4, 1990.

46.   Deitch, E. A. Simple intestinal obstruction causes bacterial translocation in man. *Archives of Surgery* 124: 699-701, 1989.

47.   Deitch, E. A., R. Berg, and R. Specian. Endotoxin promotes the translocation of bacteria from the gut. *Archives of Surgery* 122: 185-90, 1987.

48.   Deitch, E. A., and R. D. Berg. Endotoxin but not malnutrition promotes bacterial translocation of the gut flora in burned mice. *Journal of Trauma-Injury Infection & Critical Care* 27: 161-6, 1987.

49.   Deitch, E. A., and R. M. Bridges. Effect of stress and trauma on bacterial translocation from the gut. *Journal of Surgical Research* 42: 536-42, 1987.

50.   Deitch, E. A., W. Bridges, R. Berg, R. D. Specian, and D. N. Granger. Hemorrhagic shock-induced bacterial translocation: the role of neutrophils and hydroxyl radicals. *Journal of Trauma-Injury Infection & Critical Care* 30: 942-51; discussion 951-2, 1990.

51.   Deitch, E. A., L. Ma, W. J. Ma, M. B. Grisham, D. N. Granger, R. D. Specian, and R. D. Berg. Inhibition of endotoxin-induced bacterial translocation in mice. *Journal of Clinical Investigation* 84: 36-42, 1989.

52.   Deitch, E. A., W. J. Ma, L. Ma, R. Berg, and R. D. Specian. Endotoxin-induced bacterial translocation: a study of mechanisms. *Surgery* 106: 292-9; discussion 299-300, 1989.

53.   Deitch, E. A., K. Maejima, and R. Berg. Effect of oral antibiotics and bacterial overgrowth on the translocation of the GI tract microflora in burned rats. *Journal of Trauma-Injury Infection & Critical Care* 25: 385-92, 1985.

54.   Deitch, E. A., K. Sittig, M. Li, R. Berg, and R. D. Specian. Obstructive jaundice promotes bacterial translocation from the gut. *American Journal of Surgery* 159: 79-84, 1990.

55.   Deitch, E. A., R. D. Specian, and R. D. Berg. Endotoxin-induced bacterial translocation and mucosal permeability: role of xanthine oxidase, complement activation, and macrophage products. *Critical Care Medicine* 19: 785-91, 1991.

56.   Deitch, E. A., J. Winterton, and R. Berg. Effect of starvation, malnutrition, and trauma on the gastrointestinal tract flora and bacterial translocation. *Archives of Surgery* 122: 1019-24, 1987.

57.   Deitch, E. A., J. Winterton, M. Li, and R. Berg. The gut as a portal of entry for bacteremia. Role of protein malnutrition. *Annals of Surgery* 205: 681-92, 1987.

58.   DeLa Cadena, R. A., A. Majluf-Cruz, A. Stadnicki, J. M. Agosti, R. W. Colman, and A. F. Suffredini. Activation of the contact and fibrinolytic systems after intravenous administration of endotoxin to normal human volunteers: correlation with the cytokine profile. *Immunopharmacology* 33: 231-7, 1996.

59.   Dellinger, R. P. Current therapy for sepsis. *Infectious Disease Clinics of North America* 13: 495-509, 1999.

60.   DeWitt, R. C., and K. A. Kudsk. The gut's role in metabolism, mucosal barrier function, and gut immunology. *Infectious Disease Clinics of North America* 13: 465-81, x, 1999.

61.   Dinarello, C. A. Biologic basis for interleukin-1 in disease. *Blood* 87: 2095-147, 1996.

62.   Dinarello, C. A., J. A. Gelfand, and S. M. Wolff. Anticytokine strategies in the treatment of the systemic inflammatory response syndrome. *Jama* 269: 1829-35, 1993.

63.   Doig, C. J., L. R. Sutherland, J. D. Sandham, G. H. Fick, M. Verhoef, and J. B. Meddings. Increased intestinal permeability is associated with the development of multiple organ dysfunction syndrome in critically ill ICU patients. *American Journal of Respiratory & Critical Care Medicine* 158: 444-51, 1998.

64.   Eskandari, M. K., J. C. Kalff, T. R. Billiar, K. K. Lee, and A. J. Bauer. Lipopolysaccharide activates the muscularis macrophage network and suppresses circular smooth muscle activity. *American Journal of Physiology* 273: G727-34, 1997.

65.   Eskandari, M. K., J. C. Kalff, T. R. Billiar, K. K. Lee, and A. J. Bauer. LPS-induced muscularis macrophage nitric oxide suppresses rat jejunal circular muscle activity. *American Journal of Physiology* 277: G478-86, 1999.

66.   Faggioni, R., A. Moser, K. R. Feingold, and C. Grunfeld. Reduced leptin levels in starvation increase susceptibility to endotoxic shock. *American Journal of Pathology* 156: 1781-7, 2000.

67.   Fauvel, H., P. Marchetti, C. Chopin, P. Formstecher, and R. Neviere. Differential effects of caspase inhibitors on endotoxin-induced myocardial dysfunction and heart apoptosis. *American Journal of Physiology - Heart & Circulatory Physiology* 280: H1608-14, 2001.

68.   Ferguson, A. Intraepithelial lymphocytes of the small intestine. *Gut* 18: 921-37, 1977.

69.   Ferro, A. M., and B. M. Olivera. Poly(ADP-ribosylation) of DNA topoisomerase I from calf thymus. *Journal of Biological Chemistry* 259: 547-54, 1984.

70.   Fine, J. Current status of the problem of traumatic shock. *Surg Gynecol Obstet.* 120: 537-544, 1965.

71.   Fink, M. P. Gastrointestinal mucosal injury in experimental models of shock, trauma, and sepsis. *Critical Care Medicine* 19: 627-41, 1991.

72.   Fink, M. P., J. B. Antonsson, H. Wang, and H. R. Rothschild. Increased Intestinal Permeability in Endotoxemic Pigs. *Archives of Surgery* 126: 211-218, 1991.

73.   Fink, M. P., J. B. Antonsson, H. L. Wang, and H. R. Rothschild. Increased intestinal permeability in endotoxic pigs. Mesenteric hypoperfusion as an etiologic factor. *Archives of Surgery* 126: 211-8, 1991.

74.   Fink, M. P., and S. O. Heard. Laboratory models of sepsis and septic shock. *Journal of Surgical Research* 49: 186-96, 1990.

75.   Fong, Y. M., M. A. Marano, A. Barber, W. He, L. L. Moldawer, E. D. Bushman, S. M. Coyle, G. T. Shires, and S. F. Lowry. Total parenteral nutrition and bowel rest modify the metabolic response to endotoxin in humans. *Annals of Surgery* 210: 449-56; discussion 456-7, 1989.

76.   Fraser, A., and G. Evan. A license to kill. *Cell* 85: 781-4, 1996.

77.   Fukatsu, K., K. A. Kudsk, B. L. Zarzaur, Y. Wu, M. K. Hanna, and R. C. DeWitt. TPN decreases IL-4 and IL-10 mRNA expression in lipopolysaccharide stimulated intestinal lamina propria cells but glutamine supplementation preserves the expression. *Shock* 15: 318-22, 2001.

78.   Fukatsu, K., A. H. Lundberg, M. K. Hanna, Y. Wu, H. G. Wilcox, D. N. Granger, A. O. Gaber, and K. A. Kudsk. Route of nutrition influences intercellular adhesion molecule-1 expression and neutrophil accumulation in intestine. *Archives of Surgery* 134: 1055-60, 1999.

79.   Furchgott, R. F. The 1996 Albert Lasker Medical Research Awards. The discovery of endothelium-derived relaxing factor and its importance in the identification of nitric oxide. *Jama* 276: 1186-8, 1996.

80.   Grahm, T. W., D. B. Zadrozny, and T. Harrington. The benefits of early jejunal hyperalimentation in the head-injured patient. *Neurosurgery* 25: 729-35, 1989.

81.   Granville, D. J., C. M. Carthy, D. W. Hunt, and B. M. McManus. Apoptosis:

molecular aspects of cell death and disease. *Laboratory Investigation* 78: 893-913, 1998.

82.   Grossmann, J., S. Mohr, E. G. Lapentina, C. Fiocchi, and A. D. Levine. Sequential

and rapid activation of select caspases during apoptosis of normal intestinal epithelial cells.

American Journal of Physiology 274: G1117-24, 1998.

83.   Guedon, C., J. Schmitz, E. Lerebours, J. Metayer, E. Audran, J. Hemet, and R. Colin.

Decreased brush border hydrolase activities without gross morphologic changes in human

intestinal mucosa after prolonged total parenteral nutrition of adults. *Gastroenterology* 90:

373-8, 1986.

84.   Gumbiner, B. Structure, biochemistry, and assembly of epithelial tight junctions.

American Journal of Physiology 253: C749-58, 1987.

85.   Gutierrez, G., F. Palizas, G. Doglio, N. Wainsztein, A. Gallesio, J. Pacin, A. Dubin,

E. Schiavi, M. Jorge, and J. Pusajo. Gastric intramucosal pH as a therapeutic index of tissue

oxygenation in critically ill patients. *Lancet* 339: 195-9, 1992.

86.   Hamada, E., T. Nishida, Y. Uchiyama, J. Nakamura, K. Isahara, H. Kazuo, T. P.

Huang, T. Momoi, T. Ito, and H. Matsuda. Activation of Kupffer cells and caspase-3

involved in rat hepatocyte apoptosis induced by endotoxin. *Journal of Hepatology* 30: 807-

18, 1999.

87.   Harwig, S. S., L. Tan, X. D. Qu, Y. Cho, P. B. Eisenhauer, and R. I. Lehrer.

Bactericidal properties of murine intestinal phospholipase A2. *Journal of Clinical

Investigation* 95: 603-10, 1995.

88.   Hassoun, H. T., B. C. Kone, D. W. Mercer, F. G. Moody, N. W. Weisbrodt, and F. A.

Moore. Post-injury multiple organ failure: the role of the gut. *Shock* 15: 1-10, 2001.

89. Hassoun, H. T., N. W. Weisbrodt, D. W. Mercer, R. A. Kozar, F. G. Moody, and F. A. Moore. Inducible nitric oxide synthase mediates gut ischemia/reperfusion-induced ileus only after severe insults. *Journal of Surgical Research* 97: 150-4, 2001.

90. Hawkins, H. K., S. C. Heffelfinger, and D. C. Anderson. Leukocyte adhesion deficiency: clinical and postmortem observations. *Pediatric Pathology* 12: 119-30, 1992.

91. Haworth, R., N. Platt, S. Keshav, D. Hughes, E. Darley, H. Suzuki, Y. Kurihara, T. Kodama, and S. Gordon. The macrophage scavenger receptor type A is expressed by activated macrophages and protects the host against lethal endotoxic shock. *Journal of Experimental Medicine* 186: 1431-9, 1997.

92. Haziot, A., S. Chen, E. Ferrero, M. G. Low, R. Silber, and S. M. Goyert. The monocyte differentiation antigen, CD14, is anchored to the cell membrane by a phosphatidylinositol linkage. *Journal of Immunology* 141: 547-52, 1988.

93. Hebert, P. C., G. Wells, M. A. Blajchman, J. Marshall, C. Martin, G. Pagliarello, M. Tweeddale, I. Schweitzer, and E. Yetisir. A multicenter, randomized, controlled clinical trial of transfusion requirements in critical care. Transfusion Requirements in Critical Care Investigators, Canadian Critical Care Trials Group. *New England Journal of Medicine* 340: 409-17, 1999.

94. Herrmann, F. R., C. Safran, S. E. Levkoff, and K. L. Minaker. Serum albumin level on admission as a predictor of death, length of stay, and readmission. *Archives of Internal Medicine* 152: 125-30, 1992.

95. Hesse, D. G., K. J. Tracey, Y. Fong, K. R. Manogue, M. A. Palladino, A. Cerami, G. T. Shires, and S. F. Lowry. Cytokine appearance in human endotoxemia and primate bacteremia. *Surgery, Gynecology & Obstetrics* 166: 147-53, 1988.

96. Hoch, R. C., R. Rodriguez, T. Manning, M. Bishop, P. Mead, W. C. Shoemaker, and E. Abraham. Effects of accidental trauma on cytokine and endotoxin production. *Critical Care Medicine* 21: 839-45, 1993.

97. Holmes, C. L., B. M. Patel, J. A. Russell, and K. R. Walley. Physiology of vasopressin relevant to management of septic shock. *Chest* 120: 989-1002, 2001.

98. Ikejima, M., G. Marsischky, and D. M. Gill. Direction of elongation of poly(ADP-ribose) chains. Addition of residues at the polymerase-proximal terminus. *Journal of Biological Chemistry* 262: 17641-50, 1987.

99. Ikuta, N., H. Taniguchi, Y. Kondoh, K. Takagi, and T. Hayakawa. Sustained high levels of circulatory interleukin-8 are associated with a poor outcome in patients with adult respiratory distress syndrome [see comments]. *Internal Medicine* 35: 855-60, 1996.

100. Ince, C., and M. Sinaasappel. Microcirculatory oxygenation and shunting in sepsis and shock. *Critical Care Medicine* 27: 1369-77, 1999.

101. Ingalls, R. R., M. A. Arnaout, R. L. Delude, S. Flaherty, R. Savedra, and D. T. Golenbock. The CD11/CD18 integrins: characterization of three novel LPS signaling receptors. *Progress in Clinical & Biological Research* 397: 107-17, 1998.

102. Ingalls, R. R., H. Heine, E. Lien, A. Yoshimura, and D. Golenbock. Lipopolysaccharide recognition, CD14, and lipopolysaccharide receptors. *Infectious Disease Clinics of North America* 13: 341-53, vii, 1999.

103. Inoue, Y., N. J. Espat, D. J. Frohnapple, H. Epstein, E. M. Copeland, and W. W. Souba. Effect of total parenteral nutrition on amino acid and glucose transport by the human small intestine. *Annals of Surgery* 217: 604-12; discussion 612-4, 1993.

104.   Jaeschke, H., M. A. Fisher, J. A. Lawson, C. A. Simmons, A. Farhood, and D. A. Jones. Activation of caspase 3 (CPP32)-like proteases is essential for TNF-alpha-induced hepatic parenchymal cell apoptosis and neutrophil-mediated necrosis in a murine endotoxin shock model. *Journal of Immunology* 160: 3480-6, 1998.

105.   Janu, P. G., K. A. Kudsk, J. Li, and K. B. Renegar. Effect of bombesin on impairment of upper respiratory tract immunity induced by total parenteral nutrition. *Archives of Surgery* 132: 89-93, 1997.

106.   Jardin, F., M. Sportiche, M. Bazin, A. Bourokba, and A. Margairaz. Dobutamine: a hemodynamic evaluation in human septic shock. *Critical Care Medicine* 9: 329-32, 1981.

107.   Jin, H. K., Y. F. Chen, R. H. Yang, T. M. McKenna, R. M. Jackson, and S. Oparil. Vasopressin lowers pulmonary artery pressure in hypoxic rats by releasing atrial natriuretic peptide. *American Journal of the Medical Sciences* 298: 227-36, 1989.

108.   Jones, T. N., F. A. Moore, E. E. Moore, and B. L. McCroskey. Gastrointestinal symptoms attributed to jejunostomy feeding after major abdominal trauma--a critical analysis. *Critical Care Medicine* 17: 1146-50, 1989.

109.   Kameshita, I., Z. Matsuda, T. Taniguchi, and Y. Shizuta. Poly (ADP-Ribose) synthetase. Separation and identification of three proteolytic fragments as the substrate-binding domain, the DNA-binding domain, and the automodification domain. *Journal of Biological Chemistry* 259: 4770-6, 1984.

110.   Kane, T. D., J. W. Alexander, and J. A. Johannigman. The detection of microbial DNA in the blood: a sensitive method for diagnosing bacteremia and/or bacterial translocation in surgical patients [see comments]. *Annals of Surgery* 227: 1-9, 1998.

111. Katayama, M., D. Xu, R. D. Specian, and E. A. Deitch. Role of bacterial adherence and the mucus barrier on bacterial translocation: effects of protein malnutrition and endotoxin in rats. *Annals of Surgery* 225: 317-26, 1997.

112. Kennedy, M., A. G. Denenberg, C. Szabo, and A. L. Salzman. Poly(ADP-ribose) synthetase activation mediates increased permeability induced by peroxynitrite in Caco-2BBe cells. *Gastroenterology* 114: 510-518, 1998.

113. Kennedy, M., A. G. Denenberg, C. Szabo, and A. L. Salzman. Poly(ADP-ribose) synthetase activation mediates increased permeability induced by peroxynitrite in Caco-2BBe cells [see comments]. *Gastroenterology* 114: 510-8, 1998.

114. King, B. K., K. A. Kudsk, J. Li, Y. Wu, and K. B. Renegar. Route and type of nutrition influence mucosal immunity to bacterial pneumonia. *Annals of Surgery* 229: 272-8, 1999.

115. Kishimoto, T., S. Akira, M. Narazaki, and T. Taga. Interleukin-6 family of cytokines and gp130. *Blood* 86: 1243-54, 1995.

116. Krown, K. A., M. T. Page, C. Nguyen, D. Zechner, V. Gutierrez, K. L. Comstock, C. C. Glembotski, P. J. Quintana, and R. A. Sabbadini. Tumor necrosis factor alpha-induced apoptosis in cardiac myocytes. Involvement of the sphingolipid signaling cascade in cardiac cell death. *Journal of Clinical Investigation* 98: 2854-65, 1996.

117. Kudsk, K. A., G. Carpenter, S. Petersen, and G. F. Sheldon. Effect of enteral and parenteral feeding in malnourished rats with E. coli-hemoglobin adjuvant peritonitis. *Journal of Surgical Research* 31: 105-10, 1981.

118. Kudsk, K. A., M. A. Croce, T. C. Fabian, G. Minard, E. A. Tolley, H. A. Poret, M. R. Kuhl, and R. O. Brown. Enteral versus parenteral feeding. Effects on septic morbidity after

blunt and penetrating abdominal trauma. *Annals of Surgery* 215: 503-11; discussion 511-3, 1992.

119. Kudsk, K. A., J. Li, and K. B. Renegar. Loss of upper respiratory tract immunity with parenteral feeding. *Annals of Surgery* 223: 629-35; discussion 635-8, 1996.

120. Kudsk, K. A., G. Minard, S. L. Wojtysiak, M. Croce, T. Fabian, and R. O. Brown. Visceral protein response to enteral versus parenteral nutrition and sepsis in patients with trauma. *Surgery* 116: 516-23, 1994.

121. Kudsk, K. A., J. M. Stone, G. Carpenter, and G. F. Sheldon. Enteral and parenteral feeding influences mortality after hemoglobin-E. coli peritonitis in normal rats. *Journal of Trauma-Injury Infection & Critical Care* 23: 605-9, 1983.

122. Landry, D. W., H. R. Levin, E. M. Gallant, R. C. Ashton, S. Seo, D. D'Alessandro, M. C. Oz, and J. A. Oliver. Vasopressin deficiency contributes to the vasodilation of septic shock. *Circulation* 95: 1122-5, 1997.

123. Landry, D. W., H. R. Levin, E. M. Gallant, S. Seo, D. D'Alessandro, M. C. Oz, and J. A. Oliver. Vasopressin pressor hypersensitivity in vasodilatory septic shock. *Critical Care Medicine* 25: 1279-82, 1997.

124. Langkamp-Henken, B., J. A. Glezer, and K. A. Kudsk. Immunologic structure and function of the gastrointestinal tract. *Nutrition in Clinical Practice* 7: 100-8, 1992.

125. Law, N. W., and H. Ellis. The effect of parenteral nutrition on the healing of abdominal wall wounds and colonic anastomoses in protein-malnourished rats. *Surgery* 107: 449-54, 1990.

126. Lazebnik, Y. A., A. Takahashi, R. D. Moir, R. D. Goldman, G. G. Poirier, S. H. Kaufmann, and W. C. Earnshaw. Studies of the lamin proteinase reveal multiple parallel

biochemical pathways during apoptotic execution. *Proceedings of the National Academy of Sciences of the United States of America* 92: 9042-6, 1995.

127.   Lee, A., and E. Gemmell. Changes in the mouse intestinal microflora during weaning: role of volatile fatty acids. *Infection & Immunity* 5: 1-7, 1972.

128.   Leng, G., C. H. Brown, and J. A. Russell. Physiological pathways regulating the activity of magnocellular neurosecretory cells. *Progress in Neurobiology* 57: 625-55, 1999.

129.   Li, J., K. A. Kudsk, B. Gocinski, D. Dent, J. Glezer, and B. Langkamp-Henken. Effects of parenteral and enteral nutrition on gut-associated lymphoid tissue. *Journal of Trauma-Injury Infection & Critical Care* 39: 44-51; discussion 51-2, 1995.

130.   Li, J., K. A. Kudsk, P. Janu, and K. B. Renegar. Effect of glutamine-enriched total parenteral nutrition on small intestinal gut-associated lymphoid tissue and upper respiratory tract immunity. *Surgery* 121: 542-9, 1997.

131.   Li, M., R. D. Specian, R. D. Berg, and E. A. Deitch. Effects of protein malnutrition and endotoxin on the intestinal mucosal barrier to the translocation of indigenous flora in mice. *Jpen: Journal of Parenteral & Enteral Nutrition* 13: 572-8, 1989.

132.   Lipman, J., A. Roux, and P. Kraus. Vasoconstrictor effects of adrenaline in human septic shock. *Anaesthesia & Intensive Care* 19: 61-5, 1991.

133.   Lorente, J. A., L. Landin, R. De Pablo, E. Renes, R. Rodriguez-Diaz, and D. Liste. Effects of blood transfusion on oxygen transport variables in severe sepsis. *Critical Care Medicine* 21: 1312-8, 1993.

134.   Loscalzo, J. Nitric oxide and vascular disease. *New England Journal of Medicine* 333: 251-3, 1995.

135.   MacFie, J., C. O'Boyle, C. J. Mitchell, P. M. Buckley, D. Johnstone, and P. Sudworth. Gut origin of sepsis: a prospective study investigating associations between bacterial translocation, gastric microflora, and septic morbidity. *Gut* 45: 223-8, 1999.

136.   Maejima, K., E. A. Deitch, and R. D. Berg. Bacterial translocation from the gastrointestinal tracts of rats receiving thermal injury. *Infection & Immunity* 43: 6-10, 1984.

137.   Magnotti, L. J., J. S. Upperman, D. Z. Xu, Q. Lu, and E. A. Deitch. Gut-derived mesenteric lymph but not portal blood increases endothelial cell permeability and promotes lung injury after hemorrhagic shock. *Annals of Surgery* 228: 518-27, 1998.

138.   Magnotti, L. J., D. Z. Xu, Q. Lu, and E. A. Deitch. Gut-derived mesenteric lymph: a link between burn and lung injury. *Archives of Surgery* 134: 1333-40; discussion 1340-1, 1999.

139.   Magnusson, K. E., and I. Stjernstrom. Mucosal barrier mechanisms. Interplay between secretory IgA (SIgA), IgG and mucins on the surface properties and association of salmonellae with intestine and granulocytes. *Immunology* 45: 239-48, 1982.

140.   Maier, R. V., and E. M. Bulger. Endothelial changes after shock and injury. *New Horizons* 4: 211-23, 1996.

141.   Malay, M. B., R. C. Ashton, Jr., D. W. Landry, and R. N. Townsend. Low-dose vasopressin in the treatment of vasodilatory septic shock. *Journal of Trauma-Injury Infection & Critical Care* 47: 699-703; discussion 703-5, 1999.

142.   Mandir, N., and R. A. Goodlad. The effects of glutamine on intestinal epithelial cell proliferation in parenterally fed rats. *Gut* 44: 608-14, 1999.

143.   Marik, P. E., and W. J. Sibbald. Effect of stored-blood transfusion on oxygen delivery in patients with sepsis. *Jama* 269: 3024-9, 1993.

144. Martich, G. D., A. J. Boujoukos, and A. F. Suffredini. Response of man to endotoxin. *Immunobiology* 187: 403-16, 1993.

145. Martin, C., G. Perrin, P. Saux, L. Papazian, and F. Gouin. Effects of norepinephrine on right ventricular function in septic shock patients. *Intensive Care Medicine* 20: 444-7, 1994.

146. Mazen, A., J. Menissier-de Murcia, M. Molinete, F. Simonin, G. Gradwohl, G. Poirier, and G. de Murcia. Poly(ADP-ribose)polymerase: a novel finger protein. *Nucleic Acids Research* 17: 4689-98, 1989.

147. McArdle, A. H., C. Palmason, I. Morency, and R. A. Brown. A rationale for enteral feeding as the preferable route for hyperalimentation. *Surgery* 90: 616-23, 1981.

148. McCuskey, R. S., R. Urbaschek, and B. Urbaschek. The microcirculation during endotoxemia. *Cardiovascular Research* 32: 752-63, 1996.

149. McDonald, T. E., M. N. Grinman, C. M. Carthy, and K. R. Walley. Endotoxin infusion in rats induces apoptotic and survival pathways in hearts. *American Journal of Physiology - Heart & Circulatory Physiology* 279: H2053-61, 2000.

150. McGill, S. N., N. A. Ahmed, and N. V. Christou. Endothelial cells: role in infection and inflammation. *World Journal of Surgery* 22: 171-8, 1998.

151. McGilvray, I. D., and O. D. Rotstein. Role of the coagulation system in the local and systemic inflammatory response. *World Journal of Surgery* 22: 179-86, 1998.

152. Messmer, U. K., V. A. Briner, and J. Pfeilschifter. Tumor necrosis factor-alpha and lipopolysaccharide induce apoptotic cell death in bovine glomerular endothelial cells. *Kidney International* 55: 2322-37, 1999.

153. Mester, M., R. G. Tompkins, J. A. Gelfand, C. A. Dinarello, J. F. Burke, and B. D. Clark. Intestinal production of interleukin-1 alpha during endotoxemia in the mouse. *Journal of Surgical Research* 54: 584-91, 1993.

154. Meyer, T. A., J. Wang, G. M. Tiao, C. K. Ogle, J. E. Fischer, and P. O. Hasselgren. Sepsis and endotoxemia stimulate intestinal interleukin-6 production. *Surgery* 118: 336-42, 1995.

155. Miller, E. J., A. B. Cohen, S. Nagao, D. Griffith, R. J. Maunder, T. R. Martin, J. P. Weiner-Kronish, M. Sticherling, E. Christophers, and M. A. Matthay. Elevated levels of NAP-1/interleukin-8 are present in the airspaces of patients with the adult respiratory distress syndrome and are associated with increased mortality. *American Review of Respiratory Disease* 146: 427-32, 1992.

156. Minaga, T., and E. Kun. Probable helical conformation of poly(ADP-ribose). The effect of cations on spectral properties. *Journal of Biological Chemistry* 258: 5726-30, 1983.

157. Mishima, S., D. Xu, Q. Lu, and E. A. Deitch. Bacterial translocation is inhibited in inducible nitric oxide synthase knockout mice after endotoxin challenge but not in a model of bacterial overgrowth. *Archives of Surgery* 132: 1190-5, 1997.

158. Mishima, S., D. Xu, Q. Lu, and E. A. Deitch. The relationships among nitric oxide production, bacterial translocation, and intestinal injury after endotoxin challenge in vivo. *Journal of Trauma-Injury Infection & Critical Care* 44: 175-82, 1998.

159. Moncada, S., R. M. Palmer, and E. A. Higgs. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacological Reviews* 43: 109-42, 1991.

160.   Moore, E. E., and T. N. Jones. Benefits of immediate jejunostomy feeding after major abdominal trauma--a prospective, randomized study. *Journal of Trauma-Injury Infection & Critical Care* 26: 874-81, 1986.

161.   Moore, E. E., and F. A. Moore. Immediate enteral nutrition following multisystem trauma: a decade perspective. *Journal of the American College of Nutrition* 10: 633-48, 1991.

162.   Moore, F. A. The role of the gastrointestinal tract in postinjury multiple organ failure. *American Journal of Surgery* 178: 449-53, 1999.

163.   Moore, F. A., D. V. Feliciano, R. J. Andrassy, A. H. McArdle, F. V. Booth, T. B. Morgenstein-Wagner, J. M. Kellum, Jr., R. E. Welling, and E. E. Moore. Early enteral feeding, compared with parenteral, reduces postoperative septic complications. The results of a meta-analysis. *Annals of Surgery* 216: 172-83, 1992.

164.   Moore, F. A., E. E. Moore, T. N. Jones, B. L. McCroskey, and V. M. Peterson. TEN versus TPN following major abdominal trauma--reduced septic morbidity. *Journal of Trauma-Injury Infection & Critical Care* 29: 916-22; discussion 922-3, 1989.

165.   Moore, F. A., E. E. Moore, R. Poggetti, O. J. McAnena, V. M. Peterson, C. M. Abernathy, and P. E. Parsons. Gut bacterial translocation via the portal vein: a clinical perspective with major torso trauma. *Journal of Trauma-Injury Infection & Critical Care* 31: 629-36; discussion 636-8, 1991.

166.   Morrison, D. C., and J. L. Ryan. Endotoxins and disease mechanisms. *Annual Review of Medicine* 38: 417-32, 1987.

167.   Morrison, D. C., R. Silverstein, M. Luchi, and A. Shnyra. Structure-function relationships of bacterial endotoxins. Contribution to microbial sepsis. *Infectious Disease Clinics of North America* 13: 313-40, 1999.

168. Muller, J. M., U. Brenner, C. Dienst, and H. Pichlmaier. Preoperative parenteral feeding in patients with gastrointestinal carcinoma. *Lancet* 1: 68-71, 1982.

169. Murad, F. The 1996 Albert Lasker Medical Research Awards. Signal transduction using nitric oxide and cyclic guanosine monophosphate. *Jama* 276: 1189-92, 1996.

170. Nakajima, Y., N. Baudry, J. Duranteau, and E. Vicaut. Microcirculation in Intestinal Villi: A comparison between Hemorrhagic and Endotoxin shock. *Am J Respir Crit Care Med* 164: 1526-1530, 2001.

171. Natanson, C., W. D. Hoffman, A. F. Suffredini, P. Q. Eichacker, and R. L. Danner. Selected treatment strategies for septic shock based on proposed mechanisms of pathogenesis. *Annals of Internal Medicine* 120: 771-83, 1994.

172. Neutra, M., R. Weltzin, L. Winner, J. Mack, P. Michetti, L. Morrison, B. N. Fields, J. J. Mekalanos, and J. P. Kraehenbuhl. Identification and use of protective monoclonal IgA antibodies against viral and bacterial pathogens. *Advances in Experimental Medicine & Biology* 310: 179-82, 1991.

173. Neviere, R., H. Fauvel, C. Chopin, P. Formstecher, and P. Marchetti. Caspase inhibition prevents cardiac dysfunction and heart apoptosis in a rat model of sepsis. *American Journal of Respiratory & Critical Care Medicine* 163: 218-25, 2001.

174. Neviere, R. R., M. L. Pitt-Hyde, R. D. Piper, W. J. Sibbald, and R. F. Potter. Microvascular perfusion deficits are not a prerequisite for mucosal injury in septic rats. *American Journal of Physiology* 276: G933-40, 1999.

175. Nieuwenhuijzen, G. A., E. A. Deitch, and R. J. Goris. The relationship between gut-derived bacteria and the development of the multiple organ dysfunction syndrome. *Journal of Anatomy* 189: 537-48, 1996.

176. O'Dwyer, S. T., H. R. Michie, T. R. Ziegler, A. Revhaug, R. J. Smith, and D. W. Wilmore. A single dose of endotoxin increases intestinal permeability in healthy humans. *Archives of Surgery* 123: 1459-64, 1988.

177. Okamura, T., M. Toda, K. Ayajiki, and N. Toda. Receptor subtypes involved in relaxation and contraction by arginine vasopressin in canine isolated short posterior ciliary arteries. *Journal of Vascular Research* 34: 464-72, 1997.

178. Okusawa, S., J. A. Gelfand, T. Ikejima, R. J. Connolly, and C. A. Dinarello. Interleukin 1 induces a shock-like state in rabbits. Synergism with tumor necrosis factor and the effect of cyclooxygenase inhibition. *Journal of Clinical Investigation* 81: 1162-72, 1988.

179. Olson, D. M., and J. B. Hall. Is there a role for gastric tonometry in critical care? Weighing the evidence for and against the procedure. *Journal of Critical Illness* 10: 539-42, 545-6, 549, 1995.

180. Opal, S. M., and A. S. Cross. Clinical trials for severe sepsis. Past failures, and future hopes. *Infectious Disease Clinics of North America* 13: 285-97, vii, 1999.

181. Opal, S. M., and R. L. Yu, Jr. Antiendotoxin strategies for the prevention and treatment of septic shock. New approaches and future directions. *Drugs* 55: 497-508, 1998.

182. Ottaway, C. A. Neuroimmunomodulation in the intestinal mucosa. *Gastroenterology Clinics of North America* 20: 511-29, 1991.

183. Owen, R. L. Sequential uptake of horseradish peroxidase by lymphoid follicle epithelium of Peyer's patches in the normal unobstructed mouse intestine: an ultrastructural study. *Gastroenterology* 72: 440-51, 1977.

184. Page, C. P. Edgar J. Poth lecture. The surgeon and gut maintenance. *American Journal of Surgery* 158: 485-90, 1989.

185. Patel, R. T., K. I. Deen, D. Youngs, J. Warwick, and M. R. Keighley. Interleukin 6 is a prognostic indicator of outcome in severe intra-abdominal sepsis. *British Journal of Surgery* 81: 1306-8, 1994.

186. Peterson, V. M., E. E. Moore, T. N. Jones, C. Rundus, M. Emmett, F. A. Moore, B. L. McCroskey, T. Haddix, and P. E. Parsons. Total enteral nutrition versus total parenteral nutrition after major torso injury: attenuation of hepatic protein reprioritization. *Surgery* 104: 199-207, 1988.

187. Piguet, P. F., C. Vesin, Y. Donati, and C. Barazzone. TNF-induced enterocyte apoptosis and detachment in mice: induction of caspases and prevention by a caspase inhibitor, ZVAD-fmk. *Laboratory Investigation* 79: 495-500, 1999.

188. Porter, E. M., L. Liu, A. Oren, P. A. Anton, and T. Ganz. Localization of human intestinal defensin 5 in Paneth cell granules. *Infection & Immunity* 65: 2389-95, 1997.

189. Presterl, E., T. Staudinger, M. Pettermann, A. Lassnigg, H. Burgmann, S. Winkler, M. Frass, and W. Graninger. Cytokine profile and correlation to the APACHE III and MPM II scores in patients with sepsis. *American Journal of Respiratory & Critical Care Medicine* 156: 825-32, 1997.

190. Pritts, T. A., M. R. Moon, Q. Wang, E. S. Hungness, A. L. Salzman, J. E. Fischer, and P. O. Hasselgren. Activation of NF-kappaB varies in different regions of the gastrointestinal tract during endotoxemia. *Shock* 14: 118-22, 2000.

191. Pritts, T. A., R. Moon, J. E. Fischer, A. L. Salzman, and P. O. Hasselgren. Nuclear factor-kappaB is activated in intestinal mucosa during endotoxemia. *Archives of Surgery* 133: 1311-5, 1998.

192.   Pulverer, G., H. Lioe Ko, and J. Beuth. Microflora-associated defense stimulating factors. *Scandinavian Journal of Gastroenterology - Supplement* 222: 107-11, 1997.

193.   Raetz, C. R. Biochemistry of endotoxins. *Annual Review of Biochemistry* 59: 129-70, 1990.

194.   Rangel-Frausto, M. S. The epidemiology of bacterial sepsis. *Infectious Disease Clinics of North America* 13: 299-312, vii, 1999.

195.   Renegar, K. B., and P. A. Small. Immunoglobulin A mediation of murine nasal anti-influenza virus immunity. *Journal of Virology* 65: 2146-8, 1991.

196.   Ribbons, K. A., X. J. Zhang, J. H. Thompson, S. S. Greenberg, W. M. Moore, C. M. Kornmeier, M. G. Currie, N. Lerche, J. Blanchard, and D. A. Clark. Potential role of nitric oxide in a model of chronic colitis in rhesus macaques. *Gastroenterology* 108: 705-11, 1995.

197.   Rietschel, E. T., and H. Brade. Bacterial endotoxins. *Scientific American* 267: 54-61, 1992.

198.   Rodning, C. B., I. D. Wilson, and S. L. Erlandsen. Immunoglobulins within human small-intestinal Paneth cells. *Lancet* 1: 984-7, 1976.

199.   Routsi, C., J. L. Vincent, J. Bakker, D. De Backer, P. Lejeune, A. d'Hollander, J. L. Le Clerc, and R. J. Kahn. Relation between oxygen consumption and oxygen delivery in patients after cardiac surgery. *Anesthesia & Analgesia* 77: 1104-10, 1993.

200.   Rozee, K. R., D. Cooper, K. Lam, and J. W. Costerton. Microbial flora of the mouse ileum mucous layer and epithelial surface. *Applied & Environmental Microbiology* 43: 1451-63, 1982.

201.   Saito, H., O. Trocki, J. W. Alexander, R. Kopcha, T. Heyd, and S. N. Joffe. The effect of route of nutrient administration on the nutritional state, catabolic hormone secretion,

and gut mucosal integrity after burn injury. *Jpen: Journal of Parenteral & Enteral Nutrition* 11: 1-7, 1987.

202.   Salzman, A. L., M. J. Menconi, N. Uno, R. M. Ezzell, D. M. Casey, P. K. Gonzales, and M. P. Fink. Nitric oxide dilates tight junctions and depletes ATP in cultured Caco-2BBe intestinal epithelial monolayers. *The American Journal of Physiology* 268: 361-373, 1995.

203.   Salzman, A. L., H. Wang, P. S. Wollert, T. J. Vandermeer, C. C. Compton, A. G. Denenberg, and M. P. Fink. Endotoxin-induced ileal mucosal hyperpermeability in pigs: role of tissue acidosis. *The American Journal of Physiology* 266: 633-646, 1994.

204.   Schaer, G. L., M. P. Fink, and J. E. Parrillo. Norepinephrine alone versus norepinephrine plus low-dose dopamine: enhanced renal blood flow with combination pressor therapy. *Critical Care Medicine* 13: 492-6, 1985.

205.   Schlag, G., and H. Redl. Mediators of injury and inflammation. *World Journal of Surgery* 20: 406-10, 1996.

206.   Shoemaker, W. C., P. L. Appel, and H. B. Kram. Tissue oxygen debt as a determinant of lethal and nonlethal postoperative organ failure. *Critical Care Medicine* 16: 1117-20, 1988.

207.   Singh, G., R. P. Ram, and S. K. Khanna. Early postoperative enteral feeding in patients with nontraumatic intestinal perforation and peritonitis. *Journal of the American College of Surgeons* 187: 142-6, 1998.

208.   Slee, E. A., M. T. Harte, R. M. Kluck, B. B. Wolf, C. A. Casiano, D. D. Newmeyer, H. G. Wang, J. C. Reed, D. W. Nicholson, E. S. Alnemri, D. R. Green, and S. J. Martin. Ordering the cytochrome c-initiated caspase cascade: hierarchical activation of caspases-2, -

3, -6, -7, -8, and -10 in a caspase-9-dependent manner. *Journal of Cell Biology* 144: 281-92, 1999.

209. Sonoda, E., Y. Hitoshi, N. Yamaguchi, T. Ishii, A. Tominaga, S. Araki, and K. Takatsu. Differential regulation of IgA production by TGF-beta and IL-5: TGF-beta induces surface IgA-positive cells bearing IL-5 receptor, whereas IL-5 promotes their survival and maturation into IgA-secreting cells. *Cellular Immunology* 140: 158-72, 1992.

210. Souba, W. W. Nutritional support. *New England Journal of Medicine* 336: 41-8, 1997.

211. Spaeth, G., R. D. Berg, R. D. Specian, and E. A. Deitch. Food without fiber promotes bacterial translocation from the gut. *Surgery* 108: 240-6; discussion 246-7, 1990.

212. Spaeth, G., R. D. Specian, R. D. Berg, and E. A. Deitch. Bulk prevents bacterial translocation induced by the oral administration of total parenteral nutrition solution. *Jpen: Journal of Parenteral & Enteral Nutrition* 14: 442-7, 1990.

213. Spielmann, S., T. Kerner, O. Ahlers, D. Keh, M. Gerlach, and H. Gerlach. Early detection of increased tumour necrosis factor alpha (TNFalpha) and soluble TNF receptor protein plasma levels after trauma reveals associations with the clinical course. *Acta Anaesthesiologica Scandinavica* 45: 364-70, 2001.

214. Sprung, C. L., P. V. Caralis, E. H. Marcial, M. Pierce, M. A. Gelbard, W. M. Long, R. C. Duncan, M. D. Tendler, and M. Karpf. The effects of high-dose corticosteroids in patients with septic shock. A prospective, controlled study. *New England Journal of Medicine* 311: 1137-43, 1984.

215. Strocchi, A., and M. D. Levitt. A reappraisal of the magnitude and implications of the intestinal unstirred layer. *Gastroenterology* 101: 843-7, 1991.

216.   Stuber, F., M. Petersen, F. Bokelmann, and U. Schade. A genomic polymorphism within the tumor necrosis factor locus influences plasma tumor necrosis factor-alpha concentrations and outcome of patients with severe sepsis. *Critical Care Medicine* 24: 381-4, 1996.

217.   Sun, Z., X. Wang, X. Deng, A. Lasson, R. Wallen, E. Hallberg, and R. Andersson. The influence of intestinal ischemia and reperfusion on bidirectional intestinal barrier permeability, cellular membrane integrity, proteinase inhibitors, and cell death in rats. *Shock* 10: 203-12, 1998.

218.   Sun, Z., X. Wang, R. Wallen, X. Deng, X. Du, E. Hallberg, and R. Andersson. The influence of apoptosis on intestinal barrier integrity in rats. *Scandinavian Journal of Gastroenterology* 33: 415-22, 1998.

219.   Symeonides, S., and R. A. Balk. Nitric oxide in the pathogenesis of sepsis. *Infectious Disease Clinics of North America* 13: 449-63, x, 1999.

220.   Szabo, A., A. L. Salzman, and C. Szabo. Poly (ADP-ribose) synthetase activation mediates pulmonary microvascular and intestinal mucosal dysfunction in endotoxin shock. *Life Sciences* 63: 2133-9, 1998.

221.   Takahashi, A., E. S. Alnemri, Y. A. Lazebnik, T. Fernandes-Alnemri, G. Litwack, R. D. Moir, R. D. Goldman, G. G. Poirier, S. H. Kaufmann, and W. C. Earnshaw. Cleavage of lamin A by Mch2 alpha but not CPP32: multiple interleukin 1 beta-converting enzyme-related proteases with distinct substrate recognition properties are active in apoptosis. *Proceedings of the National Academy of Sciences of the United States of America* 93: 8395-400, 1996.

222. Tanamoto, K., U. Zahringer, G. R. McKenzie, C. Galanos, E. T. Rietschel, O. Luderitz, S. Kusumoto, and T. Shiba. Biological activities of synthetic lipid A analogs: pyrogenicity, lethal toxicity, anticomplement activity, and induction of gelation of Limulus amoebocyte lysate. *Infection & Immunity* 44: 421-6, 1984.

223. Teule, G. J., A. von Lingen, M. A. Verwey von Vught, A. D. Kester, R. C. Mackaay, P. D. Bezemer, G. A. Heidenal, and L. G. Thijs. Role of peripheral pooling in porcine Escherichia coli sepsis. *Circulatory Shock* 12: 115-23, 1984.

224. Thornberry, N. A., T. A. Rano, E. P. Peterson, D. M. Rasper, T. Timkey, M. Garcia-Calvo, V. M. Houtzager, P. A. Nordstrom, S. Roy, J. P. Vaillancourt, K. T. Chapman, and D. W. Nicholson. A combinatorial approach defines specificities of members of the caspase family and granzyme B. Functional relationships established for key mediators of apoptosis. *Journal of Biological Chemistry* 272: 17907-11, 1997.

225. Trinchieri, G. Interleukin-12 and interferon-gamma. Do they always go together? [see comments]. *American Journal of Pathology* 147: 1534-8, 1995.

226. Unno, N., H. Wang, M. J. Menconi, S. H. A. J. Tytgat, V. Larkin, M. Smith, M. J. Morin, A. Chavez, R. A. Hodin, and M. P. Fink. Inhibition of Inducible Nitric Oxide Synthesis Ameliorates Endotoxin-Induced Gut Mucosal Barrier Dysfunction in Rats. *Gastroenterology* 113: 1246-1257, 1997.

227. van der Hulst, R. R., B. K. van Kreel, M. F. von Meyenfeldt, R. J. Brummer, J. W. Arends, N. E. Deutz, and P. B. Soeters. Glutamine and the preservation of gut integrity. *Lancet* 341: 1363-5, 1993.

228. van der Poll, T., and S. F. Lowry. Tumor necrosis factor in sepsis: mediator of multiple organ failure or essential part of host defense? *Shock* 3: 1-12, 1995.

229.   van der Poll, T., and S. J. van Deventer. Cytokines and anticytokines in the pathogenesis of sepsis. *Infectious Disease Clinics of North America* 13: 413-26, ix, 1999.

230.   van der Waaij, D., J. M. Berghuis, and J. E. Lekkerkerk. Colonization resistance of the digestive tract of mice during systemic antibiotic treatment. *Journal of Hygiene* 70: 605-10, 1972.

231.   van der Waaij, D., J. M. Berghuis-de Vries, and L.-v. Lekkerkerk. Colonization resistance of the digestive tract in conventional and antibiotic-treated mice. *Journal of Hygiene* 69: 405-11, 1971.

232.   van der Waaij, D., J. M. Berghuis-de Vries, and W. Lekkerkerk-van der. Colonization resistance of the digestive tract and the spread of bacteria to the lymphatic organs in mice. *Journal of Hygiene* 70: 335-42, 1972.

233.   van Deuren, M., J. van der Ven-Jongekrijg, P. N. Demacker, A. K. Bartelink, R. van Dalen, R. W. Sauerwein, H. Gallati, J. L. Vannice, and J. W. van der Meer. Differential expression of proinflammatory cytokines and their inhibitors during the course of meningococcal infections. *Journal of Infectious Diseases* 169: 157-61, 1994.

234.   Walley, K. R., N. W. Lukacs, T. J. Standiford, R. M. Strieter, and S. L. Kunkel. Balance of inflammatory cytokines related to severity and mortality of murine sepsis. *Infection & Immunity* 64: 4733-8, 1996.

235.   Walley, K. R., T. E. McDonald, Y. Higashimoto, and S. Hayashi. Modulation of proinflammatory cytokines by nitric oxide in murine acute lung injury. *American Journal of Respiratory & Critical Care Medicine* 160: 698-704, 1999.

236.   Wang, Q., J. J. Wang, J. E. Fischer, and P. O. Hasselgren. Mucosal production of complement C3 and serum amyloid A is differentially regulated in different parts of the

gastrointestinal tract during endotoxemia in mice. *Journal of Gastrointestinal Surgery* 2: 537-46, 1998.

237.   Warren, H. S. Strategies for the treatment of sepsis. *New England Journal of Medicine* 336: 952-3, 1997.

238.   Weiss, M. D., V. DeMarco, D. M. Strauss, D. A. Samuelson, M. E. Lane, and J. Neu. Glutamine synthetase: a key enzyme for intestinal epithelial differentiation? *Jpen: Journal of Parenteral & Enteral Nutrition* 23: 140-6, 1999.

239.   Wenzel, R. P. Anti-endotoxin monoclonal antibodies--a second look. *New England Journal of Medicine* 326: 1151-3, 1992.

240.   Wheeler, A. P., and G. R. Bernard. Treating patients with severe sepsis. *New England Journal of Medicine* 340: 207-14, 1999.

241.   Williams, R. C., and R. J. Gibbons. Inhibition of streptococcal attachment to receptors on human buccal epithelial cells by antigenically similar salivary glycoproteins. *Infection & Immunity* 11: 711-8, 1975.

242.   Wilmore, D. W., R. J. Smith, S. T. O'Dwyer, D. O. Jacobs, T. R. Ziegler, and X. D. Wang. The gut: a central organ after surgical stress. *Surgery* 104: 917-23, 1988.

243.   Windsor, J. A., and G. L. Hill. Risk factors for postoperative pneumonia. The importance of protein depletion. *Annals of Surgery* 208: 209-14, 1988.

244.   Wiren, M., K. E. Magnusson, and J. Larsson. The role of glutamine, serum and energy factors in growth of enterocyte-like cell lines. *International Journal of Biochemistry & Cell Biology* 30: 1331-6, 1998.

245.   Wolf, B. B., M. Schuler, F. Echeverri, and D. R. Green. Caspase-3 is the primary activator of apoptotic DNA fragmentation via DNA fragmentation factor-45/inhibitor of caspase-activated DNase inactivation. *Journal of Biological Chemistry* 274: 30651-6, 1999.

246.   Xu, D., L. Qi, C. Thirstrup, R. Berg, and E. A. Deitch. Elemental diet-induced bacterial translocation and immunosuppression is not reversed by glutamine. *Journal of Trauma-Injury Infection & Critical Care* 35: 821-4, 1993.

247.   Yan, S. B., J. D. Helterbrand, D. L. Hartman, T. J. Wright, and G. R. Bernard. Low levels of protein C are associated with poor outcome in severe sepsis. *Chest* 120: 915-22, 2001.

248.   Yanagisawa, M., E. Planel, K. Ishiguro, and S. C. Fujita. Starvation induces tau hyperphosphorylation in mouse brain: implications for Alzheimer's disease. *FEBS Letters* 461: 329-33, 1999.

249.   Yang, H., J. D. Soderholm, J. Larsson, J. Permert, J. Lindgren, and M. Wiren. Bidirectional supply of glutamine maintains enterocyte ATP content in the in vitro using chamber model. *International Journal of Colorectal Disease* 15: 291-6, 2000.

250.   Yuan, J., S. Shaham, S. Ledoux, H. M. Ellis, and H. R. Horvitz. The C. elegans cell death gene ced-3 encodes a protein similar to mammalian interleukin-1 beta-converting enzyme. *Cell* 75: 641-52, 1993.

251.   Ziegler, E. J., J. A. McCutchan, J. Fierer, M. P. Glauser, J. C. Sadoff, H. Douglas, and A. I. Braude. Treatment of gram-negative bacteremia and shock with human antiserum to a mutant Escherichia coli. *New England Journal of Medicine* 307: 1225-30, 1982.

252.   Ziegler, T. R., R. J. Smith, S. T. O'Dwyer, R. H. Demling, and D. W. Wilmore. Increased Intestinal Permeability Associated With Infection In Burn Patients. *Archives of Surgery* 123: 1313-1319, 1988.

253.   Zingarelli, B., M. O'Connor, H. Wong, A. L. Salzman, and C. Szabo. Peroxynitrite-mediated DNA strand breakage activates poly-adenosine diphosphate ribosyl synthetase and causes cellular energy depletion in macrophages stimulated with bacterial lipopolysaccharide. *Journal of Immunology* 156: 350-8, 1996.