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Pathology of Septic Shock

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With 34 Figures

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Cover illustration: Symbolic space filling models of endotoxin and superantigen (SEB) representing two types of toxins often associated with the sepsis syndrome [Swaminathan et al. (1992) Nature 359: 801; Kastowsky et al. (1992) J Bacteriol 174: 4798]

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Preface

Severe sepsis and septic shock are the most serious complications of bacterial infections. Both gram-positive and gram-negative bacteria can trigger these extreme inflammatory responses and, by so doing, cause substantial morbidity and mortality. In the United States alone, over 400 000 patients suffer from septicaemia each year, and approximately 100 000 of these patients die despite optimal intensive care and modern antimicrobial therapy.

These dramatic figures have prompted intensive research to define the bacterial and host factors involved in the septic response. Scientists from many disciplines, including chemistry, physics, biology, medical microbiology, immunology, and pharmacology, have worked closely with clinicians to achieve rapid and profound progress. To translate this newly acquired knowledge into clinical practice, clinical trials have also been performed to evaluate numerous new therapeutic drugs. The disappointing results from these trials have underscored a major lesson, namely, that sepsis constitutes an extremely complex syndrome and that basic and clinical research must be greatly intensified in order to illuminate its molecular mechanisms.

At this stage, the editors of the present volume of *Current Topics in Microbiology and Immunology* considered it would be rewarding to compile a volume summarizing our present basic and clinical knowledge on sepsis. Our particular gratitude extends to those international experts who have followed our invitation and elaborated on particular areas of the basic and clinical aspects of this field.

Their contributions provide a true picture of "the state of the art" and should be a useful reference for design of new sepsis-related research projects. We also thank Doris Walker, Springer-Verlag, for her advice, professional help, and excellent guidance in editing this volume.

Bacterial infections and sepsis continue to represent major threats to human health. We live in an era in which bacterial

antimicrobial resistance is increasing and immunosuppressed patients are becoming more common, so that sepsis will have even more opportunities to injure and kill. Sepsis kills quickly and often dramatically. Civilized society should not allow this to happen.

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List of Contributors

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Definition and Pathogenesis of Septic Shock

J.L. VINCENT

1 Definition, Characteristics, and Epidemiology

Septic shock is the most severe manifestation of infection and appears to be increasingly common, especially in the intensive care unit (ICU). Its incidence is difficult to determine exactly, however, because of the many different definitions of sepsis and its associated complications and because of the varying

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incidences reported for the different populations studied. The following features are commonly seen as defining sepsis:

- Fever (temperature > 38 °C) or sometimes hypothermia (temperature <36 °C)
- Tachycardia (>90 bpm)
- Respiratory alkalosis ($PaCO_2$ < 35 mmHg), tachypnea (>20 breaths per minute)
- Low systemic vascular resistance, increased cardiac output
- Hyperleukocytosis (>12 000 cells/mm^3, with "left shift") or sometimes leukopenia (< 4000 cells/mm^3)
- Isolated thrombocytopenia or disseminated intravascular coagulation
- Increased oxygen consumption, elevated cellular metabolism
- Increased insulin requirements
- Signs of inflammation: increased sedimentation rate, elevated C-reactive protein and fibrinogen levels
- Elevated cytokine levels [tumor necrosis factor (TNF), interleukin (IL) –1, –6, –8 etc.]
- Ophthalmic and/or cutaneous manifestations
- Renal failure, adult respiratory distress syndrome (ARDS), mental obtundation, and other organ dysfunction

As in other forms of acute circulatory failure, septic shock is best described as an imbalance between oxygen demand and oxygen delivery (Fig. 1). A complex mediator network has been implicated in the three major alterations characterizing septic shock: impaired oxygen extraction, increased oxygen needs, and altered myocardial contractility. These abnormalities are described in greater detail below. Other factors than infection may augment the mediator response, such as pain, anxiety, tissue trauma, and severe heart failure (Marchant et al. 1995).

Mortality in the early stages of septic shock is generally associated with profound cardiovascular prolapse (Ruokonen et al. 1993). Despite seemingly adequate resuscitation, at least globally, many septic shock patients die at a later stage due to multiple organ failure (MOF). Ongoing oxygen defects due to incomplete resuscitation at the microvascular level are largely responsible. Splanchnic ischemia may be particularly important since the splanchnic circula-

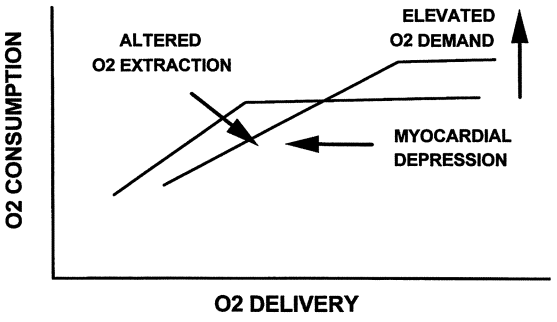

Fig. 1. Oxygen delivery and oxygen consumption in septic shock

tion is particularly at risk and the gut is an immune-rich organ. It has been proposed that splanchnic ischemia results in a breach of the gut mucosal barrier, with secondary translocation of bacteria and their products. A vicious circle of inflammation, ischemia, translocation, and heightened inflammation may be significant in the development of MOF following septic shock. The importance of translocation in humans is still under debate. Nevertheless, even in the absence of translocation the gut is capable of releasing a large quantity of inflammatory mediators, especially in the presence of gut ischemia.

1.1 Incidence

Reports of septicemia more than doubled between 1979 and 1987, from 74 to 176 cases per 100 000 persons (ANONYMOUS 1990). Although such figures depend on the definition used, a reasonable estimate is that sepsis develops in about 1% of hospitalized patients but in 20%–30% of ICU patients.

Despite our improved understanding of the mechanisms involved and the effectiveness of various treatments the incidence of septic shock continues to increase. This is largely because the ICU population contains an increasing proportion of elderly, more compromised, and immunosuppressed patients who have increased risk of infection. Furthermore, advances in modern therapies continue to push the limits of the human system and employ more invasive procedures. These factors may explain why, as some reports suggest, the severity of septic shock has increased, and why mortality from septic shock remains between 40% and 60% in most studies despite the therapeutic advances which have been made (FRIEDMAN et al. 1995).

1.2 Microbiology

Traditionally the onset of sepsis was attributed to the destructive effects of the invading micro-organism, but recent improvements in our comprehension of the immunological sequence involved have implicated a complex network of host-synthesized mediators. It should be emphasized that a similar mediator response can be triggered by other events than an infection (MARCHANT et al. 1995). Indeed it is well documented that infection can be documented in fewer than half of the patients who "look septic" in the posttrauma or injury period. Similarly, the presence of positive blood cultures is not required to establish the diagnosis of septic shock.

Older studies suggested that gram-negative organisms are more commonly associated with septic shock than are gram-positive; however, more recent studies have shown that gram-positive organisms can be just as common. This probably reflects a general change in microbiology related to ongoing antibiotic usage, with a progressive emergence of gram-positive organisms.

While both gram-positive and gram-negative organisms can participate in septic shock, there is no real difference between their influences on hemodynamic presentation or prognosis. A clinically relevant canine model of septic shock (NATANSON et al. 1989a) showed that animals with measurable endotoxemia (*Escherichia coli)* exhibited similar cardiovascular responses to those animals without measurable endotoxemia (*Staphylococcus aureus).* The presence or absence of positive blood cultures seems to have little influence on the course or prognosis of sepsis.

As with gram-negative organisms, gram-positive organisms have been used to invoke septic shock in animals (NATANSON et al. 1989a). Endotoxin has also been used in many experimental models where it has provoked biological effects similar to those observed during septic shock (fever, IL-1 and TNF elevation, complement activation; NATANSON et al. 1989b; ZHANG et al. 1995). Endotoxin has also been used to reproduce the effects of sepsis in human studies involving volunteers. Endotoxin administration resulted in a hyperdynamic cardiovascular condition with dilation and ventricular depression similar to those observed in clinical septic shock (SUFFREDINI et al. 1989). The significance of endotoxemia in patients with septic shock is disputed. About 50% of septic patients may have endotoxin levels in their blood. Endotoxemia may be transient, so that its prevalence depends on the frequency of blood sampling (DANNER et al. 1991).

1.3 Source

The source of septic shock has probably also undergone an evolution. In the 1950s and 1960s the most common sources were often due to invasive procedures. Nowadays urinary tract infections are less common and are also considered more benign. Computed tomography of the abdomen helps to diagnose abdominal complications early. In fact, pulmonary infections are now more common. This may at least in part be associated with the prolonged use of mechanical ventilation.

2 Clinical Interpretation

Acute circulatory failure frequently presents as arterial hypotension. Systolic arterial pressure is typically less than 90 mmHg. To avoid the inclusion of patients with transient hypovolemia the definition includes hypotension which is not immediately responsive to intravenous fluids therapy so that inotropic support is generally required. Signs of abnormal tissue perfusion, such as reduced diuresis (urine output less than 20 ml/h), altered mentation, and increased blood lactate levels, are also present.

2.1 Signs of Sepsis

2.1.1 Fever

Fever is not always present, and hypothermia may even be present in some cases. The degree of fever is not clearly related to prognosis, probably because a high fever may reflect both the severity of the infection and the degree of host response. On the other hand, hypothermia is associated with a poor prognosis (Clemmer et al. 1992).

2.1.2 White Blood Cell Count

Increased white blood cell count is a feature of inflammation which may result from the stress reaction which is not very specific: it is also observed in other forms of circulatory shock and in other conditions such as trauma or heart failure. The degree of hyperleukocytosis is not well correlated with prognosis. However, some patients may present with acute leukopenia, which reflects a greater peripheral margination and activation of leukocytes. Similarly, an acute fall in the white blood cell count is observed following endotoxin administration in animals and in volunteers. The presence of leukopenia is associated with a worse prognosis.

2.1.3 Tachypnea

Although the mechanism is not entirely understood, sepsis is often associated with increased respiratory rate which frequently leads to hypocapnia (respiratory alkalosis; Clowes 1974). The sign is neither sensitive nor specific to this condition. Changes in respiratory rate do not reflect well the severity of sepsis. Moreover, many patients with severe sepsis are treated with mechanical ventilation so that the respiratory rate may be at least partly controlled by the ICU physician.

2.1.4 Tachycardia

Elevated heart rate is not specific. It can also be observed in many other conditions, particularly in hypovolemia, anemia, heart failure, and stress. It may represent a mechanism to increase cardiac output or to compensate for a fall in stroke volume.

2.1.5 Reduced Vascular Tone

The loss of vascular tone and distributive blood flow alterations is a hallmark of severe sepsis and septic shock. This has been related to the release of many mediators, but the release of nitric oxide (NO) plays an important role.

2.1.6 Cytokines

Blood cytokine levels are usually elevated in septic shock, and this is related to prognosis (Casey et al. 1993; Pinsky et al. 1993). Interleukin (IL)-6 levels in particular are correlated with the severity of septic shock (Meduri et al. 1995). However, cytokine levels are no more reliable than lactate levels in evaluating the course of septic shock, and they are more expensive to measure (Marécaux et al. 1995). Blood cytokine measurements may help to identify those patients most likely to benefit from new forms of immunotherapy. For example, a study is underway to test whether high IL-6 levels help to identify patients who could benefit from anti-TNF antibodies.

2.2 Signs of Shock

2.2.1 Hemodynamic Alterations

Older studies, namely those from the 1950s, 1960s, and early 1970s, referred to two distinct hemodynamic regimes: the hyperdynamic or hyperkinetic pattern (''warm shock'') and the hypodynamic or hypokinetic pattern (''cold shock''). Investigators at that time attempted to correlate the type of pattern to the type of micro-organisms involved (gram-positive or gram-negative).

The hyperdynamic pattern is the typical picture of septic shock, where it is usually present with tachycardia, elevated or normal cardiac output, and decreased systemic vascular resistance (Parker and Parrillo 1983; Parker et al. 1984; Shoemaker 1971; Abraham et al. 1983; Groeneveld et al. 1986; Vincent et al. 1992). A hypodynamic pattern, characterized by low cardiac output, is related primarily to hypovolemia and/or myocardial failure. This pattern can be observed either early in the course of shock, that is, before fluid administration, or very late following severe myocardial failure in dying patients. In a retrospective study of 2469 consecutive ICU patients, Ruokonen et al. (1991) recorded a 73% hospital mortality rate in septic shock. Refractory hypotension was a major early cause of death and MOF was the primary factor later. Cardiac failure was the direct cause of death in only one patient.

These terms are somewhat outdated now, for two main reasons. One is that more aggressive fluid resuscitation and larger use of dobutamine as an inotropic agent has reduced the incidence of the hypokinetic pattern (Vincent et al. 1990; Metrangolo et al. 1995). The second is that regardless of the cardiac output value septic shock is seen as an imbalance between oxygen demand and oxygen supply, so that a cardiac index which is normal or high may still be insufficient to meet an elevated oxygen demand. Hence, one should consider a spectrum of presentations rather than only two extreme patterns. Also the hemodynamic pattern is not clearly related to the type of micro-organism present.

Obtaining an optimal level of cardiac output is an essential factor in the resuscitation of septic shock. The most important question is not whether car-

diac output is "high" or "low," but whether it is adequate or inadequate in relation to oxygen requirements and altered oxygen extraction capabilities (Fig. 1).

2.2.2 Myocardial Depression

Advances have been made in understanding the causes of myocardial depression. Altered cellular processes are involved in altering myocardial contractility under the influence of cytokines, including NO (Brady et al. 1992). Importantly, myocardial depression is no longer seen as a terminal event. It is now known that it can be present early, even when cardiac output is normal or high. In such cases the high cardiac output is a result of tachycardia. It is also possible that stroke volume is maintained by ventricular dilation leading to a reduced ventricular ejection fraction. Patients with septic shock have decreased left ventricular ejection fraction and right ventricular ejection fraction. The ventricles dilate to maintain stroke volume and compensate for depressed contractility (by the Frank-Starling mechanism). Interestingly, nonsurvivors are less likely to have decreased left ventricular ejection fraction, probably because they have more severe peripheral alterations, resulting in a reduced left ventricular afterload (Parker et al. 1984). However, nonsurvivors are likely to have lower right ventricular ejection fraction because pulmonary hypertension is an important feature of severe sepsis (Vincent et al. 1992). These observations have important therapeutic implications since inotropic support with dobutamine may be indicated even when cardiac output is not reduced (Vincent et al. 1990; Metrangolo et al. 1995).

2.2.3 Altered Vasculature

Vasodilating mediators can lead to the downregulation of α-receptor activity. O_2 extraction capabilities are reduced by maldistribution of blood flow. Loss of vasomotor regulation, microvascular obstruction, peripheral arteriovenous shunting, and interstitial edema formation can all contribute to the maldistribution of blood flow, with altered oxygen extraction capabilities. In the presence of a very high blood flow the microcirculatory transit time of the red blood cells may also become too short to allow oxygen extraction by the cells.

2.2.4 Altered Organ Perfusion

Shock-related disturbances in tissue perfusion are manifested by signs of altered organ function. In the most severe cases skin perfusion may be altered, as reflected by a mottled skin. However, in the typical hyperdynamic states, the skin perfusion may be normal. Reduced urine output is often an early occurrence. The insertion of a Foley catheter is vital in all patients with shock to

facilitate frequent (hourly) measurement of urine output. Reduced renal perfusion is typified by decreased urine sodium and increased urine osmolarity, but once shock is established, isosthenuria typically results from altered tubular function. Sepsis can also result in altered mentation, generally characterized by obtundation, disorientation, and confusion, which is observed in 70% of patients with (Bone 1991). This has been related by several investigators to alterations in cerebral blood flow (Bowton et al. 1989; Parker and Emerson 1977; Ekstrom-Jodal et al. 1982; Miller et al. 1987), but cytokines are probably also involved.

2.2.5 Blood Lactate

Increased lactate levels are usually indicative of a relative imbalance between the oxygen requirement of the cells and the oxygen supply to them. Anemia and hypoxemia, for example, do not readily result in hyperlactatemia because a fall in hemoglobin level or hemoglobin saturation can be compensated by an increase in cardiac output. On the other hand, hyperlactatemia can be induced experimentally by progressively reducing oxygen delivery until a critical level is reached (Zhang and Vincent 1993).

Although increased blood lactate levels are traditionally attributed to tissue hypoxia and the anaerobic mechanism, increased lactate levels in sepsis may be related to other mechanisms. Other derangements that may contribute to elevated lactate levels include altered pyruvate metabolism and increased aerobic glycolysis. In particular, endotoxin can inhibit pyruvate dehydrogenase and thereby increase lactate before tissue hypoxia develops (Vary et al. 1986). Preiser et al. (1990) demonstrated that administration of dichloroacetate, an activator of pyruvate dehydrogenase, can reduce blood lactate but does not have a significant effect on hemodynamic performance. In complex cases it may also be useful to determine pyruvate levels to assess the lactate-to-pyruvate ratio because increased glucose utilization causes pyruvate to increase whereas cellular hypoxia causes the lactate-to-pyruvate ratio to increase. Nevertheless, the clinical applications are quite limited: even patients who are profoundly septic but hemodynamically stable have normal blood lactate levels. Hence increased lactate levels should represent an alarm signal.

Indeed, several studies have shown that blood lactate levels are well correlated with the prognosis in septic shock (Bakker et al. 1995; Vincent et al. 1983; Fig. 2). Recently it has been demonstrated that repeated determination of lactate levels provides a more reliable indication of prognosis. In particular, Bakker et al. (1995) showed that repeated serial lactate measurements gives a reliable indication of not only mortality rate but also incidence and severity of organ failure. Lactate levels are a better clinical indicator than base deficit or bicarbonate level. Recent improvements in measuring instruments have facilitated the measurement of blood lactate levels, and it is to be hoped that such improvements continue so that repeated measurements of blood lactate will be widely available to assess the effects of therapy.

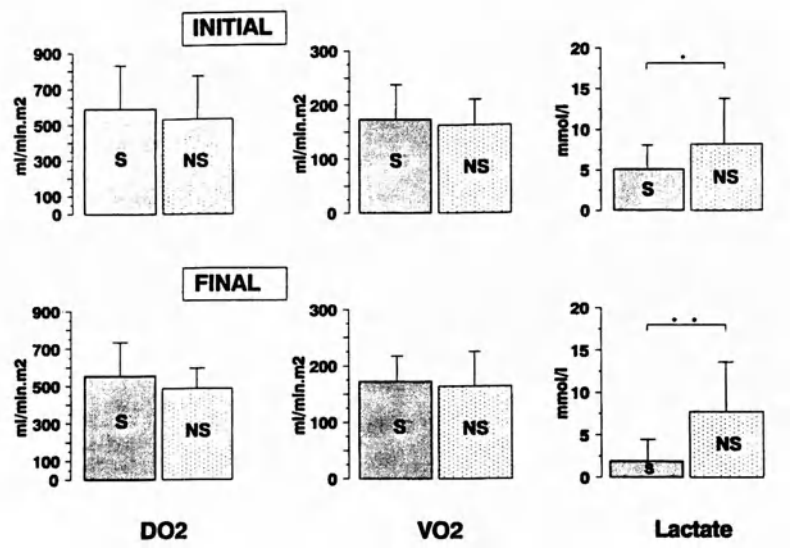

Fig. 2. Values of oxygen delivery (DO_2), oxygen consumption (VO_2), and blood lactate levels in survivors and nonsurvivors from septic shock. In contrast to blood lactate levels, DO_2 and VO_2 are poor prognostic factors in septic shock

3 Organ Dysfunction/Failure

When shock is prolonged, organ dysfunction and eventual MOF are often sustained. If fully developed, MOF is typically associated with very high mortality rates (80%–90%). Organ failure in septic shock has been related to the duration of adrenergic support (RUOKONEN et al. 1991).

In assessing the likelihood of MOF-related mortality it is important to consider both the *number of affected systems* and the *degree of dysfunction.* Table 1 presents some important but simple and widely available parameters that can be included in this evaluation. In the early and late stages of sepsis an extremely complex relationship exists between the immune response and tissue hypoxia, in which the interaction of many cytokines such as TNF-α, and IL-1 and other mediators such as NO has been implicated.

3.1 Lung

Pulmonary dysfunction can lead to ARDS and is characterized by bilateral infiltrates on the chest radiograph and by hypoxemia, requiring the use of high FiO_2, and continuous positive airway pressure. ARDS is also frequently associated with reduced lung compliance ("stiff lungs").

Table 1. Simple, widely available parameters for quantifying degree of organ dysfunction in severe sepsis

System	Parameter	Abnormal if
Respiratory	PaO2/FiO_2 or respiratory support	<400 mmHg present
Coagulation	Platelet count	<150 000/mm^3
Liver	Bilirubin level	>2mg/dl
Cardiovascular	Requirement for vasopressor agents	Present
Central nervous	Glasgow Coma Score	<15
Renal	Creatinine level or urine output	>1.2 mg/dl or <500 ml/day

3.2 Coagulation

Coagulation defects associated with severe sepsis are common, but aspecific. These range from full-blown disseminated intravascular coagulation to isolated thrombocytopenia. Altered coagulation is most commonly manifested by low platelet count and sometimes by prolonged prothrombin time or activated partial thromboplastin time.

3.3 Liver

Hepatic dysfunction often develops after a few days of septic shock and is associated with a poorer prognosis. It is typically manifested by an increase in serum bilirubin (Franson et al. 1985; Banks et al. 1982; Nolan 1981) and sometimes in liver enzymes. In lipopolysaccharide-stimulated Kupffer cells grown in coculture with hepatocytes Billiar et al. (1989a,b) demonstrated that the release of NO inhibits the function of the hepatocytes, suggesting that NO is involved in the hepatic abnormalities associated with sepsis.

3.4 Cardiovascular

As indicated above, an elevated cardiac index and reduced systemic vascular resistance are hallmarks of sepsis and cannot be considered as signs of cardiovascular dysfunction. Since treatment may vary from one institution to another and even from one physician to another, it would be preferable not to base the degree of organ dysfunction on a therapeutic requirement. Nevertheless, the requirement for vasopressor agents is probably the best factor that one may include.

3.5 Central Nervous System

Severe sepsis is often associated with altered consciousness, without organic substrate. A reduction in the Glasgow Coma Score reflects such alterations.

3.6 Kidney

Renal failure may present with or without oligoanuria. Hemodialysis or continuous hemofiltration may be required in such cases. The role of continuous hemofiltration in the removal of proinflammatory mediators is still controversial (VINCENT and TIELEMANS 1995).

4 Prognosis

The prognosis of septic shock is determined primarily by the underlying status of the patient, including physiological condition, age, type of underlying disease, presence of debilitating factors, such as hematological malignancies, liver cirrhosis, and recent chemotherapy. The prognosis is worse for fungal than for bacterial infections because the former are more common in debilitated patients. Although it has been suggested that infection with *Pseudomonas* is associated with a worse prognosis, the type of bacteria is not very discriminant. As indicated above, the presence of hypothermia or leukopenia is associated with a poorer prognosis.

Even more than the severity of infection, the adequacy of the anti-infectious management is a crucial factor influencing outcome. Appropriate antibiotic therapy and removal of the source (e.g., by surgery or drainage) are of paramount importance. The adequacy of the cardiorespiratory management are other important determinants of survival. Adequate fluid therapy and proper use of vasoactive agents are essential.

Blood levels of many cytokines, adhesion molecules, hormones, and other mediators have been shown to be related to prognosis.

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Significance and Pathogenesis of Septic Shock

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1 Definition

Septic shock is a state of inadequate tissue perfusion induced by microbial products and characterized by low blood pressure and biochemical signs of oxygen deficit. The reduced oxygen and nutrient transport to vital organs is caused by a generalized intravascular inflammatory response resulting in vasodilation. Septic shock evolves through different stages and is initially characterized by a hyperdynamic circulation, i.e., high cardiac output and after volume loading low peripheral resistance (Parillo 1993). Most patients dying of septic shock remain in this circulatory state to the end (Parillo 1993). In patients with overwhelming bacteremia caused by *Neisseria meningitidis, Streptococcus pneumoniae, Streptococcus pyogenes, Haemophilus influenzae*, and certain other bacteria the initial hyperdynamic circulation is gradually transformed into a hypodynamic circulation due to reduced cardiac performance. These patients die in a state of irreversible vasodilation combined with myocardial failure often with a terminal arrhythmia (Mercier et al. 1988; Brandtzaeg 1995).

2 Septic Shock and the Multiple Organ Dysfunction Syndrome

The altered circulation is one of several dysfunctional organ systems induced by the intravascular inflammatory response. The kidneys, lungs, liver, and the vascular system including circulating blood cells may all participate to a variable extent in the multiple organ dysfunction syndrome (MODS). Circulatory failure in connection with severe intravascular bacterial infections have been known for a long time. The increasing interest in septic shock as a clinical entity over the past 40 years has paralleled the increasing number of patients developing septic shock in modern intensive care units (ICU; Bone 1993). This cohort of patients is heterogeneous. The majority of patients developing septic shock today are characterized by a debilitated general condition related to an underlying disease, a recent operation or treatment with cytotoxic drugs, and treatment or surveillance with intravenous devices. The goals of the intensified research into the basic pathophysiological mechanisms of septic shock are to prevent or ameliorate the intravascular inflammation and restore adequate tissue perfusion thereby reducing the persistent high mortality of approximately 50% (Bone 1993; Parillo 1993).

3 Experimental Shock Research Versus Human Septic Shock

The scientific literature related to possible pathogenetic mechanisms of septic shock is overwhelming. This review addresses major well-established pathogenetic mechanisms and includes a restricted number of results obtained in primate septic shock studies. Human responses to intravenously injected lipopolysaccharides (LPS) are referred to inasmuch as they reflect phenomena observed in septic shock patients. These experimental approaches reflect disease mechanisms typically observed in acute bacteremic patients developing septic shock. Many believe that the majority of shock patients are "sensitized" by underlying diseases to shock provoking mechanisms. It is commonly believed that smaller changes in the various homeostatic systems regulating tissue perfusion may have more devastating effects in diseased than in previously healthy persons. However, we lack good models to study the effect of "sensitization by disease" in primates or other experimental animals closer to man than rodents.

4 The Microbial-Host Interaction

Septic shock develops when specific microbial components gain access to the circulation and are recognized by the immune system, which generates an exaggerated mediator response (Parillo et al. 1990; Young et al. 1991; Glauser et al. 1991, 1994; Bone 1991; Jafari and McCracken 1992; Natanson et al. 1994; Lynn and Cohen 1995). The interplay of bacterial and host factors determines the final outcome. Animal experiments indicate that the response depends on the nature and amount of bacterial components introduced and the time span of the microbial insult, for example, bolus injection versus a long-lasting infusion with the same dose.

5 The Nature of Microbial Components That Induce Intravascular Inflammation

Cell wall constituents such as LPS in gram-negative bacteria and peptidoglycan and teichoic acid in gram-positive bacteria or proteins liberated during growth (exotoxins) are the principal microbial components which induce an intravascular inflammatory reaction leading to septic shock (Rietschel et al. 1991; Chetty and Schwab 1987; Zumla 1992). They share a powerful stimulatory property when recognized by the immune system.

6 LPS in Plasma and the Development of Septic Shock

It is generally believed that LPS must be detached from the outer membrane to exert full biological effect. The toxicity of LPS is associated with lipid A interacting either with a cell receptor initiating signal transduction or host fluid phase cascade systems (Morrison and Ryan 1987; Morrison 1990). LPS is released through complement-mediated bacterial lysis or autolysis. Insertion of the aggregated terminal components 5b-(9)$_n$ (membrane attack complex) of the complement system into the outer membrane destabilize the outer leaflet. In vitro studies indicate that LPS is released through the action of complement although the membrane attack complex does not necessarily disrupt the outer membrane and kill the bacterium (Frank and Fries 1988). Several proteins secreted by neutrophils such as bactericidal permeability increasing protein and defensins may also destabilize the outer membrane (Spitznagel 1990).

Many bacteria produce surplus outer membrane material, termed outer membrane vesicles (OMV), during log phase growth. These phenomena have been studied mostly in vitro, and the relevance for the in vivo situation is unclear. Pathogenic clones of *N. meningitidis* appear to produce large amounts of OMV both in vitro and in vivo (Devoe and Gilchrist 1973; Andersen et al. 1987; Brandtzaeg et al. 1992).

The LPS-releasing effect of antibiotics has been much debated and extensively studied in model systems (Hurley 1992). However, it has never been convincingly documented that initiation of antibiotic treatment precipitates or aggravates septic shock in man (Hurley 1992). On the contrary, inadequate antibiotic treatment is a risk factor associated with mortal outcome in patients with severe bacterial infections. In fulminant meningococcal infections with high pretreatment levels of LPS the infusion of benzylpenicillin and chloramphenicol, which are both bactericidal to *N. meningitidis,* immediately reduces the circulating levels of LPS and various inflammatory mediators (Engebretsen et al. 1986; Brandtzaeg et al. 1989a,c, 1990; Waage et al. 1989).

We know very little about the physicochemical state of LPS molecules released into the circulation. Presumably they do not circulate as single molecules but as complexes due the hydrophobic lipid A region (Brandtzaeg et al. 1992). Many proteins form complexes with lipid A, including high-density lipoproteins, low-density lipoproteins, very low density lipoproteins, albumin, transferrin, and lactoferrin. Collectively these proteins appear to form a "buffer system" that partly reduces the biological effects of lipid A in LPS-responsive cells including monocytes, macrophages, and neutrophils (Flegel et al. 1989; Morrison 1990).

LPS-binding protein (LBP) – a recently discovered acute-phase reactant – forms complexes with lipid A. The LPS-binding protein–lipid A complex associates with CD14, an glycophosphatidyl inositol anchored surface protein on monocytes and macrophages, which greatly enhances the lipid A induced signal transduction to the cell nucleus (Wright et al. 1990). The basic level of LPS-

binding protein plasma (approximately 20 μg/ml) appears to be sufficient for generating a maximum response of human monocytes after a LPS challenge. The signal pathway from the cell surface to the nucleus is presently largely unknown but involves G-proteins, various protein kinases, and nuclear factor kB signal transduction pathway.

7 Gram-Positive Cell Wall Products and Septic Shock

Peptidoglycan and teichoic acid represent the major inflammatory inducing principles in the cell wall of gram-positive bacteria. Peptidoglycan is a heteropolymer consisting of 1–4 linked *N*-acetylglucosamine and *N*-acetylmyramic acid cross-linked with short peptides (Chetty and Schwab 1985). The glycan backbone structure is constant whereas the short peptide chains cross-linking the sugars vary. Peptidoglycan is generally regarded as less biologically potent on weight volume base than *Escherichia coli* LPS although this may differ between different species (Dziarski 1993). The molecular mechanisms involved in cell activation by peptidoglycan and teichoic acid are largely unknown. Recently a 70–73-kDa surface proteins which is located on various immune cells has been identified as a peptidoglycan receptor (Dziarski 1993). This serves a triple function in being a receptor for peptidoglycan, lipid A, and pertussis toxin. The signaling pathway involves a G protein signal transduction system.

We still lack methods sensitive enough to quantify peptidoglycan and teichoic acid in plasma collected from patients with gram-positive bacteremia. Consequently no systematic study in patients has been conducted linking the presence or level of gram-positive cell wall products to activation of various mediator systems. Based on the experience with overwhelming gram-negative bacteremia, high levels of cell wall products are to be expected in plasma collected from patients developing lethal *S. pneumoniae* bacteremia, a condition often linked to lack of splenic function. The spleen plays a particular important role in removing intravascular bacteria when the host lacks specific opsonins (Benacerraf et al. 1959).

8 Exotoxins Implicated in Septic Shock

Exotoxins are a group of proteins with pleiotropic effects on cell metabolism. They are secreted primarily during bacterial log phase growth. The most intensively studied exotoxins which have been implicated in septic shock are those produced by *S. aureus* (toxic shock syndrome toxin and various en-

terotoxins) and *S. pyogenes* (streptococcal pyrogenic exotoxins). These toxins are small proteins (20–30 kDa) revealing a significant amino acid homology. They function as superantigens (Zumla 1992). The staphylococcal toxic shock syndrome is usually a pure toxin-mediated disease since cultures of blood usually are negative. The exotoxins are produced outside the vascular system (in the vagina in cases of toxic shock syndrome related to tampon use) gaining access to the circulation. In the streptococcal toxic shock syndrome toxin-producing bacteria are usually present in both the blood and various tissues.

9 Bacteria Associated with Septic Shock

The spectrum of bacteria isolated from cultures of blood or infected extravascular foci in critically ill patients changes over time. Early and intensified treatment with broad-spectrum antibiotics have had a major impact on the isolation rate of live bacteria in blood cultures. New technique employing long-term intravascular devices to treat and monitor patients have contributed to the isolation of increasing numbers of gram-positive skin-related bacteria. Gram-negative bacteria, particularly *E. coli* and other gram-negative rods, however, are still the principal pathogens isolated from blood culture in septic shock patients (Glauser et al. 1994).

10 Pathophysiological Effects of Endotoxin in Man

LPS are the most intensively studied bacterial component related to the development of septic shock (Morrison and Ryan 1987). If 2–4 ng/kg [20–40 endotoxin units (EU)/kg] *E. coli* LPS is injected intravenously in healthy volunteers, profound effects occur within 30–60 min which may last for many days (Martich et al. 1993; Burrell 1994). One hour after the injection the test persons perceive symptoms associated with general malaise such as nausea, headache, myalgia, chills, and rigors which reach a maximum within 2 h (Elin et al. 1981; van Deventer et al. 1990; van Zee et al. 1995). The temperature rises to 38.5°–40 °C. The heart rate increases and the circulation becomes hyperdynamic, with elevated cardiac output, reduced peripheral vascular resistance, and dilation of the left ventricle with reduced performance; these alterations may last for several days (Suffredini et al. 1989b). The pulmonary function is altered, with a fall in PaO_2 and $PaCO_2$ and widening of the alveolar arterial gradient after fluid loading (Suffredini et al. 1992). Broncheoalveolair lavage indicates increased capillary permeability for small molecules but not for albumin (MW 69 kDa).

The coagulation system is activated by upregulation of tissue factor presumably on circulation monocytes. Concomitantly the fibrinolytic system is activated by release of tissue plasminogen activator from endothelial cells. Within 2–3 h the fibrinolysis is gradually inhibited by increasing levels of plasminogen activator inhibitor 1 (SUFFREDINI et al. 1989a; VAN DEVENTER et al. 1990). The net effect is a procoagulant state. The plasma contact system comprising coagulation factors XII and XI, prekallikerin, and high molecular weight kininogen is activated within 2 h. One consequence is the generation of bradykinin, a nonapeptide which causes profound vasodilation and increased transcapillary flux (DELA CADENA et al. 1993).

Cytokines including tumor necrosis factor-α (TNF-α), interleukins (IL) 1, 6, and 8, IL-1 receptor antagonist, and various soluble receptors neutralizing these cytokines are released in a coordinated manner (MICHIE et al. 1988; FONG et al. 1989, 1990; ZABEL et al. 1989; VAN DEVENTER et al. 1990, 1993; CANNON et al. 1990; SPINAS et al. 1990, 1992; MARTICH et al. 1991; GRANOWITZ et al. 1991, 1993; FISCHER et al. 1992; VAN ZEE et al. 1992, 1995; KUHNS et al. 1995).

Circulating neutrophils reveal a dose-dependent change in kinetics ranging from leukocytosis at low doses (0.5–1 ng/kg) to significant leukopenia at a higher dose (2–4 ng/kg) challenge (ELIN et al. 1981; VAN DEVENTER et al. 1990; KUHNS et al. 1995). The difference in kinetics is related to upregulation of endothelial and leukocyte adhesion molecules leading to a close neutrophil–endothelium interaction (KUHNS et al. 1995).

High doses of LPS have been injected into humans, with pronounced adverse effects. A 14-year-old boy developed a life-threatening septic shock and MODS after receiving 100 ml packed erythroyctes heavily contaminated with *Pseudomonas fluorescens* LPS. Plasma from the lot contained 40 000 EU/ml (FOREMAN et al. 1991). A laboratory worker injected 1 mg purified *Salmonella minnesota* LPS intravenously in a suicide attempt. The dose was 3750 times higher (15 000 ng/kg) than the one used in most human experiments. The patient developed persistent hypotension, pulmonary edema, coagulopathy, renal and hepatic impairment and needed treatment with fluid and epinephrine for 50 h. She survived (DA SILVA et al. 1993).

These observations clearly demonstrate that small doses of LPS injected intravenously induce sepsislike symptoms and activate important inflammatory mediators whereas large doses of LPS precipitate life-threatening circulatory collapse and multiple organ failure.

11 Circulating LPS and Human Septic Shock

The introduction of the *Limulus* amebocyte lysate assay into clinical medicine in 1970 made it possible to perform quantitative studies of LPS in plasma (LEVIN et al. 1970, 1972). LEVIN et al. were the first to document that clinical severity is

associated with detectable LPS in plasma. Endotoxinemia (LPS ≥ 5 ng/l; 0.05 EU/ml) in platelet-rich plasma is an early marker of a septic response in febrile patients (VAN DEVENTER et al. 1988). MCCARTNEY et al. (1983) detected LPS in plasma of all 31 patients admitted to a large ICU with septic shock of whom 21 eventually died. In patients with overwhelming gram-negative bacteremia caused by *N. meningitidis* the levels of LPS are associated with disease severity. Plasma levels of 800 ng/l (8 EU/ml or higher predict the development of persistent septic shock, impaired renal function, adult respiratory distress syndrome, massive coagulopathy, and ultimately a fatal outcome in half of the patients (BRANDTZAEG et al. 1989a, 1995). Systemic meningococcal disease is so far the only gram-negative infection in which circulating levels of LPS have been related quantitatively to activation of various mediator systems involved in the pathogenesis of septic shock and reveal a dose-dependent association with mortality (BRANDTZAEG and KIERULF 1992; BRANDTZAEG 1995; BRANDTZAEG et al. 1995). In few patients with overwhelming bacteremia caused by *H. influenzae* levels of 500–2 000 ng/l (5–20 EU/ml) have been detected (BRAZILIAN PURPURIC FEVER STUDY GROUP 1987; Brandtzaeg, unpublished results).

Low grade endotoxinemia is often observed among neutropenic patients undergoing cytotoxic therapy for hematological malignancies (SHANDS and MCKINNEY 1989; BEHRE et al. 1992). Twenty-one severely neutropenic patients undergoing treatment for leukemia or non-Hodgkin lymphoma were suspected of developing gram-negative sepsis. Of these, 17 (81%) had detectable LPS in plasma (detection limit 14 ng/l, or 0.17 EU/ml) on the first day of antimicrobial treatment, of whom 10 died. None of the four patients without detectable LPS developed septic shock, and all survived. Endotoxinemia was quantitatively associated with fever. Nonsurvivors had significantly higher maximum concentration of LPS than did the survivors. Of 11 patients with endotoxinemia and septic shock 7 (64%) died (BEHRE et al. 1992). SHANDS and MCKINNEY (1989) found no association between endotoxinemia and fever among a similar group of patients.

In 100 patients developing septic shock in an ICU 43% had detectable endotoxinemia during the shock period (DANNER et al. 1991). The development of multiple organ dysfunction occurred ten times more frequently among patients with detectable endotoxin than among those without. However, the majority of patients developing septic shock had no discernable endotoxinemia. LPS were also detected in patients with gram-positive infections (DANNER et al. 1991). GUIDET et al. (1994) studied 93 patients in a medical intensive care study fulfilling criteria of the sepsis syndrome. The 28-day mortality was 53%. Circulating LPS was detected in 47% of the patients. Endotoxinemia was associated with the development of septic shock and organ failure. Among 46 patients with confirmed gram-negative bacterial infection LPS was detected in 67%, as opposed to 28% among patients without documented gram-negative infection. Of 19 patients with gram-negative bacteremia on admission 14 (79%) had detectable LPS in the blood. For the whole group, however, detectable LPS did not predict gram-negative infection or outcome (GUIDET et al. 1994).

Summarizing the literature on the relationship between circulating LPS and the development of septic shock, two different patterns appear to exist. In patients developing acute, overwhelming gram-negative bacteremia which rapidly evolves to septic shock by organisms such as *N. meningitidis* and *H. influenzae* significantly increased levels of LPS have been documented. In patients contracting severe bacteremia caused by *Capnocytophage canimorsus* – a gram-negative rod associated with dog bites and previously termed dysgonic fermenter 2 – LPS may also play an important role in the pathophysiology. The clinical picture reveals features similar to those observed in fulminant meningococcemia (Hicklin et al. 1987). Patients contracting *N. meningitidis* and *H. influenzae* may have plasma levels of LPS up to 600 pg/ml (6 EU/ml) without developing septic shock (Brandtzaeg et al. 1989a; Brandtzaeg, unpublished results).

The majority of ICU patients developing septic shock reveal the second pattern. LPS is detectable in fewer than 50% of the patients, and quantitative measurements have no prognostic value. Circulating LPS, when detected, are often associated with bacteremia or a focal infection caused by gram-negative bacteria. A significantly larger proportion of patients with detectable LPS develop septic shock than patients with a negative result on the Limulus amebocyte lysate test. The levels of LPS are usually below 100 pg/ml (1 EU/ml), which is several orders of magnitude lower than that been measured in patients with overwhelming bacterial infections.

12 Pathogenetic Mechanisms Related to the Intravascular Inflammatory Response

Septic shock is the result of an inappropriate inflammatory response which reduces the tone of the resistance vessels and impairs myocardial performance. Two basic phenomena are related to the deficit of circulating volume: (a) relaxation of smooth muscles in the vessel wall and (b) increased endothelial permeability. The combination leads ultimately to tissue hypoxia and altered cell metabolism. It has been documented that mediators related to cascade systems in plasma, cytokines, enzymes, and oxygen radicals elicited by various cells adjacent to or in the vascular compartment play a role in septic shock. It should be stressed that our knowledge is still fragmentary, and that each decade has its favorite mediator system considered particular important in the pathophysiology of septic shock. So far, no single mediator or mediator system can explain all features of this clinical entity. Moreover, a very complex interplay appears to exist linking the various systems to each other. Many systems are activated before overt circulatory failure becomes obvious. Finally, the ultimate dysfunc-

tion occurs within cells of the vessel wall and myocardium, areas not readily accessible to investigation in patients.

13 The Proinflammatory Cytokine Response

The principal proinflammatory cytokines which have been implicated in human septic shock are TNF-α, IL-1, IL-6, and IL-8 (Waage et al. 1987, 1989; Girardin et al. 1988; Debets et al. 1989; Damas et al. 1989; Calandra et al. 1990, 1991; Offner et al. 1990; Cannon et al. 1990; Pinsky et al. 1993; Halstensen et al. 1993; van Deuren et al. 1994; Casey et al. 1993; Marty et al. 1994). Recently leukemia-inhibiting factor has been added as a putative proinflammatory cytokine of septic shock (Waring et al. 1994). Among the members of the growing cytokine family TNF-α is regarded as a major mediator of shock. This conclusion is based on extensive animal experiments, observations in human septic shock patients, and studies in human volunteers. In the baboon shock model pretreatment with TNF-α neutralizing antibodies prevents otherwise lethal septic shock. (Tracey et al. 1987). In the human endotoxin challenge model 2–4 ng/kg *E. coli* LPS induces a significant rise in plasma TNF-α, which partly induces or augments the production of other cytokines (Michie et al. 1988; Zabel et al. 1989; van Deventer 1990; Fong et al. 1990). Infusion of recombinant TNF-α reproduces pathophysiological alterations associated with human septic shock (van der Poll et al. 1990, 1991a-c, 1992). The most important production site of TNF-α after an intravenous LPS challenge is the splancnic area, possibly Kuffper cells in the liver (Fong et al. 1990). Peripheral mononuclear cells appear to contribute little to the cytokine response in plasma (Monnoz et al. 1991; Granowitz et al. 1993).

Shortly after its discovery TNF-α was identified as an important marker of severity in meningococcal septic shock (Waage et al. 1987, 1989, 1994; Girardin et al. 1988; van Deuren et al. 1994. By employing a bioassay (Wehi 164 clone 13 mouse fibrosarcoma cell line) Waage et al. (1989) documented that TNF-α circulates as a functionally active molecule, i.e., not in complex with soluble TNF-receptors in patients with overwhelming sepsis. In a few patients with exceptionally high levels of meningococcal LPS in plasma bioactive IL-1, which increases the toxicity of TNF-α, was present (Waage et al. 1989). Circulating IL-1β is usually not detected in septic shock patients and is considered a primarily local mediator.

Studies comprising a more mixed population of patients developing septic shock have disclosed that TNF-α is not uniformly present and varies over time (De Groote et al. 1989; Debets et al. 1989; Damas et al. 1989; Calandra et al. 1990; Offner et al. 1990; Pinsky et al. 1993; Casey et al. 1993). Summarizing 10 years of experience, two different patterns of TNF-α release are discernable. In acute overwhelming bacteremia caused either by gram-negative or gram-positive bacteria high levels of bioactive TNF-α are present. Effective antibacterial

therapy stops further bacterial proliferation and reduces the levels of circulating proinflammatory cytokines, including TNF-α, within few hours.

In patients developing septic shock from other causes than overwhelming bacteremia TNF-α is not universally present in plasma and may fluctuate over time. The persistence of TNF-α – more than its level – indicates a grave prognosis. Whether TNF-α is present in a bioactive form has not been well documented in the latter group of patients since most clinical studies have been performed with immunassays rather than bioassays. In many surgical patients TNF-α is biologically inactive, presumably after complex formation with soluble TNF-receptors (Waage and Aasen 1992).

14 The Anti-inflammatory Cytokine Response

The biological effects of proinflammatory cytokines are balanced by several anti-inflammatory cytokines, notably IL-10, IL-4, IL-13, and transforming growth factor-β. IL-10 has been detected in septic shock patients induced by gram-negative and gram-positive bacteria and in children with fulminant meningococcal septicemia (Marchant et al. 1994; Derkx et al. 1995).

Functional studies employing septic shock plasma containing high levels of native meningococcal LPS and proinflammatory cytokines (TNF-α, IL-6, IL-8) were unable to activate human monocytes collected from a blood donor (Brandtzaeg et al. 1996a). Removing IL-10 from the samples restored the proinflammatory effects of plasma on human monocytes. These results suggest that IL-10 is a functionally important principle counteracting the effect of LPS and proinflammatory cytokines in overwhelming bacteremia. IL-4 and transforming growth factor-β are not detected in plasma from meningococcal septic shock patients.

The lack of monocyte activation by LPS-containing shock plasma explains the dissociation between the levels of cytokines in plasma and concomitant lack of cytokine production in peripheral blood mononuclear cells, which has described in patients with the sepsis syndrome (Monnoz et al. 1991). IL-10 may partly explain the development of tolerance after LPS stimulation (Cavaillon et al. 1994). The proinflammatory cytokines are apparently counteracted by two different principles: (a) soluble receptors and receptor antagonists and (b) anti-inflammatory cytokines. Preliminary results suggest that the levels of IL-10 are 10–100 times higher and are elicited at an earlier stage than TNF-α (Brandtzaeg et al. 1996a).

15 Activation of the Kallikrein Kinin System in Septic Shock

The contact activation system of plasma comprises the coagulation, the fibrinolytic, and the kallikrein kinin systems. Coagulation factor XII (Hageman factor) circulates in complex with prekallikerin and high molecular weight kininogens and may react with negatively charged surfaces and bacterial cell wall products such as lipid A or peptidoglycans, initiating a cascade reaction. One effect is the generation of bradykinin, a nonapeptide which induces an increased transcapillary permeability, reduces arterial resistance, and causes hypotension in experimental animals (COCHRANE 1985; PROUD and KAPLAN 1988). In a classic primate shock study NIES et al. (1968) showed that endotoxin extracts from *E. coli* and *Pseudomonas pseudomallei* induce a prolonged hypotension with reduced peripheral vascular resistance and decreased cardiac output which occurs concomitantly with increased levels of bradykinin and reduced levels of kininogen. The hypotensive effect of live *E. coli* in the baboon shock model can be significantly ameliorated, converting a lethal septic shock to nonlethal hypotension by a monoclonal antibody reacting with factor XII (PIXLEY et al. 1993). Infusion of a low dose of *E. coli* LPS (4 ng/kg) in human volunteers activates the kallikrein kinin system (DELA CADENA et al. 1993). In meningcoccal septic shock low levels of prekallikrein and kallikrein inhibitor have been documented suggesting a significant and long-lasting activation (BRANDTZAEG and KIERULF 1992). Persistently low levels of factor XII and high molecular weight kininogens are associated with unfavorable outcome in patients with systemic inflammatory response syndrome (PIXLEY et al. 1995). The hypotensive effect of bradykinin is partly explained by its ability to induce nitric oxide in the endothelial cells.

16 Activation of the Complement System in Septic Shock

The complement system is a key mediator system in defending the host against intruding micro-organisms. Uncontrolled activation leading to massive generation of anaphylatoxins (C3a, C4a, and most importantly C5a) may, however, have a detrimental effect on the host. Low doses, (2–4 ng/kg) of *E. coli* LPS injected intravenously into human volunteers do not activate the complement system, indicating that the threshold is higher than for other cascade systems (MOORE et al. 1987, VAN DEVENTER et al. 1990). The importance of the complement system in the development of septic shock has been clearly documented in the baboon septic shock model. Infusion of increasing amounts of live *E.coli* leads to a dose-dependent activation of the complement system (DE BOER et al. 1993). Neu-

tralization of C5a by polyclonal antibodies converts an otherwise lethal septic shock to a sublethal septic state (HANGEN et al. 1989).

The complement system is activated in septic shock patients. Initially the observations were based on a significant decrease in the levels of complement factors (MCCABE 1973; WHALEY et al. 1979; SPRUNG et al. 1986). Complement activation has recently been monitored more closely by quantitation of activation products of key complement factors that expose neoepitopes (HACK et al. 1989; BRANDTZAEG et al. 1989b; LIN et al. 1993). By this technique the degree of complement activation and the relative contribution of the two different activation pathways have been monitored in three different groups of patients developing septic shock. In meningococcal patients the level of complement activation was closely associated with the plasma level of LPS and was significantly higher in patients with septic shock than in other clinical categories (BRANDTZAEG et al. 1989b). A follow-up study of the same patients demonstrated activation of both the alternative and the classical pathways. The massive activation in shock patients was, however, caused predominantly by alternative pathway activation (BRANDTZAEG et al. 1996a).

HACK et al. (1989) documented formation of C1 and C1-esterase inhibitor complexes as well as increased levels of C4 activation products, indicating classical pathway activation in a group of ICU patients developing septic shock. In a similar population LIN et al. (1993) concluded that the alternative pathway activation is quantitatively the most important. Septic shock is thus associated with significant complement activation, and both pathways may contribute to the activation. The alternative pathway presumably contributes the most to C3 and C5b-9 activation.

Vitronectin and clusterin, two plasma proteins inhibiting the aggregation and insertion of C5b-9 in membranes, are reduced in shock patients, suggesting consumption during activation of the complement cascade (HØGÅSEN et al. 1994). The levels are negatively correlated to levels of the terminal complement complex (vitronectin–C5b-9) and vitronectin–thrombin-antithrombin complexes. Formation of these complexes, which are rapidly removed from the circulation, may contribute to the low levels of complement inhibitors (HØGÅSEN et al. 1994). Low levels of inhibitors of the coagulation system, such as protein C and antithrombin III, have previously been demonstrated in the same patients (BRANDTZAEG et al. 1989d).

Serial studies of patients with profound meningococcal septic shock have disclosed that the complement system is increasingly activated during the first 12–24 h of treatment (BRANDTZAEG et al. 1989b). This pattern differs markedly from the rapid downregulation of bioactive cytokines (TNF-α, IL-1, IL-6, IL-8), vasoactive intestinal peptide, and markers of coagulation and fibrinolysis observed in the same patients (BRANDTZAEG et al. 1989c, 1990; WAAGE et al. 1989, 1994; HALSTENSEN et al. 1993). Intervention strategies to ameliorate complement activation in septic shock, particularly in patients with overwhelming bacteremia, should be tested in the future.

17 Activation of the Coagulation System in Septic Shock

The coagulation system is activated either via the extrinsic (tissue factor) or the intrinsic (via factor XII) pathways. The interaction of factor VIIa with tissue factor represents the physiological activation pathway of coagulation leading to formation of the prothrombinase complex (activated factors X and V on platelets lipid membranes; FURIE and FURIE 1992; LEVI et al. 1993). Prothrombin is converted to thrombin, splitting fibrinogen to monomeric fibrin and several small peptides.

When a low doses of *E. coli* LPS (2–4 ng/kg) were injected as a bolus to human volunteers, LPS was detectable in plasma for approximately 15–30 min, with a maximum of 13 pg/ml (VAN DEVENTER et al. 1990). Activation of the coagulation system was indicated by increased levels of the prothrombin activation peptides F_1 and F_2 and thrombin-antithrombin and lasted for 4–6 h. Activation of the plasma contact system via factor XII (the Hagemann factor–prekallikrein–high molecular weight kininogen complex) was not detected during this experiment.

In the baboon septic shock model activation of the coagulation system is associated with the development of shock. By inhibiting the tissue factor–factor VII–factor X–factor V–prothrombin pathway at various levels, the baboons survived otherwise lethal septic shock (TAYLOR et al. 1987, 1988, 1991; CREASEY et al. 1993). Baboon experiments which block the activation of Factor XII and thus the plasma contact system ameliorate the hypotensive response but not the activation of the coagulation system (PIXLEY et al. 1993). The mechanisms through which activation of the coagulation system influence the vascular tone is not well understood. Activation of the coagulation system enhances other inflammatory mediator systems. The use of tissue factor pathway inhibitor that inactivates the tissue factor–factor VII–factor X complex, effectively downregulates the IL-6 response in baboons challenged with *E. coli* (CREASEY et al. 1993). Principles which inhibit activation of the coagulation system protect various organs from formation of microthrombi and consequently hypoxia and impaired function.

Markers of disseminated intravascular fibrin formation and increased fibrinolysis are readily observed in patients with severe septic shock. Reduced levels of fibrinogen, platelets, various coagulation factors, protein C and antithrombin III concomitantly with increased levels of fibrinopeptide A, thrombin-antithrombin complexes, D dimers, and other fibrin split products all indicate increased coagulation and fibrinolysis. The extrinsic pathway is thought to be activated in septic shock either by upregulation of tissue factor on circulating monocytes or at the luminal side of the endothelial cells. In the baboon *E. coli.* shock model monocytes in the peripheral blood reveal procoagulant activity, i.e., upregulation of tissue factor, whereas endothelial cells do not (DRAKE et al. 1993). Circulating monocytes may represent the key cell which induces the coagulo-

pathic response in septic shock in man. They develop a transitory procoagulant state by a upregulation of tissue factor and downregulation of the fibrinolytic potential after LPS stimulation which later is reversed (KIERULF and ANDERSEN 1990). Increased levels of tissue factor have been detected in monocytes isolated from patients with fulminant meningococcemia (ØSTERUD and FLAEGSTAD 1983). Whether the endothelium really upregulates tissue factor in septic shock has so far never been compellingly demonstrated in man.

The procoagulant state induced by bacteremia appears to be a phylogenetical old pattern of reaction. It is observed in the horseshoe crab (*Limulus polyphemus*), a "living fossil." While this may be an appropriate response to wall off intruding micro-organisms locally in tissues, the response is highly inappropriate when it causes generalized intravascular fibrin formation, plugging of the microvasculature, and bleeding.

One should, however, bear in mind that the baboon experiments which compellingly document the link between coagulation per se and the general inflammatory response may reflect only the situation in overwhelming human bacteremia and not that in the majority of patients.

18 Activation and Inhibition of the Fibrinolytic System in Septic Shock

Activation of the fibrinolytic system is closely coordinated with activation of the coagulation system. When human volunteers were challenged with 2–4 ng/kg *E. coli* LPS intravenously, increased fibrinolysis was observed during the first 1–3 h (SUFFREDINI et al. 1989a; VAN DEVENTER et al. 1990). Tissue plasminogen activator released from the endothelium converts plasminogen to plasmin. Later the fibrinolysis is increasingly inhibited through the release of plasminogen activator inhibitor 1 from the endothelial cells which complexes with and inactivates tissue plasminogen activator (plasminogen activator inhibitor). The net effect of the marked inhibition of the fibrinolysis is a shift in the balance of coagulation and fibrinolysis to a procoagulant state. The same has been observed in massive endotoxinemia caused by *N. meningitidis* (ENGEBRETSEN et al. 1886; BRANDTZAEG et al. 1990). The pronounced inhibition of fibrinolysis may partly explain thrombotic organ complications in kidneys, adrenal, liver, lungs, and spleen. Postmortem examinations of baboons in septic shock and patients dying of fulminant meningococcemia reveal diffuse formation of thrombi in these organs, which apparently are dissolved at a later stage (BRANDTZAEG 1995). The reduced organ perfusion contributes to the elevated markers of tissue hypoxia and cell injury in plasma (lactic acid, creatinine, uric acid) and lowered pH (CREASEY et al. 1993).

19 Phospholipase A_2 in Septic Shock

Phospholipase A_2 induces a profound hypotensive response if injected intravenously in animals (Pruzanski and Vadas 1991). TNF-α and IL-1 enhance the production and release of phospholipase A_2. Infusion of *Salmonella abortus equi* LPS (4 ng/kg) in cancer patients as part of an experimental treatment protocol induced a 20-fold increase in circulating levels of phospholipase A_2 (Pruzanski et al. 1992). In 12 patients with septic shock the levels of PLA_2 were 14- to 260-fold higher than baseline (Vadas et al. 1988). The source of phospholipase A_2 in these patients is unknown. The levels did not correlate with the levels of pancreatic enzymes, suggesting another mechanism than that observed during acute pancreatitis. The role of phospholipase A_2 in human septic shock is still unclear, but it may play an important role in the generation of arachidonic acid metabolites and platelet activating factor.

20 Endorphins

β-Endorphin and lipotropin are elevated in the primate *E. coli* LPS septic shock model (Gurll et al. 1988). Blockade with the opiate receptor antagonist naloxone converts a lethal septic shock to sublethal hypotension in the same model. β-Endorphin is increased in plasma in human volunteers challanged with LPS (Casale et al. 1990). Treatment of septic shock in patients with naloxone has improved certain circulatory parameters but not survival (Napolitano and Chernow 1988).

21 Vasoactive Intestinal Peptide and Septic Shock

Vasoactive intestinal peptide derived from the autonomous peripheral nervous system is a potent vasodilator which is released into the circulation during canine and porcine septic shock (Bone et al. 1980; Freund et al. 1981; Revhaug et al. 1988).

The release occurs primarily in the splancnic area, and higher VIP levels are detected in the splancnic than the general circulation. The local release of vasoactive intestinal peptide may represent an effort to increase the perfusion of the gut in septic shock. It is not released during experimental porcine cardiogenic shock (Revhaug et al. 1988). Significantly increased levels of vasoactive intestinal peptide have been detected in fulminant meningococcal septic shock (Brandtzaeg et al. 1989c).

22 The Myocardial Response in Septic Shock

Contrary to previous belief, cardiac performance is basically altered in septic shock (PARRILLO 1993). Both ventricles are often dilated and reveal a decreased ejection fraction. The changes may represent a compensatory mechanism since it is more readily observed in surviving than nonsurviving patients. The changes are normalized within 7–10 days. In the human *E. coli* LPS challenge model the same changes are observed (SUFFREDINI et al. 1989b). The reason for the reduced myocardial contractility is presently unknown. A set of explanations have been proposed over the years, including altered myocyte ATP metabolism, reduced coronary flow, cardiodepressant activity of proinflammatory cytokines (TNF-α), unidentified humoral principles collectively called myocardial depressant factors, and recently nitric oxide in the myocardium (PARILLO 1993). Recent human studies suggest that the coronary flow is not significantly reduced in human septic shock and therefore cannot explain the myocardial dysfunction (CUNNION et al. 1986). A local dysregulation of the myocardial circulation may, however, exist (DHAINAUT et al. 1987).

23 Conclusion

Septic shock is a multimediator disease. We have identified a large number homeostatic systems that directly or indirectly may contribute to the vasodilation. Septic shock caused by acute overwhelming bacteremia represents one clinical entity. Most animal models developed to study septic shock reflect this clinical situation. Studies of patients with fulminant meningococcal septic shock have been particularly valuable in identifying disease mechanisms associated with this clinical entity.

We know less about the pathogenetic mechanisms associated with the development of septic shock in the majority of our patients with underlying diseases. The association with plasma LPS or other bacterial cell wall components is less consistent. Other principles may induce the inflammatory response. We lack the means to evaluate the "degree of sensitization" for bacterial components of a particular patient. New animal models should be developed which reflect this situation.

In the future we may discover that the disease mechanisms leading to hypotension, hypoperfusion, and organ dysfunction differ significantly among the different patients groups and thus require individual treatment approaches. We will undoubtedly discover new mediators that contribute to the circulatory failure and learn more about the relative contribution of each system at the different stages of septic shock. Research so far suggests that septic shock involves all component of inflammation. It is not likely that we ever will discover

one "master" mediator which we easily can manipulate to the benefit of our patients.

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Bacterial Endotoxin: Chemical Constitution, Biological Recognition, Host Response, and Immunological Detoxification*

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*Dedicated to our friend and esteemed colleague, Professor Roland Schauer, University of Kiel, Germany, on the occasion of his 60th birthday (April 8, 1996).

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1 Introduction

The discovery of endotoxin dates from the late nineteenth century when Richard Pfeiffer, then working in Berlin, characterized endotoxins as heat-stable and cell-associated molecules (Westphal et al. 1977), thus distinguishing them from the heat-labile and proteinous exotoxins which are actively secreted by bacteria (Bhakdi et al. 1994). They were first found to be produced by *Vibrio cholerae* bacteria and later by *Salmonella* and *Serratia*. Endotoxins, due to their various potent biological activities soon attracted worldwide scientific interest. Initial chemical analyses of purified endotoxin indicated that it consists essentially of polysaccharide and lipid, and it was therefore termed lipopolysaccharide (LPS). Today the terms endotoxin (Wolff 1904) and lipopolysaccharide (Shear and Turner 1943) are used synonymously for the same molecule.

Endotoxins are known to constitute amphiphilic macromolecules located on the surface of gram-negative bacteria (Raetz 1990; Rietschel and Brade 1992; Rietschel et al. 1994). Released from bacteria in vivo or administered in an isolated form, endotoxins exert both physiological and powerful pathophysiological effects in higher organisms and thus represent important virulence factors of gram-negative bacteria. With the ultimate aim of pharmacologically or immunologically controlling the endotoxic effects observed during severe gram-negative infections, various research groups attempted to characterize the nature of the biologically active region of LPS and to analyze the mode of action of endotoxin in higher organisms. Subsequently it was shown that the lipid component of LPS, termed lipid A, constitutes its toxic and immunostimulatory principle (Galanos et al. 1985; Loppnow et al. 1989), and the chemical structure of lipid A was elucidated (Zähringer et al. 1994). It was further demonstrated that certain humoral host factors interact with lipid A, and that specific cellular receptors recognize lipid A or LPS, particularly if associated with serum components (Schumann et al. 1990; Wright et al. 1990; Raetz et al. 1991; Ulevitch 1993; Morrison et al. 1993). The result of the specific interaction of LPS with cells may be the activation of cells, for example, cellular proliferation as observed with murine B-lymphocytes and, more important in the context of endotoxicity, the formation and secretion of bioactive mediators as produced by murine or human monocytes, macrophages, and vascular cells (Galanos et al. 1992; Loppnow and Libby 1989, 1990). It was also realized that the physiological and pathophysiological host response to endotoxin is mediated by such endogenous mediators which possess intrinsic biological activities (Dinarello et al. 1984; Beutler and Cerami 1989). If formed in small amounts, these mediators are important for the proper functioning of the immune system and its struggle with invading microorganisms. If, however, they are abundantly produced, they cause toxic effects such as pyrogenicity, leukopenia, and ultimately irreversible shock. With the gradual evaluation of the pathway of events: bacteria → endotoxin → mediators → toxic effects, determination of rational approaches designed to interfere with the endotoxin-induced devastating cascade of events became possible.

This chapter summarizes recent advances in our knowledge of the chemical structure, host recognition of endotoxin, and response of the host to this microbial toxin. A brief description is also presented of new immunological strategies to prevent or treat endotoxin-caused systemic inflammation.

2 Structure of Endotoxin

Over the past two decades significant progress has been made in understanding the chemistry (primary structure) and physics (mono- and multimolecular conformation) of the endotoxin molecule. Knowledge of the chemical and physical LPS structure also allowed the establishment of structure-activity relationships, some aspects of which are briefly reviewed in this chapter.

2.1 O-Specific Chain and Core

LPS of various gram-negative bacterial families are made up according to the same architectural principle, i.e., they consist of a polysaccharide portion and the covalently bound lipid A component (Fig. 1). In the classical case of Enterobacteriaceae (e.g., *Salmonella enterica, Escherichia coli*), the polysaccharide portion consists of two regions, the O-specific chain and the core oligosaccharide (LÜDERITZ et al. 1982). The O-specific chain is usually composed of a polymer of repeating oligosaccharide units. A peculiar situation is encountered in *Legionella pneumophila* serogroup 1, where up to 75 residues of a single sugar (5-acetamidino-7-acetamido-8-*O*-acetyl-3,5,7,9-tetradeoxy-D-glycero-L-galacto-

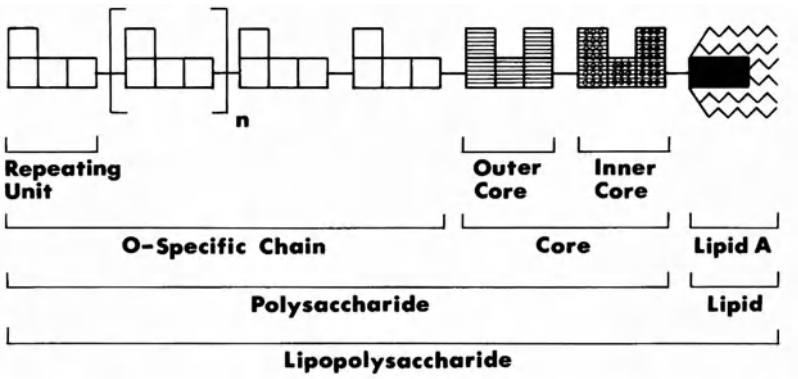


Fig. 1. Schematic structure of a *Salmonella* LPS. Phosphate and other substituents have been omitted from the structure. (From LÜDERITZ et al. 1982)

nonulosonic acid; legionaminic acid) form the O-specific chain, which in this case constitutes a homopolymer (KNIREL et al. 1994; ZÄHRINGER et al. 1995). The O-specific chain is characteristic of and unique for a given LPS and its bacterial origin (serotype). In view of the existence of thousands of serotypes, comparison of different gram-negative bacteria reveals enormous structural variability of the O-specific chain region.

The biosynthesis of the O-specific chain is determined by a cluster of genes termed *rfb*. Mutants harboring a defect in the *rfb* locus or lacking it are able to grow in vitro, indicating that the O-specific chain is dispensable for bacterial viability. In vivo, however, Enterobacteriaceae survive, i.e., resist phagocytosis and killing by bactericidal serum factors such as complement (C'), only if they express an O-specific chain. The O-chain therefore constitutes an important bacterial virulence factor and at the same time a promising target for antibacterial therapy. Recently a 262-bp DNA fragment of *Acetobacter methanolicus* phage Acm1 was found to be sufficient for the suppression of O-specific chain biosynthesis in various enterobacterial strains, including *Escherichia coli*, *Salmonella enterica,* and *Klebsiella pneumoniae* (MAMAT et al. 1991). The results strongly suggest that the Acm1-derived DNA fragment codes for a *trans*-acting regulatory antisense RNA of 97 nucleotides, designated Ibi RNA (LPS biosynthesis interfering), being obviously able to interact with conserved target RNA sequences which are linked directly or indirectly to LPS biosynthesis (MAMAT et al. 1995). This antisense RNA-mediated downregulation of O-specific side chain biosynthesis could represent a new approach for the construction of attenuated live vaccine strains applicable to a wide spectrum of gram-negative bacteria.

The core portion consisting of the O-chain proximal outer core and the lipid A proximal inner core is structurally less variable. In the case of *S. enterica* and *E. coli* the outer core exhibits some variability due to different locations and linkages of its constituents (HOLST and BRADE 1992). Nevertheless, common elements are recognized, such as the presence of the pyranosidic hexoses D-glucose (Glc), D-galactose (Gal), and 2-amino-2-deoxy-D-glucose (GlcN). Hexoses are also present in the outer core of *K. pneumoniae* (SÜSSKIND et al. 1995) and *Proteus mirabilis* (RADZIEJEWSKA-LEBRECHT et al. 1990). The inner core of all LPS-expressing gram-negative bacteria contains 2-keto-3-deoxyoctulosonic acid (Kdo) and in *Enterobacteriaceae* and many other families heptose residues of the L-glycero-D-manno configuration which are often phosphorylated. Thus the inner core region of various LPS contains a chemically similar region. In fact a common and typical core segment can be defined that is structurally conserved in all *S. enterica* and *E. coli* serotypes and various other bacteria. The schematic architecture of this core element is shown in Fig. 2. The presence of this region allowed the recent development of a monoclonal antibody (see Sect. 11.4) recognizing an epitope within this region and therefore cross-reacting with all serotypes of *S. enterica, E. coli,* and some other genera (DI PADOVA et al. 1993a,b). Shared structures which may constitute epitopes for cross-reactive and possibly cross-protective antibodies are also found in other gram-negative bacterial groups. For example, in LPS of all *Pseudomonas aeruginosa* serotypes,

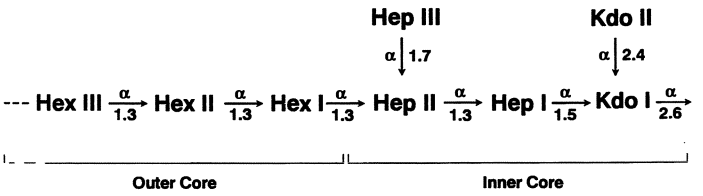


Fig. 2. Common structure present in LPS core types of E.coli and S.enterica. *Hex,* D-Glc, D-Gal, D-GlcNAc; *Hep,* L-glycero-D-manno-heptose; *Kdo,* 2-keto-3-deoxyoctulosonic acid. (From HOLST and BRADE 1992)

L-glycero-D-manno-heptose, carrying a carbamoyl residue in position 7 was identified as an ubiquitous constituent (BECKMANN et al. 1995).

The lipid A component constitutes the chemically most uniform portion of LPS, and its structure is described in more detail below.

Obviously, structural variability of LPS decreases from the surface-exposed O-specific chain over the core region to the polysaccharide-covered and membrane-embedded lipid A component. The reason for this gradient of variability may be the evolutionary pressure exerted by phagocytic cells and antibodies on gram-negative bacteria (NIKAIDO 1970). It is conceivable that the microbes evaded this pressure by changing their most exposed surface structure, i.e., the O-specific chain, and to a lesser degree the outer core.

2.2 Lipid A

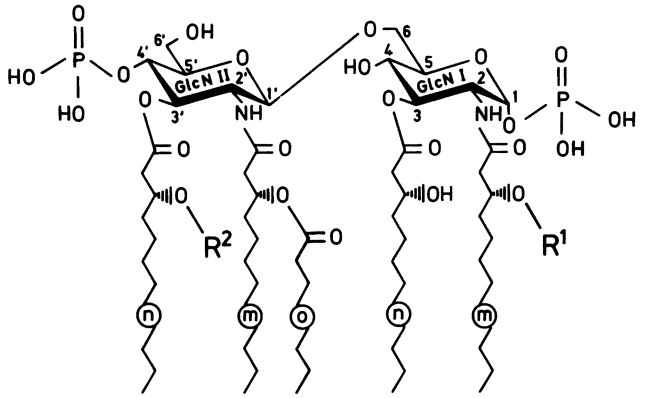
Lipid A represents the covalently bound lipid component of LPS. It can be separated from the polysaccharide region by treatment of LPS with mild acid, which preferentially cleaves the linkage between the Kdo I residue of the inner core and lipid A (Fig. 1). Lipid A has been shown to constitute the endotoxic principle of LPS (GALANOS et al. 1985). With the aim of defining those structural and conformational peculiarities which endow it with endotoxic properties, lipid A has been chemically analyzed in detail.

2.3 Chemical Structure of Lipid A

Figure 3 shows the structure of lipid A of four different types of gram-negative bacteria which all express biologically highly active LPS (*E. coli, Haemophilus influenzae, Chromobacterium violaceum,* and *Neisseria meningitidis*; ZÄHRINGER et al. 1994). Structurally, these lipid A share a 1,4'-bisphosphorylated β1,6-linked D-GlcN disaccharide (lipid A backbone) which is acylated by four (*R*)-3-hydroxy fatty acids at positions 2, 3, 2', and 3'. In each case the acyl group at position 2' of GlcN II carries at its 3-hydroxyl group a further (fifth) fatty acid. The four structures, however, differ in the location of a sixth acyl group (R1 or R2) and the

chain length of fatty acids (symbols m, n, and o). As Fig. 3 shows, lipid A of *E. coli* and *H.influenzae* carries this sixth fatty acid at GlcN II and thus possesses an asymmetric distribution of acyl groups over GlcN I and GlcN II (4+2), whereas a symmetric acyl arrangement (3+3) is present in lipid A of *C.violaceum* and *N. meningitidis*. Importantly, the average length of acyl chains is smaller in the latter group (mainly 12 carbon atoms) than in the former (mainly 14 carbon atoms).

These structural examples demonstrate that lipid A of various origins exhibits a similar architecture, but that variations exist concerning the nature and location of acyl groups. More striking examples for structural variability of lipid A are found, for example, in *Rhizobium, Chlamydia,* and *Legionella* where very long chain (C22–C32) acyl groups, also in the form of (W-1)-hydroxy or oxo-derivatives, are present (Hollingsworth and Lill-Elghanian 1989; Nurminen et al. 1985; Brade et al 1986; Moll et al. 1992; Sonesson et al. 1993). In *Rhodobacter sphaeroides* and *R. capsulatus*, part of the 3-hydroxy fatty acids is replaced by 3-oxo fatty acids (Mayer et al. 1990; Takayama and Qureshi 1992); interestingly, these latter lipid A preparations are biologically inactive (Loppnow et al. 1990;



	Nature of		Number of Carbon Atoms		
	R^1	R^2	m	n	o
Escherichia coli	H	14:0	14	14	12
Haemophilus influenzae	H	14:0	14	14	14
Neisseria meningitidis	12:0	H	14	12	12
Chromobacterium violaceum	12:0	H	12	10	12

12:0, dodecanoic acid 14:0, tetradecanoic acid

Fig. 3. Chemical structure of the lipid A component of various gram-negative bacteria. (From Rietschel et al. 1994; Zähringer et al. 1994)

QURESHI et al. 1991; SCHÖNBECK et al. 1994). Further variations of the lipid A structure concern the substitution of phosphate groups and the nature of backbone glycosyl residues (not shown in Fig. 3). Thus GlcN may be replaced by 2,3-diamino-2,3-dideoxy-D-glucose (GlcN3N), as is the case, for example, in *Rhodopseudomonas viridis, Campylobacter jejuni,* and *L.pneumophila* (for literature see ZÄHRINGER et al. 1994). Finally, phosphate groups may be absent from lipid A. This is the case, for example, in *Bacteroides fragilis* lipid A which lacks the nonglycosidic phosphate group (WEINTRAUB et al. 1989). A most remarkable phosphate-free structural variant of lipid A has been identified in *R. leguminosarum* bv. *phaseoli* (BHAT et al. 1994; PRICE et al. 1994). Here during biosynthesis the 4'-phosphate group is enzymatically removed and replaced by an α-linked D-galacturonic acid (GalA) residue. Also, the glycosidic phosphate is cleaved off, followed by oxidation of GlcN I at C1, yielding 2-amino-2-deoxygluconic acid carrying (long-chain) acyl residues in amide and ester linkage. Thus lipid A of *R.leguminosarum* carries a total of two negative charges at the nonreducing and the reducing backbone unit, however, not in the form of phosphate residues, as is the case in *E. coli* but rather as carboxylate groups.

Based on the results of chemical analyses *E. coli* type lipid A has been chemically synthesized (KUSUMOTO 1992). The demonstration of identity of bacterial and synthetic *E. coli* lipid A in all chemical, physicochemical, physical, and biological parameters unequivocally verified the previously deduced and proposed structure to be correct.

2.4 Physical Structure

Lipid A (and LPS) represents amphiphiles and therefore forms aggregates in aqueous medium above the critical micellar concentration. The availability of defined and homogenous synthetic lipid A and partial structures allowed analyses of the nature of such aggregates, i.e., their three-dimensional physical structure. According to present knowledge, biologically active lipid A adopts, at physiological ambient conditions [37 °C, pH 7, high (>90%) water content, presence of Mg^{2+}] exclusively nonlamellar structures (SEYDEL et al. 1993). These nonlamellar structures are either cubic (*S. enterica* sv. *minnesota*) or hexagonal (*R. gelatinosus*). In contrast, biologically nonactive lipid A (*R. capsulatus*) adopt lamellar structures (BRANDENBURG et al. 1993). This suggests that endotoxicity is determined by a defined conformation of lipid A or LPS. The three-dimensional structure of lipid A multimers and the shape of the corresponding monomers of active and nonactive lipid A is shown schematically in Fig. 4. Whether bioactivity of endotoxin requires the presence of a supramolecular structure (in the form of nonlamellar aggregates) is under debate at present (SHNYRA et al. 1993). More likely, however, endotoxicity is expressed by individual endotoxin or lipid A molecules possessing a conformation which, at higher concentration leads to the observed three-dimensional nonlamellar structures (SCHROMM et al. 1995). Recent studies employing disaggregated LPS support the hypothesis that en-

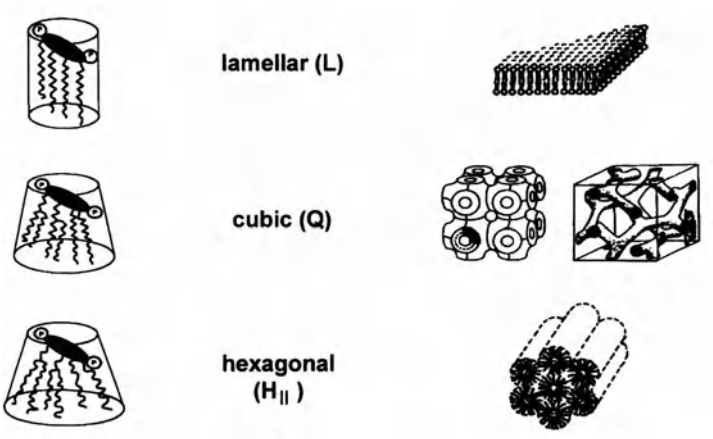


Fig. 4. Physical structure of endotoxically active and nonactive lipid A. Shown are the three-dimensional arrangements and the shape of the corresponding monomer. (From SEYDEL et al. 1993; BRANDENBURG et al. 1993)

dotoxic activity is mediated by single molecules (TAKAYAMA et al. 1994). It further appears that a peculiar spatial shape of lipid A is required for the expression of endotoxicity. We have termed this unique arrangement of lipid A the "endotoxic conformation" (RIETSCHEL et al. 1987).

The concept of a unique lipid A conformation implies that a fitting recognition molecule (receptor) for lipid A should be present either in the circulation or on endotoxin target cells. As the following sections show, the interaction of lipid A with humoral and cellular host factors, leading to a biological response, is in fact highly specific and requires a unique conformation of lipid A on the LPS side and peculiar recognition molecules on the host side.

3 Endotoxic Activity of LPS and Lipid A

The mechanisms involved in the activity of endotoxin are well understood today (MORRISON and RYAN 1987). When gram-negative bacteria multiply or die, endotoxin is released in the form of free LPS or complexed to the outer membrane protein A (FREUDENBERG et al. 1991). Circulating endotoxins constitute a particular class of toxins which induce in host organisms the production of bioactive mediators ultimately responsible for the effects observed during endotoxemia. According to present knowledge, LPS after associating with certain serum factors interacts with receptors expressed by endotoxin target cells such as granulocytes, lymphocytes, vascular cells, and in particular monocytes/macrophages. In response to LPS these cells form and secrete endogenous mediators which

are endowed with potent intrinsic bioactivities and which ultimately induce the typical endotoxin effects.

The following sections describe some of the early humoral and cellular LPS targets and some of the consequences of their interaction with lipid A.

3.1 Humoral LPS Targets

Among the humoral factors interacting with LPS figures prominently high-density lipoprotein (HDL) which is able, as is low-density lipoprotein, to attenuate LPS effects (Freudenberg et al. 1980; Ulevitch et al. 1981; Munford et al. 1982; Flegel et al. 1989). Other circulating LPS-neutralizing proteins are the bactericidal permeability-increasing protein (BPI; present in serum at a concentration of approximately 1 ng/ml) and the cationic proteins CAP 18, CAP 37, and P15A/P15B (Weiss et al. 1992; Elsbach and Weiss 1993; Marra et al. 1990; Levy et al. 1993; Larrick et al. 1991; Hirata et al. 1994). Further serum proteins which associate with LPS include albumin, transferrin, lactoferrin, hemoglobin, lysozyme and a 28-kDa mannose-binding protein. These latter proteins do not modify endotoxin bioactivity to any significant extent (Morrison 1990).

With regard to the expression of endotoxicity the most important serum protein appears to be the LPS binding protein (LBP), which dramatically augments LPS and lipid A activity, rendering femto- to picogram amounts of LPS bioactive (Tobias et al. 1986, 1988). The structure and properties of LBP are described in more detail below. A recently described class of serum proteins, termed septin, is described to possess similar properties as LBP (Wright et al. 1992). Septin could be distinguished from human LBP by means of a monoclonal antibody (Wright 1994). Further studies are, however, indicated to elucidate the peculiarities of septin in comparison to LBP.

3.2 Cellular LPS Targets

The most important target cells of endotoxin are components of the cellular immune system. Thus certain defense cells of almost all species have the ability to recognize minute amounts of LPS, thereby sensing invading micro-organisms. Four cell types can be distinguished which recognize LPS but respond to it in different ways, i.e., by phagocytosis, differentiation or proliferation, and mediator secretion.

1. Polymorphonuclear leukocytes (PMN) take up bacteria and bacterial membrane fragments including LPS, and their phagocytic capacity is greatly enhanced by LPS (Schade et al. 1987). As phagocytosis may be regarded as a first and direct measure of the host to eliminate threatening micro-organisms, the phagocytosis-augmenting property of LPS emphasizes its important role in early nonspecific steps of the host defense against microbial invasion. On the other hand, PMN have enzymes which degrade (i.e., de-O-acylate and depho-

sphorylate) LPS and lipid A to nontoxic partial structures (MUNFORD and HALL 1989; ERWIN and MUNFORD 1992). Further, PMN contain polycationic proteins which are capable of interacting with LPS. Among these BPI a 55-kDa cationic protein has been shown to be toxic for bacteria and to bind strongly to LPS, thereby efficiently neutralizing its bioactivity (WEISS et al. 1992; MARRA et al. 1990, 1992; GAZZANO-SANTORO et al. 1994; LITTLE et al. 1994; THEOFAN et al. 1994). Also, the PMN proteins CAP 18 and CAP 37 possess LPS-inactivating properties (LARRICK et al. 1991). Finally, PMN may attach to endothelial cells and, by causing damage to the endothelial lining and by penetrating through the vessel wall into the tissue, may contribute substantially to inflammatory reactions.

2. B-lymphocytes of murine origin are stimulated by LPS to proliferation, differentiation and secretion of antibodies (ANDERSON et al. 1973). This polyclonal activation may also be regarded as an early defense mechanism of the host against pathogenic microorganisms as it yields antibodies of various antibacterial specificities. Also T-lymphocytes (Th1 type) of human origin are, in a monocyte-dependent fashion, activated by LPS to proliferate and to secrete lymphokines (MATTERN et al. 1994). Thus LPS may also be involved in the cellular immunity against pathogenic microorganisms. In addition, LPS acts on a subset of murine T-lymphocytes ($CD8^+$ $CD4^-$) which are capable of suppressing the humoral immune response to bacterial polysaccharides such as pneumococcal type III polysaccharide (BAKER 1993). Lipid A has been shown to downregulate these T-suppressor cells, thereby augmenting anti-polysaccharide antibody formation (BAKER 1993).

3. Monocytes and tissue macrophages are activated by LPS to produce a large variety of bioactive protein mediators which include interleukin (IL)-1, IL-6, IL-8, macrophage migration-inhibitory factor (BERNHAGEN et al. 1994), and, in particular, tumor necrosis factor α (TNF; NATHAN 1987). Many host cells carry receptors for these mediators and are capable of responding to them, for example, by enhanced activity or by apoptosis. If produced in small amounts, these monokines help to eliminate and inactivate invading micro-organisms, such as by causing moderate fever, inducing leukocytosis, attracting defense cells to the infectious focus, activating intracellular microbicidal mechanisms, and initiating an acute-phase response (ECHTERNACHER et al. 1990; HAVELL 1989). If overproduced, however, these hormonelike mediators become a threat to the host organism by causing damage to cells and organs (TRACEY et al. 1986; WAAGE et al. 1987; VOGEL and HOGAN 1990; LI et al. 1995). Thus multiorgan failure and irreversible shock may be the result of severe gram-negative infection resulting from overwhelming mediator production. On exposure to endotoxin macrophages produce (NATHAN 1987), in addition to monokines, reduced oxygen species (superoxide anion, hydrogen peroxide, hydroxyl radical and nitric oxide), bioactive metabolites of arachidonic acid [prostaglandins, thromboxane, and leukotrienes (LT); (SCHADE et al. 1989a,b; LÜDERITZ et al. 1989) and of linoleic acid [e.g., (*S*)-13-hydroxylinoleic acid; SCHADE et al. 1987], and platelet-activating factor (BRAQUET et al. 1987). Studies using such isolated or corresponding synthetic molecules, biosynthesis inhibitors, antibodies, inactivating enzymes, and

receptor antagonists have confirmed these secondary nonprotein mediators to be intimately involved in the pathophysiology of endotoxicosis.

4. Finally, vascular cells such as endothelial or smooth muscle cells have the capacity upon stimulation by LPS to produce proteinaceous mediators such as the cytokines IL-1, IL-6, and IL-8 (Loppnow and Libby 1989, 1990, 1992; Schönbeck et al. 1995). These cells also produce a variety of other mediators, including prostacyclin, nitric oxide, platelet-activating factor, interferons, and colony-stimulating factors (Mantovani and Bussolino 1991; Libby et al. 1991). In addition to mediator release, the expression of adhesion molecules is of great importance in the regulation of inflammatory responses. The expression of adhesion molecules is induced by LPS as well as by IL-1 and TNF (Yu et al. 1986; Doherty et al. 1989). The activation by endotoxin induces expression of adhesion molecules on cultured vascular endothelial cells as potently as IL-1 (Schönbeck et al. 1994).

3.3 Cellular LPS Receptors

Some 2 decades ago the association of LPS with host cells resulting in their activation was believed to be based on an interdigitation of the lipid A acyl residues into the membrane of cellular targets (Kabir et al. 1978). Thus a rather nonspecific mechanism was thought to cause the initiation of endotoxic events. As a result of the pioneering work of Morrison, Wright, Ulevitch, Tobias, and others this view has since changed dramatically, and today different types of binding proteins and receptors are known to be involved in the specific recognition of LPS by the host.

Some of these cellular structures, such as the scavenger receptor and the CD11b/CD 18 receptors, mediate binding, uptake, and detoxification of LPS and thus are involved in early steps of host defense (Wright et al. 1989). They do not, however, participate in LPS-induced activation of cells (but see Sect. 5). Also other macrophage/monocyte membrane proteins of 18, 25, 38, and 40 kDa have been implicated in LPS binding. The biological significance of these proteins in LPS-mediated cell activation remains to be elucidated (for literature compare Ulevitch 1993; Morrison et al. 1993; Kirikae et al. 1991, 1993b).

This is also true for a 73-kDa protein first described in 1988 (Lei and Morrison 1988a,b). A large body of experimental evidence underlined the importance of the 73-kDa protein in LPS binding (Morrison 1989; Morrison et al. 1993). A recent study, however, reported this 73-kDa protein to be identical to cell surface associated albumin (Dziarski 1994). This study also showed that cell-bound albumin is not required for cell-activation, thus questioning the significance of the 73-kDa protein in endotoxicity.

The only known structure present on the surface of endotoxin-responsive cells which is unequivocally important for both binding and LPS-induced cellular activation is the CD14 molecule. This glycoprotein, the significance of which was recognized in 1990, is therefore dealt with in some detail below (Wright et al. 1990; Lee et al. 1992; Schütt et al. 1988). Its soluble form, sCD14, is also capable

of binding to LPS. It may inhibit LPS activity (Schütt et al. 1992), but interestingly LPS/sCD14 complexes are capable of inducing biological responses in certain CD14-negative cells such as endothelial cells (Frey et al. 1992; Pugin et al. 1993; Arditi et al. 1993) and smooth muscle cells (Loppnow et al. 1995).

The following sections discuss in more detail the humoral LPS-binding protein LBP and the membrane-associated LPS-binding protein CD14, together with its soluble form.

4 Lipopolysaccharide-Binding Protein

LBP is synthesized in hepatocytes as a glycosylated 58-kDa protein which is constitutively secreted into the blood stream (Schumann et al. 1990; Schumann 1992; Tobias and Ulevitch 1993). The concentration of LBP in normal serum is approximately 14–22 μg/ml with maximum of up to 200 μg/ml in acute-phase serum 24 h after induction (Tobias and Ulevitch 1993; Gallay et al. 1994a). LBP was first purified from rabbit and human plasma and more recently from murine sources (Tobias et al. 1986; Schumann 1992; Tobias and Ulevitch 1993; Gallay et al. 1993a,b, 1994a). Rabbit and human LBPs have been sequenced, and 69% homology was revealed between the two species (Schumann et al. 1990). The domain responsible for LPS binding is located in the N-terminal region. It is characterized by an accumulation of positively charged amino acids and expresses hydrophobic properties. LBP shares 44% sequence homology with human BPI, the lipid A binding region of which is also located in the N-terminal region (Schumann et al. 1990; Little et al. 1994). A putative LPS-binding domain, characterized by the presence of positively charged amino acids and located in the proximity of a hydrophobic domain is also present in the structurally well-characterized endotoxin-neutralizing protein (ENP) naturally present in the hemolymph of the horseshoe crab *Limulus polyphemus* (Hoess et al. 1993). It is tempting to speculate that ENP, BPI, and LBP adopt a similar conformation exposing cationic and neighboring hydrophobic LPS-binding sites involved in binding of lipid A. However, BPI and ENP do not (as LBP) augment but rather inhibit LPS bioactivity. These different properties of the three LPS-binding proteins appears to depend on structural features of the carboxy terminus.

The only known biological activity of LBP is its capacity to bind to LPS with high affinity (10^{-9} *M*; Tobias and Ulevitch 1993) and to augment LPS bioactivity. The well-known LPS activity enhancing effect of serum in biological systems is at least in part due to the presence and action of LBP. Thus the LPS-induced TNF production and the expression of TNF mRNA is strongly enhanced by LBP (Ulevitch et al. 1990). Also, LPS-induced responses of PMN can be greatly augmented by the addition of LBP (Vosbeck et al. 1990). On the other hand, neither LPS-induced B-cell activation nor LPS effects caused by large doses of LPS are enhanced by LBP.

The biological activity of LBP is therefore based on its capacity of associating with LPS and thereby augmenting the biological activity of minute amounts of LPS. Most likely LBP associates with LPS or lipid A in molar stoichiometry, and it is possible that LBP singles out LPS monomers from the three-dimensional aggregates described (see Sect. 2.4). LBP may thus be regarded as a biological amplifier which enables the host organism to detect small amounts of LPS that signal the invasion of gram-negative bacteria, i.e., infection. The host organism therefore uses LPS/LBP complexes to activate its defense system to deal appropriately with the invading micro-organism.

The mechanism by which LBP augments LPS activity has been elucidated in great detail in recent years. It is based on the ability of LBP to transfer and deliver LPS molecules to a cellular binding site, i.e., the CD14 protein (Hailman et al. 1994). It should be mentioned, however, that according to a recent study LBP catalytically transfers LPS not only to CD14 but also to HDL. Kinetic experiments involving LPS, LBP, and HDL show that initially the augmenting effect of LBP predominates whereas with longer incubation times LBP mediates diffusion of LPS into HDL, thus catalyzing endotoxin neutralization (Wurfel et al. 1994).

5 The CD14 Molecule

The membrane-bound CD14 molecule (mCD14) constitutes a 53-kDa glycoprotein present on the surface of myeloid cells including monocytes and macrophages (Wright et al. 1990; Ulevitch 1993; Ziegler-Heitbrock and Ulevitch 1993). It is embedded in the plasma membrane via a glycerophosphatidyl inositol (GPI) anchor (Haziot et al. 1988), a property shared by other cell surface molecules involved in cellular responses such as CD24, Thy-1, and CD58/LFA-3 (Robinson 1991).

The mCD14 molecule has been implicated as playing a role in cellular adhesion (Beekhuizen et al. 1991, 1993). Its particular significance as a primary LPS receptor was recognized only 5 years ago (Wright et al. 1990). It was then observed that CD14 serves as an LPS-binding site provided LPS has been allowed to complex to LBP (Schumann et al. 1990; Wright et al. 1990; Ulevitch 1993). LPS binding takes place via the lipid A component and is of high affinity ($K_p = 3 \times 10^{-8}$ *M*) (Kirkland et al. 1993) , and the binding domain of CD14 has been defined to reside between amino acids 57 and 64 (Juan et al. 1995; Viriyakosol and Kirkland 1995). Certain (endotoxin-free) anti-CD14 monoclonal antibodies (mAbs) have been found to mimic LPS effects in monocytes, such as the formation of reduced oxygen species and cytokines (Lauener et al. 1990). Other anti-CD14 mAbs (3C10, 60b) inhibit LPS/LBP complex induced TNF production (Wright et al. 1990) and protein tyrosine phosphorylation (Weinstein et al. 1991; see also Sect. 6). Further, certain cell types transfected with CD14 become highly reactive to LPS/LBP complexes (Lee et al. 1992).

CD14 is not a transmembrane molecule, but it is linked to the cell surface via a GPI anchor (HAZIOT et al. 1988). As this type of membrane anchor does not allow direct signal transduction, the mechanism of LPS/LBP-induced and CD14-mediated cell activation is presently not understood. Several observations bearing on this problem are mentioned below.

Monocytes of patients suffering from paroxysomal nocturnal hemoglobinuremia are unable to form a GPI anchor and therefore lack membrane-bound CD14. Nevertheless, such monocytes can be activated by LPS (with or without LBP) to form cytokines (COUTURIER et al. 1992; SCHÜTT and SCHUMANN 1993). Also, vascular cells lacking CD14 mRNA and CD14-protein respond to LPS (LOPPNOW et al. 1995). This shows that a CD14-independent pathway must exist involving another, perhaps membrane-associated receptor. One possible model therefore suggests that LPS is guided to cells by LBP, where it first associates with CD14 which subsequently interacts with or presents LPS in monomeric form to a second, so far unknown receptor which can transduce an intracellular signal. This scenario is schematically shown in Fig. 5.

CD11c/CD18 has very recently been demonstrated to function as a transmembrane signaling receptor for endotoxin which, however, appears to be operative in the absence of CD14 (INGALLS and GOLENBOCK 1995). In looking for a CD14-dependent functional LPS receptor we used a ligand blotting assay to investigate the binding of LPS to membrane proteins of the human monocytic cell line Mono-Mac-6. Among membrane proteins an 80-kDa protein was identified which binds LPS or free lipid A only in the presence of serum (SCHLETTER et al. 1995). Subsequent experiments identified the serum factors mediating binding of lipid A to the 80-kDa membrane protein as sCD14 and LBP. Thus the 80-kDa protein fulfills an important prerequisite of a putative signal transducting molecule: it recognizes LPS or lipid A only in the context of LBP/CD14. The 80-kDa membrane protein is also present in membrane preparations of human peripheral blood monocytes and endothelial cells.

Several investigators have reported CD14-independent LPS binding at high (μg/ml range) LPS concentrations (COUTURIER et al. 1992). In this case LPS may interact directly with the unknown second receptor; this latter pathway, however, requires larger amounts of LPS for cell activation. Furthermore, as with other GPI-linked surface molecules, CD14 is internalized after binding of the ligand. It is possible that this event constitutes an important step in cell activation, perhaps involving a cytosolic LPS receptor.

CD14 also exists in soluble form (sCD14). As such it is present in the circulation, at a concentration of 2–6 μg/ml (or 5×10^{-8} *M*; FREY et al. 1992). In normal serum several types of sCD14 (48-, 53-, 55-kDa) are present which either result from shedding of membrane-bound CD14 or from cellular production of GPI-free CD14 forms (BAŽIL and STROMINGER 1991; DURIEUX et al. 1994). It has recently been demonstrated that sCD14 is capable of interacting directly with LPS (HAILMAN et al. 1994). In this case LBP seems to play a catalytic role in facilitating the association of LPS with sCD14 without participating in complex formation (TOBIAS et al. 1995). The sCD14/LPS complex is capable of binding to

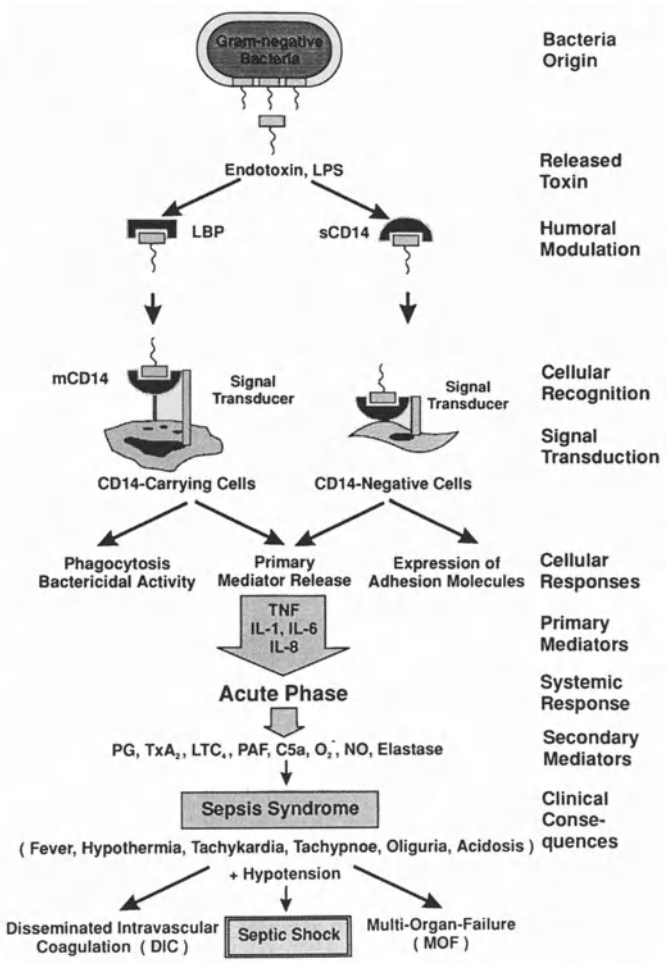


Fig. 5. Intracellular pathways involved in endotoxin-induced signal transduction in monocytes

CD14-negative cells such as endothelial cells and of activating these to produce cytokines (Frey et al. 1992; Pugin et al. 1993; Arditi et al. 1993; Haziot et al. 1993). Endothelial cells apparently possess a receptor for sCD14/LPS complexes, the nature of which awaits further characterization. It is tempting to speculate that the recently discovered 80-kDa protein (Schletter et al. 1995) which recognizes LPS in the presence of sCD14 and LBP represents this cellular receptor molecule.

Recently it has been shown that both untreated and activated monocytes shed CD14-bearing microparticles into plasma. It remains to be elucidated whether microparticle-associated CD14 participates in LPS transport and cell stimulation (Satta et al. 1994).

It should be mentioned that activation of monocytes by certain other bacterial immunomodulators such as peptidoglycan, arabinogalactan, and lipoarabinomannan (Weidemann et al. 1994; Heumann et al. 1994; Pugin et al. 1994) also proceeds via mCD14. The structural requirements of these glycoconjugates for binding to mCD14, cell activation, and cytokine secretion are presently not understood. On the other hand, monocyte activation by glycosphingolipids of *Sphingomonas paucimobilis* which share chemical and physical features with LPS (Kawahara et al. 1991) is not dependent on mCD14 (Krziwon et al. 1995).

6 LPS-Induced Signal Transduction

The signal transduction events that follow binding of LPS to target cells remain to be fully defined. Several studies suggest that treatment of macrophages with LPS results in the activation of phospholipase C as determined by the formation of inositol tris-phosphate ($InsP_3$). The response is modest and occurs 1–20 min after activation (Prpic et al. 1987; Chang et al. 1990). According to Hurme et al. (1992) and Drysdale et al. (1987) this slow turnover of $InsP_3$ is not accompanied by a rise in $[Ca^{2+}]_i$. Thus, it remains unclear whether $InsP_3$ formation constitutes an obligatory cellular event in LPS-triggered activation (Chang et al. 1990). In addition, it has been reported that responses to LPS may involve a pertussis toxin sensitive G protein (Jackway and De Franco 1986; Dziarski 1989) and activation of protein kinase C (PKC) (Liu et al. 1994; Novotney et al. 1991; Bakouche et al. 1992; Hurme and Serkkola 1991). There may be two alternative pathways by which LPS activates PKC (Chen et al. 1986). Activation occurs either via diacylglycerol and $InsP_3$ or via calpain-catalyzed cleavage of PKC, yielding the 40-kDa catalytic domain (PKM). The cleavage of PKC to PKM by calpain frees it from the regulatory constraint and releases PKM into the cytosol, where it can phosphorylate a distinct set of proteins.

LPS has been shown to induce changes in protein phosphorylation in murine macrophages, including phosphorylation of a 65-kDa protein which has been purified and characterized (Shinomiya et al. 1991). An early event triggered in monocytes/macrophages by LPS is tyrosine phosphorylation of a series of 38- to 45-kDa proteins known as mitogen-activated protein (MAP) kinases (Weinstein et al. 1992, 1993; Han et al. 1993; Liu et al. 1994; Lee et al. 1994). The p38 MAP kinase appears to be distinct from MAPK1 (44 kDa) and MAPK2 (40–42 kDa) showing sequence homologies to the products of the *Saccharomyces cerevisiae* osmosensing gene HOG1 (Han et al. 1994). Furthermore, phosphorylation of p38 occurs via the human MAP kinase kinases MKK3 and MKK4, whereas MAPK1 and MAPK2 are activated by MAP kinase kinases MEK1 and MEK2 (Derijard et al. 1994). Phosphorylation of these serine/threonine protein kinases contributes to their increased enzymatic activity. Proteins such as 90-kDa ribosomal S6 protein kinase (Chung et al. 1991; Sturgill et al. 1988) and C-jun transcription

factor (PULVERER et al. 1991) have been found to be efficient in vitro substrates of MAP kinases, and this may be indicative of MAP kinase function in vivo.

Recently we have found that endotoxin causes in human monocytes phosphorylation of two cytosolic proteins of 36 and 38 kDa which are distinct from MAP kinases (HEINE et al. 1995). Associated with the LPS-induced activation of macrophages is the change in the ADP-ribosylation state of a 33-kDa cytosolic protein (HAUSCHILDT et al. 1994). As with phosphorylation, ADP-ribosylation constitutes a covalent modification by which cells regulate protein functions. Whether this reversible protein modification is related to the functional activation of macrophages is not yet clear. We have, however, demonstrated that suppression of ADP-ribosylation inhibits phosphorylation of the 36- and 38-kDa proteins and at the same time the formation of TNF and IL-6 mRNA as well as protein production (HEINE et al. 1995). This indicates that ADP-ribosylation is involved in LPS-induced alteration of the phosphorylation state of the two proteins p36 and p38, and that the latter are involved in processes leading to monocytic cytokine formation.

Treatment of cells with LPS leads to induction of proto-oncogenes such as the tyrosine kinase, proto-oncogenes *hck* and *lyn*, and the transcription factors *myc* and *fos* (INTRONA et al. 1986; ZIEGLER et al. 1988; MÜLLER et al. 1993; STEFANOVA et al. 1994). LPS also activates nuclear factor κB and c-Rel/p50, which may activate genes in vivo by binding the κB elements located in the enhancer region (CORDLE et al. 1993).

7 Regulation of LPS-Induced Cytokine-Production in Macrophages by Lipoxygenase products

Several lines of evidence suggest that lipoxygenases are involved in the regulation of endotoxic activities (Fig. 5). For example, lipoxygenase inhibitors protect from endotoxicity in experimental models (KEPPLER et al. 1987; TIEGS and WENDEL 1988) and suppress the formation of TNF as determined in serum of LPS-treated mice (SCHADE et al. 1989b). In vitro it has been found that inhibitors of lipoxygenase (ETYA, BW 755C) but not inhibitors of cyclo-oxygenase (indomethacin, aspirin) interfere with LPS- or *Staphylococcus aureus*-induced production of IL-1 by human peripheral monocytes (DINARELLO et al. 1984). These findings suggested that product of the lipoxygenase pathway promotes the synthesis of IL-1. The compound in question, however, was not characterized. In subsequent studies other investigators analyzed various lipoxygenase products for their capacity to modulate IL-1 induction in macrophages. The results reported by different groups are at variance. For example, LTB_4 and LTD_4 have been described as stimulating the synthesis of IL-1 in human monocytes and mouse macrophages (CHENSUE and KUNKEL 1985; ROLA-PLESZCZYNSKI and LEMAIRE

1985). However, this has not been confirmed by others (BRANDWEIN 1986) who treated human monocytes with LTB_4 and failed to determine any activity on IL-1 synthesis. Therefore the question as to the nature of the lipoxygenase product involved in IL-1 synthesis remained open.

7.1 13-Hydroxylinoleic Acid and Macrophage Activation

In our laboratory it was found that lipoxygenase inhibitors also block LPS-induced TNF formation in murine macrophage cultures (SCHADE et al. 1989a,b). These results have been confirmed with other inhibitors and in different macrophage populations (BADGER et al. 1989; MOHRI et al. 1990). In analyses aiming at identifying the presumable product of lipoxygenase activity, LTB_4, LTC_4, LTD_4, or LTE_4 could not be detected in the supernatants of mouse macrophages stimulated with LPS. However, when LPS-activated mouse macrophages were hydrolyzed and extracts of the hydrolysates were analyzed, the lipoxygenase product 13-hydroxyoctadecadienoic acid (13-HODD), which is derived from linoleic acid, was identified (SCHADE et al. 1987). Analysis of the absolute configuration showed that the product is (*S*)-13-HODD, which confirms its enzymatic origin (SCHADE et al. 1987). 13-HODD was found to be enriched in LPS-activated cells. Furthermore, the effects of site-specific lipoxygenase inhibitors in comparison to less specific inhibitors were tested on LPS-induced TNF formation, zymosan-induced synthesis of LTC_4, and synthesis of 13-HODD in mouse peritoneal macrophages (SCHADE et al. 1991). The inhibitors TZI-41127 and VZ 65 reduced both TNF formation and synthesis of LTC_4 and 13-HODD (although with both substances there is a preference for inhibition of LTC_4 vs. 13-HODD). As expected, the 5-lipoxygenase inhibitors MK 886 and CGS 8515 inhibited the synthesis of LTC_4, whereas the formation of 13-HODD was unaffected. Most interestingly, neither 5-lipoxygenase inhibitors had any effect on the formation of TNF.

These results indicate a good quantitative correlation between TNF formation and the inhibition of a 15-lipoxygenase (formation of 13-HODD), but not between TNF formation and inhibition of a 5-lipoxygenase (synthesis of LTC_4). These findings are in accord with the notion that 13-HODD is produced increasingly during LPS activation, a process required for LPS-induced TNF formation (SCHADE et al. 1987, 1989b). In agreement with these results, MK 886 and other specific 5-lipoxygenase inhibitors do not impede IL-1 production in mouse macrophages stimulated with LPS (PARKAR et al. 1990).

The influence of authentic 13-HODD and other lipoxygenase products on LPS mitogenicity has recently been tested in murine spleen cell cultures (ELEKES et al. 1993). Lipoxygenase inhibitors suppressed the proliferation of spleen cells and in parallel TNF synthesis induced by LPS. It was further found that 13-HODD, unlike other lipoxygenase products, is able to counteract the inhibitors and to restore the mitogenic response. TNF was shown to have essential functions in LPS mitogenicity in murine spleen cells (JAKOBS and SCHADE 1994). It

is therefore possible that the inhibitory effect of lipoxygenase inhibitors and the augmenting effect of 13-HODD on mitogenicity is due to its action on TNF synthesis.

Summarizing these data, it appears that the linoleic acid metabolite 13-HODD plays an important role in endotoxin-induced TNF production in macrophages. Its position in the LPS-triggered signaling pathway and the molecular basis of 13-HODD action is presently under investigation.

8 Lipid A Determinants Involved in Binding to and Activation of Cells

The initial key event in endotoxin action is the interaction of the lipid A component of LPS with defined humoral and, notably, cellular recognition molecules. It is reasonable to assume that at least two parameters determine the result of the interaction between lipid A and host cells, i.e., between the endotoxic conformation of individual lipid A molecules and the host recognition system resulting in the activation of monocytes and the production of bioactive mediators. These parameters are the capacity of lipid A specifically to *bind* to the receptor and subsequently the ability of lipid A to *activate* the cell by a receptor-mediated mechanism.

Recent studies in our laboratory have shown that the hydrophilic lipid A backbone (bis-phosphorylated D-GlcN disaccharide together with two fatty acids) mediates the specific *binding* of lipid A or LPS to the cellular receptor (Kirikae et al. 1993a). On the other hand, it is the number, nature, and distribution of fatty acids, i.e., the acylation pattern which determines the *activation* capacity and mediator-inducing capacity of lipid A. These investigations also revealed that the binding of lipid A is a necessary but not sufficient event for the activation of cells. It appears that an additional receptor-mediated triggering event is required for which the acylation pattern of lipid A plays a most critical role, and which provides the signal for cellular stimulation (Rietschel et al. 1993).

9 Inhibition of Lipid A Binding to Cells and Cellular Activation

The separation of the "binding" and "activation" events suggested the possibility that endotoxically inactive LPS or partial structures may bind to target cells without activating them, thus representing possible inhibitors of LPS-induced effects.

Indeed, we and later others have demonstrated that tetraacyl lipid A (Fig. 6A), an intermediate in lipid A biosynthesis (precursor Ia) in its chemically synthesized form (compound 406), inhibits, in a dose-dependent manner the monokine production by human monocytes induced by LPS but not by *Staphylococcus epidermidis*, lipoprotein, or bacille Calmette-Guérin (Loppnow et al. 1989; Wang et al. 1991; Flad et al. 1993).These results show that the inhibitory capacity of compound 406 is highly specific for the LPS-cell interaction, and that it is not due to suppression of monokine release. Rather, as has been shown by northern blot analysis, compound 406 suppresses the LPS-induced formation of mRNA for TNF and IL-1, indicating that monokine production is inhibited before or at the level of transcription (Flad et al. 1993). Similar results have been reported for the isostructural natural precursor Ia (also termed lipid IVa) isolated from *S. enterica* sv.typhimurium (Lynn and Golenbock 1992; Kovach et al. 1990) and for nontoxic lipid A species of *R.sphaeroides* (Qureshi et al. 1991) and *R. capsulatus* (Fig. 6B; Loppnow et al. 1990, 1993). Compound 406 also inhibits LPS-but not IL-1-induced expression of the adhesion molecule ICAM-1(Schönbeck et al. 1994) and the (serum-dependent) formation of IL-6 in endothelial cells (Loppnow et al. 1993). These observations are in accordance with the previous demonstration that partially deacylated LPS is capable of inhibiting LPS-induced adherence of granulocytes to vascular endothelium (Pohlmann et al. 1987) and also with the concept that the observed inhibition is LPS specific. Very recently an LPS antagonist, the structure of which is based on that of *R. capsulatus* lipid A, was synthesized (compound E5531). This molecule protects mice from endotoxin-induced lethality and, when administered together with an antibiotic, from lethal *E. coli* induced peritonitis (Christ et al. 1995).

The mechanism of the inhibitory action of lipid A partial structures such as compound 406 has been studied in various cells types (Ulmer et al. 1992; Lynn and Golenbock 1992; Kitchens et al. 1992; Kirikae et al. 1993a; Flad et al. 1993; Heine et al. 1994; Kitchens and Munford 1995). Our and other results suggest that in the case of J774.1 cells and peripheral human monocytes inhibition is based on competitive binding to a specific LBP. This concept is supported by the finding that the degree of inhibition depends on the dose of the competitor, and that inhibition is overcome by higher LPS doses. A true competitive, i.e., antagonistic, mechanism is also suggested by the fact that if the LPS-binding data obtained in the presence or absence of an inhibitor are plotted in a double reciprocal manner (according to Lineweaver and Burk), both regression lines cross the y-axis (Heine et al. 1994). These and other results lead us to conclude that antagonistically active compounds such as precursor Ia and lipid A of *R. sphaeroides* (Kirikae et al. 1994) or compounds E5531 (Christ et al. 1995) bind to the same cellular (or humoral) recognition site as lipid A and LPS, yet lack the capability to functionally engage the receptor. Antagonistic preparations thus block the receptor effector binding site, subsequently denying access of LPS and thereby inhibiting the activation of cells.

The nature of the cellular binding molecule involved remains to be established. There is ample evidence, however, that the CD14 molecule plays a crucial

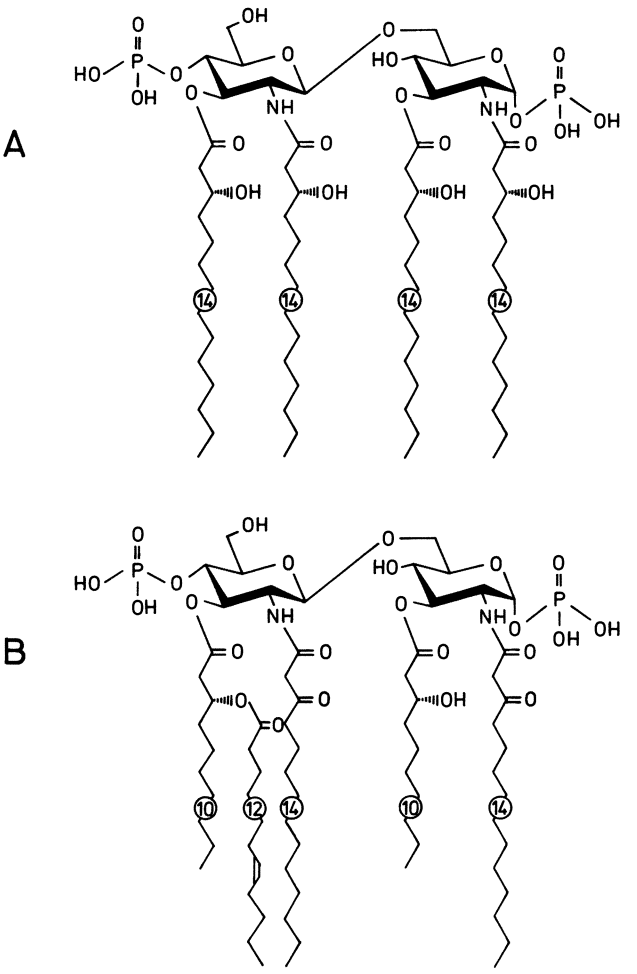


Fig. 6A,B. Chemical structure of synthetic compound 406. **A** Precursor Ia. **B** Nontoxic lipid A of *Rhodobacter capsulatus.* (From MAYER et al. 1990; ZÄHRINGER et al. 1994)

role in the antagonistic inhibitory pathway described. For example, CD14 has been shown to be capable of interacting with compound 406 (HAILMAN et al. 1994), and the antagonistic activity of compound 406 is inhibited by anti-CD14 antibodies. A CD14-dependent but nonantagonistic mechanism has been proposed in a system employing THP-1 cells (KITCHENS et al. 1992). This study showed that compound 406 interacts with CD14 without, as expected, stimulating cells, yet still allowing LPS binding to CD14. It was therefore argued that

compound 406 exerts its biosuppressive activity in THP-1 cells in a non-antagonistic fashion. One could speculate that compound 406 binds to a second structure located in close topographical neighborhood to CD14 (such as the signal transducer), thereby inhibiting the LPS-CD14 initiated signal transduction. It is also possible that the association of precursor Ia with CD 14 or an associated molecule initiates a suppressive signal, which actively counteracts the activation signal generated by the interaction of LPS with CD14 (KITCHENS and MUNFORD 1995).

10 The Pathophysiological Cascade of Events Leading to Gram-Negative Septic Shock

Considering the pleiotropic action of endotoxin and the multiplicity of endogenous mediators produced, it is difficult to define the exact mechanisms which are causally involved in the phenomenon of endotoxin-induced shock. Nevertheless, some principal reactions can be described which take place in vivo after injection of endotoxin or its release from bacteria (see Fig. 5). That portion of LPS which is not detoxified by humoral (e.g., BPI or HDL) or cellular (e.g., PMN) host components interacts with LBP, septin, or sCD14 and subsequently activates target cells to produce and release endogenous mediators or to express adhesion molecules. The primary target cells include monocytes/macrophages, endothelial cells, granulocytes, and lymphocytes. Prominent mediators such as TNF, IL-1, IL-6, and IL-8 are produced which are capable of activating susceptible (i.e., cytokine receptor carrying) cells to produce lipid mediators (including platelet-activating factor, LTs, and prostaglandins), reduced oxygen species, nitric oxide, and proteases such as elastase and collagenase (JOCHUM et al. 1992). If this inflammatory reaction cascade is limited in its capacity to produce mediators, and if it is confined to a local area, it may be highly beneficial as it helps to activate the defense system and to destroy invading micro-organisms (MASTROENI et al. 1993). However, overproduction of these mediators and release into the circulation may result in a systematic inflammatory reaction. Low mediator concentrations may also become harmful if the host organism is in a hyperreactive state to LPS. Hyperreactivity to endotoxin may be caused by exotoxins, chronic infection, and growing tumors. Important endogenously formed molecules contributing to sensitization to LPS have been identified as migration-inhibitory factor, interferons β and α and γ, and granulocyte-macrophage colony-stimulating factor (GALANOS and FREUDENBERG 1993; BENDER et al. 1993; BERNHAGEN et al. 1994; CARR et al. 1994; TIEGS et al. 1994).

Endogenous mediators subsequently stimulate receptor-carrying cells and organs, resulting in secondary and clinically relevant reactions. For example, TNF, IL-1, and IL-6 act as endogenous pyrogens on the hypothalamus to cause

fever (or hypothermia). The hypophysis releases ACTH, cortisol, and migration-inhibitory factor, and the liver responds to IL-6 with the production of acute-phase proteins (Baumann and Gauldie 1994; Steel and Whitehead 1994). Some acute-phase proteins dampen the LPS-dependent inflammatory reaction while others, such as LBP, intensify it. Blood clotting factors are consumed, and the complement system is activated, producing other potent cytomodulators. These factors as well as TNF cause leukocytes to adhere to endothelial cells, resulting in leukopenia, blood vessel injury, and capillary leakage. On the other hand, endothelial cells are stimulated to produce nitric oxide, which causes vasodilation followed by hypotension and increased cardiac output. T-cells also take part in this scenario. They release colony-stimulating factor and interferon γ, the former acting on the bone marrow causing leukocytosis and the latter activating macrophages, which in turn overproduce toxic mediators.

These effects clearly resemble the clinical picture of gram-negative septic shock, being characterized by fever, chills, leukopenia followed by leukocytosis, disseminated intravascular coagulation, hemorrhage, hypotension, tachycardia, and metabolic acidosis leading to dysfunction or failure of vital organs such as lung, kidney, liver, heart, gastrointestinal tract, and central nervous system (Glauser et al. 1991; Parillo 1993). Due to this clear association between LPS and sepsis endotoxin is implicated as a main factor involved in the pathological manifestations and consequences of gram-negative bacterial sepsis.

11 Anti-Endotoxin Antibodies

Gram-negative sepsis remains associated with high morbidity and mortality, the mortality rate being in the order of 40%–60%. These figures have not changed over the past two decades despite the development of new antibiotics and the use of improved and highly sophisticated intensive-care measures (Bone et al., 1987, 1989; Centers for Disease Control 1990; Young and Glauser 1991). The fact that in the United States alone an estimated 100 000 deaths annually result from septic shock demands intensified efforts to devise new ways in the fight against the life-threatening sepsis syndrome. Indeed, the failure of antibiotics to reduce gram-negative septic mortality below 40% is probably related in part to their ability to liberate endotoxin from infecting microbes. Experimental studies indicate that the mechanisms underlying gram-negative bacterial lethality can be altered by antibiotics, such that the resulting abrupt and sustained increases in endotoxaemia contribute even more importantly to the lethal outcome (Johnston and Greisman 1984).

New therapeutic strategies may focus on the initial stimulus (endotoxin); enhancing factors (LBP, sCD14, septin); receptors of LPS target cells (CD14); LPS-induced signal transduction leading to production of cytokines; TNF, IL-1, and IL-6; cytokine receptors of cytokine target cells; cytokine-induced signal

transduction; or on other parameters. Indeed, various approaches have recently been developed for interfering with distinct phases of the septicemic process and supplementing the (obligatory) administration of antibodies. Many of these approaches have proven effective in experimentally induced endotoxemia or sepsis; however, clinically no benefit has been observed so far (NATANSON et al. 1994).

Of particular interest are newly discovered anti-endotoxin preventive or therapeutic agents such as mAbs to endotoxin and other endotoxin-neutralizing agents. Here we deal only with immunological approaches against LPS, i.e., anti-LPS antibodies (for an update concerning other therapeutic approaches see POLLACK 1990; see also M. POLLACK and C.A. OHI, this volume). The targeting of endotoxins by immunoglobulin has several potential advantages over other approaches. These include the blockade of a very early step in the cascade leading to gram-negative sepsis, particular properties of antibodies such as long half-life, low immunogenicity, their capacity to activate complement and to interact with Fc receptors, the possibility to produce mAbs antibodies with defined specificity and of high affinity, and the choice of defined antibody classes and isotypes.

In principle, all segments of the LPS molecule are immunogenic and antigenic. Polyclonal antisera and mAbs have been prepared against the O-specific chain, the core and lipid A, and their properties have been evaluated in vitro and in vivo.

11.1 Anti-O-Chain Antibodies

Polyclonal antibodies (SAXEN et al. 1986) and mAbs (SOLWELL et al. 1984; KIRKLAND and ZIEGLER 1984; BAUMGARTNER et al. 1990) directed against determinants of the O-specific chain are highly protective in endotoxin and infection models. However, in view of the wide structural variability of the O-specific chain of those pathogenic gram-negative bacteria that are significant in clinical settings (*E. coli, Klebsiella, Pseudomonas*) and the restricted usefulness of such antisera or antibodies directed only against the homologous LPS or bacterial serotype, O-specific immunoglobulins are unlikely to gain broad use. They appear, however, to be useful in the form of multispecific, i.e., polyclonal, immunoglobulin preparations (SCHEDEL et al. 1991; DOMINIONI et al. 1991) and in exceptional and defined cases such as the prevention of *Pseudomonas* infection associated with cystic fibrosis (CRYZ et al. 1994).

11.2 Anti-Lipid A Antibodies

Lipid A has been considered a logical target for the generation of protective antibodies for two reasons. (a) Lipid A of various pathogenic gram-negative bacteria is structurally highly conserved (RIETSCHEL et al. 1994; ZÄHRINGER et al. 1994). It can therefore be expected that anti-lipid A antibodies would cross-react

with LPS of different gram-negative bacteria. (b) Lipid A constitutes the toxic principle of LPS (GALANOS et al. 1985). Therefore such antibodies could be expected to neutralize bioactivity of lipid A and endotoxin.

The immunogenicity of lipid A remained unknown for a long time because lipid A antibodies are not engendered upon immunization of experimental animals with S- or R-form bacteria or the corresponding LPS. In 1972, however, it was shown that lipid A induces specific antibodies, provided lipid A is administered in a form devoid of the LPS polysaccharide component and if complexed to a suitable carrier (GALANOS et al. 1984). Polyclonal lipid A antisera were shown to be cross-reactive with polysaccharide-free lipid A of various bacterial origin (GALANOS et al. 1984). Such antisera, however, lacked antiendotoxic and anti-infectious properties unless very peculiar in vivo assay conditions were used (RIETSCHEL and GALANOS 1977). When monoclonal anti-lipid A antibodies were available (DUNN et al. 1986; BOGARD et al. 1987), their serological properties were studied and their epitopes characterized using various partial structures of LPS, in particular, synthetic lipid A (BRADE et al. 1988). The most important results of these studies can be summarized as follows: (a) The epitopes recognized by various monoclonal lipid A antibodies reside without exception in the hydrophilic backbone region (phosphorylated disaccharide; KUHN et al. 1992; BRADE et al. 1993). (b) Fatty acids modulate the exposure of these epitopes but are not part of the determinants (BRADE et al. 1987b). (c) Lipid A mAbs cross-react with a large variety of free lipid A (i.e., polysaccharide-deprived lipid A) of distinct bacterial origin (GALANOS et al. 1984; BRADE et al. 1988). (d) Lipid A mAbs, however, do not cross-react with LPS, i.e., with lipid A carrying the polysaccharide component (GALANOS et al. 1984).

This lacking cross-reactivity is not fully understood at the present time. When present as part of LPS, the lipid A epitopes may not be expressed, suggesting that lipid A represents a neoantigen due to the exposure of determinants not present in LPS (such as the primary hydroxyl group of GlcN II which could constitute part of such an epitope). Our recent finding that synthetic lipid A preparations harboring a free primary hydroxyl group at GlcN II do cross-react with lipid A antibodies, whereas derivatives being methylated at this hydroxyl-group do not react, favors this interpretation (BRADE et al. unpublished results). On the other hand, Kdo and other LPS constituents may sterically hinder the binding of antibodies to lipid A.

Because of these properties lipid A antibodies are highly unlikely to express protective activity in endotoxic and septicemic states. Nevertheless, two types of monoclonal antibodies [Centoxin (HA-1A)] and [XMMEN-OE5 (E5)] have been described to be reactive with lipid A in the context of LPS. They have been evaluated intensively in vitro and for protective activity in experimental animals and septic patients in vivo (GREENMAN et al. 1991; ZIEGLER et al. 1991). Both antibodies are of the IgM class and are likely to be derived from CD5-positive B-cells. The lipid A epitopes recognized by either antibody are not identified (FUJIHARA et al. 1993). Cross-reactivity with LPS has been demonstrated only under very favorable conditions, i.e., with very high coating density of LPS in enzyme-

linked immunosorbent assay. Furthermore, the reactivity of mAb HA-1A is not restricted to lipid A or LPS, as it also binds to other amphiphilic compounds including gentabioseoctaacetate and chitobiose (TENG et al. 1985). Recently, mAb HA-1A has been identified as a cold agglutinin (BHAT et al. 1993).

Animal studies suggest that mAbs HA-1A and E5 are protective in endotoxemic and bacteremic models (TENG et al. 1985; YOUNG et al 1989). Other investigations, however, have failed to confirm these results (BAUMGARTNER and GLAUSER 1993). In an *E. coli* sepsis model even lethality-enhancing properties of mAb HA-1A were revealed (QUEZADO et al. 1993). Furthermore, the clinical trials performed in septic patients did not show any significant protective effect of either mAb HA-1A (BAUMGARTNER and GLAUSER 1993) or mAb E5 (WENZEL et al. 1991). It thus appears that lipid A antibodies as obtained by immunization with free lipid A or LPS do not fulfill the promise as an universal immunotherapeutic anti-endotoxic drug (BAUMGARTNER and GLAUSER 1993; WARREN et al. 1993).

11.3 Polyclonal Anti-Core Antibodies

Chemical and genetic investigations performed in the 1960s and 1970s showed that LPS of enterobacterial R mutants lacks the O-specific chain but contains (parts of) the core oligosaccharide and lipid A, thus representing a partial structure of S-form LPS (LÜDERITZ et al. 1982), and further that the constituents of the core oligosaccharide are similar or identical in various gram-negative bacterial serotypes, species, genera, and families. These findings led to the view that (a) the LPS core of various bacteria may harbor common determinants, (b) these determinants are immunogenic in R mutants, (c) they are exposed in S-form LPS and wild-type bacteria, (d) antisera against R-mutant bacteria or their LPS would cross-react with S-form LPS and S-form bacteria, and (e) such R-mutant antisera would cross-protect in endotoxemic and infection models.

Two research groups (BRAUDE and DOUGLAS 1972; MCCABE 1972) followed these ideas, one group (BRAUDE and DOUGLAS 1972) working with an Rc mutant of *E. coli* O111 (termed J5) and the other using mutants of the Re chemotype (strain R595) derived from *S. enterica* sv. *minnesota* (MCCABE 1972). The chemical structure of the Re-mutant LPS (ZÄHRINGER et al. 1985) and of the *E. coli* Rc-mutant LPS (MÜLLER-LOENNIES et al. 1994) is shown in Fig. 7. The Re-mutant LPS consists of an $\alpha 2 \rightarrow 4$ linked Kdo disaccharide which is bound to lipid A via the primary hydroxyl group of GlcN II of the lipid A backbone. The J5 (Rc-mutant) structure contains, in addition, a GlcN-carrying heptose trisaccharide and a terminal Glc residue (MÜLLER-LOENNIES et al 1994). Whereas in some studies Rc (J5) and Re (R595) antisera obtained in rabbits exhibited cross-reactivity and cross-protective activity (ZIEGLER et al. 1973; BRUINS et al. 1977), other studies have not confirmed these serological and biological data (GREISMAN et al. 1978; GREISMAN and JOHNSTON 1988; TRAUTMAN and HAHN 1985). The former studies, however, failed to define the protective epitope or the fine specificity of the

protective antibodies, thus rendering a comparison between these conflicting studies impossible.

We have recently addressed this problem by analyzing the antibody specificities present in polyclonal Re-mutant antisera using defined bacterial or synthetic partial structures as antigens (ROZALSKI et al. 1989). Surprisingly, the abundant antibody specificities were identified as being reactive with the α(2–4)-Kdo-disaccharide together with parts of the lipid A region. These antibodies bind *specifically* to Re-mutant LPS and do *not* cross-react with other R- or S-form LPS. Accordingly, they cannot be expected to provide cross-protection. In Re antisera trace amounts of antibodies were also found which are specific for a terminal α-pyranosidic Kdo residue. Of the latter specificity it is known (in the form of mAbs such as clone 20), that it cross-reacts with other LPS, i.e., those LPS which expose a lateral Kdo residue (BRADE et al. 1987a). In our hands, however, these Kdo-specific, in vitro cross-reactive antibodies do not exhibit protective activity in endotoxin test systems such as pyrogenicity in rabbits (RIETSCHEL et al., unpublished data). A similar situation was encountered with polyclonal antisera raised against Rd chemotypes. Again, the main specificity was Rd-specific and not cross-reactive with other R-mutant or S-form LPS (SWIERZKO et al. 1993, 1994). It is more than likely that the same situation holds true for the Rc chemotype, i.e., *E. coli* J5.

In view of these data it is difficult to understand how Re and Rc antisera, which must be considered as essentially chemotype-specific, should confer cross-protection. In fact in the six clinical trials performed with J5 antisera only two (ZIEGLER et al. 1982; BAUMGARTNER et al. 1983) reported a significant ther-

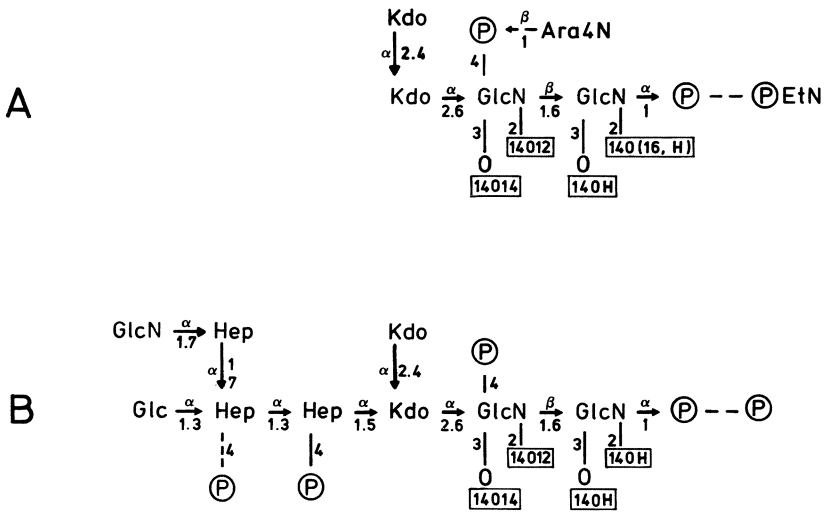


Fig. 7. Chemical structure of the LPS of immunotherapeutically important enterobacterial R mutants., **A** Re mutant of *S.enterica* serovar *minnesota* (strain R595)., **B** Rc mutant of *E.coli* 0111 (strain J5). (From ZÄHRINGER et al. 1985; MÜLLER-LEONNIES et al. 1994)

apeutic efficacy (improved survival). Whether in fact this effect was due to LPS antibodies or to other serum factors was not established. In summary, therefore, serological data render the protective approach of using polyclonal antisera obtained by immunization with R mutants highly questionable, and the vast majority of serological and biological data available do not support it.

11.4 Cross-Reactive and Cross-Protective Monoclonal Anti-Core Antibodies

Only very recently it has been shown that despite the trials and data described above, anti-core antibodies are capable of expressing cross-reactive and cross-protective properties (Di Padova et al. 1993a,b). Using O-chain defective LPS as immunogen and NZB mice, we generated broadly cross-reactive mAbs recognizing the core region of *E. coli*, *S. enterica*, and *Shigella* R- and S-form LPS. These antibodies also exhibit potent anti-endotoxic properties. One of these mAbs (WN1 222-5, IgG2a) was selected for further evaluation in vivo (Di Padova et al. 1993b). It was found to prevent effectively lethality in mice and pyrogenicity in rabbits induced by S-form LPS of different *E. coli* serotypes harboring different core types and *S. enterica* serotypes. The serological properties of mAb WN1 222-5 are quite different from those of an antibody described by Nnalue et al. (1992), as this mAb of IgM class recognizes only *Salmonella* and *E. coli* R2.

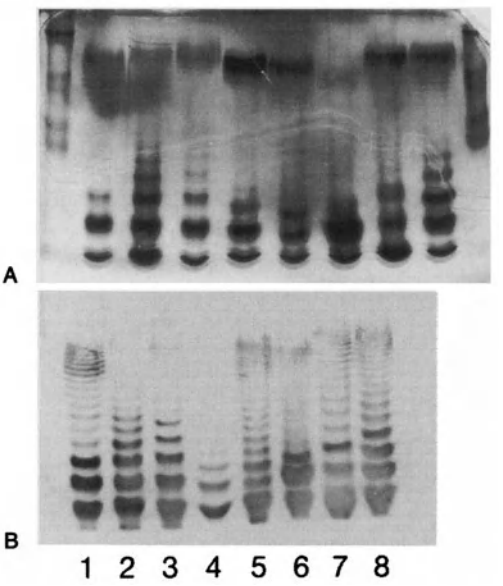


Fig. 8. Gel electrophoresis (**A**) and immunoblot (**B**) with cross-reactive and chimerized anti-LPS monoclonal antibody SDZ 219–800; 1 *E. coli* 0111; 2 *E. coli* 086; 3 *E. coli* 018; 4 *E. coli* 016; 5 *E. coli* 015; 6 *E. coli* 012; 7 *E. coli* 06; 8 *E. coli* 04. (From Di Padova et al. 1995)

To reduce the incidence of possible negative side effects such as the appearance of human anti-mouse antibodies and to increase its half-life, mAb WN1 222-5 was chimerized into a human IgG1 (SDZ 219-800), thus retaining the binding specificity and cross-reactivity of mAb WN1 222-5. Crystallographic analysis of Fab fragments of WN1 222-5 and SDZ 219-800 showed that the two V regions, identical in amino acid sequence, largely overlap (Di Padova et al. 1994). As expected, differences are observed in the constant domains. Using purified LPS and free lipid A in enzyme-linked immunosorbent assay, we showed that mAb SDZ 219-800 recognizes all S-form LPS of *E. coli* and *S. enterica*. Reactivity with the known complete core structures of *E. coli* (R1 to R4 and K-12) and *S. enterica* (Ra) was also evident (Fig. 8). The Rc core, expressed in *E. coli* J5, is the minimal structure to which mAb SDZ 219-800 binds. Some residual binding is observed with the truncated core structure of *S. enterica* sv. *minnesota* RcP^-, but no reactivity with Rd and Re structures of *E. coli* and *S. enterica* sv. *minnesota* or free mono- or bisphosphoryl lipid A was detected. In immunoblots of S-form LPS after electrophoresis in polyacrylamide gels mAb SDZ 219-800 reacts not only with the free R component but also with O-side chain substituted core structures, showing that the epitope recognized is accessible on the LPS molecule independently of the presence or absence of the O-specific side chain. This reactivity could be inhibited with an excess of heterologous LPS. These data demonstrate that the epitope is a lateral segment of the core region which has been shown to be structurally rather conserved among Enterobacteriaceae (Fig. 2).

The mAb SDZ 219-800 cross-reacts with all clinical isolates of *E. coli* and *S. enterica* tested and with other Enterobacteriaceae as well. The mAb shows biological activity in vitro as it efficiently inhibits the LPS-induced secretion of IL-6 by mouse peritoneal cells (Table 1). On a molar basis the antibody is significantly more active than polymyxin B (Bruins et al. 1977). In vivo SDZ 219-800 neutralizes the pyrogenic activity of various enterobacterial S-and R-form LPS and offers protection from the lethal effects of different LPS in D-GalN sensitized mice (Table 2) (Di Padova et al. 1993a, b, 1994).

The fact that mAb SDZ 219-800 exhibits high functional neutralizing capacity provides evidence for the accessibility of its epitope on the LPS molecule both in vitro and in vivo. mAb SDZ 219-800 does not bind to lipid A, the toxic component of the LPS molecule. Its potent anti-endotoxic properties in vitro could be based on an indirect steric effect by inhibiting binding of lipid A to its receptor or an antibody-induced change of the toxic conformation of lipid A. In addition to these factors, the neutralizing activity of this mAb in vivo may be explained by a more efficient elimination of antibody-associated LPS through the Fc receptor or the complement receptor. mAb SDZ 219-800 is therefore different from the so-called anti-lipid A IgM antibodies which have so far been tested clinically.

In conclusion, after many years of intense research, murine and chimeric mAbs of IgG class which are widely cross-reactive, and which recognize protective epitopes of the LPS molecule have been identified. The biological properties of these mAbs show that in order to neutralize endotoxicity neither a

Table 1. Monoclonal anti-LPS core antibody SDZ 219-800 inhibits IL-6 secretion of mouse peritoneal macrophages induced by *E. coli* 0111 LPS

Endotoxin *E. coli* 0111 (n*M*)	IL-6 secreted in culture supernatants in the absence or presence of mAb SDZ 219-800[a]		
	Control	MAb SDZ 219-800	
		0.1 n*M*	1n*M*
	ng/ml		
10	23.376	22.612	20.126
1	14.047	10.427	2.225
0.1	1.041	0.684	0.171
0.01	0.267	0.141	0.142
Control	0.125	0.128	0.224

[a]IL-6 in culture supernatants was determined using the B13-29 hybridoma cell line.

Table 2. Inhibition of LPS-induced lethality by monoclonal anti-LPS antibodies in mice[a]

Monoclonal antibody (1 mg/mouse)	Surviving over treated animals after challenge with endotoxin derived from	
	S. enterica sv Abortus equi (1 ng/mouse)	*E. coli* 016 (2 ng/mouse)
WN1 222-5	6/6	5/6
SDZ 219-800	6/6	5/6
HA-1A	0/6	1/6
Control	0/6	1/6

[a]mAb were administered i.v. 2 h before i.v. application of LPS and i.p. administration of D-galactosamine (16 mg/mouse) in C57BL/6 mice.

certain immunoglobulin subclass, such as the IgM class antibody, nor lipid A specificity is required. It is possible, however, that IgM antibodies of the same specificity would express stronger C'-mediated bactericidal properties in addition to similar endotoxin neutralizing capacity. The development and characterization of such mAbs with a broad spectrum of bacterial reactivity therefore offers new opportunities for the immunotherapy of patients suffering from sepsis associated with endotoxemia (Di Padova et al. 1995).

12 Final Remarks

The present chapter discusses the significance of endotoxin as a major sepsis mediator after its release from bacteria and presents new immunological pre-

ventive or therapeutic strategies against *circulating* LPS. It should be noted, however, that endotoxin contributes to the pathogenic potential of gram-negative bacteria not only in a released and soluble but also in a *bound* form, i.e., as part of the bacterial outer membrane. Endotoxin constitutes an essential component of this membrane which in turn is essential for bacterial viability. Thus mutants unable to synthesize the lipid A-Kdo domain of LPS cease to grow, and drugs interfering with its biosynthesis are bactericidal. Further, as an exposed membrane structure LPS may not only prevent the activation of complement and thereby contribute to the serum resistance of bacteria but may also act as an inhibitor of phagocytosis and intracellular destruction. In addition, the LPS-containing outer membrane protects gram-negative bacteria against certain antibiotics and bile acids. Therefore, as bacterial membrane components exposed on the cellular surface, endotoxins represent an ideal target to attack pathogenic bacteria by genetical, pharmacological, or immunological strategies. In the future a combination of strategies aimed at LPS both as a soluble toxin and as a cell-bound surface antigen may perhaps become the most promising approach in the fight against gram-negative sepsis.

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Superantigen-Mediated Lethal Shock: The Functional State of Ligand-Reactive T Cells

K. Heeg, T. Miethke, and H. Wagner

1 Superantigens

A group of bacterial and viral proteins has been termed superantigens (SAg) because of their unique mechanisms to interact with antigen-presenting cells (APC) and T lymphocytes (Herman et al. 1991a; Marrack and Kappler 1990; Herrmann and MacDonald 1991; Acha-Orbea et al. 1993). SAg are comprised of bacterial exoproducts including the staphylococcal enterotoxins, proteins encoded by viral genomes, and retroviral products such as those of open reading frame region encoded SAg of mouse mammary tumor viruses (Table 1; MacDonald et al. 1988; Marrack et al. 1991,1993; Janeway et al. 1989; Acha-Orbea et al. 1993). All SAg share the distinctive ability to bind to MHC class II molecules as intact molecules, thus bypassing rate-limiting steps during normal antigen processing, including antigen uptake, antigen degradation, and internal binding as peptide fragment to the antigen-presenting groove of class II MHC molecules of APC (Carlsson et al. 1988; Mollick et al. 1989; Fleischer and Schrezenmeier

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Table 1. Superantigens

Source		
Bacterial	*S. aureus*	Staphylococcal enterotoxins A–E
	S. aureus	Toxic shock syndrome toxin 1
	S. pyogneus	Pyrogenic toxins A,C
	M. arthritidis	MAS
Retroviral	MMTV	MIs
Viral	Rabies	nucleocapsid

1988; Torres et al. 1993; Thibodeau et al. 1994; Mottershead et al. 1995). SAg bind directly to regions of the class II molecule that are outside the physiological MHC haplotype-restricted antigen-binding groove (Fig. 1; Dellabona et al. 1990; Herman et al. 1991b; Jardetzky et al. 1994). The SAg binding region seems to be conserved within various MHC haplotypes of mammalian species since SAg bind to murine and rat as well as to human class II molecules (Herrmann and MacDonald 1991; Fraser 1989). Nevertheless a hierarchy of SAg/class II interactions is apparent in that human HLA-DR and mouse H2-IE molecules are superior binders of SAg. On the other side, individual SAg differ in their relative affinity for class II molecules, for example, *Staphylococcus aureus* enterotoxin A (SEA) binds more closely to murine class II that does staphylococcal enterotoxin B (SEB; Cantor et al. 1993). The binding affinity of SAg/class II interactions is in the range of 10^{-5} *M*, that is much lower than the binding constant of antibodies (Seth et al. 1994). As an exception to the rule, the bacterial SAg staphylococcal

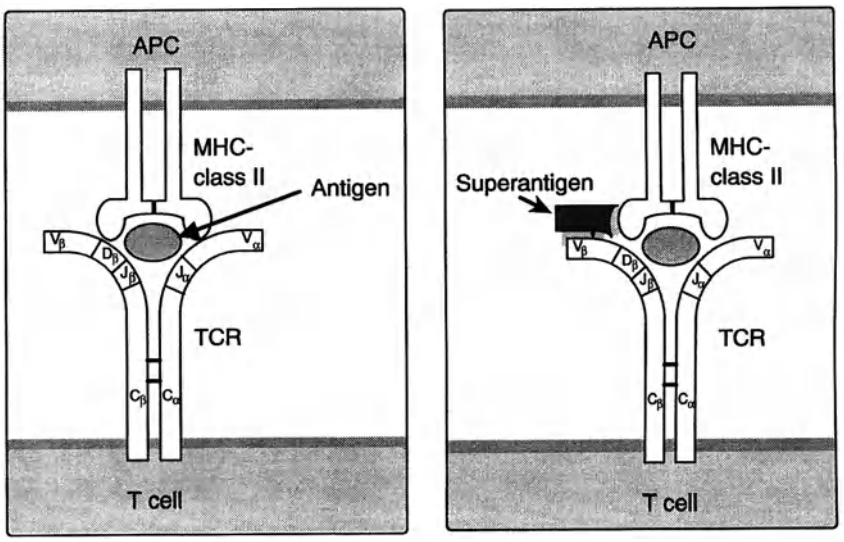

Fig. 1. Recognition of antigen and superantigen

enterotoxin C (SEC) known to bind to class II MHC also appears to bind to VCAM-1 molecules on the cell surface (CANTOR et al. 1993; AVERY et al. 1994). The extent to which other molecules can serve as SAg binding structures is unknown to date. SAg bound to class II molecules and to a lesser extent also free SAg bind to certain regions of the β-chain of the T cell receptor (TCR) that are encoded by Vβ-gene segments (CHOI et al. 1990; IRWIN et al. 1992, 1993; HAYBALL et al. 1994). Consequently a trimolecular complex of TCR, SAg, and MHC is formed which causes cross-linking and immobilization of the TCR and activation of the respective T cell (FLEISCHER and SCHREZENMEIER 1988; SETH et al. 1994; WHITE et al. 1989; KAPPLER et al. 1989). The binding affinities of the SAg–TCR interaction are in the same order of magnitude as those of the SAg–class II interaction (SETH et al. 1994). Thus SAg/TCR binding resembles at least in affinity the binding of isolated TCR to peptide–MHC complexes (MATSUI et al. 1991; WEBER et al. 1992). The binding region of the TCR β-chain for SAg is genetically encoded by Vβ segments of the TCR gene and lies outside the antigen recognition area of the TCR (WHITE et al. 1989; KAPPLER et al. 1989, 1994; HAMAD et al. 1994; HAYBALL et al. 1994). Thus binding of SAg to Vβ TCR is independent of the actual clonal antigen specificity of the TCR in question. Individual SAg interact selectively with certain Vβ encoded regions of the TCR β-chain. Because 25–50 individual Vβ segments make up the human or mouse TCR Vβ gene, an individual SAg reacts with at least 2%–5% of peripheral T cells (HERRMANN and MACDONALD 1991). Thus SAg induce a strong multiclonal yet Vβ-selective T cell activation that includes 1000 times more T cells than a T cell response toward a nominal antigen.

2 The In Vivo Response to SAg: A Complex Pattern of Reactivities

Confrontation of a mature immune system with the bacterial SAg SEB in vivo activates the reactive Vβ8-expressing $CD4^+$ and $CD8^+$ T lymphocytes leading to a complex pattern of concomitant and sequential reactivities that include hyperactivation (MIETHKE et al. 1992b; GAUS et al. 1994), deletion (KAWABE and OCHI 1991; WAHL et al. 1993; HUANG and CRISPE 1993), clonal expansion (MACDONALD et al. 1991; HEEG et al. 1993, 1995; GONZALO et al. 1992), and ligand-specific anergy (KAWABE and OCHI 1990; O'HEHIR and LAMB 1990; RELLAHAN et al 1990; MACDONALD et al. 1991; HEEG et al. 1993, 1995; BASCHIERI et al. 1993; Fig. 2). The net result of SEB-induced T cell activation includes opposite outcomes such as T cell dependent toxic lymphokine syndrome (T cell shock) or ligand-specific, long-lasting T cell anergy. This review follows up the fate of SEB-reactive T cells in vivo after SEB challenge and describes the sequential effects of a SEB-specific yet multiclonal T cell activation in vivo.

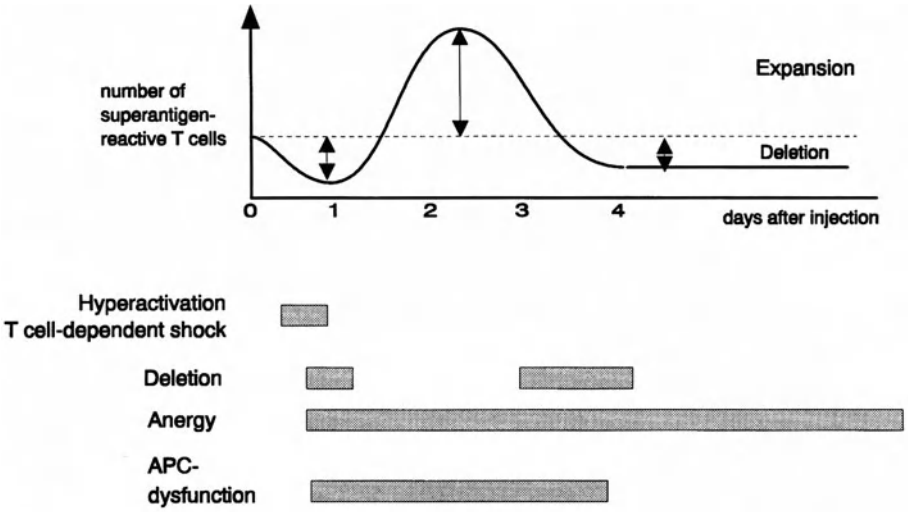

Fig. 2. In vivo response to superantigen

3 The First Hours: Hyperactivation of SAg-Reactive T Cells

SEB injected locally enters within minutes the draining lymph nodes through afferent lymphatic vessels, resulting in high local concentrations that allow binding to class II positive cells [dendritic cells (DC), macrophages (Mϕ), B cells]. Due to the concentration gradient within the lymph node and the low SEB–class II binding affinity SEB transits to the efferent lymphatic vessel, leaving SEB an effective half-time of about 30 min within a lymph node (M. VABULAS and T. MIETHKE, unpublished observations). While SEB disperses thereafter within the body fluids, it accumulates within the kidney. Overall it appears that the effective contact time of SEB with class II positive APC and T cells within a local lymph node is limited.

The short interaction time, however, is sufficient to trigger SAg-induced T cell responses. As a first sign, all SEB-reactive $V\beta8^+$ T lymphocytes in the draining lymph node shed L selectin (Mel-14) molecules from their surface within 10–15 min (MIETHKE et al. 1993c). Loss of Mel-14 is SAg-specific and Vβ-restricted, and lasts for at least 24h (MIETHKE et al. 1993c). Within 30 min $Mel\text{-}14^-$ Vβ8 T cells initiate transcription of T cell activation related mRNAs (MIETHKE et al. 1993c; GAUS et al. 1994) that include T cell-specific lymphokines [interleukin (IL)–1α, –2, –4, –6, and –10, interferon (IFN) –γ, lymphotoxin –α, and tumor necrosis factor (TNF) –α]. While $CD4^+$ $V\beta8^+$ T cells transcribe both Th_1- and Th_2-type lymphokines, in $CD8^+$ $V\beta8^+$ T cells mRNA is preferentially found encoding IL-2, IFN-γ and to a lesser extent TNF-α (GAUS et al. 1994). Transcription is followed by translation and systemic release of lymphokines. Peak values for TNF-α are

reached within 1–2 h; IL-1, -2, -4, and -6, and IFN-γ peak at 2–4 h; and IL-10 reaches ist maximum after 12–16 h (Miethke et al. 1993d; Gaus et al. 1994). The lymphokine content of serum is shut off promptly after reaching peak values, for example, TNF-α and IL-2 serum levels fall to preinjection values within 8 h (Miethke etl al. 1992b; Gaus et al. 1994). In this respect it is interesting that blockade of glucocorticoid receptors by RU 486 delays acute downregulation of cytokine production (Gonzalo et al. 1993b; Lussow et al. 1993). Since glucocorticoids are produced after SAg injection with similar kinetics as cytokines (Gonzalo et al. 1993b) (own unpublished data) glucocorticoids may act by suppressing lymphokine-specific mRNA synthesis via interaction with corticoid responsive elements in the promoter region of certain lymphokines (Daynes and Araneo 1989). The cytokine profile induced by different SAg is qualitatively not at variance; however, quantitative differences exist. Toxic shock syndrome toxin type 1 (TSST-1), for example, induces per cell higher TNF-α release as SEB (Miethke et al. 1993a).

SAg-induced production of lymphokines is strictly dependent on binding to class II molecules and on interaction with T-cells. Cyclosporin A (CsA), which blocks transcription of lymphokine mRNA, completely prevents lymphokine production induced by SEB in vivo (Vanier and Prud'homme 1992; Gonzalo et al. 1992; Heeg et al. 1993). In addition, severe combined immunodeficiency (SCID) mice lacking T cells are insensitive toward SEB and fail to respond in vivo (Miethke et al. 1992b). Finally, MHC class II deficient mice fail to respond to SAg that exclusively bind to class II molecules (SEB, SEA, TSST-1; Avery et al. 1994). In these mice only SAg (SEC1) which bind to non-MHC molecule (VCAM-1) are effective. Taken together, the first phase of a SAg-induced response of T cells in vivo is characterized by hyperacute T cell activation of all SAg-reactive T cells, leading to immediate lymphokine mRNA transcription followed by release of T cell dependent cytokines.

4 Acute Cytokine Release Causes Lethal T Cell Shock

SAg-triggered cytokine release leads to wasting of mice, associated with a transient weight loss probably due to systemic action of SAg-induced TNF and lymphotoxin (Marrack et al. 1990). When high doses (>500 μg per mouse) of SEB are injected, aged mice die within 48 h after injection (Aroeira et al. 1994). As such the mechanisms of this SAg-induced lethal cytokine syndrome resemble those of the classical endotoxic shock syndrome (Fig. 3; Glauser et al. 1991; Rietschel and Brade 1992). In the latter system bacterial-derived lipopolysaccharides (LPS) activate Mϕ which in turn release high concentrations of TNF-α, IL-1, and IL-6 (Glauser et al. 1991; Rietschel and Brade 1992; Freudenberg and Galanos 1990). These cytokines are the key players in mediating the LPS-induced lethal shock syndrome (Glauser et al. 1991; Rietschel and

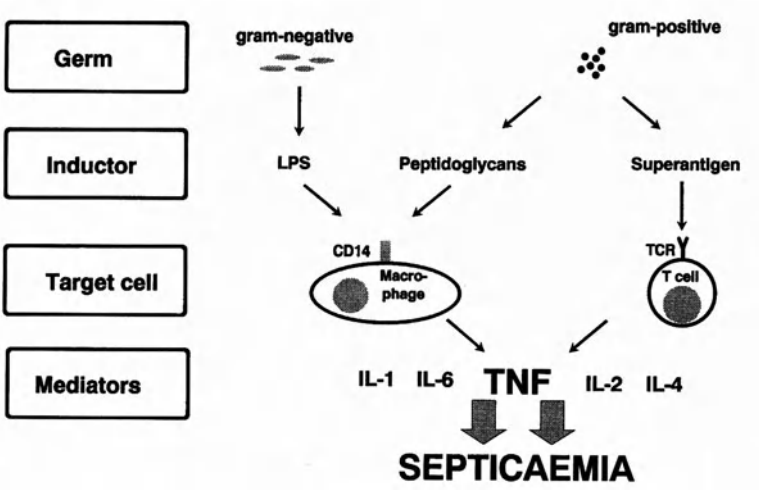

Fig. 3. Pathogenesis of lethal shock induced by endotoxin or superantigen

BRADE 1992). In contrast to primates, mice require relatively high concentrations of LPS for the induction of lethal shock, i.e., they appear endotoxin resistant (LEHMANN et al. 1987). Fortunately, mice can be sensitized for the lethal action of LPS-induced cytokines by simultaneous injection of D-galactosamine (D-GalN) together with LPS (GALANOS et al. 1979; LEHMANN et al. 1987). As a result, LD_{50} doses of LPS are reduced 100-fold, and mice thus acquire an endotoxin-sensible phenotype (GALANOS et al. 1979; LEHMANN et al. 1987).

To determine in vivo the full pathogenic potential of SAg it was deemed mandatory to analyze the consequences of SAg administration in D-GalN sensitized mice. We found that, as for LPS, the LD_{50} values for SAg in D-GalN sensitized mice are 100-fold reduced (1 μg SEB per mouse; MIETHKE et al. 1992b). This in turn allowed detailed analysis of the pathomechanisms of SAg-induced lethality. It was shown that T cells and T cell derived TNF-α are key mediatorys in the SAg-induced lethal shock syndrome since anti-TNF monoclonal antibodies efficiently prevent lethality (MIETHKE et al. 1992b). In addition, the T cell specific immunosuppressive agent CsA is protective (MIETHKE et al. 1992b). Finally, lethal shock cannot be brought about in T cell deficient SCID or nude (nu/nu) mice (MIETHKE et al. 1992b). In addition to D-GalN, at least two further "sensitizers" can synergize with SAg in inducing lethal shock, presumably via different mechanisms. First, RU 486, which blocks the action of glucocorticoids by competitive blockade of the glucocorticoid receptor, enhances SAg-induced lethality by preventing glucocorticoid-mediated downregulation of lymphokine production probably in SAg-activated T cells (GONZALO et al. 1993b). Accordingly, the amount of T cell-derived cytokines increases in RU 486 pretreated mice. Second, N^G-nitro-L-arginine methylester, which blocks action of the inducible nitric oxide synthetase (iNOS), also sensitizes mice for SAg-induced lethal shock

and seems to act distally from T cells by blocking nitric oxide dependent protective regulatory mechanisms in the periphery.

Obviously not only endotoxins but also exotoxins (SAg) induce in mice a toxic shock syndrome. Therefore the classical concept of pathogenesis of septic (bacterial) induced lethal shock can now be complemented by SAg-induced T cell activation (Fig. 3). In this unified concept endotoxin (LPS) and exotoxin (SAg) are inducers of toxic responses of host immune cells. While LPS acts on Mϕ that are activated to produce cytokines, SAg interacts with T cells which in turn are triggered to activation and cytokine production. In both situations Mϕ-derived or T cell-derived TNF-α acts as a key mediator for the induction of the lethal septic cascade (Fig. 3). According to this concept, the effector phase but not the induction phase of endotoxin- and exotoxin-mediated shock are similar to each other. On the other hand, recent, reports indicate that even the induction phases are interrelated. For example, LPS and SAg appear to synergize in the induction of lethal shock (Stiles et al. 1993) (unpublished observations). If so, synergistic interactions of endotoxin and exotoxin may condition lethal septicemia.

5 Induction of Unresponsiveness in SAg-Reactive T Cells: Anergy, Downregulation, and Deletion

While the first hours after SAg administration in vivo are characterized by hyperactivation of the responding Vβ8$^+$ T cells, after 4–6 h suppressive effects on T cells become dominant. Ligand-specific unresponsiveness (tolerance) is induced by multiple and consecutive mechanisms. First Vβ8$^+$ T cells from SEB-injected mice transit from a reactive to an anergic T cell phenotype shortly after the hyperreactive period. Vβ8$^+$ CD4$^+$ T cells removed within the first 2 h after SEB injection can be restimulated in vitro with SEB and respond with IL-2 production and subsequently enter cell cycle in vitro (Fig. 4). However, CD4$^+$ Vβ8$^+$ T cells recovered later (>6 h) fail to produce IL-2 upon restimulation with SEB in vitro and thus do not respond to SEB, i.e., these cells display an anergic phenotype (Fig. 4). Anergy is Vβ selective and confined to impaired lymphokine production in vitro. However, virtually all anergic Vβ8$^+$ T lymphocytes express IL-2 receptors (IL-2R) in vivo (Miethke et al. 1993b). In vitro, IL-2R of these anergic T cells are functional since exogenously added IL-2 induces proliferation of these cells (Miethke et al. 1993b; Heeg et al. 1993, 1995). Hence induction of anergy is SAg-reactive T cells is an immediate process that follows an initial state of hyperreactivity. It appears to be triggered directly by the TCR and clearly is not linked to cell cycle progression. T cell unresponsiveness is due not only to anergy. In vivo, anergic Vβ8$^+$ IL-2R$^+$ T cells proceed within hours through distinct levels of T cell unresponsiveness (tolerance) that may ultimately lead to T cell deletion via apoptosis. This stepwise process includes Vβ-selective TCR downregulation followed by loss of coreceptors (CD4/CD8, CD2). Apoptosis

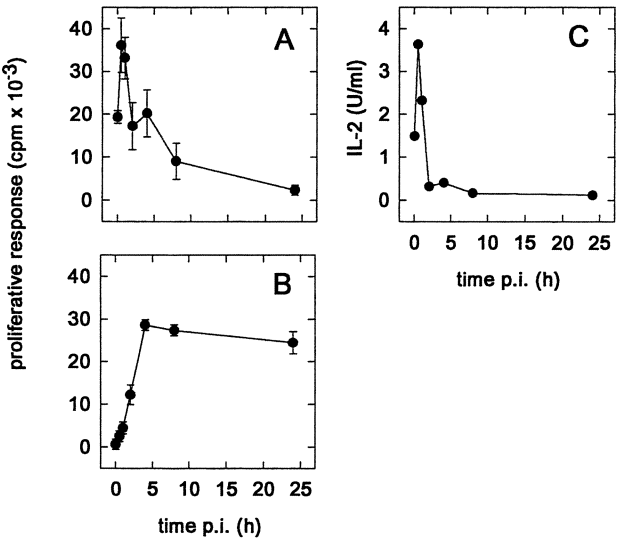

Fig. 4. SEB induces anergy. Lymph node cells from SEB-injected mice were restimulated in vitro after the indicated time periods with SEB (**A, C**) or IL-2 (**B**). Proliferative responses (**A, B**) or IL-2 production (**C**) were recorded

occurs only in a subset of anergic Vβ8$^+$ T cells. TCR downregulation may be transient, since TCR$^-$ T cells regain TCR expression, at least when transferred to cultures (Miethke et al. 1994). Since not all anergic Vβ T cells proceed to the level of tolerance defined by deletion (apoptosis), multiple levels of tolerance are observed at the same time (Miethke et al. 1994). It is not known how progression to later stages of tolerance induction is controlled. Recent experiments suggest that the dose of antigen that interacts with the responder T cells determines the level of tolerance induced. Accordingly, low concentrations of SEB induce only anergy while higher concentrations of SEB also induce TCR downregulation or even deletion (apoptosis). Experiments in TCR-transgenic mice support this hypothesis. When T cells from TCR-transgenic mice were confronted in vivo with their antigen whose expression was under control of an inducible promoter, a similar antigen dose/response relationship was observed (Schönrich et al. 1992; Hämmerling et al. 1991; Arnold et al. 1993).

Induction of apoptosis of SAg-reactive T cells is not inhibited by CsA (Vanier and Prud'homme 1992; Heeg et al. 1993), suggesting that neither T cell-derived cytokines nor Ca^{2+}-sensitive signal steps within T cells are involved in this process. In contrast, linomide can suppress apoptosis, but the mechanisms of its action are not defined (Gonzalo et al. 1993a). In fas-defective lpr/lpr mice SAg-induced apoptosis can be observed (unpublished observations), suggesting that besides fas/fas ligand-induced apoptosis other signal pathway can be utilised to induce apoptosis in SAg-reactive T cells.

According to the two-signal concept of T cell activation, the second signal provided by APC critically controls whether T cells are activated or anergized. (Bretscher and Cohn, 1970; Lafferty and Woolnough 1977). Since SEB binds to class II positive cells irrespective of whether these cells are costimulus-competent APC (DC, activated B cells) or costimulus-incompetent APC (resting B cells), it has been suggested that SEB bound to incompetent APC induces anergy and possibly even deletion of the SAg-reactive T cells. Experiments addressing this concept have been controversial. B cell knock-out mice, which lack B cells, were once shown to be indistinguishable from wildtype type strains in deleting SAg-reactive T cells after SEB administration in vivo (Miethke et al. 1995). On the other hand, concomitant injection of LPS (which polyclonally activates B cells and thus converts B cells to competent APC) prevents SAg-induced deletion (Vella et al. 1995). However, in the latter system an effect of LPS-triggered macrophage activation with subsequent cytokine release needs to be evaluated. Interestingly, apoptosis of SEB-reactive T cells can be induced in vitro under certain conditions even by costimulus-competent DC (unpublished observations). In this system transient presentation of high concentration of SEB by DC is required to induce apoptosis. We believe that a TCR signal induced by high concentrations of antigen rather than the availability of costimulatory signals controls induction of apoptosis.

6 Exhaustive Proliferation of Anergic T Cells

When TCR downregulation or T cell deletion has occurred in a subset of SAg-reactive T cells, the residual anergic V$\beta 8^+$ T cells enter cell cycle and expand clonally (Fig. 2) in vivo (Macdonald et al. 1991; Gonzalo et al. 1992; Heeg et al. 1993). The frequency of V$\beta 8^+$ T cells, which initially fell to about half of control values 14–20 h after SAg challenge, then increases dramatically. Within 2 days after SEB injection T cell areas in spleen and lymph node enlarge. SEB-reactive $CD8^+$ and $CD4^+$ V$\beta 8^+$ T cells then comprise 50% of all T lymphocytes in these secondary lymphatic organs. At the time of maximal expansion $CD8^+$ V$\beta 8^+$ exhibit SEB-specific cytolytic activity when tested directly ex vivo (Kawabe and Ochi 1990; Herrmann et al. 1992). A pathophysiological role of these cytotoxic T cells (CTL) in vivo is unlikely since the antigen, SEB, appears no longer to be available. Expansion of V$\beta 8^+$ T cells ceases after 72–96 h, and a mayor proportion of the expanded T cells again undergo apoptosis (Kawabe and Ochi 1991; MacDonald et al. 1991; Gonzalo et al. 1992; Vanier and Prud'homme 1992; Heeg et al. 1993). As a result the number of $CD8^+$ V$\beta 8^+$ T cells falls to preinjection values, while the frequency of $CD4^+$ V$\beta 8^+$ T cells is reduced to half of control values.

At present it is not known whether clonal expansion is driven by T cell derived lymphokines or by cell associated ligands. At the times of expansion SEB-reactive T cells are already anergic, and serum lymphokine levels are un-

detectable (Miethke et al. 1992b; Heeg et al. 1993). Moreover, IL-2R expression of the expanding cells has ceased (Miethke et al. 1992a, 1993b). On the other hand, CsA completely blocks $V\beta8^+$ T cell expansion (Heeg et al. 1993). $CD8^+$ T cell growth but not that of $CD4^+$ T cells can be brought about in CsA-treated mice by supplementation with exogenous added IL-2 (Heeg et al. 1993). Since cytokine production by SEB-reactive T cells and their transient IL-2R expression overlap in time, we favor the hypothesis that $V\beta8^+$ T cells becomes triggered to expansion during the initial period of a SEB-response, i.e., the time point preceding anergy induction. According, expanding T cells would starve from cytokines (exhaustion), and cytokine deprivation would initiate the second wave of clonal deletion. Recently, however, another explanation for clonal deletion has emerged. It was shown that $CD8^+$ CTL can be stimulated by $CD4^+$ T cells in a Vβ-restricted manner in vivo and in vitro, i.e., CTL appear to recognize Vβ-derived peptides on class Ib molecules of $CD4^+$ T cells (Jiang et al. 1995). These experiments suggest that regulatory $CD8^+$ CTL may be involved in the second deletion phase of SEB-reactive T cells in vivo.

7 Effects of SAg on Antigen-Presenting Cells

Although it has been shown that SAg can activate Mϕ via binding to class II molecules (Grossman et al. 1990; Chatila and Geha 1993), cytokines produced by T cells during the initial hyperreactive phase seem considerably to govern APC function. It is known that bactericidal Mϕ function is correlated with the expression of iNOS (Nathan and Xie 1994). TNF-α is a powerful inductor of iNOS (Cunha et al. 1994). We therefore analyzed iNOS expression after SEB administration. iNOS expression is observed during the first day after SEB injection, peaking at 16–24 h postinjection (unpublished observations). Moreover, resistance to lethal intraperitoneal infection with *Listeria monocytoges* is enhanced after SEB injection (unpublished observations). These data suggest that SEB-induced iNOS is functional and protective in vivo. In addition to TNF-α, other SEB-induced cytokines may alter Mϕ and APC function. For example, we observed high serum levels of IL-10 peaking 16–20 h upon challenge of mice with SEB. Since IL-10 exerts suppressive effects on APC (Ding et al. 1993; D'Andrea et al. 1993; Enk et al. 1993), we tested the ability of APC from SEB-injected mice to induce in vitro an allograft response. We found that APC function is transiently reduced 24–48 h after SEB injection (unpublished observations). Reduced APC function was not correlated with the expression of costimulatory molecules (B7) on APC. On the other hand, APC function was retained when mice were injected with SEB and CsA. We therefore conclude that T cell derived CsA-sensible cytokines (probably IL-10) are key mediators in suppressing APC function transiently. As a consequence during this phase the in vivo response toward third-party SAg (e.g., TSST-1) is completely suppressed (Miethke et al. 1993b). In

addition, IL-10 protects mice from SAg-induced lethal shock (BEAN et al. 1993). At first glance this unresponsiveness resembles LPS-induced tolerance. However, the third-party SAg-specific T cells are fully reactive in vitro when stimulated with normal APC. Thus in the course of a SAg-response a transient desensitization to SAgs is induced due to a loss of APC function of SAg-presenting cells. Desensitization is therefore not Vβ-restricted and embodies SAg independently of their intrinsic T cell specificity.

8 SAg-Induced Anergy Defined In Vitro Is Not Correlated with In Vivo Reactivity

When the second wave of apoptosis has passed, the number of $CD8^+$ $V\beta8^+$ T cells normalizes while the frequency of $CD4^+$ $V\beta8^+$ T cells remains reduced to about half of control values. $V\beta8^+$ T lymphocytes are now unresponsive to rechallenge in vitro with SEB and retain this anergic phenotype for weeks (KAWABE and OCHI 1990, 1991; O'HEHIR and LAMB 1990; RELLAHAN et al. 1990; MACDONALD et al. 1991; VANIER and PRUD'HOMME 1992; MIGITA and OCHI 1993). Detailed analyses have shown that upon restimulation anergic $CD4^+$ and $CD8^+$ T cells re-express IL-2R in vivo and in vitro but fail to produce lymphokines such as IL-2 (HEEG et al. 1993, 1995; GAUS et al. 1994). Limiting-dilution analyses have shown that virtually all $V\beta8^+$ T cells can be triggered to IL-2R-expression and proliferation in the presence of exogenous IL-2 (HEEG et al. 1995). Thus SEB-induced anergy is characterized by a selective loss of lymphokine production while other TCR-mediated signal pathways such as IL-2R expression are fully retained. On the other hand, distinct fucntional activities of anergic $CD4^+$ T cells (e.g., some forms of B cell help) seem to be maintained in vivo (OTTEN and GERMAIN 1991; LUSSOW and MACDONALD 1994; BANDEIRA et al. 1993). Further, SEB-induced anergy appears to be reversible since infection with the nematode *Nippostrongylus brasiliensis* changes the phenotype of anergic $CD4^+$ T cells which regain the ability to produce IL-4 (RÖCKEN et al. 1992).

When we evaluated SEB-induced unresponsiveness of $V\beta8^+$ T cells in vivo, the results were at variance to those obtained in vitro. While in vitro T cells from SEB-injected mice failed to produce IL-2 upon restimulation, the very same cells produced large amounts of IL-2 in vivo (HEEG et al. 1995; GAUS et al. 1994). Compared to primary stimulation with SEB in vivo, qualitatively and quantitatively the same combination of cytokines was produced with the exception that IFN-γ peak levels were five fold increased, and IL-10 peak levels were reached after only 4h (GAUS et al. 1994). Polymerase chain reaction analyses indicated that the $V\beta8^+$ T cell subset produces these cytokines upon in vivo rechallenge (HEEG et al. 1995). As a corollary, when SEB-tolerized mice were sensitized with D-GalN, the lethal shock syndrome was provoked by SEB rechallenge (HEEG et al. 1995; GAUS

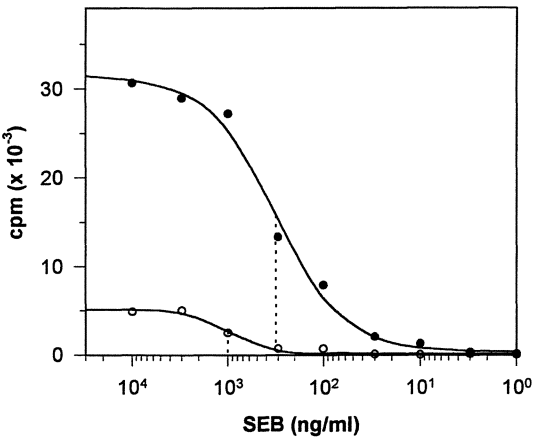

Fig. 5. Quantitative analysis of T cell triggering by SEB. $CD4^+$ T cells from normal (*closed circles*) or anergized (*open circles*) were stimulated with SEB concentrations as indicated on splenic feeder cells. Proliferative responses were recorded 5 days later. *Dashed line*, SEB concentration for half-maximal growth

et al. 1994). How can the paradox of in vitro anergy as opposed to in vivo reactivity be explained?

To answer this question we resorted to in vitro analyses and took advantage of the fact that SEB binds to class II positive cells without being processed. This in turn allows us to titrate in vitro the amount of SEB required to trigger a give T cell response, i.e., to determine the TCR threshold (BHARDWAJ et al. 1993). Further, this approach allows determination of the relative avidity of a given T cell population towards SEB (BHARDWAJ et al. 1993; HEEG and WAGNER 1995). Figure 5 details a representative analysis of this type. SEB (or SEA) titrations result in sigmoid-shaped dose-response curves that can be characterized by the amount of ligand (antigen) that induces half-maximal responses (SD_{50}). In the course of these studies it became evident that SD_{50} values depend critically on intrinsic properties of APC. For example, DC (as APC) require up to 20-fold less SEB for induction of proliferative responses of normal T cells than do splenocytes (Table 2). Resting B cells fail to induce a proliferative response at all, while activated B cells are almost as effective as DC (HEEG and WAGNER 1995). MHC

Table 2. SD_{50} values (ng/ml) for stimulation with SEB on various APC

		Spleen		Resting B cells		Activated B cells		Dendritic cells	
Responder cells	Anti-CD28	Proliferation	SD_{50}	Proliferation	SD_{50}	Proliferation	SD_{50}	Proliferation	SD_{50}
Normal	–	++	≈250	(+)	≈700	+++	≈30	+++	≈10
	+	+++	≈200	++	≈500	n.d.		n.d.	
Anergic	–	(+)	>1000	ϕ	>1000	++	≈250	+++	≈100
	+	++	≈1000	(+)	>1000	n.d.		n.d.	

class II expression and expression of a costimulatory molecules (B7) on the APC correlated with their efficacy to stimulate a response (HEEG and WAGNER 1995). In addition, cross-linking of CD28 molecules on the responding T cells by mAb substitutes, at least in part, for lacking B7 molecules on splenic APC or resting B cells without influencing SD_{50} values (Table 2, HEEG and WAGNER 1995).

In applying this technique we observed that anergic T cells (from SEB-pre-injected mice) could be reactivated provided they were challenged with higher concentrations of SEB. SD_{50} values were at least ten fold higher than those of normal T cells (Table 2). Increased SD_{50} values were evident with all types of APC tested (Table 2) and thus represent an intrinsic characteristic of anergic cells. Note that with splenic APC and resting B cells the high ligand density necessary for activation cannot be reached. Consequently $V\beta8^+$ T cells appear anergic. The critical ligand density, however, can be reached with DC or activated B cells. Thus stimulation with SEB with these APC allow reactivation of anergic cells and therefore discloses a reactive phenotype (HEEG and WAGNER 1995). Taken together, these experiments indicate that manifestation of an anergic phenotype of $V\beta8^+$ T cells depends on intrinsic and extrinsic factors. Elevated intrinsic TCR threshold (SD_{50}) values of SEB-anergized T cells can be overcome by stimulation with professional APC (extrinsic). As a result anergy can be turned into responsiveness by the use of professional APC and high ligand densities.

The observed elevation of SD_{50} values in anergic T cells can be explained by at least two mechanisms, which appear to be mutually exclusive: either the activation threshold for anergic versus normal T cells differs, or the relative avidity of the anergic T cell population toward SEB is lower than that in normal T cells. Since SEB causes apoptosis of a large proportion of SEB-reactive T cells, one could argue that the surviving T cells exhibit a lower TCR affinity, i.e, the T cells expressing high-affinity TCR for SAg would be preferentially deleted. Consequently only low-affinity TCR would escape deletion. In attempts to discriminate between these alternatives we tested the ligand dose required to induce a sustained TCR-initiated function of anergic cells, i.e., the induction of functional IL-2R (Table 3). It was shown that the SD_{50} values for induction of IL-2R expression on anergic T cells a identical to those of normal T cells (Table 3). Since the level of TCR expression is not reduced in anergic T cells, these results suggest that the relative TCR avidity of the anergic T cell population is not appreciably reduced compared to normal T cells. We therefore conclude that not TCR avidity but TCR threshold for triggering IL-2 gene activation is altered in

Table 3. TCR threshold (ng/ml) is elevated in anergic T cells

	SD_{50} for expression of functional IL-2R (APC: activated B cells + exogenous IL-2)	SD_{50} for induction of IL-2 production (APC: activated B cells)
Normal T cells	≈ 10	≈ 30
Anergic T cells	≈ 10	≈ 900

anergic cells. In fact, anergic T cells require 20- to 30-fold more ligand to trigger IL-2 production (Table 3). Hence, the refractoriness of anergic T cells is relative and can be characterised by a selectively elevated TCR threshold for induction of IL-2 production. Elevated TCR threshold can be overcome by raising the ligand concentration or by efficient stimulation with competent APC. Induction of responsiveness in SEB-anergic mice is clearly due to SEB stimulation with professional APC, thus converting anergy into responsiveness.

9 Superantigens: Pathogenic Factor and Tool to Analyze T Cell Function

Collectively, the diverse immunological responses towards SAg demonstrate their analytical and pathophysiological power and justify the tremendous interest which they have spawned in recent years. First, SAg have allowed delineation of the pathophysiology of T cell dependent shock syndrome that is triggered by hyperacute Vβ-selective T cell activation and release of T cell derived cytokines. Accordingly, a unified concept for the pathogenesis of septicemia can be formulated in which T cell derived (SAg) or Mϕ-derived (endotoxin) TNF-α act as key mediators for the induction of subsequent lethal shock. Future analyses of the interconnected action of endotoxin and SAg will allow delineation of synergy mechanisms between SAg and LPS which may be of relevance to understand the pathophysiology of septicemia in man. Second, SAg represent a tool for analyzing the mechanisms that lead to activation versus tolerization of the responding T cells. The Vβ-selective activation of T cells made it possible to follow up the fate of SAg-reactive T-cells in vivo and in vitro. It is puzzling that after an initial phase of hyperactivation distinct levels of tolerance become induced in vivo. The use of SAg will help to define the role of APC and costimulatory signals in inducing activation versus tolerization. Because of their unique presentation pathway SAg will be informative in analyzing the dose-dependent tolerization/activation signals in T cells in vivo and in vitro. Futhermore, since SAg activate a large fraction of peripheral T cells, it will now be feasible to analyze at the molecular level the signaling pathways leading either to activation or inactivation. Finally, there is an ongoing debate as to whether SAg are involved in the pathogenesis of certain human diseases in addition (Kotzin et al. 1994), including Kawasaki syndrome (Pietra et al. 1994), rheumatoid arthritis (Paliard et al. 1991), and even AIDS (Pantaleo et al. 1994; Chen et al. 1994; Lewis et al. 1994; Dadaglio et al. 1994).

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Pathogenesis of Sepsis Syndrome: Possible Relevance of Pore-Forming Bacterial Toxins

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1 Introduction

This review focuses on a group of bacterial products whose very existence is known to only a minority of clinicians, and whose potential significance as inducers of the sepsis syndrome has eluded the attention of most microbiologists. This is unfortunate because pore-forming bacterial toxins possess all the properties for contributing to the pathogenesis of local and systemic inflammatory reactions. Because pore formers generally are highly immunogenic proteins, the prospects for immune intervention are described that may eventually be of

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benefit to patients. The subject is therefore of interest not only from a theoretical but also from a practical point of view.

Pore-forming toxins are produced and secreted by bacteria as water-soluble proteins. They bind via interaction with cell surface receptors or via nonspecific absorption to lipid bilayers. After binding, the polypeptide domains insert into the target membrane to create water-filled, transmembrane pores. This process is often triggered by the oligomerization of toxin molecules in the membrane, with the formation of high molecular weight polymers that can be visualized in the electron-microscope as arc- or ring-shaped structures. It is thought that the oligomerization step provides the energy required to induce conformational changes that drive the membrane-inserting polypeptide domains into the lipid bilayer (Bhakdi and Tranum-Jensen 1987, 1988, 1991). Inhibition of oligomerization has indeed found to be an effective means to abolish the cytotoxic activity of several pore formers.

The size of transmembrane pores generated by these toxins varies between extremes. On the one hand, certain toxins such as the α-toxin of *Staphylococcus aureus* generate very small pores in the membranes of nucleated cells that are permissive only for monovalent ions (Walev et al. 1993, 1995). On the other hand, streptolysin-O and related toxins can within seconds produce pores of enormous dimensions that lead to rapid egress of proteins from the cytoplasm (Buckingham and Duncan 1983; Bhakdi et al. 1985; Alouf and Geoffrey 1991). Other toxins such as those belonging to the RTX family (with *Escherichia coli* hemolysin as the prototype) produce lesions of intermediate size (1–2 nm) that allow the flux of Ca^{2+} and nucleotides but not that of proteins through the membranes (Bhakdi et al. 1986). As is shown below, the functional size of toxin pores is a very significant factor determining the spectrum of cellular reactions that can be elicited in attacked target cells. These short- and long-range secondary effects are, in turn, probably of prime pathophysiological significance. In considering the biological effects of a pore former it is therefore always important to be aware of the respective pore size.

The list of pore formers is long and is continuously growing. Since the identification of *S. aureus* α-toxin as the first bacterial pore former only 14 years ago (Füssle et al. 1981), the majority of medically relevant pathogens have been shown to produce at least one pore-forming toxin. It is also becoming clear that these exotoxins tend to fall into categories or families of structurally and functionally related molecules. The two main families identified to date are (a) the cholesterol-binding toxins elaborated by gram-positive organisms, with streptolysin-O, pneumolysin, and perfringolysin being well-studied prototypes (Alouf and Geoffrey 1991) and (b) *E. coli* hemolysin with related toxins that have been designated the RTX (for repeat in toxin) family (Welch 1991). Another emerging family is that of the vibrio cytolysins. At a molecular level the cytolysin of *Aeromonas hydrophila* has been studied in some detail (Parker et al. 1994). Table 1 summarizes some of the major, medically relevant pore-forming toxins known today.

Table 1. Prototypes of pore-forming bacterial exotoxins

Producing Organism	Designation	Salient Features
Gram-positive bacteria[a]		
Staphylococcus aureus	α-toxin	Binds to unidentified specific sites on target cells; forms voltage-independent pores of ~1.0 nm diameter; *Identified susceptible human cells*: platelets, monocytes, lymphocytes, endothelial cells
Streptococcus pyogenes A	Streptolysin-O	Prototype of polymerizing toxin that forms large pores (up to 30 nm diameter); binds to cholesterol; all mammalian cells examined are susceptible
Gram-negative bacteria[b]		
Escherichia coli	*E. coli* hemolysin	Receptor problem unclarified, may bind nonspecifically to lipid bilayers; forms voltage-dependent, cation-selective pores of 1–2 nm diameter; potent cytocidal effects on all white blood cells and endothelial cells; triggers G protein dependent processes at low concentrations
Serratia marcescens	*Serratia* hemolysin	Forms voltage-independent pores of 1 nm diameter
Aeromonas hydrophilia	Aerolysin	Produces oligomeric pores of 1 nm diameters

[a]At least 14 other related and probably functionally similar toxins are produced by gram-positive organisms, including *Listeria monocytogenes, Streptococcus pneumoniae,* and *Bacillus cereus.*
[b]At least 10 other related toxins are produced by gram-negative organisms, including *Proteus* spp., *Morganella morganii, Pasteurella haemolytica, Actinobacillus pleuropneumoniae,* Bordetella pertussis Other pore-forming toxins are produced by *Vibrio cholerae* and *Vibrio parahaemolyticus* and *Klebsiella.*

2 *S. aureus* α-Toxin, *E. coli* Hemolysin, and Streptolysin-O: Three Prototypes of Pore-Forming Toxins

These three toxins have been extensively studied at the molecular level in this and other laboratories. They represent important prototypes, each with quite characteristic functional properties, and it is useful to review briefly some salient aspects of their structure and function.

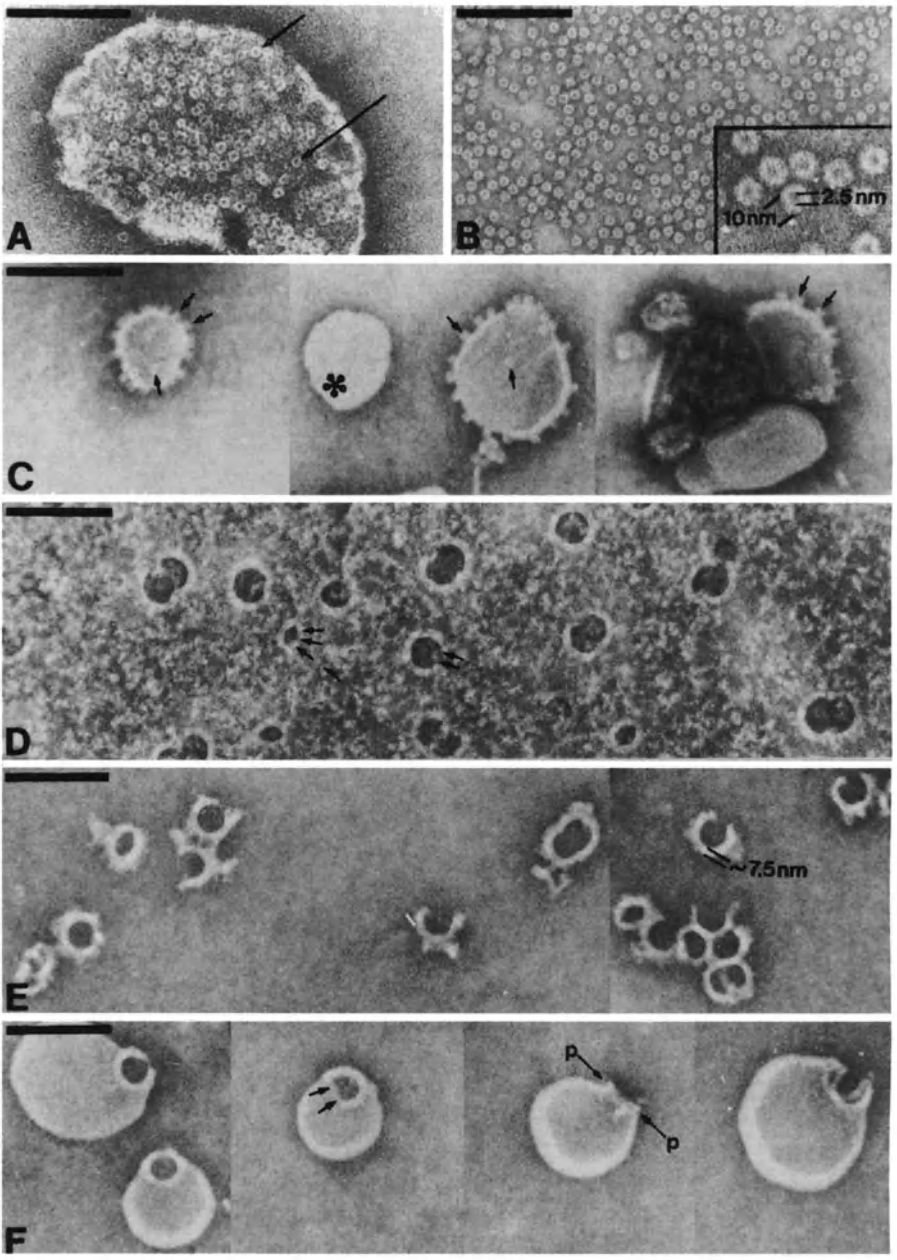

Fig. 1. A Negatively stained fragment of rabbit erythrocyte lysed with staphylococcal α-toxin. Numerous 19-nm ring-shaped structures are seen over the membrane (*arrows*). **B** Isolated toxin hexamers in detergent solution. **C** Lecithin liposomes carrying reincorporated α-toxin hexamers. The hexamers are seen as stubs along the edge of the liposomal membrane and as rings over the membrane (*arrows*). Characteristically, liposomes that escape incorporation of the toxin are

2.1 *S. aureus* α-Toxin

The toxin is secreted as a water-soluble monomer of M_r 34 000 devoid of cysteine residues. β-Sheet structures are the predominant secondary structural feature. The protein binds to as yet unidentified receptor molecules on susceptible cells in a temperature-independent manner (CASSIDY and HARSHMAN 1976; HILDEBRAND et al. 1991). Binding occurs via a conformational binding site that may involve both N- and C-terminus of the molecule. Membrane-bound toxin molecules collide in the membrane plane via lateral diffusion and form oligomers (BHAKDI and TRANUM-JENSEN 1991; Fig. 1) that appear to be heptamers (GOUAX et al. 1994). Oligomerization is accompanied by the ATP-independent insertion of the central molecular domain (encompassing approximately residues 124–138) into the bilayer. This has been delineated by the demonstration of the following: (a) Polarity-sensitive fluorescent molecules bound to these residues shift from a hydrophilic to a hydrophobic environment as toxin oligomerization occurs (VALEVA et al., 1996). (b) Biotin attached to residue 130 becomes inaccessible to streptavidin added to the external membrane face (PALMER et al. 1993a). (c) Alterations in this central region also alter the pore-forming properties of the toxin (PALMER et al. 1993b; WALKER et al. 1993; WALKER and BAYLEY 1994). (d) Derivatization of a cysteine residue placed at position 121 blocks the functional pore. (e) Removal of the blocking molecule opens the pore (VALEVA et al., 1996). Unexpectedly, it was found that the membrane-inserting region of α-toxin is not primarily hidden in the native molecule, but that it is freely accessible to modifying reagents and proteases (PALMER et al. 1993a).

When assessed in planar lipid membranes and erythrocytes, the size of α-toxin pores is approximately 1–1.5 nm, i.e., large enough to accommodate divalent ions and nucleotides (BHAKDI et al. 1984; MENESTRINA 1986). Unexpectedly, the size measured in susceptible nucleated cells such as keratinocytes, monocytes, and fibroblasts is slightly but significantly smaller. In these cases we have found that Ca^{2+} does not freely permeate the lesions (WALEV et al. 1993, 1995). Hence, when applied at physiological concentrations, α-toxin generates pores that are selective for monovalent ions in many cell targets.

impermeable to the stain. **D** Negatively stained erythrocyte membrane lysed by streptolysin-O showing numerous curved rods 25–100 nm long and approximately 7.5 nm wide with inner radius of curvature of 13–16 nm. Most rods are approximately semicircular, often joined in pairs at their ends. Dense accumulations of stain are seen at the concave side of the rods. When these do not form closed profiles, the stain deposit is partly bordered by a "free" edge of the erythrocyte membrane (*arrows*). **E** Negative staining of isolated streptolysin-O oligomers, showing, numerous curved rod structures identical to those found in toxin-treated membranes. **F** Purified streptolysin-O complexes reincorporated into cholesterol-free lecithin liposomes. The toxin oligmers form holes in the liposomes (*unlabeled arrows*); *p*, lesion seen in profile. *Scale bars*, 100 nm in all frames. **B–F** Sodium silicotungstate was used as negative stain; **A** uranylacetate (From BHAKDI and TRANUM-JENSEN 1988)

2.2 *E. coli* Hemolysin

The family of RTX toxins encompasses at least 14 members of homologous proteins elaborated by gram-negative organisms (Welch 1991). *E. coli* hemolysin is the best studied member thereof, and it has several singular properties that probably extend to other toxins in this family. *E. coli* hemolysin is a single-chain polypeptide devoid of cysteine with a M_r of 107 000. The toxin contains a hydrophobic stretch of amino acids in the N-terminal half of the molecule. It requires acylation with fatty acids at two lysine residues to obtain pore-forming activity (Issartel et al. 1991; Stanley et al. 1994). The following properties are remarkable. First, all mammalian cells tested thus far are susceptible to toxin action, but certain cell types including granulocytes, monocytes, endothelial cells, and renal epithelial cells are particularly vulnerable. The molecular basis for this phenomenon is unknown. Binding studies conducted in our laboratory in collaboration with V. Koronakis and C. Hughes speak against the presence of specific binding sites on cells that would account for their susceptibility. It appears that binding occurs quite unspecifically, possibly via interaction of the hydrophobic toxin domain with lipid. This interaction does not depend on the presence of covalently bound fatty acids, and toxin mutants lacking the acylation sites can therefore still bind to target cells. Remarkably, the binding step is accompanied by triggering of G protein dependent reactions that have been well studied in human granulocytes. These include the stimulation of the respiratory burst, secretion of vesicular constituents, and production platelet-activating factor, processes that are related to inositol 1,4,5-triphosphate (IP_3) generation, and that can be partially inhibited by pertussis toxin (Grimminger et al. 1991b; Bhakdi and Martin 1991). The events underlying the potent triggering of IP_3 related events is not known. They are not related to pore formation, and they can be evoked by nonacylated toxin mutants that create no pores. We speculate that the hydrophobic toxin domain interacts directly with elements of the G protein cascade to evoke the known spectrum of reactions that are normally provoked by the binding of chemokines to their receptors.

Pores created by *E coli* hemolysin are permissive for calcium and nucleotides such as ATP and GTP, but are too small to permit egress from the proteins from the cells (Bhakdi et al. 1986, 1989a; Menestrina et al. 1987). The pore-forming domains of *E. coli* hemolysin have not yet been identified.

2.3 Streptolysin-O

Toxins of this family are single-chain polypeptides of M_r 50 000–70 000, containing one cysteine residue whose chemical modification generally leads to inactivation. A stretch of hydrophobic amino acids is located near the cysteine in the highly conserved C-terminus of the protein. This hydrophobic sequence is involved in the primary binding event to cholesterol molecules in target membranes. For these toxins to bind efficiently to membranes the lipid bilayers must

have a high content of cholestrol (over 20 mol% of lipid; BERNHEIMER 1974; ALOUF 1980; OHNO-IWASHITA et al. 1988; ALOUF and GEOFFREY 1991). Virtually all mammalian cells are susceptible to their action, whereas bacteria are not. The membrane-bound toxin monomers are not cytotoxic; pore formation occurs when they aggregate laterally with each other to form noncovalent polymers (Fig. 1). The polymers are heterogeneous in size and probably accommodate 25–80 toxin molecules. In analogy to α-toxin, polymerization is accompanied by the insertion of as yet unidentified domains into the bilayer with formation of very large pores (BUCKINGHAM and DUNCAN 1983; BHAKDI et al. 1985). Mutant toxin preparations that have lost the ability to polymerize are also devoid of pore-forming properties.

3 Consequences of Pore Formation in Target Cells

3.1 Cell Death

Cell death is the most obvious and inevitable consequence of transmembrane pore formation if a lesion is not removed or repaired. Death ensues because the cells are rapidly depleted of ATP, and because they are unable to counteract the deleterious effects of ionic disequilibrium. Cell death can have immediate detrimental consequences. Necrosis of tissues generates niches for bacterial survival and multiplication. Death of phagocytes fosters microbial persistence and invasion (BHAKDI et al. 1989a). Endothelial cells are generally highly susceptible towards the action of pore formers; their death can lead to major perturbations in the microcirculation, for example, in the pulmonary vasculature (SEEGER et al. 1990). Damage to renal epithelial cells evoked, for instance, by *E. coli* hemolysin is probably directly responsible for renal dysfunction that is typical of acute pyelonephritis (KEANE et al. 1987). The cytotoxic action of pneumolysin and streptolysin-O on epithelial cells lining the round window membrane may contribute to sensineural hearing loss during acute otitis media caused by pneumococci because of breakdown of the round window membrane permeability barrier (ENGEL et al. 1995). There is also increasing evidence that the cytotoxic action of pore formers on epithelial cells of the gastrointestinal tract contributes to pathogenesis of diarrhea. These examples serve to underline the fact that cytotoxicity itself can cause organ dysfunction. It is noteworthy that, although most pore-forming toxins have been designated hemolysins because of their easily detectable hemolytic action in vitro, their in vivo significance is certainly due in the main to their action on susceptible nucleated cells and platelets.

3.2 Secondary Cellular Reactions

It cannot be sufficiently emphasized that secondary reactions mounted by cells under attack by a pore former are of prime importance. It is useful to differ-

entiate four main categories or reactions that are provoked by the different actions of pore-forming toxins.

3.2.1 Reactions Provoked by Egress of Macromolecules and Lysosomal Contents from the Cells

Such reactions are evoked, for example, by streptolysin-O and other toxins of this group. Biologically active molecules may be released from the cytoplasm that then act on cells in the immediate or less immediate surroundings. A simple example is the release of basic fibroblast growth factor from fibroblasts that are killed by streptolysin-O (Walev et al., unpublished). Such events surely contribute to cellular reactions in the infected sites. Another example is the massive liberation of lysosomal enzymes by the cells permeabilized with streptolysin-O. This may be the reason for the observed cleavage and release of interleukin-6 receptor and CD14 from cells bearing these molecules. Release of interleukin-6 receptor and CD14 may be highly significant events in the pathogenesis of septic shock because of transignaling events that render cells primarily devoid of the receptor to become susceptible to this interleukin (Walev et al., unpublished).

3.2.2 Ca^{2+}-Dependent Reactions

These occur when Ca^{2+}-permissive pores are generated that are too small to permit simultaneous leakage of cytoplasmic proteins. Many cellular mechanisms remain functionally intact over a short time period that respond to the rapid influx

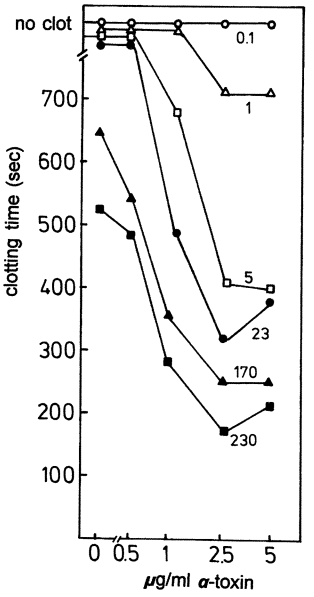

Fig. 2. Reduction in clot times of recalcified human plasma by *S. aureus* α-toxin. Citrated plasma samples containing the depicted numbers of platelets (per nanoliter) were given 12 m*M* Ca^{2+} and α-toxin at the given final concentrations. Marked reductions clot times were noted at toxin concentrations of 1–2.5 μg/ml, dependent on the presence of platelets in the plasma sample. *No clot,* lack of clot formation within 900 s. (From Bhakdi et al. 1988)

of Ca^{2+} ions. Some well-studied phenomena include secretion, activation of phospholipases with generation of eicosanoids, and contraction of cytoskeletal elements. Thus by stimulating secretion of procoagulatory substances from platelets α-toxin exerts potent procoagulatory effects (Bhakdi et al. 1988) when added to human blood (Fig. 2). *E. coli* hemolysin provokes the secretion of granular constituents from human granulocytes; the uncontrolled liberation of elastase may be pathophysiologically relevant (Bhakdi et al. 1989a). *E. coli* hemolysin potently induces generation of biologically active lipid mediators in target endothelial cells and granulocytes (König et al. 1986; Grimminger et al. 1990a,b, 1991a,b; Seeger et al. 1989; Suttorp et al. 1985, 1993). Both α-toxin and *E. coli* hemolysin provoke cytoskeletal dysfunction in endothelial cells, and cellular contraction leads to the formation of intercellular gaps that cause leakage of macromolecules across a confluent monolayer (Fig. 3; Suttorp et al. 1988, 1990). Such processes certainly contribute to organ dysfunction that can be

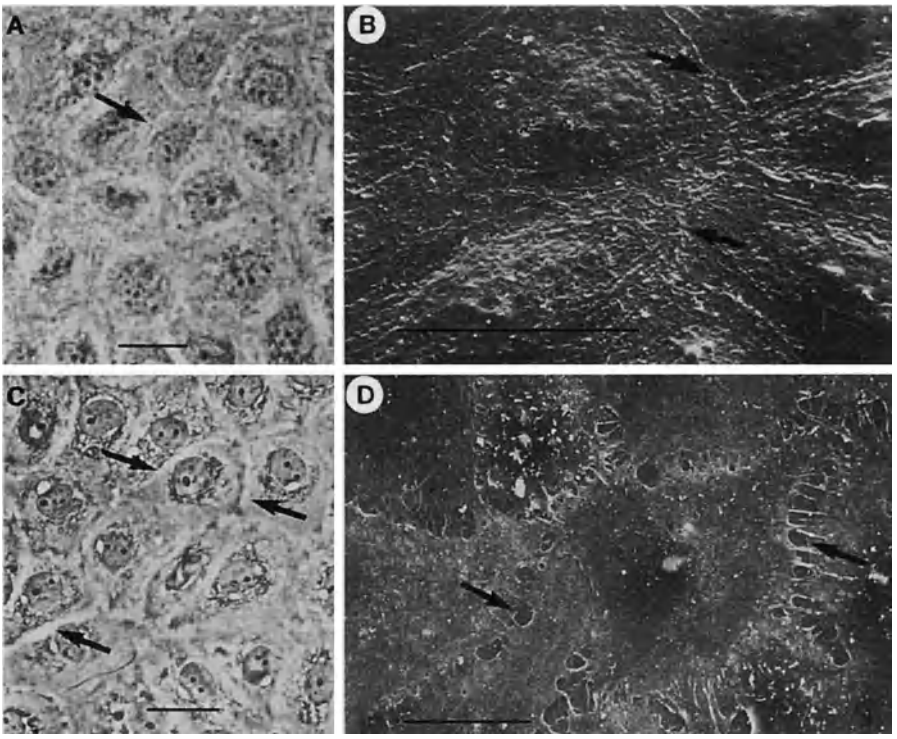

Fig. 3A–D. Intercellular gap formation in cultured endothelial cells induced by a pore-forming toxin. **A,** and **B** Sealed endothelial monolayer of pig pulmonary artery grown on gelatine-coated cover slips and exposed for 90 min to a hydrostatic pressure of 10 cm H_2O. Examination by phase contrast **(A)** and scanning electron microscopy **(B)** shows that virtually all interendothelial junctions (intercellular space) are closed. **C,** and **D** Endothelial monlayer exposed for 90 min to 1 μg/ml staphylococcal α-toxin. Note large interendothelial gaps (→) that are seen by both phase-contrast **(C)** and scanning electron microscopy **(D)**. *Bar*, 20 μm. (From Suttorp et al. 1988)

provoked in isolated organ systems, and that are observed when animals are challenged with these toxins.

3.2.3 Short-Circuiting of G Protein Dependent Signaling Pathways

This property is characteristic of *E. coli* hemolysin and probably of other RTX toxins. For unknown reasons the initial interaction of *E. coli* hemolysin with the

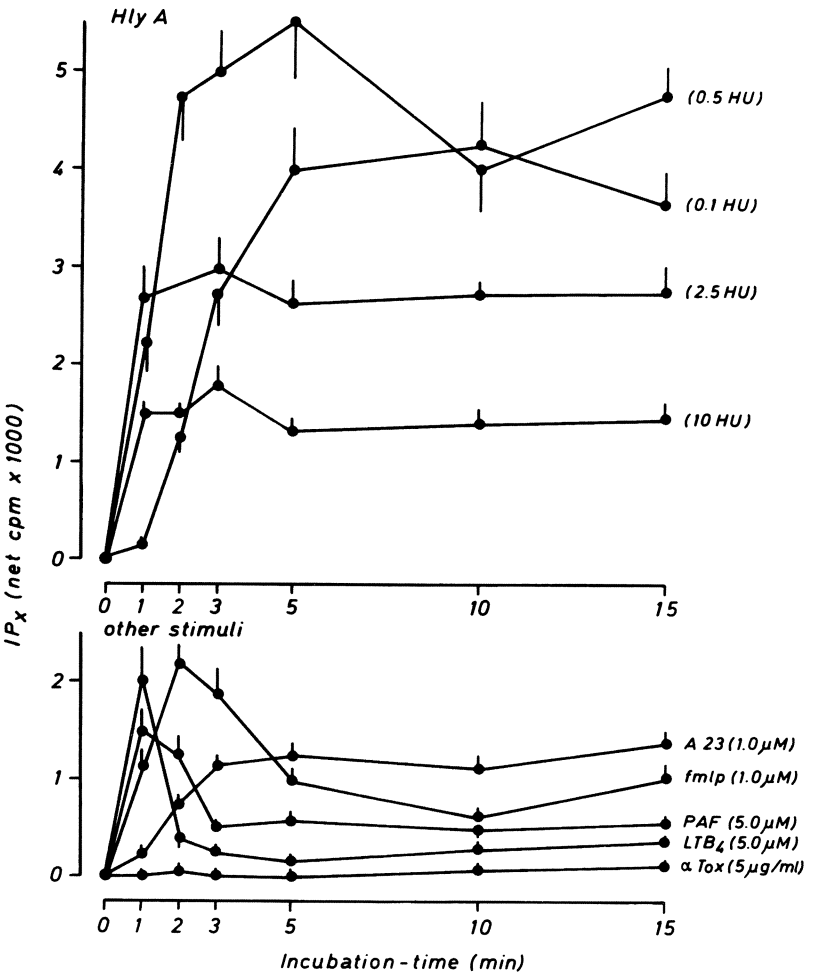

Fig. 4. Time course of neutrophil inositol phosphate generation evoked by *E. coli hemolysin* (H*ly A*) in comparison with various other stimuli. Human neutrophils (10^7) prelabeled with [^{3}H]inositol were incubated with various concentrations of Hly A (*top*) or other stimuli at optimum concentrations (*bottom*) for various time periods. Extracted inositol phosphates were separated by anion-exchange chromatography. Ip_x, IP_3, IP_2, and IP_1, corrected for baseline levels in nonchallenged cells (net cpm; baseline range between 540 and 1100 cpm). Means ± SEM of five (*top*) and four (*bottom*) independent experiments are given. (From Grimminger et al. 1991b)

target membrane is linked to the triggering of G protein dependent pathways and IP_3 generation (Fig. 4). Granulocytes challenged with toxin molecules that can bind but not form pores respond with reactions typical of those normally provoked by chemokines, i.e., secretion and production of reactive oxygen metabolites. Indeed, *E. coli* hemolysin has been identified as the most potent proteinaceous inducer of phosphoinositide hydrolysis known to date. As outlined above, we currently speculate that the triggering of these G protein dependent processes is related to the spontaneous insertion of the hydrophobic domain of *E. coli* hemolysin into the bilayer.

3.2.4 Reactions Provoked by Selective Flux of Monovalent Ions

These have recently been discovered in the course of our studies with α-toxin, which produces small pores in target membranes of many nucleated cells that are not Ca^{2+} permissive. Two striking phenomena have been observed. First, programmed cell death (apoptosis) is triggered in T-lymphocytes (Jonas et al. 1994). An important requisite is that the membranes are not permeable for Ca^{2+}: when α-toxin is administered at high concentrations, Ca^{2+} -permissive lesions are generated, and the extent of programmed cell death (PCD) diminishes (Fig. 5). Remarkably, PCD can be totally inhibited if cells are suspended in KCl rather than in Nacl. Hence internucleosomal DNA degradation ensuing through

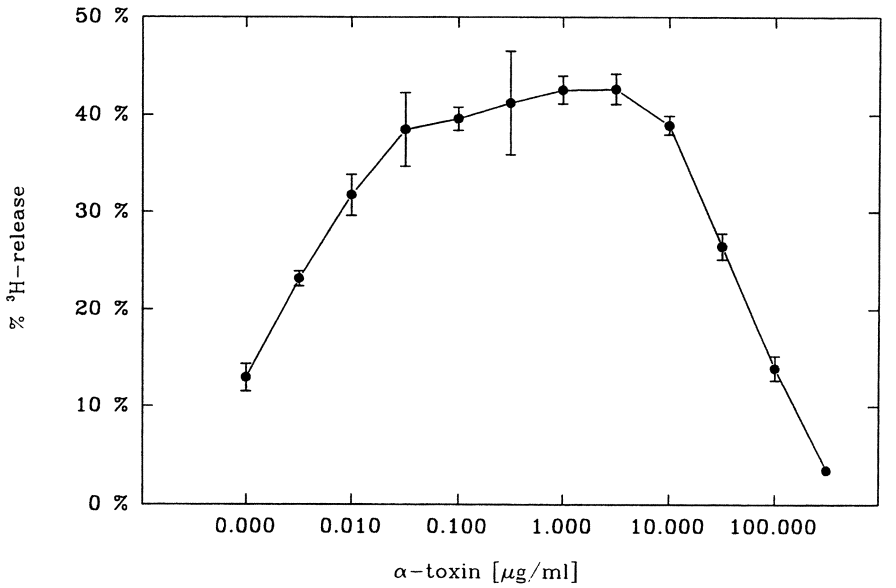

Fig. 5. Dose-independent induction of DNA fragmentation in T-lymphocytes by staphylococcal α-toxin. T-cells were activated with phythemagglutinin and labeled with [^{3}H]thymidine. They were treated with α-toxin at the depicted concentrations and release of [^{3}H]thymidine was measured after 4 h. Note the bell-shaped dose-dependent curve of DNA degradation evoked by the toxin. (From Jonas et al. 1994)

DNAse activation appears to somehow be coupled to perturbance of Na^+/K^+ homeostasis.

A second process of potential significance is the activation of interleukin-converting enzyme (ICE) in human monocytes (WALEV et al. 1995). This leads to rapid processing and secretion of interleukin-1β (Fig. 6). Recent studies have shown that ICE is controlled directly or indirectly by K^+. Thus any agents that cause cellular K^+ depletion also activate ICE. Conversely, the activation of ICE can be abrogated or suppressed in permeabilized cells by their transfer to K^+-rich media. Since an ICE-related protease may be involved in triggering apoptosis, the parallel findings made with α-toxin with regard to the triggering of PCD and the activation of interleukin-1β secretion may be linked. The study of secondary reactions evoked by pore-forming toxins has thus uncovered a novel phenom-

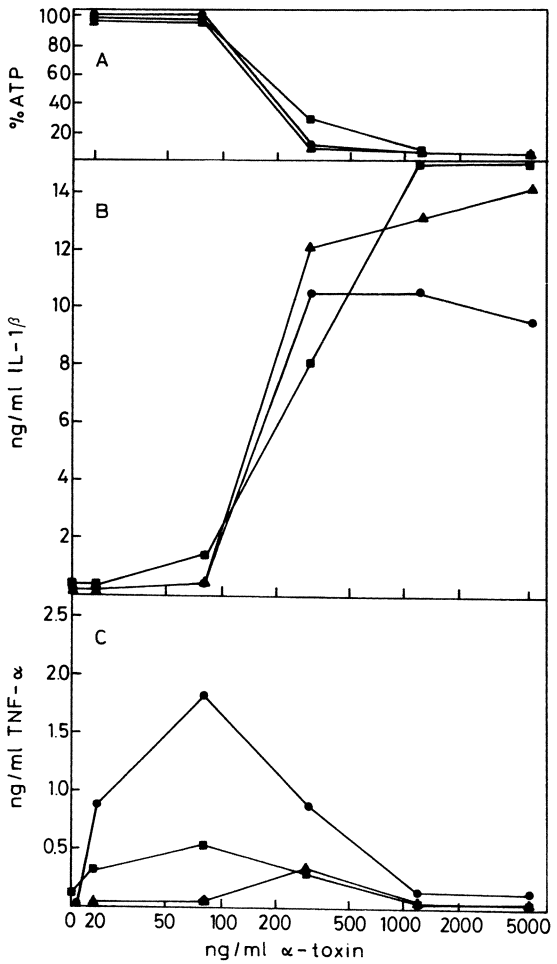

Fig. 6A–C. Action of α-toxin on monocytes in the presence of 10% autologous serum. **A** Cellular ATP content, expressed as a percentage of untreated control levels. **B, C** Concentrations of interleukin-1β IL=1β; **B** and tumor necrosis factor-α (TNF-α **C**) in the cell supernatants. The incubation period with α-toxin was 6 h at 37 °C. Each plot depicts the results obtained from one individual. (From BHAKDI et al. 1989c)

enon in cell biology: the role of K^+ in the regulation of vitally important cellular proteases (WALEV et al. 1995).

As a postscript, it is noteworthy that a limited number of small pores created by α-toxin can be repaired in certain nucleated cells such as fibroblasts. The repair process fails at lower pH (< 7) and seems to involve closure of the pores in the membranes (WALEV et al. 1994). Another interesting aspect pertains to the action of pore-forming toxins within cells that have ingested toxin-producing micro-organisms. As has been elegantly shown in the case of listeriolysin, this pore former enables the bacteria to escape from endolysosomes by the disruption of the lysosomal membrane (PORTNOY et al. 1992).

Taken together, secondary reactions by pore-forming toxins are thus very diverse, and it is important to realize that they are probably responsible for an array of short- and long-range effects in the macro-organism. The spectrum of these cellular reactions are indeed at least as wide as those elicited by endotoxins.

4 Organ Dysfunction and Lethal Effects

Since pore formers can evoke functional disturbances in target cells, it is easy to understand that they will also elicit acute organ dysfunction and are lethal in experimental animals. In these respects they are no less potent than endotoxins. Indeed, in some experimental models such as in the isolated lung they are much more toxic than their lipopolysaccharide counterparts. Detailed investigations have been performed in the latter model with both α-toxin (SEEGER et al. 1984, 1990) and *E. coli* hemolysin (GRIMMINGER et al. 1990a, b; SEEGER et al. 1991). Each of these toxins rapidly evoked profound pathophysiological alterations in the pulmonary microvasculature. This subject has been reviewed in sufficient detail recently (BHAKDI et al. 1994a), and it suffices here to say that subnanomolar concentrations of either α-toxin or *E. coli* hemolysin administered to the recirculating perfusion fluid leads to development of irreversible pulmonary edema. The underlying mechanisms are complex but encompass the direct toxic action on endothelial cells and the production of eicosanoids that in turn foster pulmonary arterial hypertension. Overall, it is evident that pore-forming exotoxins have the propensity to evoke acute pulmonary failure such as is typically found in patients presenting with adult respiratory distress syndrome during severe bacterial infections.

That pore-forming toxins are lethal when administered low amounts has long been known for α-toxin and streptolysin-O (ALOUF 1980; BERNHEIMER 1974). Experiments with *E. coli* hemolysin have extended this to the group of RTX toxins. In the case of α-toxin and *E. coli* hemolysin it is noteworthy that animals succumb to the action of both pore formers with symptoms that may be explained from the in vitro observations. For example, α-toxin provokes acute

thrombocytopenia and pulmonary edema in rabbits and monkeys (BHAKDI et al. 1989b). Rabbits challenged with *E. coli* hemolysin present with acute leukopenia and hemorrhagic pulmonary edema (VAGTS et al. 1993). These facts support the view that attack by pore formers on susceptible cells, coupled with detrimental cellular reactions, can lead to multiorgan failure and death.

5 Synergism Between Pore Formers and Other Toxins

Importantly, there may be synergism between endotoxin and pore-forming toxins. It has recently been demonstrated that the priming of pulmonary cells in isolated lungs with endotoxin does potentiate vascular abnormalities in response to α-toxin and *E. coli* hemolysin (WALMRATH et al. 1993, 1994). Such synergism and the resulting vascular abnormalities may be relevant to the pathogenesis of organ failure in systemic infections. We anticipate that similar synergisms are operative between superantigens (FLEISCHER 1991) and pore formers.

6 Pore-Forming Toxins: Candidates for Immune Intervention?

Pore-forming toxins usually fall within a narrow range of related protein families exhibiting antigenic cross-reactivity. Therefore development of vaccines against the major toxins is basically feasible. Many toxins have been cloned, and it is easy to generate inactive, site-directed mutants. In the case of α-toxin, for example, the replacement of a single amino acid residue can result in immunogenic but perfectly nontoxic proteins that elicit high antibody titers in animals (JURSCH et al. 1994; BHAKDI et al. 1994b). High-titered human antibodies against α-toxin have also been produced with use of a conventional toxoid. These antibodies are capable of neutralizing detrimental effects of α-toxin in vitro and in vivo (BHAKDI et al. 1989b). Apart from the possibility of passive immunization with such antibodies in patients with severe infections, a theoretical option is to vaccinate patients who are to undergo high-risk operations, for example, with a combination of potentially relevant vaccines. Such a prophylactic regimen would probably be cheap and safe and may also prove to be effective.

7 Conclusion

In contrast to endotoxins, pore-forming bacterial exotoxins continue to receive little attention as possible contributors to the pathogenesis of sepsis syndrome and septic shock. This is inappropriate since they are produced by the majority of important bacterial pathogens, and their toxic action has been extensively documented in defined cell systems, isolated organs, and animals models (e.g., WELCH et al. 1981; O'REILLY et al. 1986; PATON et al. 1993). Mutant bacterial strains that have selectively lost their capacity to produce important pore formers such as α-toxin, *E. coli* hemolysin, and pneumolysin also exhibit marked reduction in virulence in various animal models. Antibodies against α-toxin have been shown to significantly protect animals against various infections with *S. aureus*. Considering the wide spectrum of cellular disturbances that pore-forming toxins can evoke in target cells, these findings are in no way unexpected.

Unfortunately, pore-forming toxins are very difficult to quantify in biological fluids and tissues, Quantitation of the proteins is feasible only if methods can be devised to release them from the target cells. At present no satisfactory solution to the problem is available. In this regard the state of the art corresponds to the situation in clinical endotoxin research approximately 10 years ago, when the problem of endotoxin measurements had not yet been solved. Furthermore, pore-forming toxins probably do not have to be released in measurable quantities into body fluids to exert their action. Very few molecules are generally required to generate a membrane lesion, and attack on a cell may occur within a small, circumscribed areas as bacteria gain contact with their target. For example, seeding toxin inducing *E. coli* onto monocytes at the ration of only 1:1 causes death of the monocytes in the absence of detectable toxin in the cell supernatants (BHAKDI et al. 1990). Similarly, perfusion of isolated lungs with hemolytic *E. coli* also caused massive derangements in pulmonary vasculature in the absence of detectable circulating toxin (SEEGER et al. 1991). For these reasons it will prove difficult to obtain conclusive evidence that pore-forming toxins contribute to the development of sepsis syndrome. Nevertheless, the suggestive evidence at the present is already quite overwhelming, and it is highly appropriate to consider these very widespread bacterial products as probable contributors to sepsis and septic shock.

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The Cellular Response in Sepsis

D. Pajkrt and S.J.H. van Deventer

1 Introduction

The introduction of new medical technologies has resulted in the invasive and more aggressive treatment of diseases that in the past would have remained untreated. As a consequence the incidence of invasive bacterial infections has increased. Sepsis, which can be characterized as an ill-regulated host inflammatory reaction during infection, is a major cause of mortality in critically ill patients. Sepsis is not a disease but encompasses a set of clinical and laboratory findings that can be observed in critically ill patients and are known to be associated with high mortality. Despite various attempts to define sepsis (also known as sepsis syndrome, severe sepsis and systemic inflammatory response syndrome) to date the diagnostic and therapeutic implications of such definitions remain unclear. Recently completed large intervention studies using monoclonal antibodies or recombinant proteins aimed at blocking the inflammatory response in sepsis have not resulted in increased survival, and in some cases such interventions may even be harmful. These data have added to the uncertainty in respect to the clinical relevance of definitions of sepsis and have raised doubt

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about the future of immunotherapy in sepsis. It is of considerable interest that, in contrast to the failure of clinical applications of immunotherapy, animal models have demonstrated impressive results of immunotherapy which clearly indicate "proof of principle" for various intervention strategies, such as interleukin-1 receptor antagonist (IL-1Ra), anti-tumor necrosis factor (TNF) antibodies, and platelet-activating factor (PAF) antagonists.

In contrast to the evolving knowledge of soluble mediators, surprisingly little is known about the cellular response in clinical sepsis. Such knowledge is essential because the outcome of sepsis eventually depends on the result of interactions of cells of the immune system and nonimmune cells. A clear example of such interaction is the binding of leukocytes to the inflamed endothelium, which is necessary for successful clearance of local bacterial infections but also may result in damage to the lungs. This chapter reviews data on abnormal cellular responses in sepsis. Although sepsis is regularly complicated by damage to the parenchyma of various organs, this review is restricted to the cells that are thought to be most intimately involved in systemic inflammatory responses, i.e., leukocytes, endothelial cells, and blood platelets.

2 Leukocytes

Exposure to bacterial components such as endotoxin results in the production of various endogenous inflammatory proteins, cytokines, and lipid mediators that initiate an inflammatory response. Several animal studies have identified proinflammatory cytokines as important components of the host defense response, in part by their ability to activate leukocytes, and by guiding recruitment of primed neutrophils to local sites of inflammation. In this respect monocyte- and macrophage- derived TNF and IL-1β, the chemokines, and endothelial cell bound PAF have been characterized as crucial players by providing either chemotactic stimuli and/or regulating the expression of adhesion molecules and their ligands.

2.1 Neutrophilic Granulocytes

Neutrophils are distinguished from other white blood cells by their ubiquitous availability and by their rapid recruitment. Neutrophils are the first phagocytic cells to arrive at sites of local infection. In sepsis and in experimental endotoxemia early neutropenia is usually followed by late neutrophilia. The neutropenia in sepsis results from sequestration of the neutrophils into several organs such as lungs, liver, and spleen (Grisham et al. 1988). It is generally assumed that following sequestration the activation of neutrophils by endotoxin or cytokines contributes to the local injury by causing the release of reactive

oxygen species and proteinases. Neutrophil-induced endothelial cell damage is a major pathogenic mechanism in the pathogenesis of pulmonary damage (adult respiratory response syndrome, ARDS) in sepsis, and in ischemia-reperfusion injury. In addition to the classical proinflammatory cytokines, granulocyte-colony stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (Gm-CSF) can prime neutrophils and may thus influence the resulting recruitment of the neutrophils (Balazovich et al. 1991; de Haas et al. 1994).

Neutrophils are major effectors of microbial killing. Opsonized bacteria are recognized by neutrophil Fc receptors and by complement receptors and subsequently engulfed by enclosure within the neutrophil cell membrane. The thus formed phagosome fuses with the intracellular primary and secondary granules, and through a NADPH-oxidase dependent mechanism a reduction of oxygen to superoxide takes place. This superoxide is transformed into hydrogen peroxidase which eventually kills the micro-organism. Several other proteins such as myeloperoxidase and lysozyme which are readily available in the granules of neutrophil granulocyte also contribute to microbial and fungal killing. Neutrophils also have potent nonoxidative ways of killing bacteria, which include the endotoxin-binding proteins CAP-18 and bactericidal permeability protein (BPI). The latter protein is a 55-kDa molecule contained within the neutrophil azurophilic granules. In sepsis and experimental endotoxemia the circulating BPI concentration may increase threefold, nonetheless remaining too low to have significant endotoxin neutralizing effects (von der Möhlen et al. 1995b). Recombinant aminoterminal fragments of BPI ($rBPI_{23}$ and $rBPI_{21}$) have been shown potently to neutralize the biological effects of endotoxin in animal models of sepsis and in experimental endotoxemia in human volunteers (von der Möhlen et al. 1995a). In contrast to the relatively low circulating BPI levels, BPI can be present at considerable concentrations in local abscesses and within the inflamed bowel and would therefore be expected to exert its major effects at the local tissue level (Marra et al. 1994; Opal et al. 1994; Monajemi et al., in press).

Endotoxin is known to prime neutrophils and to stimulate the oxidative burst (Forehand et al. 1989). However, continuous stimulation of neutrophils causes functional alterations that include the loss of lysosomal granules and shedding of Fcγ receptors from the cell membrane. Possibly as a consequence of continuous stimulation, under pathological circumstances such as burns and injury both the neutrophil oxidative burst and chemotaxis become impaired (Alexander and Wixson 1970), and this results in an increased susceptibility for secondary infections. Indeed, in patients with septic ARDS circulating neutrophils have been reported to have a diminished capacity for respiratory bursts and adherence to pulmonary microvascular endothelial cells (Fein et al. 1991). Although inhibition of neutrophil/endothelial interactions would seem an interesting target for (immuno) therapy in sepsis, and several interventions indeed have shown efficacy in preventing ARDS in ischemia-reperfusion injury, such therapy may harm the infected patient. The crucial importance of neutrophils for effective clearance of bacteria has been demonstrated in several animal models of localized infection in which blockade of neutrophil recruitment by administration of anti-CD18 anti-

bodies caused decreased endotoxin clearance and increased mortality (Nathanson, personal communication)

Few studies have investigated the potential harmful effects of anti-selectin antibodies in models of infection, but because of the apparent redundancy of the various selectins less interference with neutrophil recruitment and little effect on bacterial phagocytosis would be anticipated.

In conclusion, neutrophils are thought to be involved in the organ damage that may occur in critically ill patients. Interference with neutrophil recruitment by targeting either CD11b or CD18 may prevent pulmonary damage in ischemia-reperfusion injury. Such interventions may potently interfere with the clearance of loci of bacterial infection and thus seem contraindicated in most patients with sepsis.

2.2 Mononuclear Cells

The main targets of endotoxin are monocytes and macrophages and many clinical symptoms observed during sepsis are mediated by monocytes and macrophage-derived cytokines. Several proteins present in serum under normal circumstances mediate the interaction between endotoxin and monocytes/macrophages. Lipopolysaccharide (LPS)-binding protein (LBP) is an acute-phase protein synthesized by hepatocytes that binds the endotoxin lipid A moiety. LBP is important in deaggregating circulating endotoxin particles and presenting endotoxin to the GPI-anchored CD14 on neutrophils and monocytes. It should be noted that, although LPS binding to CD14 is undisputed, other endotoxin signal transduction pathways exist because monocytes from patients suffering from paroxysmal nocturnal hemoglobulinuria that lack CD14 on their outer membrane can be activated by LPS. On the other hand, the absence of LBP or the presence of excess soluble CD14 (sCD14) significantly reduces the ability of LPS to induce cytokine synthesis.

It should be noted that various other membrane-expressed molecules, including the scavenger receptor (or scavenger receptor related protein) and the CD11b/CD18 complex can also bind LPS (Wright et al. 1989). Binding of endotoxin to these molecules may be important for endotoxin clearance but has no important role in inducing cytokine synthesis. The control of LPS binding to membrane-bound CD14 is complex because even in healthy individuals relatively high levels of sCD14 are present in the serum (up to 3 μg/ml). An increase in sCD14 levels can be found in a variety of conditions such as severe burns, after trauma, rheumatoid arthritis, and psoriasis. Although the mechanism of elaboration seems to differ, in sepsis sCD14 levels have been reported to correlate with C-reactive protein. It should be noted that sCD14 is able to recognize LPS/LBP complexes and to present these to cells that lack the membrane-bound CD14 (Frey et al. 1992). On the other hand, an excess concentration of sCD14 is able to bind and neutralize endotoxin-LBP complexes and prevent activation of monocytes and macrophages (Schütt et al. 1992). Further complexity is added

by the recent observation that LBP may also function to transport endotoxin molecules into high-density lipoprotein (HDL) particles (Wurfel et al. 1994). Hence the circulating concentration of HDL particles would be important for endotoxin removal, low levels driving the reaction toward CD14 binding. Indeed, addition of reconstituted human HDL to human blood decreases the amount of cytokines release after endotoxin stimulation (Casas et al. 1995). Furthermore, treatment with rHDL has been shown to increase survival in animal models of endotoxemia (Hubsch et al. 1993; Cué et al. 1994).

It has recently been recognized that in patients suffering from the sepsis syndrome a new subset of $CD14^+/CD16^+$ monocytes appears within the circulation. In healthy humans the concentrations of these cells are extremely low (9%). This amount can increase up to 50% in some septic patients, and a significant correlation has been shown between increased $CD14^+/CD16^+$ monocytes and the circulating IL-6 concentrations. In addition, these double-positive monocytes express decreased CD11b and CD33 antigen, suggesting a state of advanced differentiation. It is presently unclear whether these double-positive monocytes are responsible for the increased IL-6 production, or whether increased IL-6 levels are responsible for the changed phenotype of the monocytes in sepsis (Fingerle et al. 1993). Future studies need to elucidate the question of whether monocytes present in the circulation of septic patients change in their adhesion molecule expression, and which monocyte subpopulation is responsible for the increased cytokine production in sepsis.

3 Blood Platelets

Disseminated intravascular coagulation (DIC) is a frequent complication in septic patients. Coagulation activation, platelet activation, and platelet aggregation may all contribute to the pathogenesis of DIC. In contrast to conventional teaching, it is now known that platelets can specifically adhere to intact endothelium when activated by either TNFα, IL-1, or endotoxin (Grau and Lou 1993). Although thrombocytopenia in sepsis may be multifactorial, including decreased synthesis and immune complex mediated depletion, adhesion of platelets to intact endothelium may be one of the causes. Transient thrombocytopenia also may be observed in endotoxin-challenged volunteers, in which situation other causes are unlikely. The adherence of platelets to intact endothelium induces contact-dependent functional changes that are thought to be mediated by platelet membrane-expressed IL-1 (Hawrylowicz et al. 1991), which is importantly upregulated by thrombin (Bevilacqua et al. 1984). Of considerable interest is the finding that platelets may also express CD11a/CD18 and ICAM-1, which have been shown to be important for the binding to endothelial cells (Piguet et al. 1993; McCaffery and Berridge 1986). Blocking the CD11a/CD18 complex with anti-LFA-1 monoclonal or anti-ICAM-1 antibodies

dramatically reduces the hemorrhagic necrosis and platelet invasion into the skin seen in a mouse model.

We have recently demonstrated that the interaction of platelets with intact human umbilical vein endothelial cells (HUVEC) monolayers results in increased IL-8 production, and increased transmigration of neutrophils (Jansen and van Deventer, unpublished results). Others have reported that the platelet-induced upregulation of endothelial cell IL-8 production is blocked by IL-1Ra (Kaplanski et al. 1993). When activated by TNF, platelets have the remarkable ability to fuse with endothelial (Yamazaki et al. 1992) and subsequently damage the endothelial vessel wall.

In conclusion, the interaction of thrombin-activated platelets with the intact endothelium seems to upregulate local inflammation and neutrophil recruitment. This observation underscores the complex interactions between coagulation activation, neutrophils, platelets, and endothelial cells in sepsis.

4 The Vascular Endothelium

Long considered to be a passive lining of the vascular system, the endothelium is now known to be a very active player in inflammatory processes. Several functions of endothelial cells importantly change in sepsis, and these include its role in the process of blood coagulation and its ability to interact with circulating leukocytes.

4.1 Coagulation and Fibrinolysis

Endothelial cells have the capacity to influence blood coagulation by controlling local thrombin generation and by either promoting or inhibiting fibrinolysis. In most inflammatory conditions thrombin formation is enhanced, and this may cause the symptoms and signs of DIC that may further compromise the impaired tissue perfusion in sepsis. Although DIC has been reported in recent studies to occur in up to 40% of patients, the diagnosis has been usually based on a combination of a decreased platelet count and prolonged prothrombin time, the former being aspecific and the latter nonsensitive for abnormal thrombin production. Furthermore, septic patients frequently have underlying disease, such as cancer and trauma, that may activate the coagulation system independently. Nonetheless, the role of the vascular endothelium in blood coagulation activation in sepsis is now rather well defined by studies performed with cultured endothelial cells, in endotoxin- and TNF-infused volunteers, and by various specific interventions in primate endotoxemia and bacteremia (Bauer et al. 1989; van der Poll et al. 1990, 1994a,b).

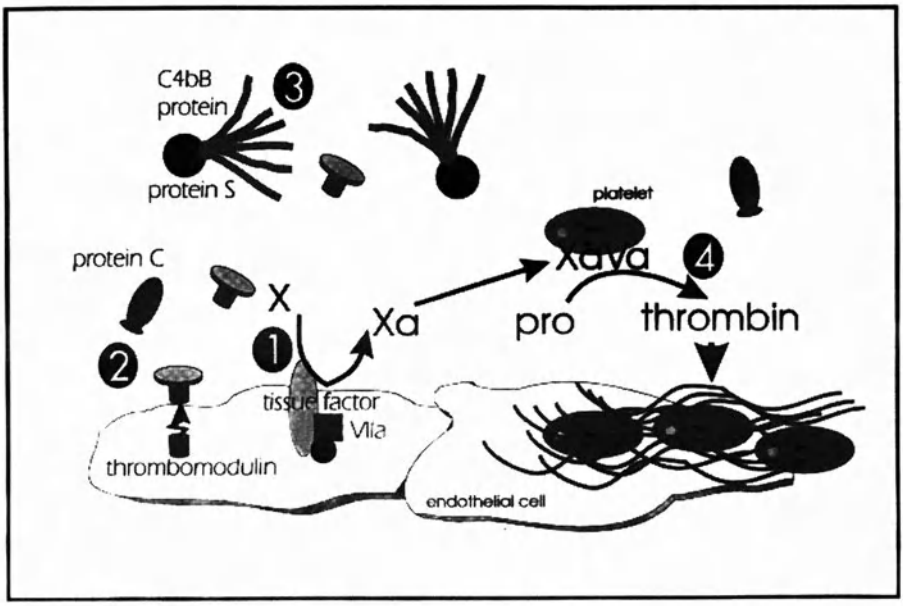

Fig. 1. Coagulation activation in sepsis. Thrombin generation in sepsis is mainly a result of increased expression of tissue factor by monocytes and endothelial cells (*1*). The tissue factor VIIa complex activates factor X, and subsequently the XaVa complex together with several cofactors causes prothrombin conversion (*4*). Thrombin generation leads to fibrin formation. Protein C is a potent inhibitor of the common pathway of coagulation activation. In order to be biologically active protein C needs to be activated by the thrombomodulin/thrombin complex on endothelial cells (*2*). Thrombomodulin expression by endothelial cells is sepsis is reduced, in part because of proteolytic cleavage. Protein S, cofactor for protein C, circulates in part as a free protein, and in part is bound to C4b-binding protein. In inflammatory states the serum concentration of C4b-binding protein increases, thereby potentially reducing protein S availability (*3*)

Although the contact system ("intrinsic system") of blood coagulation is activated in sepsis, it is now generally believed that its main consequence is the generation of proinflammatory mediators such as bradykinin, which may be involved in the pathogenesis of hypotension, and not thrombin formation. Consequently the main route of blood coagulation activation in sepsis is now thought to be initiated through the extrinsic pathway (Fig. 1). Resting endothelium in vitro and in vivo does not promote thrombin formation, but cultured human umbilical cord endothelial cells may be induced to express tissue factor, which is the main inducer of the extrinsic pathway of blood coagulation activation by endotoxin, IL-1, or TNF (Prydz and Pettersen 1988; Brox et al. 1984; Bevilacqua et al. 1984). Remarkably, tissue factor is expressed predominantly at the basal membrane by endothelial cells, which implies that its interaction with factor VIIa, which subsequently activates the common pathway of coagulation activation, must take place below the endothelial surface. It is conceivable that such a subendothelial coagulation process is facilitated by the increased permeability for plasma proteins that occurs in sepsis, but this hypothesis has not been experimentally

tested, It should further be noted that circulating monocytes from septic patients also express tissue factor and hence importantly contribute to coagulation activation.

One of the main inhibitors of thrombin formation is protein C. Protein C is activated by thrombomodulin after binding thrombin at the endothelial surface. The expression of thrombomodulin by endothelial cells is decreased by proinflammatory cytokines, both by a suppression of thrombomodulin mRNA transcription and by shedding of membrane-expressed thrombomodulin (Conway and Rosenberg 1988). In sepsis thrombomodulin shedding indeed occurs, as reflected by an increase of the circulating thrombomodulin concentration. The most dramatic example of DIC in sepsis is meningococcal septic shock, which is characterized by a severe reduction of protein C levels (Fijnvandraat et al. 1994), and it has recently been reported that the circulating thrombomodulin concentration increases in this disease. However, in a recent study the protein C concentration as expressed by antigen and activity was correlated very well, which suggests decreased protein C synthesis, or increased clearance, rather than a reduction in protein C activation by thrombomodulin, as the cause of the coagulopathy in meningococcal septic shock (Fijnvandraat et al. 1995).

Another important function of the vascular endothelium is modulation of fibrinolysis. In vitro, endothelial cells can be induced to release both tissue plasminogen activator (t-PA) and plasminogen activator inhibitor (PAI-1). In vivo, injection of endotoxin into healthy volunteers causes an initial increase in t-PA release, which is followed by a rise of PAI-1 plasma levels. These changes have been demonstrated to result in an initial increase and subsequent reduction in fibrin formation, as reflected by D-dimer plasma concentrations (Suffredini et al. 1989). It should be noted that the observed changes in fibrinolysis resulted from a single injection of a rather low dose of endotoxin, whereas septic patients are usually exposed to endotoxin, TNF, or IL-1 for a prolonged period. Moreover, in severe DIC in sepsis the depletion of various coagulation proteins may become rate limiting. Nevertheless, plasma PAI-1 concentrations in sepsis have been demonstrated to be correlated well with survival, suggesting a causal role for impaired fibrinolysis in sepsis.

In conclusion, sepsis importantly alters the normal anticoagulant function of the vascular endothelium. In fact, some endothelial surfaces may become strongly procoagulant, causing local fibrin deposition and therefore interfering with tissue perfusion.

4.2 Neutrophil–Endothelial Cell Interactions

The recruitment of inflammatory cells to local tissue sites is necessary for a normal host defense and results from interactions of surface molecules expressed by leukocytes and the vascular endothelium. This process is importantly modulated by several small cytokines (chemokines) and PAF. In sepsis, neutrophils adhering to the endothelium or transmigrating into tissues

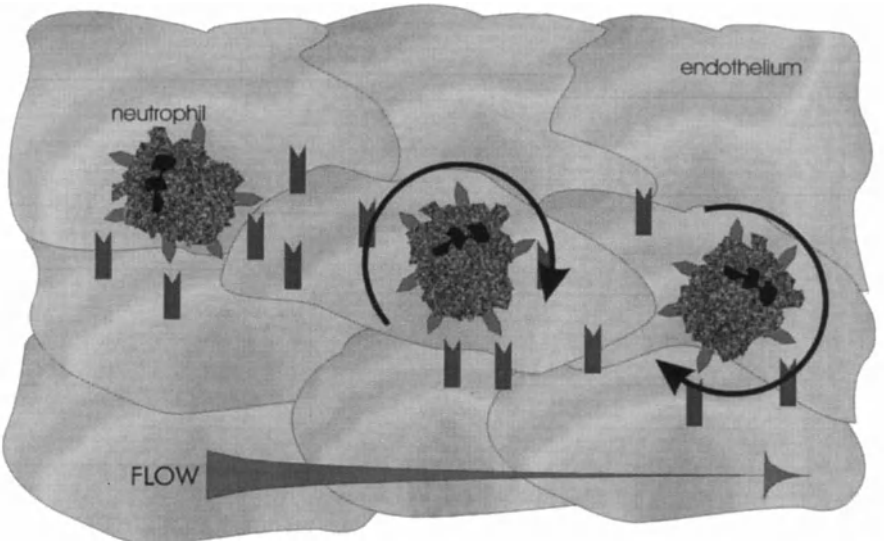

Fig. 2. Neutrophil rolling. In normal conditions few neutrophils are in contact with the vascular endothelium. Inflamed endothelial cells express adhesion molecules of the selectin family (E-selectin, P-selectin) which induces rolling of a substantial fraction of the circulating neutrophil pool

are considered to be involved in organ failure through the production of reactive oxygen species and proteinases. The endothelial adhesion molecules consist of the selectins (E-selectin, formerly known as ELAM-1; P-selectin, formerly known as GMP-140) and molecules that belong to the immunoglobulin superfamily (ICAM-1, ICAM-2, VCAM-1). Elegant in vivo studies using novel imaging techniques have demonstrated that within the microvasculature a minority of the circulating neutrophils is in transient contact with the endothelium. This process, known as "rolling" is a result of the opposed forces of shear stress and endothelial "stickiness," which in large part depends on the expression of the selectin-group of adhesion molecules. The physiological role of unstimulated neutrophil "rolling" remains to be defined, but it may serve to facilitate the normal circulation of phagocytic cells through noninflamed tissues. In inflammatory states the rolling of neutrophils is known to increase dramatically, which depends in part on increased expression of P-selectin, and it has recently been demonstrated that neutrophils may roll not only over endothelial cells but also over already adhering neutrophil monolayers (Fig. 2). At local inflammatory sites rolling neutrophils come to a halt, forming tight adhesions with endothelial cells. This tight endothelial-neutrophil interaction is a consequence of an interaction of the neutrophil β_2-integrins, in particular CD11b/CD18, with ICAM-1 (Fig. 3).

This CD11b/CD18 complex is expressed constitutively on the surface of leukocytes but is normally in an "inactive" state. Transformation to the active state occurs following stimulation by chemotactic factors such as endothelial-

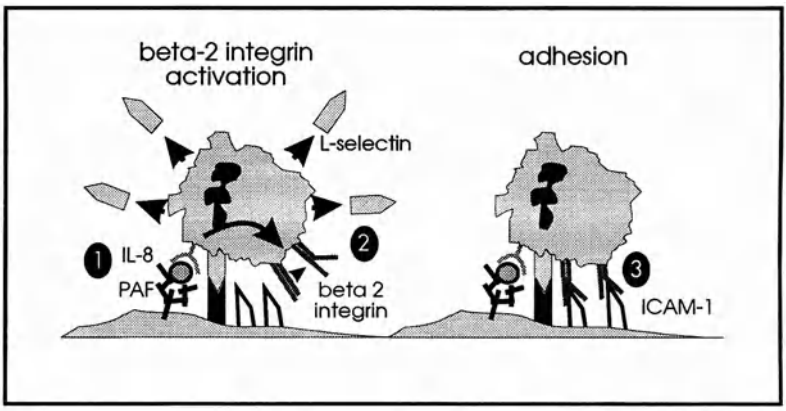

Fig. 3. Neutrophil adhesion. Rolling of neutrophils is not sufficient for thight adhesion and hence does not lead to transmigration. Close contact of neutrophils with the vascular endothelium causes activation of β_2-integrins (*2*), which is in part mediated by IL-8 and PAF (*1*), that bind to ICAM-1 on endothelial cells (*3*). In this process neutrophils shed L-selectin

bound IL-8, G-CSF, and PAF. The endothelial-neutrophil interaction is further facilitated by increased expression of ICAM-1 that results from stimulation by endotoxin or cytokines. The important role of β_2 integrins for the adherence of neutrophils to the endothelium cells is underscored by the sometimes dramatic clinical consequences of the leukocyte adhesion deficiency syndrome, a disease caused by absent expression of all three members of the leukocyte integrins. Several investigators have shown that blocking the integrins with monoclonal antibodies can reduce aggregation and adherence to endothelial cells (Arnaout 1990). Neutrophil CD11b/CD18 expression becomes highly elevated in low-dose endotoxemia in volunteers (unpublished), and it is now understood that this increased expression is induced at least partly by TNFα, for the CD11/CD18 upregulation can be reduced with anti-TNFα monoclonal antibodies (Windsor et al. 1993). In contrast to the considerable knowledge of neutrophil-endothelial cell interactions in vivo, the data on neutrophil-endothelial cell interactions in sepsis are scant. Increased sequestration of neutrophils into the lungs of patients with septic ARDS has been demonstrated using lung scans with ^{111}In-labeled neutrophils (Warshawski et al. 1986), but more detailed descriptions of adhesion receptor activation at the alveolar endothelium are still missing.

Clearly, neutrophils do not transmigrate as long as they remain bound to the luminal site of endothelial cells, and this implies that binding must be reversible. The exact molecular mechanisms involved in transmigration of neutrophils through endothelial cells remain to be characterized, but in vivo studies using HUVEC have demonstrated a role for PAF and IL-8. In this respect the observations that endothelial cells express a membrane-bound PAF, or PAF-like activity, and that IL-8 is associated with endothelial cells by heparan sulfate, seem to be of importance. Moreover, endothelial cell production of IL-8 is po-

larized, resulting in a preferential basal secretion and thus a concentration gradient that promotes neutrophil transmigration.

In conclusion, neutrophil-endothelial cell interactions have been fairly well characterized at the molecular level. Obviously it is difficult to study these interactions in septic patients, and indeed very few studies have addressed this problem.

4.3 NO Production

One of the main determinants of vascular tone in both health and disease is nitric oxide (NO). Endothelial cells maintain vascular tone by constitutive production of NO as a result of synthesis from arginine resulting from the activity of a constitutive NO synthetase (NOS; Fig. 4.). Endothelial cells are capable of synthesis of arginine from citrulline by means of an incomplete urea cycle and citrulline transport into endothelial cells is negatively influenced by glutamine. Hence, although infusion of large amounts of arginine may cause a drop in blood pressure, the changes in circulating amino acids may affect NO synthesis in a more complex manner. Moreover, various cytokines may induce other types of NOS (collectively known as iNOS), thus enhancing NO production (Moncada et al. 1991). No doubt increased NO production in sepsis occurs, and inhibition of NO

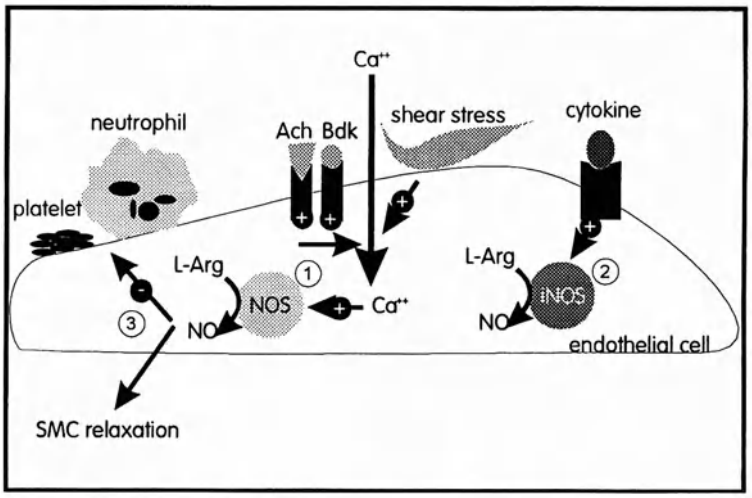

Fig. 4. NO production by endothelial cells in sepsis. Constitutive NO production from L-arginine by endothelial cells is dependent on the function of NOS (*1*). The activity of NOS is Ca-dependent and regulated by shear stress, acetylcholine, and bradykinin. Cytokines can induce another NOS, commonly known as iNOS (*2*). NO is an important regulator of vascular tone, and increased NO production is thought to be a major pathogenic factor for the development of irreversible hypotension in sepsis. However, complete inhibition of NO may not be beneficial in sepsis because NO inhibits both CD18-dependent neutrophil adhesion and adhesion of blood platelets to endothelial cells (*3*)

synthesis by the administration of NOS blockers may reverse hypotension, albeit at the expense of decreased tissue perfusion. It is beyond the scope of this chapter to extensively discuss the role of NO in sepsis, but we would like to stress that increased endothelial NO production may have a protective role. For example, inhibition of NO production has been shown to facilitate adhesion of platelets to endothelial cells in vitro (Katusic 1994), and the abnormally high rate of adhesion of neutrophils to canine coronary arteries following ischemia-reperfusion injury was reversed by the administration of an NO-donating compound. In fact, severe myocardial ischemia may occur in endoxemic animals after blockade of NO production, and this may exaggerate the late-phase hypotension that has been observed in such models.

5 Conclusion

Mononuclear cells, neutrophils, and the vascular endothelium all become activated in sepsis. Although it is popular to think of neutrophils and monocytes as the major effector cells in sepsis, the vascular endothelium being a prime target, organ damage in sepsis should be considered the result of complex cellular interactions. In this respect it is clear that the endothelium may become an important proinflammatory surface by enabling neutrophil adhesion and by facilitating intravascular coagulation activation. Although the induction of various cytokines in sepsis is now relatively well characterized, there is an important lack of knowledge of the cell activation processes in critically ill patients. For example, it remains unclear what cells contribute to cytokine production in sepsis, and no clinically useful methods exist that would enable the characterization of neutrophil-endothelial interactions in septic patients. Hence most knowledge of cell activation processes in sepsis is derived from data on soluble adhesion molecules and circulating cells, which may differ importantly from the resident or adhering populations. Thus far, coagulation activation and impaired fibrinolysis are among the best characterized consequences of activation of monocytes and the vascular endothelium, and inhibition of tissue factor and administration of protein C seem to be promising therapeutic strategies. Inhibition of neutrophil–endothelial cell adhesion events is now feasible, but the results of such strategies in experimental sepsis cast considerable doubt about their efficacy in reducing mortality. Finally, NO has been characterized as an important endothelial cell derived gas that is involved in the pathogenesis of irreversible septic shock. Although inhibition of NO may be an attractive strategy in septic shock, such an intervention may have adverse effects by increasing endothelial-neutrophil interactions and by promoting neutrophil adhesion.

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Cytokines as Mediators in the Pathogenesis of Septic Shock

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1 Introduction

During septic shock the host produces several proinflammatory cytokines which have been implicated as playing a critical role in the pathogenesis of the disease. The production of these cytokines is initiated by the organisms themselves (phagocytosis) or by soluble products of the organisms. For example, the lipopolysaccharide endotoxins (LPS) of gram-negative bacteria, the protein exotoxins of gram-positive bacteria, and the cell-wall glycopeptides such as teichoic acids and muramyl peptides. Of course, LPS is by far the most potent soluble product of bacteria which induces cytokine production, and therefore most information about cytokine induction is derived from studies using LPS in vitro and in vivo. However, it is important to recognize that the cytokine production in septic shock is neither specific nor unique. The cytokines which contribute to pathological changes in septic shock are not unique to infection. Multiple trauma, ischemia-reperfusion injury, acute transplant rejection, antigen-specific immune responses, and various acute inflammatory states (acute hepatitis and pancreatitis) initiate the same cytokine cascade and result in both systemic and local inflammatory processes.

However, special consideration exists for septic shock since no other disease is associated with such high mortality, despite our ability to provide patients with septic shock with appropriate antibiotics and supportive therapy. Gene deletion, neutralizing antibody studies, and specific receptor blockade of cytokines have revealed their pivotal role in the pathogenesis of spetic shock, at least in animal studies. This chapter reviews the cytokines most likely to participate in the pathological events of septic shock and discusses the mechanisms by which these cytokines contribute to the shock syndrome. In addition, we consider the evidence that nature did not leave us without counterregulatory mechanisms to oppose the effects of proinflammatory cytokines. Anti-inflammatory cytokines and specific naturally occurring inhibitors of cytokines are produced in health as well as in disease. These inhibitors likely reduce the severity of inflammation induced by proinflammatory cytokines. Therefore an emerging concept is that in a given individual, the net biological response of pro-and anti-inflammatory cytokines (sometimes called "balance" in this chapter) affects the outcome of a particular disease process. The systemic inflammation of septic shock is no exception to this concept. The preexisiting status of the patient may affect the nature of the pro-and anti-inflammatory cytokine response. Genetic factors also play an important role in this balance.

In infectious diseases the micro-organism induces a cytokine profile which is distinct from that induced during a response to foreign-tissue antigens. For example, during bacterial infections there is little or no production of interferon (IFN)-γ or interleukin (IL)-2 whereas these cytokines are prominent components of the cytokine profile during transplant rejection and immunologically mediated

diseases. In both situations and tumor necrosis factor (TNF) are produced and function primarily as proinflammatory cytokines.

Biologically IL-1 and TNF are closely related, although the structure and receptors for IL-1 and TNF are clearly distinct. IL-1 and TNF are active in the low pico- and femtomolar range. Based on short-term blockade of IL-1 and TNF receptors in humans and animals and recent data on IL-1β and TNFα deficient mice, there is no evidence that these cytokines play a critical role in development or normal homeostasis such as metabolism, hematopoiesis, renal and hepatic function, or regulation of blood pressure. During inflammation, injury, immunological challenge, or infection IL-1 and TNF are produced. One concludes from those studies that biological properties of IL-1 and TNF mimic host responses to infection, inflammation, injury, or immunological challenge. In animal models of systemic inflammation (such as in septic shock), specific blockade of *either* IL-1 or TNF results is a reduction in the severity of the inflammation. Moreover, IL-1 and TNF act synergistically in nearly every in vitro and in vivo model of local or systemic inflammation. When *both* cytokines are specifically blocked, the severity of inflammation is reduced further.

Understanding how IL-1 and TNF manifest so many different biological properties can be focused on relatively few mechanistic pathways, mostly those involving changes in constitutive and inducible gene expression or numbers of surface receptors for biologically active molecules. For example, inducible phospholipase A_2(PLA_2) and cyclo-oxygenase, genes controlling increased synthesis of inflammatory leukotrienes (LTs) and prostaglandins (PGs), are highly relevant to understanding the multiple effects of IL-1 and TNF. Another gene is inducible nitric oxide (NO) synthase. Therefore many of the pleiotropic effects of IL-1 and TNF are ameliorated by cyclo-oxygenase or nitric acid synthase inhibitors. Some biological effects of IL-1 and TNF are not mediated by intermediate production of eicosanoids or NO but rather by increased gene expression for other cytokines or regulation of celluar receptors for cytokines. A very prominent cytokine response to IL-1 and TNF is the production of the chemokine family of inflammatory cytokines (Baggiolini et al. 1994). These are highly active molecules which increase the ability of lymphocytes, monocytes, and neutrophils to attach to endothelial cells and migrate into the extravascular space. In septic shock the neutrophil plays an important role in tissue damage, which can be reduced by blocking receptors for IL-8.

2 Biological Effects of IL-1 and TNF Relevant to Septic Shock

2.1 Local and Systemic Effects

A distinction is made between the local effects of IL-1 and TNF and the consequences of their systemic levels. If the function of host defense is the elimination of the invading organism or destruction of foreign tissue, inflammation is the price which is paid for an effective defense. Therefore in systemic inflammation large amounts of IL-1 and TNF are released into the circulation, inducing hypotension and shock which can be lethal in experimental animals. Humans are particularly sensitive to the pyrogenic and hyportensive properties of IL-1 and TNF; a single intravenous injection of 10 ng/kg IL-1 or TNF induces fever (39 °C), whereas hypotension is consistently observed at doses of 100 ng/kg; 300 ng/kg is the maximal dose tolerated because of the severe fall in blood pressure (Chapman et al. 1987; Crown et al. 1991; Smith et al. 1992; van der Poll et al. 1990, 1991).

More common than the systemic inflammatory response to IL-1 and TNF are the local effects which result in release of lipid-derived mediators such as platelet-activating factor (PAF) and eiscosanoids. At sites of local injection or production of IL-1 and TNF neutrophil, monocyte, and lymphocyte emigration takes place. This emigration, which is the hallmark of local inflammation, is due to two events: (a) the induction of chemokines and (b) the upregulation of cell adhesion molecules. The chemokine family is best characterized by IL-8. For the purposes of this chapter, the entire family of chemokine peptides are represented by IL-8. IL-1 and TNF are potent inducers of IL-8 synthesis from monocytes, fibroblasts, and endothelial cells. Concentrations of 1 pg/ml IL-1 or TNF induce IL-8 production in fibroblast cultures. In monocytes stimulated with bacterial endotoxin 50% of the IL-8 produced is via an intermediate action of IL-1 (Porat et al. 1992), and IL-8 produced from endothelial cells stimulated with activated platelets is an IL-1-dependent pathway (Kaplanski et al. 1993).

2.2 Synergistic Actions of IL-1 and TNF

The synergistic activities of IL-1 and TNF are as follows:

IL-1 plus TNF
- Hemodynamic shock and lactic acidosis in rabbits
- Radioprotection
- Generation of Shwartzman reaction
- Luteal cell $PGF_{2\alpha}$ synthesis
- PGE_2 synthesis in fibroblasts

Galactosamine-induced hepatotoxicity
Sickness behavior in mice
Circulating nitric oxide and hypoglycemia in malaria
Nerve growth factor synthesis from fibroblasts
Insulin release and islet β-cell death
Insulin resistance
Loss of lean body mass
IL-8 synthesis by mesothelial cells

IL-1 plus bradykinin
Angiogenesis
PGE_2 synthesis in gingival fibroblasts
Arachidonic acid release from synoviocytes
$PGF_{2\alpha}$ synthesis in uterine decidua
IL-6 production from hepatoma cells and fibroblasts

IL-1 plus IL-6
Antigen-induced T-cell IL-2 production
Hepatic synthesis of acid glycoprotein and C3
Hepatic synthesis of LPS binding protein
Endothelial cell synthesis of Mn superoxide dismutase

IL-1 plus fibroblast, platelet-derived, epithelial, or α-transforming growth factor
PGE_2 synthesis in dermal fibroblasts
PGE_2 synthesis in synovial cells
Chemotaxis for fibroblasts
Phospholipase A_2 release from synoviocytes
Degradation of articular cartilage
PGE_2 synthesis in osteoblastic cells

The synergism between IL-1 and TNF is highly consistent and a frequently reported phenomenon. In addition, the synergism between IL-1 and TNF is also observed in vivo whereas the synergism between IL-1 and IL-6, IL-1 and bradykinin, or IL-1 and the various growth factors is mostly on prostanoid synthesis and primarily an in vitro finding. The mechanism for IL-1 synergism in the synthesis for PGE_2 likely involves the ability of one cytokine to release arachidonate and of the ability of IL to stimulate cyclo-oxygenase type 2(COX–2) synthesis. The mechanism for synergism may also involve receptor modulation; however, in the case of IL-1 and TNF synergism receptors for TNF are downregulated by IL-1 (Brakebusch et al. 1994; Holtmann and Wallach 1987). Could the synergism be explained at the level of signal transduction? Although this is an attractive hypothesis, no pathway of IL-1 or TNF signal transduction appears unique to either cytokine at the present time to account for synergism. In fact, since signal mechanisms appear similar, additive rather than synergistic effects should be observed.

The signal transduction of IL-1 appears to be similar to that of TNF. Although the signaling pathway of the IL-1 postreceptor binding remains unclear, several mechanisms have been proposed. These include activation of a GTP-binding

protein with no associated increase in adeny1cyclase (O' Neill et al. 1990), activation of adeny1cyclase (Mizel 1990; Munoz et al. 1990), hydrolysis of three phospholipids by nonphosphatidylinositol phospholipase C (Kester et al. 1989; Rosoff et al. 1988), release of ceramide from sphingomyelin following activation of sphingomyelinase (Mathias et al. 1993), and release of arachidonic acid from phopholipids via cytosolic PLA_2 and activation of PLAP (Clark et al. 1991; Gronich et al. 1994). In addition, some of the kinases that are activated by IL-1 are also activated in cells stimulated with TNF (Freshney et al. 1994; Guesdon et al. 1993, 1994; Kracht et al. 1994).

2.3 Expression of Various Genes in Cells Exposed to IL-1 and TNF

A fundamental property of IL-1 and TNF in the pathogenesis of septic shock is the ability to induce a variety of genes which affect the vasculature and the local tissue environment. In most cases IL-1 and TNF induce new transcripts in cells which express these genes only during disease. There are several examples, but the most dramatic appear to be other members of the cytokine family and inducible enzymes regulating low molecular weight mediators. Mediators such as PGs, LTs, and NO require cellular enzymes to covert precursors to active molecules. IL-1 and TNF are potent inducers of these enzymes. For PGE2 and LT synthesis IL-1 and TNF stimulate inducible PLA2, which contributes to the synthesis of PAF from lipid precursors. There is also COX-2 gene expression. The genes coding for IL-8 and other members of the chemokine family are not expressed in most cells in health, but picomolar concentrations of IL-1 and TNF trigger gene expression and protein expression in monocytes, fibroblasts, and endothelial cells. IL-1 and TNF induce the expression of their own genes. However, it is important to note that IL-1 and TNF suppress the transcription of the genes coding for albumin and the cytochrome P450 family.

2.4 Effects Mediated by Prostanoids

Many changes induced by IL-1 and TNF are mediated by PGs, particularly PGE2 (Tables 1 and 2). In fact, the use of cyclo-oxygenase inhibitors for a variety of inflammatory conditions is often a therapeutic strategy to reduce IL-1- and TNF-induced PGE2. Humans injected with IL-1 or TNF experience fever, headache, myalgias, and arthralgias, each of which is reduced by coadministration of cyclo-oxygenase inhibitors. One of the more universal activities of IL-1 and TNF is the induction of gene expression for type2 PLA2 and COX-2. IL-1 and TNF induce transcription of COX-2, and neither cytokine increases production of COX-1. Moreover, once triggered, COX-2 production is elevated for several hours, and large amounts of PGE2 are produced in cells stimulated with IL-1 or TNF. It comes as no surprise that inflammation is reduced by administration of cyclo-

Table 1. Cyclo-oxygenase *dependent* activities of IL-1 and TNF

In vivo
Fever
Natriuresis
ACTH and growth hormone release
Suppression of T-cell mitogenesis
Decreased pain threshold
Suppression of appetite and weight loss
Hypotension and shocklike syndrome
Increased melanoma bone marrow metastases
Decreased water intake
In vitro
Suppression of cell proliferation
Increased corticotropin-releasing factor
Formation of osteoclasts
Collagenase and stromelysin
Increased relaxation of arterial vessels
Inhibition of bone mineralization
Induction of leukocyte inhibition factor in fibroblasts

Table 2. Cyclo-oxygenase *independent* activities of IL-1 and TNF

In vivo
Slow-wave sleep
Non-rapid eye movement sleep
Neutrophilia
Lethality in adrenalectomized mice
Decreased mast cell degranulation
Production of colony-stimulating factor
Hypoferremia; hypozincemia
Hepatic acute-phase protein synthesis
Hypoglycemia
IL-6 production
Migration of neutrophils into tissues
In vitro
Gene expression and synthesis of cytokines
Destruction of islect β-cells
Augmentation of T-cell responses
Corticosterone synthesis (direct)
Collagenase/stromelysin gene expression
Induction of NO synthase
Hematopoietic stem cell activation
Enhancement of NO synthase (IL-1-induced PGE2 may augment gene expression and/or synthesis of some IL-1-induced genes, for example other cytokines and NOS via increase cAMP)
Suppression of gastric secretion
Increased progesterone synthesis
Increased nerve growth factor in astrocytes (augmented by addition of cyclo-oxygenase inhibitors)
Increased production of granulocyte-macrophrage colony-stimulating factor, (augmented by addition of cyclo-oxygenase inhibitors)

oxgenase inhibitors, and that many biological activities of IL-1 and TNF are actually due to increased PGE2 production.

There appears to be selectivity in cyclo-oxygenase inhibitors in that some nonsteroidal anti-inflammatory agents are better inhibitors of COX-2 rather than COX-1. Similar to COX-2 induction, IL-1 and TNF preferentially stimulate new transcripts for the inducible form (type 2) of PLA2 which cleaves the fatty acid in the number 2 position of cell membrane phospholipids. In most cases this is arachidonic acid. The release of arachidonic acid is the rate-limiting step in the synthesis of PGs and LTs.

2.5 Effects Mediated by Nitric Oxide

The generation of NO in inflammatory disease appears to be a fundamental event (reviewed in Moncada et al. 1991). Several studies have demonstrated that IL-1 and TNF initiate transcription and translation of the inducible form of nitric oxide synthase (iNOS). This has been observed in a variety of cells, for example, osteoclaste, murine macrophages, pituitary cells, mast cells, osteoblasts, glial cells, insulin-producing β-cells in the pancreas, smooth muscle cells, chondrocytes, myocytes, and mesangial cells. In mesangial cells IL-1β induced NOS is augmented by elevated levels of cAMP (Kunz et al. 1994). As with induction of COX-2 and type-2 PLA2, induction of NO likely accounts for a considerable number of biological effects of IL-1 and TNF. In experimental septic shock the fall in mean arterial pressure and the decrease in systemic vascular resistance are thought to be mediated by the induction of NO from smooth muscle cells. LPS injection into animals increases NO in several tissues, and when treated with IL-1 receptor antagonist, there is a 70% decrease in NO (Szabo et al. 1993). In vitro, low concentrations of IL-1 (1pg/ml) are sufficient to induce NO synthase.

In rabbits the blockade of constitutive and inducible NOS with *N*-nitro-L-arginine methyl ester led to IL-1-induced slow-wave sleep, but fever was not inhibited (Kapas et al. 1994). However, in the rat IL-1-induced fever was inhibited (Reimers et al. 1994), as were effects of IL-1 on the endocrine pancreas. In the rat, blocking the generation of NO augmented the IL-1 induced release of ACTH (Rivier and Shen 1994). In vitro, blocking NO production reduced PAF release from mast cells, inhibition of proteoglycan synthesis by chondrocytes, and apoptosis of the insulin-producing β-islet cell. In a murine macrophage line the induction of COX-2 by LPS was blocked by the NOS inhibitor *NG*-monomethyl-L-arginine and thus has been linked to a requirement for NO generation for PGE2 synthesis (Salvemi et al. 1993). IL-1β induced PGE2 on fibroblasts was enhanced by the NO donor, nitroprusside, and its enhancement was inhibited by hemoglobin (Salvemi et al. 1993). IL-1 induced NOS is not inhibited by indomethacin (Palmer et al. 1994).

2.6 Effect on Receptor Expression

Whether at the level of gene or surface expression, an important biological effect of IL-1 and TNF is the regulation of cytokine receptors, receptors for growth factors, and cell adhesion molecules. In general, changes in the binding of ligands to their respective receptors are not due to altered affinities but rather to a change in the number of surface receptors. The expression of surface receptors is usually reflected at the level of receptor gene expression, although there are examples where decreases in surface expression is due to increased receptor shedding. IL-1 and TNF-mediated upregulation of adhesion molecule expression on cultured endothelial cells is a well-established method for to study these adhesins. Low concentrations (1–10 p*M*) of IL-1 alone increases gene and surface receptors expression for ELAM and ICAM-1. In contrast, an increase in IL-2 (p55) receptors by IL-1 requires a costimulant. The p55 TNF receptor is downregulated by IL-1 (Holtmann and Wallach 1987); however, closer examination shows this effect of IL-1 to be due to shedding of the receptor as well as suppression of gene expression (Brakebusch et al. 1994).

2.7 IL-1 and TNF Infusion Mimics Septic Shock

Many of the biological effects of IL-1 and TNF are similar to those observed during a septic event; however, recent studies in humans have confirmed data from animal experiments. IL-1α or IL-1β has been administered to humans in phase I trials. Systemic administration of intravenous IL-1 from 1–10 ng/kg has produced fever, sleepiness, anorexia, generalized myalgias, arthralgias, and headache. However, the most dramatic biological response to IL-1 was observed at doses of 100 ng/kg or higher; in these patients a rapid fall in blood pressure takes place (Smith et al. 1992). Because of these results the dose limiting toxicity for IL-1 of hypotension has been set at 300 ng/kg. In some patients receiving 1 μg/kg stage IV hypotension was reported (Smith et al. 1992). The subcutaneous route is associated with fewer side effects.

TNF infusion into humans is similar to that observed for IL-1 (Chapman et al. 1987). However, TNF also induces a coagulation cascade (van der Poll et al. 1990), which has not been observed in humans injected with IL-1. In addition, low doses of TNF in humans induces neutrophilia whereas higher doses result in a leukopenia (van der Poll et al. 1991).

In the rabbit a single intravenous injection of 10μg/kg recombinant human IL-1β resulted in a shocklike state with hypotension, neutropenia, and thrombocytopenia (Okusawa et al. 1988). This has been confirmed in studies using human IL-1α in baboons (Fischer et al. 1991).

The mechanism for the hypotensive effect of IL-1 appears to be due to the generation of at least three low molecular weight meditators: cyclo-oxygenase products (Okusawa et al. 1988), PAF, and NO (Beasley et al. 1991). The fall in circulating leukocytes and platelets is thought to be due to the stimulation of

endothelial adhesion molecules. The effects of IL-1 in inducing a shocklike state are potentiated by coinfusion of TNF. The potentiation of IL-1 and TNF has been observed in both anesthetized (Okusawa et al. 1988) and conscious rabbits (Tredget et al. 1988). In the conscious rabbit coinjection of IL-1 and TNF induced a fall in mean arterial pressure, onset of lactic acidosis, and glucose intolerance. Many effects of IL-1 and TNF are synergistic in a variety of models in vitro and in vivo.

2.8 Effects on the Vascular Wall

In a now "classical" experiment on the local inflammatory response, the roles of IL-1 and TNF were studied for their role in leukocyte infiltration and extravastion of red cells and platelets using intradermal injection of endotoxin. Small doses of endotoxin induce a capillary leak and leukocyte infiltration within hours. The local Swartzman reaction takes place when a systemic injection of endotoxin is given 24 h later. In the hours following the systemic injection of endotoxin there is neutrophil emigration, hemorrhage, and necrosis at the "priming" site of initial the intradermal injection. Using either IL-1 or TNF only, no significant Swartzman reaction was observed as each cytokine was incabable of replacing the "priming" injection of endotoxin. However, when the combination of small doses of IL-1 plus TNF were injected as the priming agent, the full-blown local inflammatory response characteristic of the local Shwartzman reaction was observed (Movat et al. 1987). The proinflammatory effect of IL-1 and TNF was similar to that of acute vasculitis (Movat et al. 1987). Contributing to this event are the effects of IL-1 and TNF on endothelial cells. These include increased production of procoagulant activity, tissue factor, eicosanoids, PAF, and plasmingen activator inhibitor (Dejane et al. 1987; Rossi et al. 1985).

2.9 Comparison with IL-6

The most consistent correlations of clinical severity in inflammatory, autoimmune, or infectious disease with plasma cytokine levels are clearly those with IL-6, not IL-1 or TNF. The best correlation of plasma cytokine levels with mortality from septic shock has been found with IL-6 (Casey et al. 1993). IL-6 levels but not TNFα levels were found to predict fatal outcome in patients with septic shock (Fisher et al. 1993). A recent trial of anti-TNF monoclonal antibodies for the treatment of septic shock observed benefit (significant decrease in 28-day all-cause mortality) only in patients who entered the trial with elevated plasma IL-6 levels (Reinhart et al. 1995). Similar to other intervention studies, there was no correlation with benefit when patients were stratified according to their entry score on the Acute Physiologic and Chronic Health Evaluation.

Therefore, one can conclude that elevated levels of IL-6 in patients with septic shock represent the net effect of *biologically active* IL-1 and TNF. In other

words, due to natural inhibitors of IL-1 and TNF (see below) it is the biologically active concentrations of these cytokines which are best correlated with disease severity. It is nearly impossible to measure biologically active plasma IL-1 and TNF levels because plasma contains large concentrations of the IL-1 receptor antagonist (IL-1Ra) and soluble receptors for the two TNF receptors (TNFsRp55 and TNFsRp75; see below). Because of the presence of these inhibitors it is equally impossible to measure the synergistic effect of IL-1 and TNF plasma from patients with septic shock. However, one can use the surrogate marker of IL-6 levels which refect the net, biologically active concentrations of IL-1 and TNF in these patients. In patients with septic shock a 3-day infusion of IL-1Ra resulted in a dramatic, dose-dependent fall in IL-6 levels compared to patients given placebo (Fisher et al. 1994b).

It is important to emphasize, that unlike IL-1 and TNF, there is no evidence that IL-6 is itself an inflammatory cytokine. IL-6 does not induce PGE2 (Dinarello et al. 1991) but rather suppresses IL-1 inducible cyclo-oxygenase (Hauptmann et al. 1991). IL-6 does not cause shock in mice or primates regardless of the amount given, either alone or with TNF. In humans the intravenous administration of IL-6 at 30μg/kg does not produce hypotension whereas at 100 ng/kg IL-1 induces a fall in blood pressure in nearly all patients (Smith et al. 1992). IL-6 has been given to humans in very high concentrations (100μg/kg) without hypotension; the only sign was headache and fever. Thus human and animal experiments support the concept that IL-6 does not have a causal role in septic shock.

In some models the production of IL-6 appears to be under the strict control of IL-1 and TNF; for example, mice subjected to an inflammatory event induced by intramuscular turpentine fail to produce IL-6 when pretreated with anti-IL-1 receptor antibodies (Gershenwald et al. 1990). In baboons injected with *Escherichia coli* anti-TNF antibodies prevent the production of IL-6 in the circulation (Fong et al. 1989). Mice deficient in IL-1β do not produce IL-6 nor serum amyloid A proteins following an inflammatory event due to subcutaneous turpentine injection (Zheng et al. 1995). In vitro, blocking IL-1 receptors reduces the amount of endotoxin-stimulated IL-6 produced by human monocytes (Granowitz et al. 1992a, b). A reduction in IL-6 levels has also been observed in mice injected with LPS (Henricson et al. 1991).

3 Experimental Sepsis Affected by Reducting Production of IL-1 and TNF

3.1 Corticosteroids

Of the drugs that suppress the production of IL-1 and TNF the most studied are corticosteroids. Corticosteroids inhibit the transcription of IL-1, TNF, and nearly all cytokines, and hence they are not specific in this regard. There is also some

indication that steroids reduce the secretion of IL-1. In human volunteers injected with corticosteroids just prior to an intravenous injection of endotoxin there are reduced levels of circulating IL-1β, TNF, and IL-6 (Rock et al. 1992; Santos et al. 1993). These reductions in IL-1β, TNF, and IL-6 take place without suppressing IL-1Ra production (Santos et al. 1993). Relevant to these experiments are studies showing that IL-1 and TNF induce endogenous corticosteroids, and that adrenalectomized mice have a profound decrease in their resistance to IL-1 or TNF-mediated lethality (reviewed in Fantuzzi and Ghezzi 1993). Therefore some studies suggest that IL-1 and TNF-induced endogenous corticosteroid production acts as an intrinsic anti-inflammatory negative feedback mechanism.

3.2 Inhibition of Lipoxygenase

Inhibitors of 13-lipoxygenase (Schade et al. 1987) but not 5-lipoxygenase (Sirko et al. 1991) reduce TNFα and IL-1β transcription. Earlier studies had implicated as the lipoxygenase product triggering IL-1 and TNF synthesis. However, using specific inhibitors of 5-lipoxygenase, the importance of LTB4 in the production of cytokines is unlikely. Nevertheless, the role of lipoxygenase products in stimulating IL-1 and TNF production is supported by several controlled studies in humans consuming dietary supplements of eicosapentaenoic (ω-3) fatty acids or a diet rich in these fatty acids (see below). When ω-3 fatty acids are incorporated into cell membranes, the cyclo-oxygenase and lipoxygenase products following phopholipase-mediated hydrolysis of membrane phospholipids are not PGE2 and LTB4 but rather PGE3 and LTB5. This change alters signal transduction pathway induced by exogenous stimulants and results in an attenuation in the synthesis of proinflammatory cytokines.

3.3 Inhibition by Dietary ω-3 Fatty Acids

Several clinical trials have demonstrated a beneficial effect of dietary supplementation of ω-3 fatty acids (reviewed in Meydani and Dinarello 1993). Controlled studies have consistently shown a 50%–60% decrease in IL-1, IL-6, and TNF production in peripheral blood mononuclear cells (PBMC) of subjects ingesting ω-3 fatty acid supplements compared to PBMC taken before this dietary intervention (Endres et al. 1989b; Meydani et al. 1990) One study reported a selective decrease in cell content of IL-1β (Molvig et al. 1991). The phenomenon can also be demonstrated by measuring cytokine production in whole blood (Cooper et al. 1993). The source of the ω-3 fatty acid does not have to be high-dose dietary supplementation. In fact, a significant suppression of in vitro IL-1 and TNF production was observed in volunteers consuming low fat diets using fish as the primary source of protein (Meydani et al. 1993). When the major

source of protein was changed to nonfish foods (poultry), there was no reduction in cytokine production (MEYDANI et al. 1993).

3.4 Effect of Cyclo-oxygenase Inhibitors on the Production of IL-1 and TNF

Despite several studies there is no clear answer as to whether PGs suppress IL-1 production in cultured PBMC in vitro. This is probably due to the type of stimulant used and to the contribution of endogenous IL-1 production (IL-1 induced IL-1) to the total IL-1 synthesized (GRANOWITZ et al. 1992a). In general, adding cyclo-oxygenase inhibitors to LPS-stimulated PBMC can suppress, augment, or have no effect on IL-1 production. On the other hand, under the same culture conditions, LPS-induced TNF gene expression and synthesis is exquisitively sensitive to suppression by PGE2 and PGI2. In humans injected with LPS and pretreated with oral cyclo-oxygenase inhibitors the circulating levels of TNF and IL-6 are higher than in controls not given cyclo-oxygenase inhibitors (SPINAS et al. 1991). This observation is consistent with the mechanism that PGI2/PGE2-induced TNF suppression is via elevation in cAMP (ENDRES et al. 1991) and explains why drugs such as pentoxiphylline suppress TNF production. IL-1 induces cAMP via the intermediate induction of PGI2 (BEASLEY and MCGUIGGIN 1995). An increase in cAMP can also be accomplished by triggering the H_2 receptor on moncytes with histamine and without the intermediate production of PGE2 (VANNIER et al. 1991b).

In the absence of an external stimulus, raising intracellular levels of cAMP has a slight or no effect on IL-1β gene expressin (VANIER AND DINARELLO 1993). However, the secretion of meture IL-1β is suppressed by elevated PGE2; therefore cyclo-oxygenase inhibitors or IFNγ increases the secretion of IL-1β (SCHINDLER et al. 1990a). It is presently unclear whether this effect is due to decreased myristoylation (STEVENSON et al. 1992b, 1993), decreased IL-1β converting enzyme (ICE) activity or a reduction in the secretory ("channel") pathway proposed for IL-1β (SINGER et al. 1993). In contrast to PBMC stimulated with LPS, when these cells are stimulated with IL-1, cyclo-oxgenase inhibitors suppress IL-1 and IL-6 production. To confirm this observation the addition of either exogenous PGE2 or histamine to cell cultures increase intracellular cAMP and results in enhanced IL-1-induced IL-1, IL-6, and IL-8 production (VANNIER and DINARELLO 1993, 1994; VANNIER et al. 1991a). In terms of noninfectious diseases cyclo-oxygenase inhibitors likely reduce IL-1-induced cytokine production. One can conclude that IL-1 induced cytokine synthesis is positively influenced by prostanoid-induced cAMP, but that LPS-induced cytokines are either unaffected or slightly negatively influenced. The effect of prostanoids on TNF production is, however, clearly negative.

3.5 Inhibition of IL-1 and TNF Processing Enzymes

Studies on the production of IL-1β and TNFα have established that synthesis of the precursors of each cytokine and release into the extracellular compartment are distinct events. IL-1β is unique in that the precursor lacking a leader sequence is barely active and remains in the cytosol until cleavage and release. TNFα has a weak leader sequence, appears to be associated with the Golgi, and exists in a cell membrane form before being cleaved and released (KRIEGLER et al. 1988). The precursors for IL-1β (STEVENSON et al. 1993) and TNFα (STEVENSON et al. 1992a) undergo myristoylation on lysines, which is thought to contribute to membrane localization. Although the primary N-terminal amino acids for extracellular IL-1β and TNFα have been known for several years, the way in which the respective precursors are cleaved and transported out of the cell was poorly understood.

ICE is a constitutively produced intracellular cysteine protease which appears to be the sole enzyme for cleaving precursor IL-1β between aspartic acid (116) and alanine (117). ICE is stored in cells in an inactive form but becomes enzymatically active by the same cell stimuli which induce the synthesis of IL-1β. Substrate inhibitors of ICE have been used to prevent cleavage of the 31-KDa precursor IL-1β and release of mature 17-Kda in human blood and animal monocytes (CHIN and KOSTURA 1993). Reduction in inflammatory disease activity also takes place when ICE is inhibited.

Serine proteases cleave the 26-Kda TNFα precursor between alanine (76) and valine (77), yielding the 17-Kda mature TNFα (McGEEHAN et al. 1994; MOHLER et al. 1994). Unlike ICE, a putative, specific TNFα-converting enzyme is presently unknown, although it appears to be in the general class of metalloproteinases with a zinc binding motif of HEXGH (GEARING et al. 1994; McGEEHAN et al. 1994). In vitro, metalloproteinase inhibitors and zinc chelators suppress the processing of TNFα from human blood monocytes and murine macrophages but do not affect the release of lymphotoxin-α from T-lymphocytes or the release of other cytokines (GEARING et al. 1994; McGEEHAN et al. 1994; MOHLER et al. 1994). These metalloproteinsase inhibitors do not affect the production of IL-1β or IL-6 in whole human blood incubated with LPS (GEARING et al. 1994). Even membrane-associated cytokines such as macrophage colony-stimulating factor and transforming growth factor (TGF)α were unaffected. The proteinase inhibitors used in these studies are not specific since they can cleave other proteins. However, when administered to rats or mice, these metalloproteinase inhibitors reduce circulating levels of LPS-induced TNFα (GEARING et al. 1994; MOHLER et al. 1994).

Other examples of cell-associated cytokines requiring a proteolytic cleavage step include macrophage colony-stimulating factor, TGFα and endothelin-1. Of relevance to inflammation, the precursor of IL-1α is vulnerable to cleavage by a calcium-dependent, membrane-associated serine protease (calpain; KOBAYASHI et al. 1990, 1991; WATANABE and KOBAYASHI 1994). However, unlike TNFα and IL-1β, IL-1α is primarily an intracellular molecule; precursor IL-1α is active as a mem-

brane form and requires cell-cell contact for biological activity (KAPLANSKI et al. 1994). Processing and release of IL-1α requires the special circumstance of activating calpain using calcium ionophores or stimulating cells with very large numbers of bacteria. Ordinarily LPS or cytokine stimulation of human moncytes is accompained by the spontaneous release of IL-1β and TNFα but not of IL-1α (ENDRES et al. 1989a; LONNEMANN et al. 1989; SCHINDLER et al. 1990b).

Do these cytokine cleaving proteases play a critical role in the progression of inflammation? In ICE-deficient mice there is prolonged survival following a lethal challenge of endotoxin and decrease in vitro release of IL-1α (LI et al. 1995). In another strain of mice deficient in ICE a reduction in IL-1α, TNF, and IL-6 production was observed (KUIDA et al. 1995). There was a reduction in the near 100% lethality of mice pretreated with one of the TNFα-processing inhibitors before receiving galactosamine and challenged with LPS (MOHLER et al. 1994). Figure 1 summarizes the processing enzymes for IL-1β, IL-1α, and TNFα.

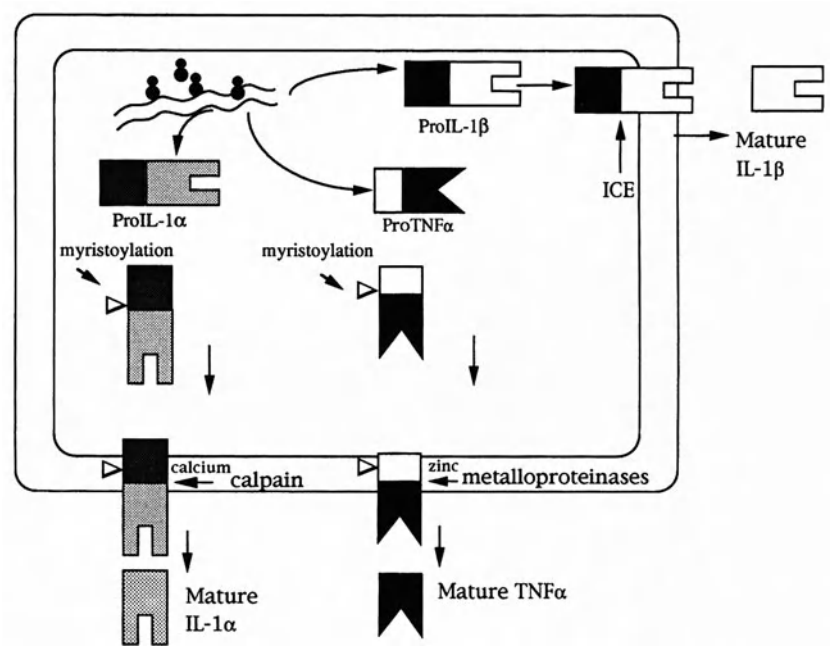

Fig. 1. Processing of IL-1β, IL-1α, and TNFα by proteases. The 31-kDa precusor for IL-1β (*proIL-1β*) is initially translated and found in the cytosol. It is transported to the vicinity of the cell membrane where the intracellular IL-1β converting enzyme (*ICE*) cleaves proIL-1β and the mature (17-kDa) is secreted into the extracellular space. ICE must be activated before it can cleave proIL-1β. Precursor IL-1α (*proIL-1α*) is also found in the cytosol. It is myristoylated and inserted into the cell membrane. The cell membrane associated calpains are activated by calcium and cleave proIL-1α. The mature (17-kDa) IL-1α is then secreted into the extracellular space. The precursor for TNFα is also myristoylated and found in the cell membrane. Zinc-dependent metalloproteinases cleave membrane-bound TNFα, and mature 17-kDa TNFα is released

3.6 Cytokine-Suppressing Anti-inflammatory Drugs

Recent studies have taken advantage of pyridinyl-imidazol compounds which block the synthesis of IL-1β and TNFα without affecting transcription or their steady-state levels of mRNA. mRNA levels for either IL-1 or TNFα in PBMC stimulated with LPS in the presence of these compounds are indistinguishable from those in PBMC stimulated with C5a, hypoxia, or adherence. In other words, there is ample cytokine mRNA but no cytokine protein. These are interesting compounds which have entered clinical medicine. Some are cyclo-oxygenase/lipoxygenase inhibitors because, by classification, they block these enzymes. Hence they are often called "dual inhibitors." However, their mechanism of action in suppressing IL-1 and TNF have never been linked to their ability to suppress cyclo-oxygenase/lipoxygenase (Sirko et al. 1991). These and other imidazol-like drugs have recently been called "cytokine-suppressing anti-inflammatory drugs" or CSAIDs (Young et al. 1994).

Two agents have received careful study for their ability not to affect transcription but rather to inhibit translation (Olivera et al. 1993; RordorfAdam et al. 1994). Recent studies suggest that the mechanism by which CSAIDs reduce IL-1 and TNF translations their ability to bind to and inactivate two related mitogen-activating protein (MAP) kinases (Lee et al. 1994). As with most MAP kinases, the novel kinases are serine-threonine kinases. These kinases phosphorylate proteins are required for translation of cytokine mRNAs into their respective proteins (Lee et al. 1994). These MAP kinases also have the same nucleotide sequences as those of the IL-1 and TNF signal-associated MAP kinase p38 (Galcheva-Gargova et al. 1994; Han et al. 1994). The p38 MAP kinase is a homologue of the yeast *HOG-1* gene. The cytokine synthesis inhibiting drugs bind and inactivate these MAP kinases in cells stimulated with LPS or hyperosmolarity (Galcheva-Gargova et al. 1994; Han et al. 1994). A *HOG-1* gene related p38 MAP kinase is part of the IL-1 and TNF signal transduction phosphorylation cascade (Freshney et al. 1994; Kracht et al. 1994). Recently it has been shown that the initiation factor eIF-4E requires a MAP-like phosphorylation step in order to dissociate from PHAS-I, a translational regulatory molecule (Lin et al. 1994). Although speculative, an IL-1, LPS, or TNF-directed MAP kinase phosphorylation of PHAS-I may be related to the MAP kinases inhibited by CSAIDs.

4 The Anti-inflammatory Cytokine Network in Sepsis

In most situations cytokine responses are self-limiting. In some patients sepsis is also a limited event, even without the intervention of antibiotics. Several mediators of the inflammatory response contribute to the downregulation of

cytokine production and activity. For example, PGE2 suppresses a variety of immune responses such as IL-2 production. On the other hand, in some infectious diseases inflammation persists because the infectious agent is still present, and cytokine production persists. Proinflammatory cytokines, which are produced in response to an infection, in turn induce the synthesis of other cytokines which have the ability to suppress the inducing cytokine. For example, IL-1 stimulates a cascade of inflammatory mediators as well as a cascade of cytokines which act to suppress further production of IL-1. This is simple negative feedback, a well-established biological process. Only recently has the extent and complexity of the "anti-inflammatory cytokine" network been investigated. The evidence that "anti-inflammatory cytokines" do in fact play a role in downregulating inflammation comes from (a) experiments in which neutralizing antibodies to certain cytokines worsen the inflammation and (b) gene deletion studies in mice reveal a role for a particular cytokine as an anti-inflammatory agent. For example, antibodies to IL-1Ra worsen colitis (Ferretti et al. 1994) and mice deficient in IL-10 develop spontaneous inflammatory bowel disease (Moore et al. 1993).

4.1 IL-4, IL-10, IL-13, and TGFβ

IL-4, IL-10, IL-13, and TGFβ each suppress gene expression and synthesis of IL-1, TNF, and other cytokines. In vitro, these cytokines can reduce endotoxin-induced gene expression and synthesis of IL-1 and TNF as much 90%, and when given to mice or rats, can reduce lethal endotoxemia. As such they are potentially useful in some clinical situations. IL-10 appears to be particularly useful because, unlike IL-4 and TGFβ, IL-10 has no clinical side effects. A randomized, double-blind, placebo-controlled trial (phase I) in healthy human volunteers demonstrated the absence of clinical toxicity and also studied the effect of a single intravenous injection of IL-10 on cytokine production (Chernoff et al. 1995). Blood was removed before and 3, 6, 24, and 48 h after the injection and incubated in vitro with endotoxin, and the amounts of IL-1β, TNFα, IL-6, IL-8, IL-1Ra, and TNF soluble receptor p55 (TNFsRp55) were measured. At doses of 10 and 25 μg/kg there was a 90% reduction in IL-1β, TNFα, and IL-6 production in blood taken 3 and 6 h after the injection; at 25μg/kg a 50% reduction IL-1β, TNFα, and IL-6 production was present after 24 and 48 h. In contrast, there was no suppression of IL-1Ra or TNFsRp55.

A 40%–60% reduction in circulating lymphocytes expressing CD4, CD8, and CD3 was observed after infusion of IL-10 (Chernoff et al. 1995). The proliferative response to the mitogen phytohemagglutinin was suppressed in PBMC from volunteers given 10 or 25 μg/kg IL-10 and was not reversed by using higher concentrations of the mitogen. It is unclear whether the suppression of lymphocyte responses was due to low numbers of CD3 cells or to an intrinsic reduction in response to a proliferative signal. Although CD2 is the major receptor for mitogenic responses, CD2-bearing lymphocytes did not change fol-

lowing IL-10 (Chernoff et al. 1995). These human studies confirm the in vitro effects of IL-10 and suggest that IL-10 may be useful in suppressing inflammatory cytokine production in selected diseases.

IL-4 and IL-13 also suppress LPS -induced IL-1 and TNF gene expression and synthesis. In addition, they increase IL-1Ra production (Vannier et al. 1992). IL–4 and IL-13 share the same receptor complex on monocytes, and hence similar biological effects for both cytokines are often observed. There are, however, few if any receptors for IL-13 on T-lymphocytes, and hence the immunological suppressive effects of IL-4 and IL-10 are not observed for IL-13. Similarly to IL-4 –IL-10, and IL-13, TGFβ suppress gene expression and synthesis of IL-1 and TNF and also increase IL-1Ra production (Chantry et al. 1989). However, TGFβ, which has profound immunosuppressive effects, is a growth factor for normal and neoplastic cells.

4.2 IL-6 and Other Ligands for the gp130 Signal Transducer

In mice LPS induces higher circulating levels of TNF in mice deficient in producing IL-6 than in wild-strain mice (Fattori et al. 1994). This experiment suggests that IL-6 production during systemic inflammation downregulates TNF production, and that IL-6 functions as an anti-inflammatory cytokine. Other evidence supports the view that IL-6 may be a naturally occurring anti-inflammatory cytokine. Infusions of IL-6 into humans results in high levels of IL-1Ra and high levels of TNFsRp55 (Tilg et al. 1994a). In vitro, IL-6 suppresses LPS - and TNF-induced IL-1 production (Aderka et al. 1989; Schindler et al. 1990b). The ability of IL-6 to function as an anti-inflammatory molecule is consistent with it being part of the cytokine family, which use the gp130 signal apparatus. IL-6 shares its signaling event with leukemia-inhibitory factor (LIF), ciliary neurotropic factor (CNTF), IL-11, oncostatin M, and cardiotropin. CNTF suppress IL-1 stimulated IL-8 and PGE2 synthesis (Shapiro et al. 1994). In vivo, IL-6 stimulates the release of corticosteroids and synthesis of the full spectrum of acute-phase proteins, including many antiproteases. One interpretation of the biological significance of IL-6 is the anti-inflammatory property of these anti-proteases (Tilg et al. 1994a).

A specific receptor for IL-6 binds the ligand and the IL-6/IL-6R complex triggers homodimerization of gp130. Recent studies indicate that in various animal models of sepsis and inflammation the adminstration of IL-11 or LIF reduces the severity of disease (Waring et al. 1995). Similarly to IL-6, CNTF binds to its specific soluble receptor and triggers dimerization of gp130 (with the LIF receptor). When incubated with human blood monocytes, the CNTF/soluble receptor complex supresses LPS-induced PGE2 and IL-1α synthesis (Shapiro et al. 1994). In addition, the CNTF/soluble receptor complex suppresses IL-1α-induced IL-8 and PGE2 synthesis (Shapiro et al. 1994). In general, inhibition is 50%, and it is unclear whether this is at the transcriptional or posttranscriptional level. The anti-inflammatory effects of LIF in some cells are similar to those of IL-6 and CNTF since they use the same gp130 signal transduction pathways. However,

for LIF the signaling through gp130 does not require a soluble receptor since dimerization occurs between gp130 and the LIFβ receptor.

The above studies suggest that signaling of gp130 may provide an insight into the mechanism of inhibiting IL-1 mediated events. Following dimerization of gp130, tyrosine phosphorylation, and translocation of p91 a well-known nuclear factor (NF) associated with the induction of acute-phase proteins in the liver, appears . p91 is one of three NFs making a complex which initiates gene expression. Several acute phase proteins preferentially induce IL-1Ra over IL-1 in vitro (Tilg et al. 1993). In hepatocytes the same signal that induces hepatic acute-phase protein gene expression also inhibits transcription of albumin and cytochrome p450. Therefore this pathway, although studied for increased gene expression of hepatic proteins, also leads to events which decrease gene expression of IL-1 inducible genes. Therefore a concept has developed that IL-6 and related gp130 signaling cytokines lead to an anti-inflammatory state to counter the proinflammatory state initiated by IL-1 and TNF (Tilg et al. 1993).

4.3 Interferons

IFNγ is not part of the circulating cytokine profile in humans injected with LPS. IFNγ has been shown to increase receptors for TNF and thus can act to enhance the biological effects of TNF. The role of IFNγ in septic shock has been reviewed by Billiau and Vandekerckhove (1991). No clear role for IFNγ emerges from these studies. However, in some clinical situations IFNs are thought to exert their effects by acting as anti-inflammatory agents. Early studies showed that INFγ suppresses LPS or IL-1 induced PGE2 production in human monocytes. Although it is well-known that IFNγ increases IL-1 and TNF production induced by LPS (Ucla et al. 1990), using IL-1 as a stimulant, IFNγ suppresses IL-1 induced IL-1 (Ghezzi and Dinarello 1988)> Subsequently, it was shown that IFNα also suppresses IL-1-induced IL-1 synthesis due to reduced transcription (Schindler et al. 1990a). Similarly to CNTF (Shapiro et al. 1994), suppression of IL-1 induced IL-8 transcription by IFNγ has been reported to be mediated by NFκB (Oliveira et al. 1994). Of considerable importance is the finding that tyrosine phosphorylations following triggering of IFN receptors result in a kinase cascade similar to those described for gp130. Thus, depending on the signal, the phosphorylation of p91 can lead to suppression of IL-1 induced gene expression.

5 Naturally Occurring Inhibitors of IL-1

5.1 IL-1 Receptor Antagonist

IL-1Ra is produced primarily from macrophagic cells as a 22-kDa glycosylated protein. Unlike IL-1α and IL-1β, which lack a leader peptide, IL-1Ra possesses a leader sequence and is synthesized, processed, and secreted from the cell (reviewed in AREND 1993). The three-dimensional structure of each member of the IL-1 family (IL-1β, IL-1α, and IL-1Ra) is comprised of all β strands, and each member binds to the same IL-1 type I receptors (IL-1RI). However, IL-1Ra binds to IL-1RI with virtually the same affinity as IL-1α or IL-1β but does not trigger a response (DRIPPS et al. 1991). The cytokine is thus the naturally occurring inhibitor or IL-1. IL-1Ra has nearly the same affinity (approx. 200 pM) for the IL-1RI as that of human IL-1α and IL-1β (EISENBERG et al. 1990; HANNUM et al. 1990; SECKINGER et al. 1987).

In animal studies the administration of IL-1Ra reveals that IL-1 plays an important role in the pathogenesis of inflammatory and immunologically mediated disease, including animal models of septic shock. Many studies report a reduction in severity or mortality of at least 50%, but in many the amelioration of pathological changes is complete. One consistent observation is the reduction in the number of infiltrating neutrophils associated with local inflammation, and this effect of IL-1Ra may be due to preventing IL-1 induced synthesis of cell adhesion molecules, PAF, IL-8, and related chemokines. Only a few IL-1RI need be occupied to trigger a biological response, and therefore it is necessary to sustain a high level of IL-1Ra to block unoccupied receptors. When exogenous IL-1Ra is injected into animals, high plasma levels (10–20 μg/ml) are needed before one observes a reduction in disease. In humans similar levels of IL-1Ra are needed to block the hematological response to LPS (GRANOWITZ et al. 1993).

5.2 IL-1Ra Production in Sepsis

It is not unusual to measure high and more sustained levels of IL-1Ra than IL-1β in patients with septic shock (FISCHER et al. 1992). For example, in health volunteers injected intravenously with a low dose of *E. coli* endotoxin circulating IL-1Ra levels are at a 100-fold molar excess (peak level of 6000–7000 pg/ml) to those of IL-1β (70–80 pg/ml) and are significantly elevated above the baseline levels for over 24 h (GRANOWITZ et al. 1991). In patients with septic shock, juvenile rheumatoid arthritis, or inflammatory bowel disease a similar ratio can be observed, and elevated IL-1Ra levels may be correlated with the severity of disease (HYAMS et al. 1994). In patients with thermal burns the levels of IL-1Ra are correlated with the burn surface area, and the highest levels of IL-1Ra have been measured in non-survivors (MANDRUP-POULSEN et al. 1995). The intravenous injection of 30 ng/kg of IL-1α into humans induces 25–30 ng/ml of IL-1Ra (TILG et

al. 1994b), which is fourfold higher than that induced by LPS. Injection of IL-1β into humans results in an 86-fold increase in plasma IL-1Ra after 1 h (BARGETZI et al. 1993). In humans, endogenous TNF production during endotoxemia contributes to IL-1Ra production (VAN DER POLL et al. 1994).

In patients with acute Lyme arthritis the duration of joint inflammation is shortest in those patients with the highest joint fluid levels of IL-1Ra whereas it is prolonged in those with low levels of IL-1Ra (MILLER et al. 1993). Is the amount of IL-1Ra produced in disease sufficient to dampen the response to IL-1? Using specific, neutralizing antibodies to mouse IL-1Ra, an increase in the formation of SCHISTOSOMA egg granulomata was observed when endogenous IL-1Ra was neutralized (CHENSUE et al. 1993). In rabbits with immune complex colitis the infusion of a neutralizing antibody to rabbit IL-1Ra resulted in exacerbation and prolongation of the colitis (FERRETTI et al. 1994). At the present, the phenotype of an IL-1Ra deficient mouse is unknown, but neutralizing endogenous IL-1Ra appears to worsen inflammations.

5.3 IL-1Ra in Experimental Endotoxemia in Humans

IL-1Ra given intravenously to healthy volunteers is without side effects or changes in biochemical, hematological and endocrinological parameters, even when peak blood levels reach 30 μg/ml and are sustained above 10 μg/ml for several hours (GRANOWITZ et al. 1992b). To evaluate the effect of IL-1 receptor blockade on clinical disease under controlled experimental conditions, healthy volunteers were challenged with intravenous endotoxin and administered an infusion of 10 mg/kg IL-1Ra at the same time. There was no effect on endotoxin-induced fever, although blood levels of IL-1Ra were not significantly elevated until 1 h after the bolus injection of endotoxin. In animal studies peripheral endotoxin induces fever by triggering IL-1 induction of IL-6 synthesis in the central nervous system (LEMAY et al. 1990). Since IL-1Ra does not cross the blood-brain barrier, this may account for the inability of IL-1Ra to diminish endotoxin fever (DINARELLO et al. 1992). However, there was a 50% reduction in the endotoxin-induced neutrophilia and a reduction in the circulating levels of granulocyte colony-stimulating factor compared to subjects injected with endotoxin plus saline (GRANOWITZ et al. 1993).

Endotoxin injection suppresses the mitogen-induced proliferative response of PBMC in vitro. However, in volunteers injected with endotoxin plus IL-1Ra there was no suppression of the response (GRANOWITZ et al. 1993). Mitogen- and antigen-induced proliferation is a well-established parameter of immunocompetence and is associated with decreased production of IL-2. Similarly to experimental endotoxin injection, this suppression is observed in patients with multiple trauma, sepsis, and cardiopulmonary bypass. In experimental endotoxemia and the above clinical conditions, treatment with cyclo-oxygenase inhibitors restores these cell-mediated immune responses (MARKEWITZ et al. 1993). This effect of cyclo-oxygenase inhibitors is consistent with the well-

known suppressive effects of PGE2 on IL-2 production and T-cell proliferation. Since IL-1 is a potent inducer of COX-2, it is not surprising that blocking IL-1 receptors during endotoxemia reduces IL-1 induced PGE2 production during endotoxemia. Thus, the studies establish that under conditions of low-dose endotoxemia it is possible to block IL-1 mediated responses with IL-1Ra. Those host response parameters which are unaffected by IL-1Ra are likely due to other cytokines such as TNF or IL-6 or the combination of these cytokines with IL-1.

5.4 Clinical Trials of IL-1Ra in Septic Shock

IL-1Ra has been in given to patients with septic shock. The initial (phase II) trial was a randomized, placebo-controlled, open-label study in 99 patients. Patients received either placebo or a loading bolus of 100 mg followed by a 3-day infusion of 17, 67, or 133 mg/h IL-1Ra (Fisher et al. 1994b). A dose-dependent improvement in 28-day mortality was observed; mortality was reduced from 44% in the placebo group to 16% in the group receiving the highest dose of IL-1Ra (p=0.015). In this study, there was a dose-related fall in the circulating levels of IL-6 24 h after the initiation of IL-1Ra infusion. This fall is consistent with the well-established control of circulating IL-6 levels by IL-1 (Fischer et al. 1991; Gershenwald et al. 1990) and the correlation of disease severity and outcome with IL-6 levels (Casey et al. 1993). The mean plasma level of IL-1Ra was 25–28 μg/ml in the high-dose group, and this order of magnitude of curculating IL-1Ra concentration is measured in animals which benefit from IL-1Ra during experimental shock (Aiura et al. 1993).

A large phase III trial in 893 patients revealed a trend but without a statistically significant reduction in 28-day mortality (Fisher et al. 1994b). However, a retrospective analysis of 563 patients with a predicted risk of mortality of 24% or greater (Knaus et al. 1993) revealed a significant reduction in 28-day mortality (45% in the placebo group and 35% in patients receiving 2 mg/kg per hour for 72 h, p=0.005; Fisher et al. 1994b). Similar improvement was observed when patients were scored on the basis on organ failure at entry. Circulating levels of thromboxane B2, PGI2, LTC4, LTD4, and LTE4 were attenuated (p<0.05) at 72h in patients receiving the high dose of IL-1Ra whereas in patients receiving the placebo these eicosanoids were increased at 72 h (Friedman et al. 1994). A second phase III trial using 10 g IL-1Ra infused over 3 days was undertaken but terminated during an interim analysis because a reduction in overall 28-day mortality would not likely reach statistical significance. Similarly to other trials in septic shock using strategies of cytokine or endotoxin blockade, the efficacy of IL-1Ra in reducing 28-day all-cause mortality is not easily demonstrated despite the impressive results in animal models of septic shock and death. Patient heterogeneity versus animal homogeneity is thought to contribute to a failure to bridge the gap between animal and clincal data in sepsis.

5.5 Soluble IL-1 Receptors

The extracellular domains of the type I and type II receptors are found in the circulation of healthy humans and are further elevated under conditions of infection and inflammation. The concentration of soluble type I receptor (IL-1sRI) in health is approximately 50–100 pM (Arend et al. 1994; Giri et al. 1994) whereas the concentration of the soluble form of the type II receptor (IL-1sRII) in health is approximately 100–200 p*M* (Arend et al. 1994; Orencole et al. 1994). Therefore there is a rather high level of IL-1 soluble receptors present in health, and their circulating concentrations are certainly at two-threefold molar excesses of the concentrations of circulating IL-1 produced in severe disease. The tissue levels (interstitial fluid) of these receptors are unknown, and therefore the degree to which they reduce the response to locally produced IL-1 is presently unclear.

IL-1sRI has been used in several models of inflammatory and autoimmune disease. Administration of murine IL-1sRI to mice has increased the survival of heterotopic heart allografts and reduced the hyperplasic lymph node response to allogeneic cells (Fanslow et al. 1990). In a rat model of antigen-induced arthritis the local instillation of the murine IL-1sRI reduced joint swelling and tissue destruction (Dower et al. 1994). When a dose of soluble receptor (1 μg) was instilled into the contralateral, unaffected joint, a reduction in the degree of tissue damage was observed in the affected joint. These data suggest that the amount of IL-1sRI given in the normal, contralateral joint acted systemically. In a model of experimental autoimmune encephalitits the IL-1sRI reduced the severity of this disease (Jacobs et al. 1991). Administration of IL-1sRI to animals has also been reported to reduce the physiological response to LPS, acute lung injury, and delayed-type hypersensitivity (Dower et al. 1994). To date there are no data on the efficacy of in animal models of disease.

5.6 Soluble IL-1 Receptor Type I in Human Studies

Recombinant human IL-1sRI has been administered intravenously to healthy humans in a phase I trial without side effects or changes in physiological, hematological, or endocrinological parameters. Thus, similarly to infusions of IL-1Ra, IL-1sRI appears safe and reinforces the conclusion that IL-1 does not have a role in homeostasis in humans. Administration of IL-1sRI in humans has been effective in reducing the delayed hypersensitivity skin reaction to recall antigens. Patients with known antigen hypersensitivity received an intradermal injection of a specific antigen and also an injection of either placebo or IL-1sRI near the site. In addition, IL-1sRI was injected locally at a contralateral site. As the amount of IL-1sRI increased, there was a progressive decrease in the inflammatory lesion due to the allergen. However, there was also a decrease in the lesion size as the amount of IL-1sRI injected at the contralateral site was increased to 10–100 μg (Dower et al. 1994). These findings are similar to observations in rats receiving a contralateral instillation of IL-1sRI during antigen-induced arthritis.

6 Reducing the Activity of TNF

6.1 Soluble TNF Receptors

Unlike IL-1, a naturally occurring receptor antagonist to TNF has not been found. However, soluble receptors to TNF are present in the circulation of healthy humans and may act as naturally occurring inhibitors of TNF activity. The situation is similar to that of soluble IL-1 receptors. There are two cell surface TNF receptors: p55 and p75 (Engelmann et al. 1989, 1990a,b; Olsson et al. 1993). The extracellular domains of each TNF receptor are shed from the cell surface by a serine protease associated with cell activation (Brakebusch et al. 1994) and are found in the circulation of healthy humans. The concentration of the p75 is approximately 300 p*M* and is threefold greater than that of the p55 form (Chikanza et al. 1993; Girardin et al. 1992). However, increases in the circulating levels of soluble receptors to TNF during disease states appears greater than that for suluble IL-1 receptors. For example, endotoxemia induces the release of both TNF receptors into the circulation and the increase is several-fold that of the concentration in healthy subjects (Shapiro et al. 1993; van Zee et al. 1992). TNF itself induces the release of its soluble receptors (Jansen et al. 1995). Soluble TNF receptors are also elevated in patients with cancer, and the levels are correlated with the tumor burden or extent of the metastases (Aderka et al 1991). Other studies have documented the presence of soluble TNF receptors in the circulation or joint fluid in a variety of autoimmune and inflammatory diseases.

The soluble receptors for IL-1 inhibit the action of IL-1 in a dose dependent fashion. In contrast, soluble TNF receptors can act as "carriers" of TNF in certain experimental models. This phenomenon was first shown by adding increasing amounts of soluble TNF receptors to cells exposed to TNF. The biological acitivity of TNF was enhanced at low molar ratios of receptor to ligand (Aderka et al. 1992). At higher molar ratios of soluble receptor to TNF the activity was decreased, and there was dose-dependent inhibition of TNF activity (Aderka et al. 1992). Therefore at low molar ratios, the soluble TNF receptors protect the TNF from degradation and destabilization. One likely mechanism for the stabilization of TNF by the soluble receptors is to maintain the trimer structure of TNF since monomeric TNF is biologically inactive. The amount of natural inhibition or natural "stabilization" of TNF by the soluble receptors during inflammation is unclear. Mice deficient in the the p55 TNF receptor do not manifest increased susceptibility to infection or inflammation. However, the p75 receptor is thought to function as the natural carrier of TNF compared to the p55 receptor.

In several animal models of sepsis and inflammatory disease the administration of recombinant forms of soluble p55 TNF receptor has reduced inflammation or prolonged survival (Bertini et al. 1993; Lesslauer et al. 1991a,b; Porat et al. 1995; van Zee et al. 1992). The subject has recently been reviewed (Olsson et al. 1993). Although a chimeric receptor to TNF p75 has been observed

in human sepsis, the survival at higher doses was without benefit (reviewed in Opal 1996). This may be due to prolongation of the half-life of TNF due to trimer stabilization. In general, clinical trials for soluble forms of the p55 TNF receptor are presently underway for sepsis, inflammatory bowel disease, and rheumatoid arthritis. The use of soluble forms of the p55 TNF receptor in these clinical situations is based upon the beneficial effects of monoclonal antibodies to TNF in several trials. Therefore it is anticipated that soluble forms of the p55 TNF receptor will be used to treat acute and chronic graft rejection, sepsis, graft versus host disease, and a variety of inflammatory diseases.

6.2 Neutralizing Antibodies to TNF

From a historical viewpoint, the first experiment which implicated the importance of endogenous cytokines in the pathogenesis of septic shock was the demonstration that neutralizing antibodies to TNF (also called cachetin) reduce the lethality of LPS in mice (Beutler et al. 1985). This study was then expanded to primates and employed live *E. coli* organisms (Tracey et al. 1987) and in rabbits using LPS (Mathison et al. 1988). A protective role was again observed. Furthermore, anti-TNF antibodies had a dramatic effect in reducing the circulating levels of IL-1 and IL-6 (Fong et al. 1989). Following these studies many reports confirmed a role for TNF in the lethality of endotoxin shock in animals. Only a few studies, namely those using cecal ligation as a model, have shown that anti-TNF antibodies do not affect outcome. Nevertheless, animal studies formed the basis for using anti-TNF antibodies in humans with septic shock. Clinical trials have yielded mixed results, not dissimilar to those reported in patients treated with IL-1Ra.

A large, randomized, placebo-controlled, double-blind multicenter study of a murine anti-human TNFα monoclonal antibody in 971 patients with the sepsis syndrome (Abraham et al. 1995). There were two doses of anti-human TNFα, a single infusion of 7.5 mg/kg or one of 15 mg/kg. There was no overall benefit in 28-day all-cause mortality in patients receiving the antibody. However, in a subset of 478 patients who had septic shock upon entry into the study there was reduction in 3-day all-cause mortality compared to matched placebo controls (44% reduction at 15 mg/kg, p=0.01; 48% reduction at 7.5 mg/kg, p=0.004). At 28 days there were no differences in mortality in patients treated with the different doses of the antibody. Similar results have been reported in smaller studies (Fisher et al. 1993). Short-term benefits on left ventricular function have been observed in patients with septic shock treated with anti-TNF (Vincent et al. 1992).

Why do these trials fail to show overall efficacy in reducing the mortality in these patients? The topic of heterogeneity of patients as well as that of disease causation has been used to explain these results (Opal 1995). Is there anything wrong with the concept that blocking (or reducing) TNF activity or production should reduce the mortality of septic shock, as has been observed in the vast

majority of animal studies? The best test for efficacy an anti-TNF in terms of patient heterogeneity are studies in patients with rheumatoid arthritis. This patient group is not as heterogeneous as the group with septic shock. In a multicenter, placebo-controlled study monclonal anti-TNF reduced the both severity and the biochemical markers of rheumatoid arthritis (ELLIOTT et al. 1994).

Therefore one may conclude that for patients with septic shock to show a benefit with anti-TNF treatment a better set of entry criteria need to be selected. Using plasma IL-6 levels as the surrogate marker of biologically active TNF (and IL-1) may provide a better selection criterion (REINHART et al. 1995). An alternate approach is to use patients in which the underlining disease does not contribute to 28-day mortality other than that due to the sepsis episode. This approach, however, will require a far greater amount of time to complete a large pivotal study.

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Tumor Necrosis Factor and the Systemic Inflammatory Response Syndrome

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1 Introduction

Tumor necrosis factor (TNF) was discovered in the middle 1970s by the research group of Old (Carswell et al. 1975; Old 1988) as the result of a long search for the mechanisms underlying endotoxin-induced antitumor effects. However, it was only after the cloning of the genes for TNF in 1984–1985 (Pennica et al. 1984; Wang et al. 1985; Fransen et al. 1985; Marmenout et al. 1985; Shirai et al. 1985) that sufficient amounts of pure material became available for detailed investigation of its effects. Studies in a syngeneic murine melanoma model confirmed the antitumor effectiveness of TNF, especially in combination with interferon (IFN)-γ, but also showed that when the homologous murine (m) TNF is used, the effective dose is close to the lethal dose in systemic administrations

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(Brouckaert et al. 1986). Subsequent clinical trials have largely confirmed the major conclusions drawn from those preclinical experiments. When locoregional treatments such as the isolated limb perfusion technique are used for treatment of melanoma, a rise in the complete response rate from 50% to 80%–100% is observed when TNF and IFN-γ are added to the classical treatment with melphalan (Lejeune et al. 1994). Also in the case of sarcomas a 91% limb salvage rate has been obtained (Eggermont et al. 1993). Systemic treatments with these cytokines, however, are still impossible due to the severe systemic inflammatory response syndrome (SIRS) that occurs when even subtherapeutic doses of TNF are administered. Hypotension and hepatotoxicity are the most prominent dose-limiting toxicities observed in clinical trials (Fraker et al. 1995). This chapter discusses only the role of TNF as an inducer and mediator of SIRS. Current knowledge regarding the antitumor effects of TNF has recently been reviewed elsewhere (Brouckaert et al. 1993a, 1994; Fiers 1995).

The role of TNF as a mediator of endotoxic shock was first described by Beutler et al. (1985a,b) and Tracey et al. (1986), who cloned TNF as the result of their search for a cachexia-inducing factor. Subsequent studies in animals, patients, and human volunteers have yielded a large body of evidence for such a role of TNF. This has been reviewed extensively (e.g., Tracey and Cerami 1993; Beutler and Grau 1993). This chapter focuses on observations that question the early paradigm that TNF is both a necessary and a sufficient mediator of sepsis. In fact, since 1985 quite a number of studies have shown that the outcome of a challenge with TNF is highly dependent on the presence or absence of other factors. More recent results obtained in transgenic TNF receptor knockout mice even raise the question of whether TNF is indeed a necessary and important factor in endotoxic shock.

The data that form the basis for our knowledge of the relationship between TNF and SIRS originate mainly from two distinct research areas: the development of TNF as an anticancer agent and the development of TNF inhibitors as a treatment for sepsis. To understand the approaches taken in different studies it is important to note some major differences between the two fields. When administering TNF as an anticancer drug, it is (at least theoretically) possible to avoid synergistic factors. Moreover, drugs that are active only when administered before TNF remain very valuable and useful, and this is in contrast to the situation in sepsis. On the other hand, inhibitors that completely destroy the activity of TNF might be applicable in sepsis, while this is obviously not valid in antitumor treatment.

2 TNF: Structure and Mechanism of Action

The mRNA for TNF codes for a presequence of 76 amino acids (79 amino acids in the mouse) followed by a mature protein of 157 amino acids (156 amino acids

in the mouse) and is transcribed from a single gene located in the middle of the MHC locus (Spies et al. 1991). It is expressed as a 26-kDa integral membrane protein from which a 17-kDa mature TNF protein subunit is released by proteolytic cleavage. Although the protease responsible has not yet been identified, it has recently been shown that synthetic metalloproteinase inhibitors can specifically interfere with this step, both in vitro and in vivo (Gearing et al. 1994; McGeehan et al. 1994; Mohler et al. 1994). Both the membrane-bound form and the secreted form of TNF are biologically active (Perez et al. 1990). The native TNF molecule is a trimer consisting of three identical subunits held together by hydrophobic forces (Wingfield et al. 1987) which can be dissociated to inactive monomers by mild detergents (Smith and Baglioni 1987) or suramin (Alzani et al. 1993). The three-dimensional structure of TNF has been solved (Jones et al. 1989; Eck and Sprang 1989), and the receptor-binding site has been located in the lower half of the cleft between two subunits of this triangular pyramid (Yamagishi et al. 1990; Van Ostade et al. 1991), implying the existence of three receptor-binding sites on each TNF molecule.

In addition to activated macrophages, which form the major source of TNF, a number of other cell types are also able to produce TNF: T lymphocytes, natural killer (NK) cells, endothelial cells, mast cells, glial cells, Kupffer cells, granulosa cells, and smooth muscle cells. The production of TNF is regulated at multiple levels: transcription, mRNA stability, and translation (Beutler et al. 1992; Kruys et al. 1993). Pentoxifylline (Beutler et al. 1992) and thalidomide (Sampaio et al. 1991) inhibit synthesis at the transcriptional level, while glucocorticoids are active mainly at the posttranscriptional level, as glucocorticoid-treated macrophages accumulate the TNF message in abundance but fail to produce the TNF protein (Beutler et al. 1986). In addition to glucocorticoids, which are themselves induced by TNF, a number of other endogenous molecules suppress the induction of TNF: prostaglandin E_2 (Beutler et al. 1992), interleukin (IL)-4 (Hart et al. 1989), transforming growth factor-β (Flynn and Palladino 1992), and most predominantly IL-10 (Fiorentino et al. 1991).

TNF exerts its activities by clustering two distinct types of receptor: TNF-R55 and TNF-R75 (Vandenabeele et al. 1995a). Most interestingly, human (h) TNF does not interact with mTNF-R75 (Lewis et al. 1991). Mutants of hTNF have been engineered that recognize selectively either hTNF-R55 or hTNF-R75 (Van Ostade et al. 1993, 1994; Loetscher et al. 1993). The biological significance of these receptors is discussed in a later section. In the liver a third type of TNF-R has been reported, but its significance remains unclear (Schwalb et al. 1993).

Both TNF-Rs can give rise to circulating TNF-binding proteins following proteolytic cleavage at the base of their extracellular domains. These circulating proteins, called soluble (s) TNF-R, play a major role in the clearing of TNF. Depending on the situation, they may act as an inhibitor or as a reservoir. It should be noted that while the interaction between TNF and TNF-R55 is rather stable, that between TNF and TNF-R75 is very labile. This has important consequences for the use of sTNF-R immunoglobulin constructs as potential inhibitors of TNF-induced pathology in vivo.

Activation of the receptors, which is brought about by binding of the trimeric TNF and subsequent clustering, results in both cytoplasmic and nuclear effects. The way in which the signals are transduced is currently being unraveled (VANDENABEELE et al. 1995a). Evidence has been presented that proteins associate with the intracellular domains of TNF-R55 and TNF-R75, and that both receptors can be phosphorylated by an associated kinase (DARNAY et al. 1994a,b). Also, two new putative signal transducers, TNF-R associated factors 1 and 2 have been identified as being associated with the cytoplasmic domain of TNF-R75 (ROTHE et al. 1994).

A number of intracellular events have been described to occur after triggering of TNF-Rs. It is unclear at present, however, which ones are particular for a given cell line, which are epiphenomena, and what the relationship between the various signals is. In addition to the activation of phospholipases, sphingomyelinases, and kinases, especially of the mitogen-activating protein kinase pathways, the perturbation of the electron transport chain in mitochondria may be of particular relevance for the situation in SIRS (SCHULZE-OSTHOFF et al. 1992, 1993). TNF also induces the production and/or activation of a number of transcription factors such as nuclear factor κB, AP-1, interferon-regulator factor 1, nuclear factor–IL-6, cAMP response element binding protein, and serum-responsive factor (FIERS 1995). This in turn leads to gene induction and synthesis of a number of proteins which might well be involved in SIRS: other cytokines (such as IL-1, IL-6, IL-8, granulocyte-macrophage colony-stimulating factor, granulocyte colony-stimulating factor), adhesion molecules (such as ICAM-1, E-selectin, CD11/18), proteins involved in the coagulation and fibrinolytic pathway (such as tissue factor, urokinase, plasminogen-activator inhibitor types 1 and 2), inducible nitric oxide (NO) synthase and proteases, to name only a few (FIERS 1995).

These intracellular events lead to the activation of the target cells. Although almost every cell type bears receptors for TNF, leukocytes and endothelial cells may be considered to be the most important target cell types for the SIRS-inducing effects of TNF. The activation of these two cell types leads to a number of pro-inflammatory and procoagulant changes. TNF induces the expression of E-selectin, ICAM-1, and VCAM-4 and promotes leukocyte-endothelial cell adhesion (BEVILACQUA el al. 1987; GAMBLE et al. 1992). It also induces neutrophil activation. It contributes to the procoagulant climate by induction of tissue factor and tissue plasminogen activator inhibitor and by the inhibition of protein C and tissue plasminogen activators (BEVILACQUA et al. 1986; CLAUSS et al. 1992). Also important is the induction of NO in endothelial cells and vascular smooth muscle cells (GENG et al. 1992), which is responsible for the observed hypotension.

In addition to its role is SIRS and as a potential antitumor therapeutic, TNF is also involved in the pathogenesis of several other diseases, such as cerebral malaria and rheumatoid arthritis (TRACEY and CERAMI 1994). Further details about the structure and function of TNF and its activities on various cell types can be found in a recent review (FIERS 1995).

3 TNF Is Involved in SIRS

There is ample evidence for the role of TNF as an inducer of SIRS and as a mediator of SIRS induced by, for example, bacteria or α-CD3 treatment. When injected into animals, TNF can induce a number of symptoms that closely mimic those observed in sepsis. TNF injected into rats leads to hypotension, tachycardia, tachypnea, lactic acidosis, hemoconcentration, hyperkaliemia and hyperglycemia, followed by hypoglycemia (TRACEY et al. 1986). Studies in other animals and in human volunteers receiving TNF have largely confirmed that TNF can induce metabolic and pathological effects very similar to those observed after endotoxin (TRACEY and CERAMI 1993; BEUTLER and GRAU 1993).

Although early reports suggested a relationship between TNF levels and the severity of sepsis (WAAGE et al. 1987; GIRARDIN et al. 1988), this has not been confirmed by further studies. A review of the available data shows that TNF levels do not discriminate between surviving and nonsurviving patients (LOWRY et al. 1994). Moreover, patients treated with TNF using the isolated limb perfusion technique had in fact much higher levels of TNF in their general circulation than septic shock patients, but the resulting toxicity does not mimic septic shock (LEJEUNE et al. 1994). In particular, neither adult repiratory distress syndrome nor multiple organ failure were observed, and all toxicities were readily reversible. This may be due to the fact that these patients received high bolus amounts of TNF in circulation while sepsis patients could be exposed to a more prolonged production of lower amounts of TNF. It has been suggested that it is indeed the more sustained presence of low doses of TNF that is correlated with a fatal outcome in sepsis (OFFNER et al. 1990). Another element contributing strongly to the observed differences is without doubt that in the case of sepsis a number of other synergistic or sensitizing factors such as endotoxin, IL-1, or IL-12 are also present. It is worth noting that also in patients suffering from leakage during isolated limb perfusion no correlation is observed between levels of TNF in the general circulation and severity of clinical symptoms (LEJEUNE et al. 1994)

The administration of monoclonal antibodies against TNF prevents lethality in a number of sepsis models (TRACEY and CERAMI 1993). sTNF-R immunoglobulin fusion proteins also provide protection, but important differences have recently been observed between the effects of such constructs with sTNF-R55 vs. sTNF-R75. Because of the higher dissociation rate of the sTNF-R75/TNF it did not prevent lethality in an animal model of gram-negative sepsis (EVANS et al. 1994a). Whether such approaches or the use of inhibitors of the synthesis or the maturation cleavage of TNF will be of value in clinical sepsis remains to be determined. It may also be recalled that in models of bacterial peritonitis TNF proves to be a protective rather than a deleterious factor (SHEPPARD et al. 1989; ECHTENACHER et al. 1990)

4 Two Receptors for TNF

Our knowledge regarding the function of each of the receptor types for TNF is derived from four types of experiments. Studies in mice and on murine cells exploit the fact that hTNF is a selective agonist of mTNF-R55 as it does not interact with mTNF-R75 (Lewis et al. 1991). The knowledge that TNF-R become activated by clustering allowed studies with agonistic monoclonal antibodies. Finally, the engineering of TNF mutants that are selective agonists of either hTNF-R55 or hTNF-R75, and of mice carrying a targeted deletion in either the TNF-R55 or TNF-R75 gene are two important tools that have recently become available.

The earliest results were obtained in in vivo models in mice (reviewed in Brouckaert et al. 1993b). It was observed that hTNF is lethal only at 50-fold higher concentrations than mTNF (Brouckaert et al. 1992b). Hence it seemed that triggering of TNF-R55 is not sufficient to induce lethality in mice. This, however, holds only for healthy mice receiving TNF as a single agent. Coadministration of agents such as galactosamine (GalN; Lehmann et al. 1987; Brouckaert et al. 1992b), the glucocorticoid receptor antagonist RU38486 (Brouckaert et al. 1992a), IL-1 (Everaerdt et al. 1989), or lipopolysaccharide (LPS; Rothstein and Schreiber 1988), or the presence of some infections or some tumors (see Sect. 5.1) considerably reduces the lethal dose of hTNF and abolishes partially or completely the species specificity. When one analyzes more closely the changes in quantitative parameters induced after TNF administration, one observes that the initial peak changes are very similar after hTNF and mTNF, but that while hTNF induces only transient changes, mTNF induces sustained, irreversible effects (Fig. 1; Brouckaert et al. 1992a; Takahashi et al. 1995a).

Studies in TNF-R55$^{0/0}$ mice (Pfeffer et al. 1993; Rothe et al. 1993) led to the conclusion that such mice are resistant to an otherwise lethal challenge with TNF or LPS in GalN-sensitized mice or with the combination of mTNF and IL-1. This is in agreement with our previous findings since in these situations no differences between hTNF and mTNF were observed. Remarkably, TNF-R55$^{0/0}$ mice turned out to be equally sensitive to the lethal effects of endotoxin as control mice (Rothe et al. 1993), which raises the question of whether TNF is really an essential factor for lethality in endotoxic shock. TNF-R75$^{0/0}$ mice were marginally resistant to endotoxin and were dramatically less sensitive, but not completely resistant, to the lethal effects of mTNF (Erickson et al. 1994). The data, however, do not report the degree of resistance in a quantitative way. TNF-R75$^{0/0}$ mice are also resistant to TNF-induced skin necrosis. This observation has been confirmed by the administration of inhibiting antibodies against TNF-R75 (Sheehan et al. 1995).

Although several reports show that, except in T lymphocytes, the triggering of TNF-R55 is sufficient to obtain all TNF-inducible in vitro effects in a qualitative way, more elaborate studies show that interaction with TNF-R75 substantially enhances TNF-R55 mediated effects. Regarding important proinflammatory ef-

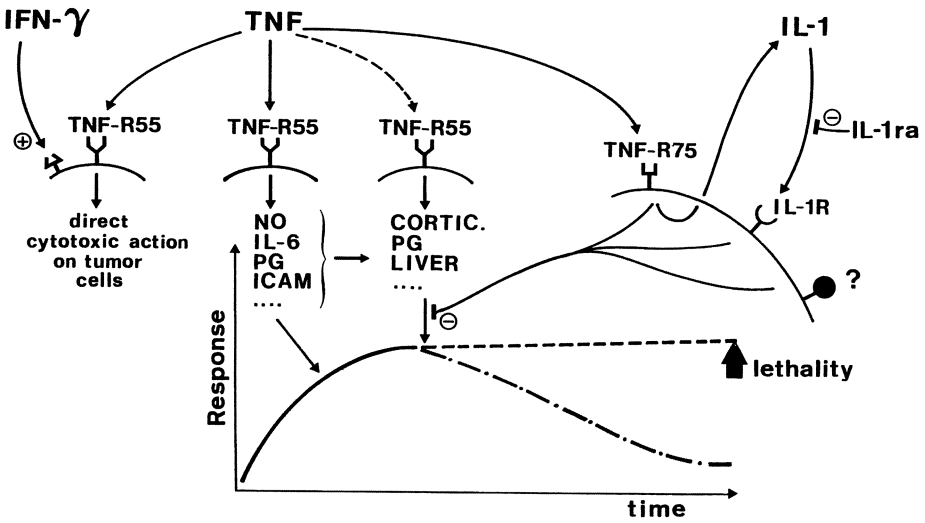

Fig. 1. Different kinetics of induction of mediators following administration of either hTNF or mTNF. Induced mediators, such as IL-6, NO, prostaglandins (*PG*) etc., reach peak levels after about 2–3 h following hTNF treatment, which acts only on mTNF-R55. Because of feedback mechanisms, at least partially based on induced glucocorticoids, the levels of these mediators subsequently start to decrease. After administration of mTNF, however, which also triggers TNF-R75, or when the IL-1 receptor is triggered, the feedback mechanism becomes inoperative, and the concentration of mediators, such as IL-6, plateaus or continues to increase until death ensues. Triggering of TNF-R55 is sufficient to cause cytotoxicity in many malignant cells, and this effect is strongly enhanced by IFN-γ cotreatment. (From FIERS 1995)

fects on endothelial cells and neutrophils, it was shown that although triggering of TNF-R75 alone had no effect, mutants of hTNF that interact only with TNF-R55 induce maximal effects only at almost 100-fold higher doses than wild-type TNF (Barbara et al. 1994). This cooperation may be the consequence either of ligand passing, where TNF-R75 traps TNF and transfers it to TNF-R55 (TARTAGLIA et al. 1993), or of true intracellular cooperation between both receptor-induced signaling pathways (VANDENABEELE et al. 1995b). On T lymphocytes TNF-R75 seems to be the major signaling receptor (VANDENABEELE et al. 1992). Whether TNF-R75 has a signal-transducing function on other cell types, especially as regards cytotoxicity, is subject to controversy.

Mutants of hTNF that are selective agonists of TNF-R55, are being developed as potential anticancer agents. The rationale is based on studies with mTNF in TNF-R75$^{0/0}$ mice and with hTNF in normal mice which indicate that TNF-R55 selective agonists cause lethality only at much higher doses than wild-type TNF. Furthermore, they retain their antitumor effect against human tumors in nude mice (VAN OSTADE et al. 1993). Studies in baboons, however, reveal that they display a different pharmacokinetic profile, and that their half-life in circulation is three times longer than that of wild-type TNF (VAN ZEE et al. 1994). This not only reveals an important function for sTNF-R75 in clearing TNF but also makes the interpretation of other data more difficult (BROUCKAERT et al. 1994).

The slower clearance may be an important advantage, as prolonged exposure of malignant cells to TNF is required for maximal cytotoxic efficacy.

5 Seed and Soil: Other Factors Determine the Outcome of a Challenge with TNF

Although TNF undoubtedly can induce SIRS, albeit not identical to that seen in patients with clinical septic shock, it is the condition of the host rather than the dose of TNF that determines the final outcome of a challenge with TNF. This is the case not only in cancer patients (Sect. 3) but also in animal models. Depending on the condition or on the presence or absence of other substances, the LD_{50} of hTNF for mice can be as low as 0.1 μg/mouse or exceed 500 μg/mouse. The strongest sensitizer described is GalN, a hepatotoxin depleting UTP in the liver (LEHMANN et al. 1987). It should be noted, however, that although presented as a model for endotoxic shock in many papers, the lethality induced in GalN-sensitized animals is mediated by a fulminant hepatic necrosis due to massive apoptosis of individual hepatocytes (LEIST et al. 1995a,b), very similar to what is observed after the administration of α-Fas (OGASAWARA et al. 1993), and as such is of little significance for clinical sepsis. Rather, it may be considered a model for certain forms of hepatitis.

The importance for the outcome of which TNF-R type is triggered is discussed in Sect. 4. Section 6 describes the role of four particular cytokines (IL-1, IL-6, IL-12, and IFN-γ). Here we discuss some of the more complex situations such as sensitization and tolerance.

5.1 Sensitization

Mice carrying certain types of tumor or suffering from bacterial infections are extremely sensitive to the lethal effects of TNF. This is a problem for the treatment of cancer patients with TNF, but especially the sensitization induced by infection is a situation which might be quite relevant for the septic syndrome. Indeed, in this condition TNF is present in a host which may have been infected since some time. Recent studies have provided important new information on the possible mechanisms by which infection induces such a sensitization. It has been observed that IFN-$\gamma R^{0/0}$ mice can no longer be sensitized to the lethal effects of either LPS (KAMIJO et al. 1993) or TNF (CAUWELS et al. 1995) by an infection with bacillus Calmette-Guérin (BCG). However, these mice remain as sensitive to the lethal effects of mTNF in uninfected animals as controls (CAUWELS et al. 1995). IL-12 was found to be a sufficient and necessary mediator (CAUWELS et al., 1996). It therefore seems that BCG and other infections bring about this sensitization by an IL-12 driven production of IFN-γ in NK cells

(CAUWELS et al. 1995, 1996). The target of INF-γ is presently unknown, but it seems that upregulation or activation of adhesion molecules is a central feature since α-Leukocyte-function associated antigen (LFA)-1 is able to prevent TNF-induced lethality in sensitized mice, although not in healthy mice challenged with mTNF (Cauwels et al., submitted). Most unexpectedly, this is not due to an inhibition of LFA-1/ICAM-1 interaction since ICAM-$1^{0/0}$ mice remain as sensitive as control mice (Cauwels et al., submitted). Neither E-selectin nor P-selectin seems to be involved in this process (Cauwels et al., submitted).

When sensitization is induced by the presence of some tumors, such as Lewis lung carcinoma, α-LFA-1 can still protect (Cauwels et al., submitted), but neither NK cells, IL-12, nor IFN-γ seem to be involved (CAUWELS et al. 1995, 1996).

5.2 Tolerance

The repetitive administration of rather low doses of TNF to mice results in a condition in which mice have become tolerant to the lethal effects of mTNF (TAKAHASHI et al. 1991). This tolerance is selective as some effects of TNF are still inducible in tolerant animals. Importantly, the antitumor effect of the combination of TNF and IFN-γ (TAKAHASHI et al. 1991, 1995b) and hypertriglyceridemia (GRUNFELD et al. 1989) are retained in tolerant mice.

When changes in quantitative parameters are determined, one observes in the tolerant mice a pattern that closely resembles the differences seen after the administration of hTNF or mTNF. In tolerant animals the initial peak changes in hypothermia, IL-6 levels, hematocrit increase, and NO production are indistinguishable from those in control animals. However, while these changes are reversible in tolerant animals, they are irreversible in control mice and eventually result in death.

Regarding the mechanism of tolerance, trivial explanations such as changes in the bioavailability of TNF, the involvement of soluble receptors, neutralizing antibodies, and changes in the number of receptors can be excluded (TAKAHASHI et al. 1994). We have recently proposed that the mechanism of tolerance must be found in the functional ablation of (part of) the signaling pathway induced by triggering of TNF-R75 (TAKAHASHI et al. 1995a). Tolerance itself is induced by TNF-R55, while triggering of TNF-R75 or the addition of IL-1 or RU38486 inhibit tolerance induction, although they do not break an acquired tolerance (TAKAHASHI et al. 1995a).

5.3 Desensitization

A single administration of TNF or IL-1, given several hours before the administration of an otherwise lethal dose of TNF, can protect against TNF-induced lethality (WALLACH et al. 1988; LIBERT et al. 1991b). In contrast to what is observed

in the case of tolerance, this insensitivity is short-lasting. It is mediated by protein synthesis in the liver, but IL-6 is not involved (Libert et al. 1991b). Whether α_1-acid glycoprotein (AGP; see Sect. 8) is the protein responsible for desensitization remains to be established.

6 TNF and Other Cytokine Mediators of SIRS

TNF is part of a cytokine network. It induces other cytokines which may not only mediate part of its in vivo effects but also work in concert with it. Regarding the SIRS-inducing effect of TNF, four cytokines – IL-1, IL-6, IL-12, and IFN-γ – have been studied in detail. Other cytokines such as transforming growth factor-β, IL-4, IL-8, and IL-10 may also affect this syndrome, but as their in vivo relationship with the effects of TNF is less well characterized, we do not discuss them here.

6.1 IL-1

IL-1 is a cytokine which has overlapping effects with TNF but also distinct effects. It is suspected to play an important role in sepsis since its levels are correlated with the severity of the disease (Waage et al. 1989), and since in animal models IL-1ra, a natural IL-1 receptor antagonist, reduces lethality in various shock models (Ohlsson et al. 1990; Alexander et al. 1991; Fischer et al. 1992). Recently it has also been shown that mice deficient in IL-1βconverting enzyme, which are defective in the production of mature IL-1β and also produce much less IL-1α, have a decreased sensitivity to the lethal effects of endotoxin (Li et al. 1995). Phase III clinical trials with IL-1ra in sepsis patients, however, have not confirmed the promising protective results obtained in pilot trials.

The influence of IL-1 on the toxicity of a challenge with TNF depends strictly on the sequence and timing of treatment. Administration of IL-1 several hours after a challenge with TNF does not influence the outcome (Everaerdt et al. 1989). Pretreatment with IL-1 protects against a subsequent challenge with TNF (Libert et al. 1991b). When given simultaneously, an important synergism in inducing lethality is observed (Waage and Espevik 1988; Everaerdt et al. 1989). Furthermore, in the presence of IL-1 the triggering of TNF-R55 is sufficient to cause lethality, as the species-specific toxicity of TNF is abolished (Everaerdt et al. 1989). This was confirmed in TNF-R55$^{0/0}$ mice (Rothe et al. 1993). Regarding the mechanism of this synergism, we have observed that the glucocorticoid receptor antagonist RU38486 mimics closely IL-1 or TNF-R75 triggering in its sensitizing activities to the IL-6 inducing and lethal effects of TNF (Brouckaert et al. 1992a). It is noteworthy that IL-1ra protects against a lethal challenge with *Escherichia coli* but does not alter the host response to sublethal endotoxemia (Fischer et al. 1992). This argues for a common effect of IL-1 and TNF-R75

triggering, both of which constitute a "lethal switch" from a reversible, nonlethal syndrome to an irreversible, lethal syndrome (BROUCKAERT et al. 1993b). Regarding the possible involvement of TNF-induced IL-1 in the effects of TNF, it has been observed that recombinant IL-1ra protects somewhat against the lethal effects of an LD_{100} challenge with mTNF. In infection- or tumor-sensitized mice, however, IL-1ra was without effect (EVERAERDT et al. 1994).

6.2 IL-6

This cytokine, which has important activities on B cells, bone marrow cells, and hepatocytes, is induced by TNF as well as by LPS or IL-1 (BROUCKAERT et al. 1989a,b, 1992a; LIBERT et al. 1990, 1991a). Its production is highly correlated with lethality, both in patients with sepsis and in animal models (WAAGE et al. 1989; BROUCKAERT et al. 1989b; LIBERT et al. 1990, 1991a; LOWRY et al. 1994). Nevertheless, from a theoretical point of view, IL-6 has generally been considered an anti-inflammatory cytokine since it is a potent inducer of glucocorticoids and of acute-phase proteins with antiprotease activity. Several groups, however, have observed that monoclonal antibodies directed against IL-6 or IL-6R diminish rather than enhance the lethality induced by TNF or LPS (STARNES et al. 1990; LIBERT et al. 1992; HEREMANS et al. 1992). In contrast, mice carrying a targeted deletion of the IL-6 gene are equally sensitive to the lethal effects of TNF or LPS as the controls (LIBERT et al. 1994a). A possible explanation for this apparent discrepancy is that the antibodies act as a reservoir rather than a neutralizing agent (HEREMANS et al. 1992) and so provide a much longer exposure to IL-6. Since soluble IL-6R (α-chain) promotes rather than neutralizes the function of IL-6, the same could hold for the antibodies directed against IL-6R. In any case, these results should inspire caution in interpreting data obtained with neutralizing antibodies or using mice with a targeted gene deletion.

6.3 IL-12

IL-12 is a heterodimeric cytokine mainly produced by phagocytic cells (TRINCHIERI 1995). It acts as an activator of NK cells and CTL and shifts the immune response to a T_h1-controlled response. As such it is a major inducer of IFN-γ (GATELY et al. 1994). Also TNF is a mediator of the activities of IL-12, although only at higher doses or levels of IL-12 (ORANGE et al. 1995). Recently IL-12 has been implicated as the priming factor of the Shwartzman reaction (OZMEN et al. 1994). Whether the antitumor properties of IL-12 are mediated by TNF and IFN-γ remains to be established.

We have observed that IL-12 is a necessary and sufficient mediator in sensitization to the lethal properties of TNF induced by a BCG infection (CAUWELS et al., 1996). Pretreatment with IL-12 can render mice extremely sensitive to the lethal effects of TNF; in fact it reduces the LD_{50} of hTNF at least 100-fold. Also,

this method of sensitization can be alleviated by either methylene blue or α-LFA-1 treatment. The requirements regarding dose and kinetics of the pretreatment indicate that this sensitizing effect is mediated by the induction of IFN-γ, probably in synergism with endogenous TNF. Mice with a targeted deletion of the IFN-γ receptor were fairly, although not absolutely resistant to this IL-12 induced sensitization to hTNF. The conclusion is that all conditions which bring about the production of IL-12 render the host extremely sensitive to TNF-induced lethality.

6.4 IFN-γ

IFN-γ (Farrar and Schreiber 1993) is a cytokine produced by T lymphocytes and NK cells. In addition to its antiviral effects, it functions mainly as an immune stimulator. It activates macrophages and induces intercellular adhesion molecules. Endothelial cells may also be an important target for the actions of this cytokine. IFN-γ is a mediator of the Shwartzman reaction (Heremans et al. 1990; Ozmen et al. 1994) and is an important contributor to the LPS-induced lethality in sensitized animals (Kamijo et al. 1993).

IFN-γ synergizes with TNF for quite a number of effects (Fiers 1995). IFN-γR$^{0/0}$ mice are resistant to the lethality induced by hTNF in mice sensitized by an infection with BCG but not by a growing tumor. They are equally as sensitive as wild-type mice to the lethality induced by mTNF in healthy animals. It may be concluded that (a) IFN-γ can sensitize the host to the shock-inducing properties of TNF, (b) the role of IFN-γ as a sensitizer in LPS-induced shock cannot be reduced to an enhanced production of TNF, and (c) TNF-induced IFN-γ does not play a role in the lethality observed after administration of mTNF. The mechanism by which IFN- γ causes sensitization is at present unknown, but our current research points to effects on endothelial cells rather than to effects on macrophages.

7 TNF and Nitric Oxide

Rightly or wrongly, hypotension has always been considered as the central feature of sepsis-induced lethality and as the dose-limiting toxicity of TNF. The discovery of NO as the mediator of TNF-induced hypotension (Kilbourn et al. 1990) was considered a major breakthrough which would lead to a quick solution for the problems of sepsis and TNF-based anticancer treatment. As expected, inhibitors of the synthesis of NO can inhibit or even revert the hypotension caused by LPS or TNF, both in animal models and in septic patients (Kilbourn et al. 1990; Thiemermann and Vane 1990; Petros et al. 1991; Geroulanos et al. 1992).

The unpleasant surprise however, was, that this led to no improvement in survival. In contrast, in several animal models the addition of NO synthase inhibitors rather worsened the observed lethality and pathology (Cobb et al. 1992; Evans et al. 1994b; Wright et al. 1992). Indeed, both hepatic damage (Harbrecht et al. 1992) and glomerular thrombosis (Shultz and Raij 1992) are increased when NO synthesis is inhibited. Furthermore, such inhibition increases leukocyte adherence and extravasation (Kubes et al. 1991). Recently it was shown that NO downregulates the expression of IFN-γ and TNF (Florquin et al. 1994). This has inspired several investigators to propose that NO is also a molecule with both deleterious and protective properties.

To separate these activities and to obtain a selective inhibition of the deleterious activities of NO most groups attempt specifically to inhibit the inducible NO synthase (reviewed in Griffiths et al. 1994). We took another approach and tried to inhibit in a selective way part of the effects of NO, in particular those mediated by soluble guanylate cyclase, which includes NO-mediated hypotension. For this we studied the effects of methylene blue in TNF-related murine models and observed that methylene blue protects against the lethality caused by mTNF in healthy mice and by hTNF in mice sensitized by a BCG infection, by a tumor, or by a pretreatment with IL-12 (Cauwels et al., submitted). When an inhibitor of NO synthase is added to the treatment with methylene blue, the protection is abated in the cases of mTNF-induced lethality and hTNF-induced lethality in tumor-sensitized mice. These results strongly suggest that NO, in addition to its deleterious effects, has protective properties that seem indispensable to survive a challenge with TNF. These protective effects operate by mechanisms different from those by which guanylate cyclase is activated. In mice sensitized by IL-12 or BCG the protection provided by methylene blue is not reverted by NO synthase inhibitors. However, in these models the inhibitors are less effective, and considerable amounts of NO are still produced; this in fact adds further evidence to a necessary protective role for NO. Methylene blue has been shown to abate the hypotension in sepsis patients (Schneider et al. 1992; Preiser et al. 1994). In mice, however, we observed no protection by methylene blue against the lethality induced by endotoxin (unpublished results). The reason for this difference between the effects of methylene blue against challenges with TNF or endotoxin are unclear. It illustrates again that the mechanisms underlying sepsis cannot be reduced to the effects of TNF.

8 α_1-Acid Glycoprotein

Unlike some of the inhibitors mentioned above, AGP, an acute-phase protein, protects mice from the lethal shock induced by either TNF or endotoxin in galactosamine-sensitized mice and the lethality induced by mTNF, but not endotoxin, in healthy mice (Libert et al. 1994b). It also prevents lethality in BCG- or

tumor-sensitized mice (unpublished observations) and as such is one of the most potent and broadest inhibitors known. AGP is a highly glycosylated, polyanionic protein with an apparent molecular mass of about 40 kDa. It is induced in hepatocytes by TNF, IL-1, and other cytokines. Glucocorticoids are a permissive factor in its induction. The physiology of AGP is poorly understood (Baumann et al. 1993).

AGP not only protects against lethality but also provides a complete inhibition of all TNF-induced metabolic changes: fall in body temperature, release of liver enzymes, and enhanced clotting time (Libert et al. 1994b). Whether AGP is really the protective liver protein responsible for the desensitization phenomenon (Sect. 5.3) or the protein that forms the relevant target for the sensitizing activity of GalN is not yet clear. The mechanism by which AGP exerts its protective activity still needs to be elucidated.

9 Conclusions and Prospects

Shortly after the cloning of TNF, a number of important observations were made that seemed to provide a clear mechanism of sepsis. Since TNF is induced by endotoxin and mimicks the cardinal features of sepsis, and since specific antibodies capable of neutralizing TNF prevent lethality in various shock models, it was proposed that endotoxin induces TNF, and that this TNF in turn causes the pathology and lethality of sepsis. Subsequent research has shown that the condition of the host and other factors induced by endotoxin and/or bacteria to a large extent determine the outcome of a challenge with TNF. Indeed, both LPS and IL-1 are potent synergistic factors. IFN-γ and IL-12 sensitize the host substantially to the lethal effects of TNF. ICAM-$1^{0/0}$ mice are resistant to the lethal effects of endotoxin but not to those of TNF despite TNF levels induced by endotoxin that are comparable to those in control mice (Xu et al. 1994). On the other hand, some endogenous factors, such as AGP, inhibit the lethality induced by TNF, but not by endotoxin. Methylene blue also protects against lethality by TNF, but not by endotoxin. More recently another important observation has questioned even the role of TNF as a necessary mediator. Mice with a targeted deletion of TNF-R55, which are resistant to the lethal effects of TNF, remain as sensitive as the control animals to the lethal effects of endotoxin. Moreover, mice deficient in IL-1-β converting enzyme are resistant to endotoxic shock, despite high levels of LPS-induced TNF in circulation (Li et al. 1995).

It is quite probable that TNF is involved in the pathogenesis of septic shock. Sepsis should, however, not be reduced to the effects of this single contributor. Studies using various kinds of inhibitors suggest an important role for various cytokines, such as TNF, IL-1, and IFN-γ, in the events leading to lethality. It may be that at a minimal dose of the challenge sufficient to cause 100% lethality, death is the result of the synergistic activity of various cytokines which are all

limiting, but that at higher doses the redundancy of the cytokine effects becomes more prominent, and therefore inhibition of a single cytokine no longer prevents lethality. Strategies aiming at interfering with common intracellular effects of these cytokines may possibly be more effective. Another problem that may hamper anticytokine strategies in sepsis is the fact that at least some of these cytokines, albeit at lower levels than observed in sepsis, may have indispensable protective properties.

Regarding SIRS induced as a side effect of antitumor treatment with TNF, it should be noted that it displays a number of features which are distinct from those observed in sepsis. Because fewer factors are involved in TNF-induced SIRS than in sepsis, and because preventive measures can be applied as the moment of the challenge is known, it may be more suitable for effective remedying. The use of receptor-selective mutants and the induction of tolerance can, at least in mice, effectively broaden the therapeutic margin, showing that the antitumor and SIRS-inducing effects of TNF are not necessarily linked. Recently we have identified two new potent inhibitors of TNF-induced SIRS: AGP and methylene blue. Their influence on the antitumor efficacy of TNF is under study, and it is hoped that they, or similar agents, will allow the systemic administration of therapeutic doses of receptor-specific TNF.

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Mediators: Nitric Oxide and Other Toxic Oxygen Species

T.J. Evans and J. Cohen

1 Introduction

Amongst the many mediators that have been implicated in the pathophysiological changes seen in sepsis and septic shock, nitric oxide (NO) is one of the most recent to be described. Its role in mammalian cell biology is enormous (reviewed in Nathan 1992; Bredt and Snyder 1994), but this chapter focuses on its role in sepsis. While the functions of NO are legion, we concentrate on a few central roles that NO plays in sepsis. In particular, we discuss its hypotensive, cytotoxic, immunomodulatory, cardiovascular, and cerebral effects. In common with other mediators in sepsis there are problems in extending the results obtained in animal models to humans. We therefore discuss the clinical data per-

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taining to the role of NO in sepsis separately. NO is one example of a potentially toxic oxygen compound, and many others have been identified as possible mediators in sepsis which we do not have space to review. However, it is clear that NO can react with a number of these compounds to generate very toxic molecules, which are described here.

2 Historical Background

A number of different lines of research have revealed the important roles that NO plays in mammalian cell biology. Since 1977 it has been known that NO activates soluble guanylate cyclase, producing a rise in intracellular cyclic GTP (Arnold et al. 1977). This in turn can produce vascular smooth muscle cell relaxation and accounts for the vasorelaxatory properties of a number of different compounds such as organic nitrates and sodium nitroprusside (Gruetter et al. 1979; Ignarro et al. 1981). The mechanism of action of other vasodilators which do not themselves generate NO remained obscure but was shown to be dependent on an intact endothelium, leading to the concept of an endothelium-derived relaxing factor (EDRF; Furchgott and Zawadzki 1980). Further work by two groups led to the discovery that the chemical nature of EDRF is in fact NO, or a closely related oxide of nitrogen (Palmer et al. 1987; Ignarro et al. 1987).

A completely separate area of research concerned the generation of inorganic oxides of nitrogen by mammalian cells, a process thought to be of potential importance in carcinogenesis. Both rodents and humans were found to have endogenous mechanisms responsible for the generation of nitrate which increased in intercurrent infection (Green et al. 1981a,b). This activity was found also to reside in macrophages removed from suitably treated animals (Stuehr and Marletta 1985); biochemical studies showed that the generation of such reactive nitrogen intermediates depends on the oxidation of the terminal guanidino nitrogen atom of L-arginine (Iyengar et al. 1987). In addition, such reactive nitrogen intermediates were also found to be responsible for the cytostatic and tumoristatic activities of activated rodent macrophages (Hibbs et al. 1987). Subsequent to the identification of EDRF as NO it was demonstrated that the reactive nitrogen intermediate released from activated murine macrophages is NO, which subsequently forms nitrate and nitrite ions in aqueous solution (Stuehr et al. 1989).

Thus NO was found to be of key importance in vasodilatation and macrophage killing of micro-organisms and tumor cells. Subsequent work has shown that NO is important in mediating a diverse number of biological effects, including acting as a neurotransmitter in non-adrenergic, non-cholinergic nerves (Bult et al. 1990), inhibiting platelet aggregation (Mellion et al. 1981), and acting as a negative inotrope and chronotrope (Finkel et al. 1992). The chemical nature of EDRF has also been analysed more thoroughly. More precise chemical analyses have established that the main oxide of nitrogen produced by mammalian

cells is NO, although undoubtedly under certain conditions other reactive nitrogen oxide species may be produced (IGNARRO 1990). In addition, longer lasting 'storage' forms of NO exist, such as S-nitroso protein adducts (STAMLER et al. 1992).

3 Reduced Oxygen Species

A large number of other toxic oxygen metabolites have been suggested as potential mediators of tissue damage in sepsis. These include superoxide ($O_2^{\cdot-}$), hydroxyl radical ($OH^{\cdot}$), and hydrogen peroxide (H_2O_2). We do not have space here to review these areas in detail (see BRIGHAM 1991; FLOHÉ and GIERTZ 1987). However, it is clear that some of these compounds can react with NO to produce even more toxic molecules (see Sect. 5.2). Moreover, in the absence of arginine, brain NO synthase can actually form superoxide and hydrogen peroxide (POU et al. 1992). Thus there is considerable interaction between the systems generating reactive nitrogen and reactive oxygen species.

4 Nitric Oxide: Mechanisms of Production

4.1 Enzymes Producing Nitric Oxide

NO is produced by a family of enzymes known as NO synthases (SESSA 1994). There are three NO synthases, each encoded by a separate gene. Direct comparisons of protein sequence similarity between the different human isoforms shows that they have about 60% sequence identity and share a number of similar properties which reflect their structural similarities (FORSTERMANN et al. 1994). All of the enzymes catalyse the same reaction, which is the oxidation of L-arginine by molecular oxygen to L-citrulline and NO. In each case this involves the transfer of electrons from the co-factor NADPH. In addition, each of the enzymes contains tightly bound flavins, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), as well as tetrahydrobiopterin, heme and calmodulin, which are involved in the intramolecular transfer of electrons from NADPH and tetrahydrobiopterin. These similarities in enzymatic action are reflected in the presence of common structural motifs within the proteins, which possess consensus sequences for the binding of FAD, FMN, NADPH and calmodulin. The presence of binding sites for both FMN and FAD is relatively unusual; the only other example of a mammalian enzyme with such properties is cytochrome P450 reductase. Indeed, the C terminal portion of the NO synthases has about 30% identity with this cytochrome (BREDT et al. 1991; SESSA 1994).

The NO synthases differ most significantly in their dependence on calcium ion concentration and their cellular and tissue distributions. Two of these NO synthases, the brain and endothelial isoforms, are constitutive, that is, they are constantly expressed in tissues where they are present. These isoforms are also highly dependent on calcium ion concentration for their enzymatic activity (Bredt and Snyder 1990; Pollock et al. 1991). In common with most calmodulin-binding enzymes, calmodulin is bound to these isoforms only when free calcium levels are elevated. Hence NO synthesis from the brain and endothelial isoforms occurs only when calcium levels within the cell are elevated, with EC_{50} values for Ca^{2+} of approximately 0.2–0.4 μM (Nathan 1992). Thus in cells possessing these isoforms the concentration of calcium within the cell provides a virtually instantaneous control mechanism over NO synthesis.

This is not the case with the third isoform of NO synthase, the inducible form. This was originally identified within macrophages (Stuehr and Marletta 1985), but it is now known that a huge variety of different cells can make this isoform under suitable conditions (Xie and Nathan 1994). Under resting conditions cells do not produce the inducible NO synthase, but after suitable stimulation with agents such as pro-inflammatory cytokines and lipopolysaccharide (LPS) they can make large amounts of this enzyme. Importantly, although this NO synthase isoform contains very tightly bound calmodulin, under most conditions it appears that this binding is essentially independent of calcium ion concentration (Cho et al. 1992; Evans et al. 1992a). Thus, at least at the calcium ion concentrations pertaining within the cell, inducible NO synthase activity is not affected by changes in calcium ion concentration. Once synthesised, the inducible NO synthase can make large amounts of NO for extended periods of time; in cell culture the NO output is limited only by the depletion of arginine within the tissue culture medium (Vodovotz et al. 1994).

4.2 Inducers of Nitric Oxide Synthesis

The inducible isoform of NO synthase is responsible for most of the increased output of NO during sepsis (Green et al. 1981b; Buttery et al. 1994). Once synthesised, this isoform can make large amounts of NO over a long period (Vodovotz et al. 1994). Thus the induction of this form of the enzyme is a critical step in the production of NO in sepsis. An enormous number of different agents can act as inducers, but many of these depend on the synthesis of more proximal activators.

4.2.1 Proinflammatory Cytokines

Sepsis is characterised by the release of a large number of cytokines which initiate and augment the inflammatory response (Glauser et al. 1991). Principal amongst these are tumour necrosis factor (TNF)-α, interleukin (IL)-1, and interferon (IFN)-γ. Combinations of two or more cytokines are much more effective

inducers of inducible NO synthase (iNOS) synthesis, with marked synergy often demonstrable within a variety of different cell types (NATHAN 1992). Human cells particularly require the presence of a number of different pro-inflammatory cytokines to produce significant iNOS activation (NUSSLER et al. 1992).

4.2.2 Bacterial Products

LPS is an extremely potent activator of iNOS production, both on its own and as an agent synergising with pro-inflammatory cytokines (STUEHR and MARLETTA 1985; NAKAYAMA et al. 1992). In vivo of course many of its effects are due to the intermediate synthesis of a variety of cytokines. Products of gram-positive organisms can also induce iNOS synthesis. These include cell wall components such as lipoteichoic acid (LONCHAMPT et al. 1992) as well as secreted extracellular toxins such as the staphylococcal protein toxic shock syndrome toxin-1 (ZEMBOWICZ and VANE 1992). The intracellular pathways involved in the induction of iNOS by these agents are not well characterised.

4.2.3 Down-Regulation of iNOS Activation

A number of counter-regulatory cytokines such as IL-4 (BOGDAN et al. 1994) and transforming growth factor-β (VODOVOTZ et al. 1993) can prevent iNOS induction if present at the same time as the inducing agents. Corticosteroids are also very effective at preventing iNOS induction (RADOMSKI et al. 1990). None of these agents appears to have a significant effect on iNOS activity once it has been induced.

5 Nitric Oxide: Pathophysiological Effects

5.1 Hypotension

As outlined above, NO has been identified as the chemical identity of EDRF, an extremely potent vasodilator. NO is able to relax vascular smooth muscle by activation of the enzyme guanylate cyclase by nitrosation of its heme group (ARNOLD et al. 1977). This in turn leads to increased levels of cGMP within the cell. cGMP has many effects within cells, but its critical action in vasorelaxation depends on its activation of cGMP-dependent kinase, which in turn increases the conductance of a potassium (K^+) channel within the smooth muscle cell membrane, the calcium-dependent K^+ channel (ARCHER et al. 1994). Increased K^+ efflux from the cell then leads to a hyperpolarisation of the cell membrane which inhibits muscle contraction. NO is a potent vasodilator, active at concentrations as low as 10^{-8} *M* (PALMER et al. 1987). In a whole animal therefore vasorelaxation

leads to decreased peripheral vascular resistance and a fall in systemic blood pressure.

Hypotension is of course one of the cardinal features of septic shock and leads to ischaemic organ damage and multi-organ failure (GLAUSER et al. 1991). What evidence is there that the overproduction of NO in sepsis causes this vasodilatation and hypotension? Four lines of evidence in experimental animals support this assertion; the evidence for the role of NO in human sepsis is considered separately (Sect. 6).

1. Animals which have been treated with inflammatory agents such as LPS or infected with live bacteria show an increased production of nitrite and nitrate, stable end-products of NO in aqueous solution (GREEN et al. 1981b; EVANS et al. 1992b). This output is correlated with the development of hypotension and multi-organ failure in treated animals. Thus, increased NO production does occur in animals with septic shock.

2. The source of this increased NO production is from iNOS. In cell culture LPS and pro-inflammatory cytokines are potent stimuli for the induction of iNOS which can produce large amounts of NO (NATHAN 1992). This can occur not only in inflammatory cells such as macrophages but also directly in vascular smooth cells, where the local production of NO leads to rapid vasodilatation and hypotension (BUSSE and MÜLSCH 1990). In whole-animal models of sepsis the administration of LPS or live bacteria can also increase iNOS expression. This has been demonstrated by enzyme assays for iNOS which show marked induction of the enzyme in a wide variety of tissues, notably the liver, lung, heart and gut (KNOWLES et al. 1990). Immunochemical techniques have allowed detailed localisation of the sites of iNOS production (BUTTERY et al. 1994; SATO et al. 1995). Although induction of the enzyme within tissue macrophages and hepatocytes has been easily demonstrated, the production of iNOS within vascular smooth muscle cells following LPS or live bacterial treatment of animals has been more difficult to demonstrate. Indeed, a number of studies have failed to show the presence of iNOS in vascular smooth muscle following LPS treatment of whole animals. However, in cultured vascular smooth muscle cells (BUTTERY et al. 1994) or whole arterial preparations (THORIN TRESCASES et al. 1995) treated with LPS and cytokines such immunoreactive iNOS can be demonstrated. An additional source of NO of relevance in the development of hypotension in shock is the vascular endothelium, which in addition to the constitutive NOS can also produce the inducible isoform following stimulation with cytokines and/or LPS (GROSS et al. 1991).

3. Pharmacological blockade of NO production has demonstrated its importance in the hypotension which develops in animals following LPS or bacterial injection. A number of different inhibitors of the NO synthases have been developed (MARLETTA 1995). Most work has utilised drugs which are N-substituted L-arginine derivatives, such as N^G-monomethyl-L-arginine (L-NMMA) and N^G-nitro-L-ariginine-methyl ester (L-NAME). Although these do differ in their rank order of potencies of inhibition on the different NOS isoforms, they effectively do not discriminate between them. More recently a novel NOS inhibitor has been de-

veloped which does have some selectivity for the inducible isoform – this is S-methylisothiourea sulfate, which is 10 to 30-fold more potent an inhibitor of iNOS than the endothelial form (SOUTHAN et al. 1995).

A large number of experiments using different inhibitors, animals and LPS doses have been performed. The results are often confusing, with significant differences between species. However, a number of common themes have evolved. Firstly, these inhibitors can attenuate the hypotension consequent upon LPS or bacterial injection into an animal (THIEMERMANN and VANE 1990). However, the effects on overall survival are much more variable and depend critically upon dose of the inhibitor used and the animal species (WRIGHT et al. 1992; NAVA et al. 1992; EVANS et al. 1994). In part this is due to the relative non-selectivity of most of the inhibitors used, so that not only the iNOS isoform is inhibited but also the endothelial and brain isoforms. Since a basal level of NO production from the constitutive endothelial isoform is essential for maintaining basal vasorelaxation and hence organ perfusion, it is not surprising that inhibition of all NO production can lead to the vascular collapse and rapid death of treated animals that has been seen in some models (WRIGHT et al. 1992; NAVA et al. 1992). In addition, inhibition of brain NOS may account for the CNS toxicity of some of these drugs (COBB et al. 1992). The selective agent S-methylisothiourea sulfate not only reverses the LPS-induced hypotension in rats but also improves their long-term survival, suggesting that such targetted NO inhibition may be beneficial clinically (SZABO et al. 1994).

4. The final and most direct evidence that NO production from the iNOS isoform is essential to the development of hypotension following LPS challenge comes from experiments using transgenic mice in which the gene for iNOS has been disrupted (MACMICKING et al. 1995; WEI et al. 1995). Such animals make none of the inducible enzyme on LPS challenge and do not show any increase in nitrite/nitrate production, compared to the large increases seen in the wild-type parent strain. Blood pressure in the knock-out mice does not fall significantly following LPS administration, in marked difference to the wild-type animals. In addition, with large doses of LPS the knock-out mice are protected from its lethal effects compared to the wild type. Interestingly, when these mice are sensitised to the effects of LPS with *Propionibacterium acnes*, they show no protection from the massive hepatic necrosis following LPS challenge or any improvement in survival compared to the wild-type strain. These experiments provide the most direct evidence that NO production from the iNOS is essential to the production of hypotension following LPS treatment. They also demonstrate, however, that while NO is an important mediator of the toxicity of LPS, under certain circumstances it clearly is not pivotal in determining survival.

5.2 Cytotoxicity

The potentially toxic effects of NO were recognised in the early phases of its discovery. As a product of activated macrophages it was found to be cytotoxic to certain tumour cells (HIBBS et al. 1987) and also to participate in the killing of a

number of different micro-organisms (NATHAN and HIBBS 1991; OSWALD et al. 1994). However, the toxic effects of NO may not just be beneficial, but in addition may be damaging when overproduced during sepsis. The evidence for the production of NO in sepsis is reviewed above. What evidence is there for NO-mediated toxicity being important in the pathology of sepsis?

NO cytotoxicity occurs through a number of different mechanisms. At high enough concentrations NO can inhibit a range of critical enzymes which possess iron-sulfur centres, by forming iron-sulfur-nitrosyl derivatives. Thus its inhibition of the mitochondrial enzymes aconitase and NADPH:ubiquinone oxidoreductase leads to cessation of oxidative metabolism in the affected cell and prevents protein synthesis (STADLER et al. 1991). A more potent mechanism of cytotoxicity, however, can occur through the reaction of NO with superoxide anions. Superoxide ($O_2^{-\cdot}$) is produced by activated macrophages and a variety of epithelial and endothelial cells under very similar conditions which induce the production of NO (BRIGHAM 1991). Superoxide itself can be directly toxic, but in fact it has limited bioreactivity (BECKMAN et al. 1990). However, a number of studies have shown that intravenous treatment with superoxide dismutase ameliorates endothelial injury seen in a number of models of sepsis (FREEMAN et al. 1985). This indicates that superoxide plays a role in this process. Combination of NO with superoxide leads to the production of the potent oxidising agent, peroxynitrite ($ONOO^-$). Peroxynitrite may be a crucial intermediate in the induction of oxidant damage in sepsis (BECKMAN et al. 1990, 1994).

Endothelial injury is critical in many areas of sepsis, particularly in the lung, where the breakdown of the endothelial barrier that this injury produces leads to the increased influx of fluid and inflammatory cells that is typical of adult respiratory distress syndrome (ARDS). Peroxynitrite has a number of different toxic effects. It can initiate lipid peroxidation in biological membranes (RADI et al. 1991a) and produce nitration of aromatic amino residues (ISCHIROPOULOS et al. 1992) as well as oxidation of protein sulfhydryl groups (RADI et al. 1991b). It is more potent than superoxide in producing alveolar type II cell injury and damage to surfactant apoproteins. As well as being directly toxic, peroxynitrite can decompose under acid conditions to yield the very reactive hydroxyl radical, $OH^{\cdot}$ (BECKMAN et al. 1994). All these effects combine to make peroxynitrite a very attractive candidate as a mediator of the oxidative damage to lung in sepsis.

One method of analysing for the production of peroxynitrite is to assay for the presence of nitrotyrosine, since only peroxynitrite and not NO or superoxide can nitrate the phenolic ring of this aminoacid (ISCHIROPOULOS et al. 1992). Specific polyclonal antibodies to nitrotyrosine have been used to immunostain the lungs of patients with ARDS and rats that have sustained hyperoxic damage (HAUSCHILDT et al. 1992). In both cases a clear increase in nitrotyrosine staining was seen over control sections. Additional studies have shown increased levels of nitrotyrosine in the lungs of rats after LPS treatment (WIZEMANN et al. 1994). These data demonstrate that peroxynitrite is generated in experimental animal models of lung damage and in human cases of ARDS and may play a significant role in its pathology.

5.3 Immunomodulation

NO production is stimulated by pro-inflammatory cytokines produced during sepsis. NO itself may also influence the production of these cytokines. Production of different cytokines by T cells has allowed two functional subsets to be defined, at least in mice: those that secrete cytokines such as IL-2 and IFN-γ, known as T_h1 cells, and those that produce IL-4 and IL-5, known as T_h2 cells (Mosmann et al. 1986). In work on the immune response to infection with the pathogen *Leishmania* it is apparent that T_h1 cells produce NO which exerts a negative feedback effect on the same cells to inhibit the production of IL-2 and IFN-γ (Liew et al. 1991; Taylor-Robinson et al. 1994). Such a negative regulatory pathway may also operate in sepsis, such that the production of NO down-regulates the production of T_h1 cytokines, which include the important mediators IFN-γ and TNF-α.

Most evidence for the operation of this feedback pathway has come from the use of NOS inhibitors. In a model of gram-negative sepsis in mice, NOS inhibition did not alter the levels of TNF-α following infection (Evans et al. 1994) although other studies in models of gram-negative infection have observed increases in TNF-α levels. The effect of NO in these systems may depend on the degree to which the cytokine cells are stimulated; clearly, it there is maximal stimulation, NO inhibition does not produce any additional effect. Important mediators of gram-positive sepsis include a number of bacterial toxins which can act as potent stimulators of a large percentage of the T cell pool, so-called 'superantigens'. In mice given the staphylococcal superantigen staphylococcal enterotoxin B, NOS blockade markedly increased production of IFN-γ and TNF-α both in vitro and in vivo (Florquin et al. 1994). The increased cytokine production in these mice was associated with an increased mortality.

Further work supporting these data has come from the use of transgenic mice in which the iNOS gene has been disrupted (Wei et al. 1995). Splenocytes from such animals produce more IFN-γ following antigenic challenge with *Leishmania major*. Concentration of NO may be important, however, since at low concentrations NO can enhance T cell proliferation. The pathophysiological effects of such feedback roles of NO on cytokine production are far from clear, especially in human studies; further work is necessary to establish the importance of this pathway and whether it limits the usefulness of NOS inhibitors in the treatment of sepsis.

5.4 Myocardial Effects

Although the majority of patients with septic shock have a normal or increased cardiac index, there is a sub-population who have reduced cardiac output (Nishijima et al. 1973). Moreover, in patients with normal or increased cardiac output there is often profound reduction in ejection fraction with concomitant compensatory dilatation of the ventricles (Parker et al. 1984). These abnormalities return

to normal within 7–10 days after resolution of sepsis. The clinical significance of this myocardial depression is uncertain. In patients who have profound vasodilatation, depressed cardiac performance contributes to the difficulties of increasing blood pressure and tissue perfusion. In fact, however, no difference has been found between survivors and non-survivors of septic shock with respect to the degree of myocardial depression (Parker et al. 1984). Although further work is required to clarify the exact role that myocardial depression plays in the outcome from septic shock, it is certainly a significant phenomenon which contributes to the altered cardiovascular responses seen in sepsis.

Initial studies using sera from patients with septic shock identified a circulating factor which reduced the extent of contraction of spontaneously beating neonatal rat cardiac myocytes (Parrillo et al. 1985). The discovery of pro-inflammatory cytokines which are produced in large quantities in sepsis made these agents likely candidates for this myocardial depressant factor. The first report of the direct effect of pro-inflammatory cytokines on myocardial function found that cytokines such as TNF-α were able to produce a rapid (2–3 min) reduction in contractility of isolated hamster papillary muscle (Finkel et al. 1992). This effect was blocked by inhibitors of NOS, suggesting that the negative inotropic effect was mediated by NO. A separate study, however, found that the immediate effects of TNF-α in adult cat myocytes do not depend on increased NO output (Yokoyama et al. 1993).

Such rapid effects of cytokines suggested that the induction of iNOS is unlikely to be involved. Certainly it is known that the resting cardiac myocyte contains constitutive NOS activity (Forstermann et al. 1994), and it is possible that these effects result from activation of this isoform. More recent studies have analysed the properties of myocytes isolated from endotoxin-treated animals or treated for more prolonged times with cytokines. In endotoxin-treated rats, isolated cardiac myocytes show a reduction in contractility that is NO dependent (Brady et al. 1992). NO is also involved in mediating the negative chronotropic effect of IL-1β on neonatal rat cardiac myocytes (Roberts et al. 1992). In both these cases the iNOS isoform was involved. A more detailed study of NO output of individual adult rat cardiac myocytes found that the sustained rise in NO output following treatment with IL-1β or IFN-γ is due to induction of the iNOS (Balligand et al. 1994). Production of NO by this isoform is responsible for reducing the contractility of the muscle cells. Taking these results together, it would seem that the major effect of cytokines on cardiac myocytes is to induce iNOS. Production of NO by this isoform then mediates negative inotropic and chronotropic effects. Clearly under some conditions, however, cytokines can produce rapid effects which are likely mediated by the constitutive NOS(s).

The role that cardiac NO plays in the whole animal is also somewhat confusing. Studies using NOS inhibitors in LPS-treated animals have demonstrated in a number of cases clear reductions in cardiac output following inhibitor treatment (Cobb et al. 1992). Obviously, if NO is a mediator of a negative inotropic effect in sepsis, the predicted effect of NOS inhibitors would be to in-

crease cardiac output. Negative inotropic effects of NOS inhibitors have also been found in humans with septic shock (see Sect. 6). It is difficult to explain this apparent contradiction. Either compensatory cardiovascular reflexes produce cardiac depression on blocking NO synthesis, or NO production actually protects the heart by suppressing its metabolic activity, such that on removing NO production damaging factors produced in sepsis can further reduce cardiac output.

5.5 Cerebral Effects

NOS is widely distributed within the central nervous system, where it plays a number of roles (Bredt and Snyder 1994). One of these is in the synaptic transmission of the neurotransmitter glutamate, via *N*-methyl-D-aspartate subtype receptors. Excess levels of NO are neurotoxic, and it is possible therefore that the excess production of NO in sepsis has deleterious effects on brain function (Izumi et al. 1992; Iadecola et al. 1995).

There are not large amounts of data which address this question directly. Following exposure to LPS and/or inflammatory cytokines both astrocytes and neurones can produce iNOS (Wallace and Bisland 1994; Galea et al. 1994; Minc Golomb et al. 1994). Thus there is potentially a source a high-output NO production in the brain in sepsis, although immunochemical studies have not shown appreciable CNS staining for iNOS in a number of animal models of sepsis (Buttery et al. 1994; Sato et al. 1995). NOS inhibitors can block the neural damage seen after cerebral artery occlusion in a number of animals. No specific study has been made of pathophysiological brain changes in sepsis following NOS inhibition. However, the NOS inhibitor *N*-aminoarginine produces seizure activity and increased mortality in a model of sepsis in dogs (Cobb et al. 1992). Clearly, CNS effects of NOS inhibition may limit the usefulness of this approach in the therapy of sepsis; human data are at present lacking.

6 Nitric Oxide: Clinical Data in Sepsis

The clinical evidence that NO is involved in the pathology of sepsis comes from two sources; first, direct measurement of NO (or, more usually, surrogate markers of its presence) in septic patients, and, second, data from clinical trials in which various NO blocking drugs have been used.

6.1 Measurement of Nitrite/Nitrate in Sepsis

Three studies have made direct measurements of nitrite/nitrate (the stable end-products of NO biosynthesis) in septic patients. Ochoa et al. (1991) obtained

sequential samples from a total of 39 critically ill patients: 17 general surgery patients who were clinically septic or had a severe inflammatory process, 14 trauma patients who had an uneventful recovery, and 8 trauma patients who became septic. The average NO_2^-/NO_3^- level in the septic patients was indeed higher than that the healthy controls (63.1 ± 6.5 μmol/l versus 28.9 μmol/l, $p<0.02$). Curiously, however, levels in the 14 trauma patients without complications (12.8 ± 1.5 μmol/l, $p<0.001$) were significantly *lower* than the controls, and even more surprisingly, NO_2^-/NO_3^- levels did not rise (15.6 ± 1.9 μmol/l) in trauma patients who developed sepsis. To determine whether increases in NO_2^-/NO_3^- were correlated with measurable changes in vascular tone, the levels in the septic patients were compared with the systemic vascular resistance index (SVRI) in the (unspecified) subset of patients in whom invasive hemodynamic monitoring had been carried out. In patients with NO_2^-/NO_3^- levels greater than 42 μmol/l there was a significant reduction in SVRI, although no effect on cardiac index. Interestingly, serum endotoxin levels were also significantly elevated in this subgroup of patients.

The second study (Evans et al. 1993) was performed in 12 septic patients, 7 of whom were receiving inotropes at the time that blood was drawn. The mean arterial pressure (MAP) of the 12 patients ranged between 47 and 73 mmHg, and the mean score on the Acute Physiological and Chronic Health Evaluation (APACHE) II was 24 (range 6–32). The mean NO_2^-/NO_3^- level in the septic patients was 124 μ*M* (range 20–187) compared to 36.4 μ*M* in healthy controls ($p<0.001$). Of interest, a third group of 7 postoperative patients with no evidence of sepsis also had elevated NO_2^-/NO_3^- levels (87.3 μ*M*), intermediate between the other two groups.

The third of this group of studies was similar in design and investigated two groups of septic patients (with or without shock), with a group of non-septic patients as controls. The findings were consistent with those from the other two reports: septic patients with shock had significantly higher NO_2^-/NO_3^- levels (mean 52.9 ± 9.15 nmol/ml) than either the non-shock or non-septic groups. Furthermore, within the subgroup of 13 septic shocked patients, there was significant correlation between NO_2^-/NO_3^- levels and plasma endotoxin, and a negative correlation with systolic blood pressure. In the 8 patients in whom hemodynamic measurements were made NO_2^-/NO_3^- levels were also correlated with cardiac output and SVRI.

The only other study which attempted to correlate NO_2^-/NO_3^- levels with the clinical response was carried out not in sepsis but in patients receiving IL-2 as tumour immunotherapy (Ochoa et al. 1992). Toxicity to IL-2 is manifest principally by the vascular leak syndrome, hypotension, and a hyperdynamic response with low systemic vascular resistance. In 12 patients plasma nitrate levels were significantly elevated, and the authors concluded that NO was a likely cause of the toxicity.

In summary, these data indicate that plasma NO_2^-/NO_3^- levels are elevated in patients with septic shock, and that the levels are correlated directly with physiological variables such as blood pressure and systemic vascular resistance.

However convincing this may seem, the data do not prove causation; nitrate levels are also elevated in healthy controls as well as non-shocked patients, and they are not elevated in shocked trauma patients. Patients with sepsis are extremely complex to evaluate, and it is notoriously difficult to identify cause and effect. This problem was well illustrated by the initial report that plasma nitrate levels showed a highly significant correlation with APACHE III scores, suggesting to the authors that NO_2^-/NO_3^- levels could be used as a predictor of morbidity in postoperative patients (VAN DISSEL et al. 1994). However, MACKENZIE et al. (1994) later pointed out that this finding is probably just an artefact of the correlation of nitrate with renal function and the fact that renal function and age make a very significant contribution to the calculation of the APACHE score.

The best way to address the issue of cause is to study the effects of specific inhibitors of NO in patients with sepsis.

6.2 Clinical Studies with Inhibitors of Nitric Oxide

PETROS et al. (1991) were the first to describe the use of arginine analogues in two patients with severe septic shock. Both L-NMMA and L-NAME produced a rise in MAP lasting between 10 min and several hours, although in the second patient this was accompanied by a fall in cardiac index (CI) from 4.5 to 2.7, $l\,min^{-1}\,m^{-2}$ and the patient died with multi-organ failure. Independently, SCHILLING et al. (1993) also tried L-NMMA in a patient with refractory septic shock, and they too noted a rise in MAP accompanied by a 30% fall in CI, although their patient survived.

Since these early anecdotal reports two more substantial studies of arginine analogues have been completed. PETROS and colleagues (1994) carried out a randomised, double-blind, placebo-controlled investigation of L-NMMA in 12 patients with septic shock. Since the numbers were small, comparison of the two groups is rather unhelpful; it is not surprising that there were major differences between them. The 6 patients receiving active drug were given two bolus injections of 0.3 mg/kg, then 1 mg/kg, followed by a continuous infusion of 1 mg/kg per/hour for 6 h. The findings confirmed the earlier observations, in that there was a sustained rise in MAP and systemic vascular resistance, accompanied by a fall in heart rate and cardiac output.

In the second study LORENTE et al. (1993) carried out a more detailed investigation using a slightly different inhibitor, N^{ω}-nitro-L-arginine. The protocol they used was interesting: the first group of 8 patients were given a bolus injection of N^{ω}-nitro-L-arginine (20 mg/kg) followed by a bolus injection of L-arginine. The second group (7 patients) received L-arginine alone. NO is derived from the terminal guanidyl molecule of L-arginine, and to give L-arginine to a septic patient might be thought unwise. However, it is not as surprising as it seems at first sight. First, the early work of Petros and others had suggested that inhibiting NOS is potentially a "two-edged sword"; the rise in MAP is beneficial, but the fall in cardiac output is a significant disadvantage. Second, there

was experimental evidence that L-arginine prevents death in animal models of sepsis (MADDEN et al. 1988; GIANOTTI et al. 1993). Hence the protocol adopted in this study was designed to address directly the causative role of NO in the hemodynamic changes that occur in sepsis, and indeed the results are very clear. The administration of N^{ω}-nitro-L-arginine caused a sustained rise in MAP from 89 ± 8 mmHg to a maximum of 140 ± 12 mmHg, accompanied by fall in CI from 3.51 ± 0.39 to 2.65 ± 0.21 l $min^{-1}m^{-2}$. These changes were reversed by the administration of L-arginine. In the second group of patients, who received L-arginine alone, there was transient hypotension and an increase in CI from 3.57 ± 0.15 to 4.74 ± 0.54 l $min^{-1}m^{-2}$.

Arginine analogues such as L-NMMA are competitive inhibitors of NOS; an alternative approach to limiting the effect of excess NO is to block soluble guanylate cyclase, the cellular target of NO. The dye methylene blue acts on soluble guanylate cyclase and has been in use for many years for the treatment of nitrate poisoning; therefore several investigators reasoned that it might also have a role in septic shock. SCHNEIDER et al. (1992) gave methylene blue to two patients in advanced septic shock. In each there was a modest rise in MAP (59–87 mmHg) and a fall in CI (7.8–6.6 l $min^{-1}m^{-2}$). Both patients died a few days later. In the second study (PREISER et al. 1995) 14 patients in shock were given an infusion of 2 mg/kg of methylene blue, and 6 of them were given a second dose 90 min later. The drug produced short-lived increases in MAP (from 61.1 ± 7.6 to 71.7 ± 12.0 mmHg) without any significant effect on heart rate or cardiac output. No adverse effects were seen, but 11 of the 14 patients subsequently died as a consequence of multi-organ failure.

To what extent then do these clinical studies with NO inhibitors of various types provide firm evidence implicating NO in the pathology of septic shock? Ideally, to be able to answer that question we need to know: (a) that the drugs specifically block the production (or the effect) of NO, and that they have no other pharmacological effect at the doses employed; (b) that they can be shown to significantly reduce the plasma levels of nitrite/nitrate; (c) that any haemodynamic changes can be confidently attributed to the intervention, and that no other changes in treatment occur simultaneously; and (d) that there is a sustained effect on the clinical course (ideally, although not necessarily associated with improved survival).

How do the studies described above match up to these goals? The various arginine analogues are specific inhibitors of NO synthase, although they are not specific for the inducible isoform which many believe is principally responsible for the excess NO production in sepsis. Methylene blue is clearly non-specific. The lack of nitrite/nitrate measurements is perhaps the most surprising omission from these clinical studies. None of the papers reviewed here included data showing that treated patients had lower NO_2^-/NO_3^- levels than controls or healthy normals; given the fact that such measurements are feasible (see above), this is an oversight which should be remedied. Indeed, the only *direct* evidence that the drugs were used at a clinically relevant concentration was in the two patients given methylene blue, in whom plasma cGMP levels were

reduced (SCHNEIDER et al. 1992). Probably the best evidence that haemodynamic changes are the direct result of the intervention comes from the study by LORENTE et al. (1993), although in all the others the authors were careful to emphasise that no other changes to the treatment were made during the period of experimental observation.

Finally, what about efficacy? It is not our purpose here to discuss in detail the rationale for NO as a therapeutic target or to compare specific forms of treatment. However, in assessing whether there is good evidence that NO plays an important role in the disease or is present merely as a bystander, we must look at the effect these interventions have had. There is a consistent theme in the reports discussed above which suggests that modification of NO activity is reflected in changes in the haemodynamic state in septic patients. The fact that these changes are often merely transitory and may be accompanied by unwanted effects represent real challenges for the pharmacologist and clinician, but nevertheless do suggest that there is a true cause and effect relationship.

In summary, the clinical data presently available do suggest that excess NO is produced during septic shock, and that it is responsible for some of the clinical features of the disease. The data are not perfect; we have alluded to some of the caveats above. There is a lack of histochemical data in patients showing NOS activity in patients with shock, and the absence of specific, effective therapy limits the conclusions that can be drawn. Given the active state of investigation in this field, most of these issues are likely to be resolved in the near future.

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Multiple Organ Failure in Septic Shock

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1 Introduction

In the late 1960s several reports appeared describing remote organ failure (e.g., pulmonary or liver failure) as a complication of severe sepsis (CLOWES et al. 1968; SKILLMAN et al. 1969). Stimulated by the observation of TILNEY et al. (1973) that sequential failure of initially uninvolved organ systems may follow operations for ruptured aortic aneurysm, the concept emerged that severe injury can result in damage to distant organ systems. In a classical editorial by BAUE (1975) entitled "Multiple, Progressive or Sequential Systems Failure: A Syndrome of the 1970s," this concept was formulated as the basis of a "new" clinical syndrome. Several terms were coined thereafter, such as multiple organ failure (EISEMAN

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et al. 1977), multiple system(s) organ failure (Border et al. 1976), and multiple organ system failure (Bell et al. 1983), to describe this evolving clinical syndrome of otherwise unexplained progressive physiological failure of several interdependent organ systems. More recently the term multiple organ dysfunction syndrome has been proposed as a more appropriate description (Bone et al. 1992).

Major advances in the field of surgical and medical treatment and resuscitation techniques, widespread introduction of monitoring and life-support technologies, and the evolution of intensive care units (ICUs) have allowed the survival of critically ill patients who previously would have died. However, it also became increasingly evident that in a number of patients, in particular septic patients, progressive deterioration of multiple organ function occurs, notwithstanding optimal therapeutic efforts and interventions.

The increasing incidence of morbidity and mortality caused by multiple organ failure (MOF) has paralleled improvements in life-support technologies and their application to an increasingly high-risk patient population. The emergence of the MOF syndrome, usually defined as failure of at least two organ systems, has therefore been a direct consequence of the evolution of ICU capabilities.

MOF is presently the leading cause of death in the ICU and is responsible for 50%–80% of all ICU deaths, with a major impact on ICU and hospital resources (Barton and Cerra 1989). Critically ill patients today rarely die as a consequence of the disease process progressing to the point that admission to the ICU is required but rather from MOF, which is increasingly recognized as the final common pathway leading to death in these patients (Marshall 1994). Initial studies stressed the relationship between uncontrolled sepsis and remote organ dysfunction (Polk and Shields 1977; Fry et al. 1980). It became increasingly evident, however, that remote organ system dysfunction can evolve in the absence of indentifiable infection (Meakins et al. 1980; Faist et al. 1983; Goris et al. 1985). MOF has now been documented as a complication in a variety of noninfectious conditions such as acute pancreatitis, major surgery, severe trauma, burns, and hypovolemic shock. No obvious differences in clinical signs and symptoms of MOF can be identified between patients with or without a bacterial infection (Goris et al. 1985). Moreover, a similar syndrome can be reproduced in the animal model by infusion of various mediators of inflammation (Tracey et al. 1986). These observations have led to the concept of a generalized inflammatory host response to variable insults as the pathophysiological basis of MOF. The fact that organ failure occurs simultaneously or in a certain sequence also suggests a common or at least similar pathogenesis. In addition, a complex interrelationship among individual organ systems may promote a domino effect in that failure of one organ may establish an amplification process that hastens injury to another (Eiseman et al. 1977; Matuschak and Rinaldo 1988; Callery et al. 1991).

2 Definitions

Although the term MOF has been widely used since its introduction, the criteria used to characterize it emerged in an arbitrary and retrospective fashion and differed widely among the various studies. Many authors regard organ *failure* as a dichotomous event, either present or absent, and define the severity of MOF by the number of failing organ systems (Fry et al. 1980; Bell et al. 1983; Knaus et al. 1985; Henao et al. 1991). This concept of organ failure reflects insufficient physiologic function to sustain the normal needs of the host without exogenous intervention (Baue 1975).

Others, however, support the concept that organ *dysfunction* is a continuum with incremental degrees of physiologic derangements in individual organ systems, a process rather than an event (Goris et al. 1985; Marshall et al. 1988). Alteration in organ function can vary widely from some mild degree of organ dysfunction such as a moderate elevation in creatinine or bilirubin levels, which have no clinical consequences, to frank organ failure such as severe respiratory failure or coma. The degree of organ dysfunction therefore has a major clinical impact.

Recently the term *multiple organ dysfunction syndrome* (MODS) has been proposed, defined as a clinical syndrome characterized by the development of progressive but potentially reversible physiological dysfunction in two or more organs or organ systems induced by a variety of acute insults, including sepsis (Bone et al. 1992).

The term dysfunction identifies this process as a phenomenon in which organ function is not able to maintain homeostasis and recognizes it as a dynamic continuum of deranged physiological function rather than an event which is either present or absent. In the continuous process of the host inflammatory response MODS may be understood to represent the more severe end of the spectrum of the severity of illness (Bone et al. 1992). If MODS represents a continuum of severity, ICU mortality would be related not only to the number of failing organs but also to the degree of dysfunction of any particular organ system (Marshall 1994). Support for this concept has been provided by the observation of a strong correlation between increasing dysfunction within organ systems and the probability of death (Marshall 1994, 1995). There is, however, no clear consensus on what specific dysfunctioning (or failing) organ systems should constitute the syndrome. If looked for, abnormalities in physiological function can be found in virtually any organ system in critically ill (septic) patients. Moreover, there is no consensus on the best descriptors of organ dysfunction or the most appropriate assessment of the degree of dysfunction. Also, if the term MODS is generally accepted, it remains to be decided whether the term organ failure should still be used for the most severe degrees of dysfunction. Authors have used a wide variety of indicators of organ dysfunction, including (biochemical) markers reflecting functional organ abnormalities with various cutoff points, the necessity of mechanical support (e.g., mechanical ventilation, he-

modialysis) or pharmacological support (e.g., inotropes), and even disease categories such as cholecystitis and stress ulcer. Therapeutic measures as a descriptor of organ dysfunction are subject to different medical policies and local practices. Also, diagnostic categories may be based on subjective criteria, and one may question whether they really reflect remote organ dysfunction. Objective, indisputable, and quantifiable variables, widely used in clinical practice, therefore appear to be the best candidates to assess organ dysfunction.

In a review of 30 papers related to the subject MARSHALL (1995) showed that seven systems (respiratory, renal, gastrointestinal, hepatic, central nervous system, hematological, and cardiovascular) were included in the definition of MOF in more than half of the reports. It appeared that, although criteria for organ dysfunction varied markedly, definitions were based primarily on physiological abnormalities and to a lesser extent on the basis of therapeutic interventions. In this analysis valid single variables reflecting physiological dysfunction appeared to be available for five organ systems: PaO_2/FiO_2 ratio for the respiratory system, serum creatinine level for the renal system, serum bilirubin level for the hepatic system, platelet count for the hematological system, and the Glasgow Coma Score for the central nervous system. Descriptors of dysfunction for the cardiovascular system and the gastrointestinal system seem at present suboptimal (MARSHALL 1995). A single variable has the advantage of simplicity and provides a measure that can be very sensitive to progressive degrees of organ dysfunction, but it may lack specificity, is prone to measurement error, and can be influenced by therapeutic intervention (MARSHALL 1995).

The importance of this detailed analysis of the literature is that it provides a basis for more uniformly accepted definitions and criteria for MOF/MODS. Given the lack of uniform definitions, the term organ failure for specific organ systems is used below on the basis of the definitions used by the authors, which implies obvious limitations for conclusions.

3 Risk Factors and Mortality

Several studies have demonstrated that advanced age, preexisting chronic disease (including malnutrition and alcohol abuse), and sepsis are major (independent) risk factors for the development of MOF (PINE et al. 1983; KNAUS et al. 1985; TRAN et al. 1990). Underlying chronic disease and advanced age are usually associated with a depression in functional reserve of individual organs, reducing the tolerance to injury (GEE et al. 1990). Advanced age and preexisting chronic disease are in turn risk factors for sepsis (TRAN et al. 1990). In a large multicenter study it appeared that patients with septic shock (and those with cardiac arrest) had a much higher chance to develop MOF than patients with other diagnoses (KNAUS et al. 1985). A case-control study in a surgery and trauma patient population showed that hypovolemic shock, sepsis, and the time before

adequate resuscitation were independent risk factors for MOF (Henao et al. 1991). Therefore, sepsis and shock seem to be the most common conditions leading to MOF.

Mortality of patients with MOF is extremely high, ranging from 30% to 100%. Mortality is related significantly to the number of failing organ systems (Fry et al. 1980; Pine et al. 1983; Knaus et al. 1985; Goris et al. 1985; Tran et al. 1990; Henao et al. 1991; Hebert et al. 1993) and to the duration of organ failure (Knaus et al. 1985). Mortality almost uniformly approaches 90%–100% when three or more organ systems fail. In a large-scale multicenter study 99 patients had three or more failing organ systems after 72 h of intensive therapy, of whom only two survived (Knaus et al. 1985). Most of these patients (80%) were non-operative admissions with diagnoses such as septic shock or cardiac arrest. In addition to the number of failing organs, advanced age and preexistent chronic disease are major determinants of overall mortality (Knaus et al. 1985; Tran et al. 1990).

4 Incidence and Prevalence

Depending on the type of ICU and its case mix, MOF can be observed to develop in about 10%–15% of the patients (Faist et al. 1983; Knaus et al. 1985). However, much higher incidences have been reported, even as high as about 40% (Tran et al. 1990). There is a remarkable paucity of published epidemiological data and of studies focusing specifically on the incidence of MOF in patients with septic shock. Most studies have addressed MOF as an entity more or less regardless its cause. However, the prevalence of sepsis in most series is high, ranging from 50% to 90% (Eiseman et al. 1977; Fry et al. 1980; Henao et al. 1991).

In a study in patients with positive blood cultures who exhibited clinical signs of the sepsis syndrome the course of disease was complicated by the adult respiratory distress syndrome (ARDS) in 15%, by disseminated intravascular coagulation (DIC) in 5.4%, and by other manifestations of MOF (acute renal and hepatic failure) in 36% (Kieft et al. 1993). A recent prospective evaluation of the natural history of the systemic inflammatory response syndrome (Bone et al. 1992) demonstrated that the incidence of end organ dysfunction increased with the more severe forms of sepsis (Rangel-Frausto et al. 1995). In patients with septic shock the attack rate of ARDS was 18%, of DIC 38%, and of acute renal failure 51% (Table 1). A higher incidence of organ failure was observed in another study in 52 septic patients: ARDS 79%, acute renal failure 67%, liver failure 65%, and 38 patients died of at least three organ failures (Esen et al. 1992). It is not clear in these studies, however, how many patients developed dysfunction of two or more organ systems. In a survey of 393 patients with bacteremia over a 7-year period MOF was present in 22.6% of the patients

Table 1. Clinical trials in sepsis

Study	Type of patients	*n*	Organ failure (%)				
			Respiratory	Renal	Hematological	Hepatic	Neurological
Ziegler et al. (1991) (HA-IA study 1)	Gram-negative, bacteremia with, sepsis syndrome	200	10.5**	40.5	19.5*	22.5	–
Greenman et al. (1991) (E5-study 1)	Gram-negative sepsis	316	21.5**	22.5	27 *	–	–
	No shock	137	14.0	15.0	16.0		
	Shock	179	27.0	28.0	35.0		
Fisher et al. (1994) (IL-1ra study)	Sepsis syndrome	893	25.0**	30.0	14.0*	26.0	–
Dhainaut et al. (1994) (anti-PAF study)	Severe gram-negative sepsis	120	67.0	30.0	29.0	28.0	32.0
Brun-Buisson et al. (1994) (HA-1A study 2)	Sepsis syndrome	600	83.0	44.5	14.0	–	6.0
Abraham et al. (1995) (Norasept anti-TNF study)	Sepsis syndrome	971	19.5**	9.0	32 *	6.0	7.5
Bone et al. (1995) (E5 study 2)	Gram-negative sepsis (no shock)	530	9.5**	13.5	7.5*	–	–
Cohen and Carlet (submitted) (Intersept anti-TNF study)	Septic shock	420	19.5**	18.0	49 *	4.5	6.0
Rangel-Frausto et al. (1995)	Sepsis	649	6 **	19	16 *	–	–
	Severe sepsis	469	8 **	23	18 *	–	–
	Septic shock	110	18 **	51	38 *	–	–

*ARDS, **DIC

(Uzun et al. 1992). In a report from a medical ICU including 196 patients with sepsis, 27 (14%) did not have any organ failure at any time during their ICU stay, 48 had single-organ failure, and 121 (62%) developed MOF, but cardiovascular failure was included in the definition of organ failure (Tran et al. 1990). A study of 48 patients with severe septic shock found the mean number of failing organ systems 48 h after admission to be 3.3 for survivors and 4.0 for nonsurvivors (Ruokonen et al. 1991).

The recently completed clinical trials in sepsis provide a large data base, including the prevalence of organ dysfunction at the time of entry into the study (Table 1). There is, however, no published information available on the prevalance of MOF, i.e., simultaneous failure of at least two organ systems and some degree of organ dysfunction was usually included in the inclusion criteria. In the French HA-1A study 33% of patients had three or more organ failures at study entry (Brun-Buisson et al. 1994). Moreover, limited details are available on the development of organ dysfunction in the placebo group in these studies. In the second E5 study 9% of the placebo group patients developed ARDS, 10%

acute renal failure, 7% DIC, 18% central nervous dysfunction, and 6% hepatobiliary dysfunction after enrollment in the study (Bone et al. 1995). In the Intersept study 33.6% of the patients in septic shock had dysfunction of two organ systems, 10.3% of three and 2.3% of four are more organ system at the time of inclusion (data kindly provided by Bayer-Miles Inc.). Therefore, taken together, 46.2% of the patients had multiple organ failure early in the course of septic shock (Cohen and Carlet, submitted). During a 28-day follow-up an additional 31.2% of the placebo group developed acute renal failure, 14.3% ARDS, 23.4% DIC, 14.1% liver dysfunction, and 6.3% neurological dysfunction (Bayer-Miles). Although the definition of organ failure varied, the conclusion is warranted that the incidence of organ failure in sepsis and particularly septic shock is high, and this complication seems to occur mainly early in the course of disease. Because only the more severe forms of organ dysfunction are commonly included in the definition, the true incidence of organ dysfunction is most likely underestimated.

5 Is There a Sequence of Organ Failure?

It has been suggested that the evolution of MOF generally follows a predictable course, beginning with the lungs and followed by hepatic, intestinal, and renal failure, in that order (Deitch 1992). This may be particularly clear in polytrauma patients but is often less so in other patient categories. The exact temporal pattern of organ dysfunction in MOF differs somewhat between studies (Eiseman et al. 1977; Fry et al. 1980; Goris et al. 1985). The temporal sequence of organ failure can be significantly modified by the presence of preexistent disease and by the nature of the precipitating event. For example, in patients with chronic renal disease the kidney tends to be the first organ to fail. This illustrates that the physiological reserve of a specific organ system is an important determinant of its failure following an acute insult. Also, acute renal failure may be an early event in patients who sustain a prolonged period of circulatory shock. Nevertheless, the respiratory system in general is almost invariably the first organ system to fail.

The pattern of failing organ systems may furthermore change over time. Nowadays, bleeding due to stress ulcers, in various reports a criterion for gastrointestinal failure, has become a rare event in the ICU, most likely due to improved resuscitation policies (Cook et al. 1994). There is only very limited information in the literature on the time sequence of failing organ systems in patients with septic shock. In this condition severe cardiovascular failure is by definition present from the very start. In a study in 48 patients with severe septic shock all had respiratory failure, defined as the clinical need for mechanical ventilation at the onset of shock (Ruokonen et al. 1991). After 48 h the prevalence of acute renal failure was 50%, gastrointestinal failure 64%, hepatic failure 14.3%, and central nervous system failure 50%. This study, again, illus-

trates that organ failure may occur early and simultaneously in various organ system, especially in severe septic shock. In general, respiratory failure and ARDS are early complications of septic shock, followed by acute renal failure, whereas clinical jaundice is usually a late complication. Also, (severe) thombocytopenia can occur early in the course of septic shock, in some cases associated with DIC. Central nervous system dysfunction also may occur very early in the course of septic shock. (Bolton et al. 1993) but is often difficult to evaluate as many patients are mechanically ventilated.

6 Clinical Manifestations

Circulatory failure, i.e., septic shock, complicates invasive bacterial infection in about 40%–50% of cases and may be the most impressive initial clinical expression of organ failure in many of these patients. Circulatory failure by itself can contribute significantly to the development of organ failure (e.g., acute renal failure) and may therefore also be considered a cause of MOF. There is, however, a wide variety in the time course and severity of complicating circulatory failure in septic patients, ranging from mild circulatory instability somewhere in the course of a septic period to severe progressive shock as its earliest and often fatal manifestation. MOF and circulatory failure however, are, often dissociated, and multiple organ dysfunction may complicate severe sepsis without clinical signs of shock. Also, before circulatory shock develops, sepsis-related dysfunction such as thrombocytopenia can already be observed, and acute respiratory distress may be its earliest manifestation (Vito et al. 1974). These observations emphasize that sepsis-induced multiple organ dysfunction is the result of a systemic disorder with varying clinical expression and severity, among other things related to preexisting chronic disease and/or limited organ physiological reserve.

Patients with septic shock usually respond initially to therapeutic management. However, some may die within 72 h of progressive nonresponsive hypotension (Parker et al. 1987; Ruokonen et al. 1991). Most patients survive the acute hypotensive episode with gradual improvement in clinical state (Jordan et al. 1987). In the days following resuscitation the clinical condition can deteriorate due to increasing distant organ dysfunction. Several clinical stages in the development of MOF have been described in postoperative patients, starting with a phase with relative hemodynamic stability in which only very mild abnormalities can be detected, followed by stages of progressive clinical and laboratory deterioration (Carrico et al. 1986; Barton and Cerra 1989). A more fulminant course may be followed in septic shock, and MOF is often an early complication (Ruokonen et al. 1991). Nevertheless, in patients surviving the early acute episode progressive multi-organ involvement becomes the primary clinical problem and cause of mortality (Parker et al. 1987; Ruokonen et al. 1991). It appears

therefore that in general the septic patient can exhibit both an acute phase with severe physiological derangements in a few organ systems, which resolve when the patient responds to treatment, and a more chronic phase with functional deterioration of multiple organ systems which may resolve upon adequate therapy but often becomes progressive and resistant to treatment (JORDAN et al. 1987). These patients are usually hypermetabolic and have a hyperdynamic circulation with an elevated cardiac output and a low systemic vascular resistance (DEITCH 1992; BARTON and CERRA 1989). This hypermetabolism–organ failure syndrome (BARTON and CERRA 1989) is characterized by persistent (low-grade) fever, leukocytosis, tachycardia, tachypnea, hyperglycemia, hyperlactatemia, increased urea production, and increased total body oxygen uptake.

6.1 Adult Respiratory Distress Syndrome

Septic shock is the major risk factor for ARDS (PEPE et al. 1982), and this complication occurs in 20%–60% of cases. The basic pathogenetic mechanism of ARDS is pulmonary microvascular injury with increased permeability and resultant flooding of alveoli with protein-rich fluid which in addition damages surfactant. This has major pathophysiological consequences: alveolar collapse, increased intrapulmonary shunting, progressive hypoxemia, decreased compliance, decreased functional residual capacity, and increased work of breathing.

Tachypnea and hyperventilation with respiratory alkalosis occur almost uniformly in early septic shock and do not necessarily indicate significant pulmonary damage. However, increased pulmonary microvascular leakage of protein-risk fluid may occur even at this stage. Clinical signs of evolving ARDS are progressive dyspnea, use of auxilliary respiratory muscles, flaring of the nostrils, and central cyanosis. ARDS may develop within hours after the onset of septic shock. The chest roentgenogram shows diffuse alveolar infiltrates compatible with pulmonary edema, but significant changes in pulmonary gas exchange usually occur before pulmonary edema becomes radiographically visible. When not supported by mechanical ventilation, respiratory muscle fatigue develops, resulting in progressive (hypercapnic) ventilatory failure and death. The increased work of breathing, diminished oxygen supply to respiratory muscles, and metabolic factors are its pathogenetic basis.

6.2 Renal Dysfunction

Oliguria is a very common initial sign in septic shock and is often included in its definition. It is a functional compensatory response to hypotension with sodium retention and a rise in serum urea in excess of serum creatinine. Proteinuria is not uncommon and is often of tubular origin (RICHMOND et al. 1982). In some patients, inappropriate polyuria is observed, which may be related to a little understood tubular dysfunction (LUCAS 1976). When shock is not readily and

adequately reversed, acute tubular necrosis with progressive uremia ensues. Most cases of acute renal failure in septic shock are oliguric, but increasing incidence of nonoliguric cases is observed (KLEINKNECHT 1990). Renal hypoperfusion is the most important pathogenetic mechanism, also in septic acute renal failure, with decrease in outer cortical blood flow and other intrarenal hemodynamic alterations (GROENEVELD 1994). However, nonhemodynamic factors including sepsis-induced humoral mediators (e.g., thromboxane A_2, endothelin) and activated cellular elements (e.g., granulocytes, coagulation products) may contribute significantly to the development of renal dysfunction (GROENEVELD 1994).

6.3 Central Nervous System Failure

Changes in mental state may be an early and sometimes the sole initial sign of severe sepsis. Elderly patients seem to be more prone to central nervous system dysfunction, which ranges from mild disorientation, impaired orientation and concentration, agitation, confusion, or lethargy to frank obtundation (GLECKMAN and HIBERT 1982; HOLLOWAY and REINHARDT 1984). These clinical symptoms are often included in the definition of septic shock as a sign of organ hypoperfusion. Focal signs and seizures may occur but are rare. In a prospective study YOUNG et al. (1992) observed encephalopathy in 70% of a group of 69 septic patients, being severe in most. Mild clinical abnormalities were observed even within hours of a positive finding on blood culture. Severe encephalopathy manifesting as delirium and then coma was evident within 2 weeks.

The liquor cerebrospinalis usually does not show abnormalities except for a mildly elevated protein level. The EEG is a sensitive indicator of encephalopathy and may show any one of four main patterns of increasing severity (BOLTON et al. 1993). The pathogenesis of septic encephalopathy has not been clarified and is presumably multifactorial. Cerebral hypoperfusion, hypoxemia, dysfunction of the blood-brain barrier, direct effects of cytokines on brain function, focal thrombosis (or bleeding), vasogenic cerebral edema, and disturbances of amino acid metabolism have been implicated (BOLTON et al. 1993). Multiple microabcesses in the brain have been demonstrated in a significant number of patients (JACKSON et al. 1985), but it is unclear whether these can account for the observed mental state abnormalities.

6.4 Hematological Failure

Leukocytosis with a left shift, a common finding in septic shock, reflects the release of less mature leukocytes from bone marrow storage pools. Leukopenia is sometimes observed in the early phase of severe septic shock. Microvascular aggregation of granulocytes apparently then predominates, a phenomenon that may herald the development of ARDS (THOMMASEN et al. 1984). Varying degrees

of leukocytosis or leukopenia have been included in the definition of hematological failure. However, most often DIC or (severe) thrombocytopenia is used as a marker of hematological failure. Isolated thrombocytopenia due to increased platelet turnover is a frequent companion of septic conditions and can be an early clue to sepsis. Thrombocytopenia can be extremely pronounced and is clinically apparent by petechiae and (mucosal) bleeding. In cases of renal failure these clinical signs can develop at higher platelet counts due to uremic thrombocytopathy. The main cause of isolated thrombocytopenia is probably endothelial cell injury resulting in a procoagulant surface with platelet aggregation. Sepsis is the most common cause of acute DIC, a syndrome characterized by intravascular clotting and microvascular fibrin deposition and consumption of clotting factors. Prolonged clotting times, hypofibrinogenemia, thrombocytopenia and the presence of fibrin degradation products are laboratory signs of the syndrome. Both hemorrhagic complications and thrombotic small vessel occlusions are its clinical manifestations.

6.5 Liver Dysfunction

Liver dysfunction is a virtually universal occurrence in patients with severe sepsis and may range from minor elevation of liver enzymes to pronounced jaundice in the late stages. A rising bilirubin level disproportional to levels of hepatocellular enzymes or alkaline phosphatase appears to be associated with a poor prognosis (Banks et al. 1982; Barton and Cerra 1989). It has been suggested in fact that the development of clinical hepatic failure defines MOF (Barton and Cerra1989). Mild elevations in serum bilirubin may be noticed even early in septic shock (Franson et al. 1985). Characteristically bilirubin is almost entirely of the conjugated type, but nonconjugated bilirubin can appear in associated hemolysis (Zimmerman et al. 1979). In some cases marked elevations of alkaline phosphatase are observed (Fang et al. 1980). Pathological examination of the liver may reveal Kupffer's cell hyperplasia, focal hepatic necrosis, and cholestatic changes (Zimmerman et al. 1979). The pathogenesis of liver dysfunction in sepsis is clearly multifactorial and includes such mechanism as liver ischemia, direct injury from endotoxin, and sepsis-induced mediators, drug toxicity, parenteral nutrition, and bacterial overgrowth.

7 Pathophysiology–Pathogenesis

The similarity in clinical, pathophysiological, and biochemical abnormalities in patients with sepsis-induced MOF and those with MOF associated with severe noninfectious conditions has led to the concept that MOF is the result of a systemic widespread inflammatory host response. A major conceptual change

has been that the host is not an innocent bystander whose tissues are ravaged by invading bacteria but instead is an active participant in this destructive process (Deitch 1992). The phrase "generalized autodestructive inflammation" has been coined to describe this process (Goris et al. 1985). This shift in view has important therapeutic implications: if bacteria or their products directly cause tissue injury, the goal of immunomodulation would be to increase the host's immunoresponse, but if an uncontrolled inflammatory reaction damages tissues, inhibition of this response would limit tissue injury.

Sepsis is initiated by a nidus of infection which causes a local inflammatory response with local activation of macrophages and neutrophils, which is intended to contain and eradicate infecting micro-organisms and to clear damaged tissues of cell debris. Although generally beneficial to the host, inflammatory processes are intrinsically destructive to surrounding tissues (Deitch 1992). When this infection cannot be controlled locally, bacteria or bacterial products may induce a generalized and uncontrolled inflammatory response with the vascular endothelium as essential target (Bone 1991). The clinical correlates are sepsis, severe sepsis, or septic shock, indicating clinical syndromes of progressive severity which can all be complicated by MOF (Bone et al. 1992; Rangel-Frausto et al. 1995).

In septic shock excessive generation of *mediators* and microcirculatory *ischemia* are considered the main mechanisms in the generation of tissue injury. These two processes are closely interrelated and amplify each other. Septic shock itself is the clinical expression of an excessive inflammatory host response which sets the scene for further propagation and amplification of processes leading to multiple organ dysfunction or failure. It is therefore not surprising that MOF often complicates septic shock. Widespread endothelial inflammation and dysfunction, abnormalities in vascular tone, myocardial depression, metabolic derangements, coagulation abnormalities, and resulting organ tissue damage are hallmarks of severe septic shock.

7.1 Mediators

A large number of humoral mediator cascades and products released by various cells are involved in the exaggerated host response which causes most if not all of the clinical symptoms of sepsis and its more severe form septic shock. Invasive (bacterial) infection is a trigger that sets in motion an orchestra of mediators (Hack and Thijs 1991), which activates cell-cell interactions and induces metabolic cellular alterations, processes that ultimately lead to tissue damage and MOF. The major (initial) players in this orchestra are proinflammatory cytokines (tumor necrosis factor, TNF; interleukins, IL), the complement system, the contact system and the extrinsic pathway of coagulation, the fibrinolytic system, and cellular elements such as mononuclear cells, neutrophils, endothelial cells, and platelets. Activation of these cellular and humoral systems results in the release of bioactive products such as prostaglandins, leukotrienes, platelet ac-

tivating factor (PAF), nitric oxide (NO), endothelin, oxygen free radicals, and proteinases. The neuroendocrine and the autonomous nervous system are activated in addition, as part of the general stress response resulting in changes in hormonal levels, and release of mediators such as endorphins. Significant metabolic alterations induced, among others, by cytokines and hormonal changes are part of the syndrome. However, the pathogenesis of the sepsis syndrome and consequently of MOF is far from elucidated. Biological effects of these mediator systems are diverse and complex, many can prompt release of others (and some also of themselves), or have synergistic effects, many may have different effects in combination than they have alone, and many interactions occur that may either augment or limit the inflammatory response (Shalaby et al. 1985; Beutler et al. 1986; Dinarello et al. 1988; Balk et al. 1988; Butler et al. 1989; Petrak et al. 1989; Elias et al. 1990). Moreover, actions of a mediator may differ from setting to setting (Elias et al. 1990), and the preexistent clinical condition of the patient can modify the effects of mediators (Bone 1991). Nevertheless, several mediator systems including cytokines may, when excessively and protactedly produced, directly or indirectly cause tissue injury and establish an self-amplification process which can progress to MOF (Tracey and Lowry 1990).

7.1.1 The Cytokine Network

Bacterial products once shed into the circulation can activate macrophages and monocytes with subsequent release of proinflammatory cytokines (TNF, IL-1, IL-6, IL-8) together with other products. In particular *TNF* and *IL-1* are considered the most important proximal mediators of the generalized inflammatory response. These have considerable overlapping actions although they bind to different receptors and act synergistically in many respects. They have strong biological activities. The interactions of TNF and IL-1 with endothelial cells, leukocytes, and macrophages are in particular important to understand why these cytokines can be instrumental in the induction of tissue injury. These cytokines exert direct toxic effects on *endothelial cells* and enhance permeability (Martin et al. 1988; Schuger et al. 1989; Robaye et al. 1991).

TNF and IL-1 can induce expression of tissue factor and downregulation of thrombomodulin, which reduces the anticoagulant properties of the endothelial surface and enhances its procoagulant activity (Bevilacqua et al. 1986; Nawroth and Stern 1986; Lentz et al. 1991) as well as synthesis of plasminogen activator inhibitor (PAI-1; Nachman et al. 1986; van Hinsbergh et al. 1988). In vivo TNF can induce coagulation and fibrinolysis followed by inhibition of fibrinolysis resulting in a procoagulant state (van der Poll et al. 1990, 1991; van Hinsbergh et al. 1990). Both TNF and IL-1 can promote expression of adhesion molecules in endothelial cells such as ICAM-1, ELAM-1, and VCAM-1, which mediate the adherence of leukocytes to the endothelium (Gamble et al. 1985; Mantovani and Dejana 1989; Briscoe et al. 1992).

Interaction of these cytokines with *endothelial cells* results in the production of hematopoietic growth factors, IL-1, IL-6, IL-8, monocyte chemoattractant protein 1 (MCP-1), PAF, and the prostaglandins E_2 and I_2 (prostacyclin) as well as the generation of vasodilating substances via induction of NO synthase and of the vasoconstrictor peptide endothelin-1 (Kawakami et al. 1986; Nawroth et al. 1986; Kaushansky et al. 1988; Sica et al. 1990a,b; Beasley et al. 1991; Marsden and Brenner 1992). These activities set the scene for amplification processes of mediators that have profound microcirculatory effects. Interaction of TNF with *neutrophils* triggers an increased expression of surface adherence glycoproteins, augmentation of superoxide production, phagocytosis, enzyme release, and aggregation of these cells (Klebanoff et al. 1986; Le and Vilçek 1987; Larrick et al. 1987; De Forge et al. 1990). TNF is able to activate and degranulate neutrophils in vivo (van der Poll et al. 1992). The effects of IL-1 on neutrophil function are less clear. Interaction of TNF with *macrophages* increases their cytotoxic capacity and the production of oxygen radicals and induces the release of other cytokines such as IL-1, IL-6, IL-8, and PAF and prostaglandin E_2 (De Forge et al. 1990; Rock and Lowry 1991). Recently it has been demonstrated that TNF has myocardial depressant activities (Pagani et al. 1992; Finkel et al. 1992; Yokoyama et al. 1993) which may be mediated by NO (Finkel et al. 1992). Also IL-1 is able to induce nitric oxide in myocardial cells (Tsujino et al. 1994). Infusion of TNF and IL-1 in the animal model results in hemodynamic, metabolic, and histopathological abnormalities as observed in severe sepsis (Tracey et al. 1986; Butler et al. 1989). Moreover, anti-TNF antibodies prevented the development of shock and vital organ failure in a sepsis model (Tracey et al. 1987), indicating the pivotal role of this cytokine in the systemic inflammatory response. In vivo TNF can induce the release of IL-1, macrophage, and granulocyte-macrophage colony-stimulating factors (Kaushansky et al. 1988), IL-6 (van der Poll et al. 1992), IL-8 (van Deventer et al. 1993), and IL-10 (van der Poll et al. 1994). TNF is also able to activate the complement and contact systems (Nurnberger et al. 1993), although the underlying mechanisms are far from clear.

Increased plasma levels of TNF and IL-1, albeit inconsistently and variably, can be observed in septic patients (see review by Thijs and Hack 1995), but a correlation with the development of MOF has not been clearly established. Plasma concentration may not precisely reflect activation in tissues. However, persistent elevation of TNF levels have in some studies been associated with MOF (Pinsky et al. 1993; Martin et al. 1994), and high levels of TNF and IL-1, higher than in plasma, have been found in the bronchoalveolar lavage (BAL) fluid of patients with ARDS (Suter et al. 1992). IL-6 can be released by many cell types and has a number of biological activities, of which perhaps the most important is the induction of the synthesis of acute-phase proteins by the liver (Geiger et al. 1988). It is most consistently elevated in the plasma of patients with sepsis and related to outcome (Hack et al. 1989a), but its role in the pathophysiology of sepsis has not been defined. IL-8 is produced by a variety of cells including monocytes, macrophages, neutrophils and endothelial cells and shows a remarkable specifity for neutrophils. IL-8 has chemoattractant activity,

is able to induce degranulation, and to elicit a respiratory burst (Collins et al. 1991, Schröder 1989). In addition, IL-8 may promote adherence of neutrophils to endothelium (Carveth et al. 1989; Huber et al. 1991) whereas, in contrast, under certain conditions this cytokine can also inhibit this process (Gimbrone et al. 1989). Elevated levels of this cytokine which are related to outcome have been found in the plasma of septic patients (Marty et al. 1994; Hack et al. 1992) and in the BAL fluid in patients with ARDS (Miller et al. 1992), indicating that local production of IL-8 may contribute to pulmonary damage. High plasma levels of IL-8 have recently been demonstrated in patients with sepsis-associated MOF, which was much more pronounced than in MOF not related to infection (Marty et al. 1994). The role of IL-8 in the pathogenesis of sepsis and its sequelae is, however, not clear.

7.1.2 Complement System

Bacteria and their products can activate the complement system and result in the generation of anaphylatoxins (C3a, C4a, C5a) which have strong biological activities. C5a is the most powerful anaphylatoxin, and binding of C5a to neutrophils stimulates an oxidative burst, degranulation, and enhanced adhesion of these cells to each other and to endothelium (Jacob et al. 1980). Complement-stimulated neutrophils damage endothelium by the release of reactive oxygen species and proteases (e.g., elastase, collagenase, cathepsins), favoring the lung as a target. Complement activation stimulates the release of other substances such as histamine, serotonin, PAF, prostaglandins, leukotrienes, and cytokines (TNF, IL-1, IL-6) by the interaction with neutrophils, platelets, mast cells, and monocytes (Cavaillon et al. 1990; Scholz et al. 1990). The pathophysiological consequences are among others vasodilation, cell aggregation, and increased microvascular permeability. Animal studies have demonstrated that complement activation may produce severe inflammatory responses with microvascular injury in the lungs and other organs (Goris et al. 1986; Nuytinck et al. 1986a). In neutropenic animals such changes could not be produced or were dramatically attenuated (Hosea et al. 1980; Hohn et al. 1980), indicating the important role of activated leukocytes in the generation of tissue damage.

A polyclonal antibody to C5a can markedly attenuate markers of ARDS in septic primates (Stevens et al. 1986). Some studies have suggested that complement activation per se is unable to fully simulate the effects of endotoxin, and that other factors are necessary to express its effects (Henson et al. 1982). Although activated complement factors are elevated in the plasma of most septic patients (Hack et al. 1989b), the relationship to the development of ARDS is inconsistent. Some investigators (Slotman et al. 1986; Tennenberg et al. 1987; Parsons et al. 1989) have found such a relationship, but others (Weinberg et al. 1984; Hack et al. 1989b; Ketai and Grum 1986) have not. It has been suggested that persistence of an elevated C4a level is associated with MOF (Heideman and Hugli 1984), but no correlation between the development of ARDS and this complement activation product was observed in another study (Langlois and

Gawryl 1988). Anaphylatoxin levels may, however, underestimate the extent of complement activation since they are rapidly cleared from the circulation. Instead, the more stable fluid-phase terminal complement complex could be a more useful marker of this process. Higher levels have been found in (septic) patients with ARDS than in those without ARDS (Langlois and Gawryl 1988; Heideman et al. 1988) but not in all series (Parsons and Giclas 1990). Activated complement products can be found in the BAL fluid of patients with ARDS (Modig et al. 1986; Zheutlin et al. 1987) which may be more sensitive markers of the effects of anaphylatoxins at the tissue level than blood levels.

7.1.3 Contact System, Coagulation, Fibrinolysis

The contact system of coagulation consists of the proteins factor XII, prekallikrein, high molecular weight kininogen, and factor XI. The activation products of the contact system have important biological effects: enhancement of vascular permeability, decrease of vascular tone, and aggregation and degranulation of neutrophils. Bradykinin, liberated from kininogen by plasma kallikrein is one of the most potent vasodilators (Colman 1989). The contact system is activated in sepsis and ARDS, indicated by lowered levels of factor XII and prekallikrein (Nuijens et al. 1988; Carvalho et al. 1988), which are correlated with blood pressure (Hack et al. 1990). Monoclonal antibodies to factor XII can reverse irreversible hypotension in a lethal sepsis model in baboons, indicating that bradykinin significantly contributes to the hypotensive response in sepsis (Pixley et al. 1993). Coagulation disorders and abnormalities of fibrinolysis are common in septic patients. Sepsis is the most common cause of acute DIC, and this syndrome is a frequent complication of sepsis. Widespread microvascular thrombosis in various organ systems is a feature of lethal septic shock (Coalson 1986) and can contribute to organ failure (Tanaka et al. 1990). DIC in septic patients is associated with MOF and is a predictor of both the occurrence of this syndrome and death (Fourrier et al. 1992).

Coagulation is initiated in vivo by tissue factor expressed on endothelial cells and monocytes (Furie and Furie 1992). This is followed by a sequence of proteolytic activation resulting in thrombin formation and fibrin generation. Thrombin-antitrombin complexes are sensitive markers of in vivo thrombin generation and high thrombin-antithrombin complex levels are common in septic patients (Hesselvik et al. 1991; Thijs et al. 1993; Lorente et al. 1993). Also, fibrinolysis is activated, resulting in the generation of plasmin and plasmin-antiplasmin complexes, indicators of this process, are found in plasma of septic patients (Thijs et al. 1993). Fibrinolysis is, however, inhibited by the release of PAI-1 (Pralong et al. 1989) probably resulting in impaired fibrinolysis and an imbalance between coagulation and fibrinolysis (Thijs et al. 1993). High levels of PAI-1 may in fact be associated with the development of MOF (Hesselvik et al. 1989). Also in the BAL fluid of patients with ARDS such a dysbalance was demonstrated (Idell et al. 1989). The role of coagulation in ARDS has recently been reviewed (Hasegawa et

al. 1994). Importantly, modulation of coagulation in experimental sepsis protects the animals from organ damage and lethal effects (Taylor et al. 1987, 1988).

7.1.4 Neutrophils

A central mechanism in the pathogenesis of sepsis-induced tissue damage is the interaction between neutrophils and endothelial cells. Inflammation is associated with effusion and recruitment of neutrophils and monocytes and in sepsis these processes are generalized and take place in various organ systems, especially in the lung. The direct and indirect involvement of neutrophils in ARDS and MOF has been established by many studies (Flick et al. 1981; Nuytinck et al. 1986a). However, ARDS can occur in severe neutropenic patients (Ognibene et al. 1986) and in some animal studies neutrophil depletion had no effect on the degree of endotoxin-induced tissue injury (Winn et al. 1987). Other pathways may apparently also lead to tissue injury. Cytokines (TNF, IL-1) and endotoxin can induce expression of endothelial leukocyte adhesion molecules (ELAM-1, ICAM-1) which serve to bind neutrophils. ELAM-1 expression is one of the possible preconditions that result in leukocyte-related tissue damage. Endothelial expression of ELAM-1 has been demonstrated to occur in vivo in several organ systems after endotoxin administration in the experimental model (Engelberts et al. 1992a). Also, in patients with peritonitis skin biopsies revealed substantial endothelial ELAM-1 expression (Engelberts et al. 1992b). Neutrophils can be activated by a variety of mediators operative in sepsis, including cytokines (TNF, IL-1, IL-8, granulocyte-macrophage colony-stimulating factor), complement activation products (C5a), contact activation products (kallikrein, factor XIIa), and bacterial products (endotoxin). Neutrophil activation triggers upregulation of neutrophil adherence molecules, especially of the CD11b/CD18 complex that serves as a ligand for ICAM-1 on endothelial cells, a process which promotes adhesion of neutrophils to endothelium (Gamble et al. 1985).

In ARDS patients neutrophils circulating in the pulmonary artery show increased CD11b/CD18 expression and increased oxygen radical production (Simms and D'Amico 1991). Recently it was demonstrated that also circulating neutrophils in patients with sepsis show increased expression of CD11b/CD18 (Astiz et al. 1995). In the experimental model organ injury can be ameliorated by inhibition of CD18 dependent neutrophil adherence (Mileski et al. 1990). Also lymphocytes and monocytes bind to the endothelium (Arnaout et al. 1988). Cells of monocyte lineage can be activated by the potent MCP-1 which is released by various cells. Elevated concentrations of MCP-1 have recently been found in plasma of septic patients (Bossink et al. 1995). Activated neutrophils (and monocytes) attached to activated endothelium degranulate and release digesting proteases such as elastase, collagenase, and cathepsin G and toxic oxygen radicals which in concert with other products (e.g., leukotrienes, prostaglandin E_2, PAF) act to increase vascular permeability by damaging endothelial cells (Smedley et al. 1986; Varani et al. 1989). Prostaglandin E_2 strongly increases vascular permeability in vitro induced by C5a and neutrophils (Wedmore and

Williams 1981). In vivo TNF mediates leukocyte-endothelial adherence associated with microvascular protein leakage (Edwards et al. 1993).

This toxic damage of endothelial cells seems to be a self-perpetuing process since a microenvironment is created where toxic products act locally, and plasma inhibitors of elastase (α_1-antitrypsin, α_2-macroglobulin) gaining accesss to this area are inactivated by reactive oxygen radicals and other toxic products (Beatty et al. 1980; Reddy et al. 1989). In this way elastase can exert its local activity for a longer period despite the presence of large amounts of inhibitors in plasma. There is no doubt that elastase is essential for neutrophil-mediated endothelial cell injury (Smedley et al. 1986; Henson and Johnston 1987). Administration of elastase in the animal model results in pulmonary leukostasis, DIC, and increased pulmonary vascular resistance (Stokke et al. 1986). Leukostasis in several organs is a prominant histopathological sign of the endothelial-leukocyte interaction as demonstrated in experimental hyperdynamic sepsis (Redl et al. 1991). Activation of neutrophils in vivo can be assessed by plasma levels of elastase–α_1-antitrypsin complexes and lactoferrin. In septic patients these levels are elevated and related to outcome (Duswald et al. 1985; Nuijens et al. 1992). In trauma and sepsis patients, levels of elastase have been found to be correlated with the development and the severity of MOF (Nuytinck et al. 1986b; Tanaka et al. 1991). In fact, all available studies show a positive relation between elastase and severity of sepsis, ARDS, and MOF (Goris 1990). In patients with sepsis ARDS may be the first and often most important explicit clinical sign of endothelial damage. There is little doubt that increased pulmonary permeability is mediated by the toxic interaction between neutrophils and pulmonary endothelium presumably with a concerted action of other mediators such as thromboxane A_2, leukotriene B_4, PAF, activated complement products, and cytokines. It is likely that similar mechanisms are operative in other organ systems, although presumably the exact processes may vary. This, however, remains at present speculation as it has not been demonstrated in patients with sepsis.

7.2 Ischemia and Reperfusion

Several lines of evidence indicate that ischemia and reperfusion are strong mechanisms that can cause tissue injury. The impressive work by Shoemaker et al. (1992) has demonstrated that adequate tissue oxygenation is of paramount importance in maintaining homeostasis and preserving organ function. In one study the calculated oxygen deficit in high-risk surgical patients during and immediately after surgery appeared to be related to the development of MOF. Septic shock is characterized by a low systemic vascular resistance and usually an elevated cardiac output to meet the tissue's metabolic needs (Groeneveld et al. 1986). However, a defect in effective oxygen extraction as manifested by a low arteriovenous oxygen difference may limit adequate supply of oxygen to the cells. In animal models of sepsis a diminished oxygen extraction capability has

been demonstrated, not only for the whole body but also in various organ systems, with a decline in critical oxygen extraction and an increase in critical oxygen delivery (NELSON et al. 1988; ZHANG et al. 1995).

The concept of diminished oxygen supply to cells in severe sepsis has been challenged as increased levels of skeletal muscle pO_2 have in fact been found, suggesting that oxygen utilization is impaired rather than oxygen supply (BOEKSTEGERS et al. 1994). However, most investigators consider tissue ischemia an important pathogenetic mechanism, particularly in septic shock. Inadequate oxygen availability leads to cellular dysfunction, production of lactic acid, injury, and ultimately cell death. In clinical practice, blood lactate levels are used to globally assess the severity and duration of tissue ischemia. Increases of oxygen delivery may increase oxygen uptake even far above the normal critical oxygen delivery. This pathological supply dependency of oxygen uptake in the presence, but not in the absence of lactic acidemia during septic shock, suggests than an elevated lactate concentration is associated with an oxygen deficit and anaerobic metabolism in tissues (GILBERT et al. 1986; VINCENT et al. 1990; DE BACKER et al. 1994). This phenomenon is the main argument for human septic shock being accompanied by an oxygen deficit in the tissues. Admittedly, however, this supply dependency phenomenon is not observed in other studies and is certainly not beyond debate (see review GATTINONI et al. 1995). Other mechanisms, in addition to tissue ischemia such as a depressed activity of pyruvate dehydrogenase may, however, be involved in the rise in blood lactate concentration (VARY et al. 1986; HOTCHKISS and KARL 1992). Nevertheless, serial blood lactate levels are important in predicting the development of MOF in septic shock (BAKKER et al. 1996).

The mechanisms of impaired oxygen extraction in sepsis are not completely unraveled. Most likely the maldistribution of microcirculatory blood flow resulting in regional over- and underperfusion relative to demand and perfusional shunting can account for the paradox of a high total body oxygen supply and a low oxygen uptake for tissue needs in the presence of an inappropriately low oxygen extraction and pathological supply dependency of oxygen uptake (CAIN 1984; HOTCHKISS and KARL 1992). At least three mediator-induced mechanisms seem to be operative and to act in concert at the microcirculatory level: inappropriate vasodilation and vasoconstriction, microembolisation, and endothelial cell injury (CAIN 1984). Excessive, inappropriate vasodilation and loss of vascular reactivity may cause maldistribution of flow not adapted to local tissue needs (GROENEVELD et al. 1987). Mediator-induced microaggregation of neutrophils, platelets, and fibrin results in loss of autoregulation, limitation of recruitable capillary surface, increased inhomogeneity of flow, and diminished reactive hyperemia. Experimental embolization with microspheres abolishes reactive hyperemia, reduces oxygen extraction, and induces supply dependency of oxygen uptake (ELLSWORTH et al. 1981; CAIN et al. 1988). A reduced reactive hyperemia has been observed in septic animals (NELSON et al. 1988) as well as in patients (HARTL et al. 1988; ASTIZ et al. 1995). Endothelial cell injury may cause endothelial swelling and increases microvascular permeability with subsequent tissue edema, which further impairs

oxygen diffusion. Therefore, these concerted microcirculatory abnormalities are most likely mechanisms by which oxygen supply to tissue cells is impaired.

In the animal model marked interorgan redistribution of blood flow induced by sepsis is observed, and this probably also occurs in humans (see review by THIJS 1994). Such a redistribution may not subserve adequate adaptation of oxygen supply to tissue needs in specific organ systems. The splanchnic tissues seem to be an important "target" in septic shock. Several studies have demonstrated an increase in splanchnic blood flow in hyperdynamic sepsis and shock (DAHN et al. 1987; FINK 1991; RUOKONEN et al. 1993). However, increases in splanchnic oxygen demand, related to sepsis-induced metabolic processes such as synthesis of acute-phase proteins and gluconeogenesis, may exceed the increase in oxygen supply (DAHN et al. 1987; RUOKONEN et al. 1993), which makes the splanchnic tissues very sensitive to alterations in oxygen supply. The ultimate balance between oxygen supply and demand determines the occurrence and severity of ischemia (ARVIDSSON et al. 1991; RASMUSSEN and HAGLUND 1992; BREUILLE et al. 1994). The villous countercurrent system present along the entire gut renders the gut mucosa extremely sensitive to ischemia (FALK et al. 1985; FINK 1991). Gut ischemia may damage its barrier function, thereby promoting bacterial or endotoxin translocation (discussed elsewhere in this volume). In septic patients a reduced gastric intramucosal pH has been found, even when global hemodynamics seemed adequate, suggesting splanchnic ischemia in spite of relatively high cardiac output (SILVERMAN and TUMA 1992; MARIK 1993; GUTIERREZ et al. 1994). This index of local ischemia may be a better predictor of MOF and death during sepsis than commonly used oxygen-derived variables (MARIK 1993).

Although reperfusion is necessary for restoration of cellular metabolic activity, it can by itself trigger or aggravate the extent of ischemic tissue injury (DEITCH 1992). Reperfusion injury is an important additional mechanism of tissue injury by the generation of toxic reactive oxygen species (GRANGER 1988; REILLY et al. 1991). During ischemia there occurs the breakdown of high-energy phosphates to hypoxanthine, which in the presence of oxygen delivered with reperfusion is converted by xanthine oxidase into oxygen radicals. Endothelium is rich in the precursor enzyme xanthine dehydrogenase. High-energy phosphate degradation products have been found in the circulation of patients with septic shock (GRUM et al. 1985). Oxygen free radicals react with all biological substances, but most susceptible are polyunsaturated fatty acids in cell membranes, and this reaction leads to lipid peroxidation and ultimtely to membrane disintegration. Elevated plasma levels of lipid peroxides and depressed concentrations of antioxidants have been found in patients with septic shock and with ARDS (OGILVIE et al. 1991; RICHARD et al. 1990). The respiratory condensate from mechanically ventilated patients with ARDS contain elevated concentrations of hydrogen peroxide (BALDWIN et al. 1986; SZNAJDER et al. 1989), indicating that oxygen radicals may be involved in the pathogenesis of the syndrome.

Hypoxia can also activate macrophages to produce cytokines such as TNF and IL-1 (COLLETTI et al. 1990; GHEZZI et al. 1991), IL-8 (METINKO et al. 1992),

induce complement activation (Bengtsson et al. 1987), and production of prostaglandins (Paterson et al. 1989). These mediators may initiate or amplify injury also in organs remote from the site initially involved (Colletti et al. 1990). Hence, reperfusion may exert a direct injury effect on the specific area that was reperfused, as well as an indirect injury on organs remote from the ischemic tissue due to excessive mediator generation.

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Involvement of Bacteria/Endotoxin Translocation in the Development of Multiple Organ Failure

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1 Introduction

The progression of multiple organ failure (MOF) in patients with various underlying diseases continues to pose a life-threatening problem in intensive care units. Despite being the subject of an exponentially increasing number of experimental and clinical investigations during recent decades, the precise mechanisms leading to the development of MOF are not yet clearly understood. While several authors had earlier suggested that systemic sepsis due to uncontrolled infection is the leading cause of MOF (Baue 1975; Fry et al. 1980; Polk and Shields 1973), it has become increasingly clear that an infectious focus cannot be identified in all patients with MOF (Carrico et al. 1986; Faist et al. 1983; Goris et al. 1985).

Although the pathogenesis of MOF is most likely multiprincipal, gut barrier failure has recently been considered to play a key role. The concept that a breakdown of the intestinal mucosa, a vital component of body's defenses against endogenous infection, may lead to the access of intraluminal pathogens into the body, contributing to morbidity and mortality of systemic sepsis, was

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developed by FINE in the 1950s (FINE et al. 1959). Fine's theory of endogenous infection was not generally accepted (CULBERTSON et al. 1959) until the 1980s, when BERG's and DEITCH's groups reappraised this concept in a complementary manner. In a series of studies DEITCH and coworkers demonstrated experimentally the entry of gut-associated bacteria into the lymphatic systems after burns, trauma, malnutrition, and starvation (DEITCH et al. 1985, 1987a,c,d; DEITCH and BRIDGES 1987; DEITCH and BERG 1987b). These authors also showed, that endotoxin promotes translocation of bacteria from the gut (DEITCH et al. 1987b). These and other similar reports (KOZIOL et al. 1988), renewed interest in the intestinal tract as a source of bacterial infection and led Meakins and Marshall to suggest that "the gut is the motor of multiorgan failure" (CARRICO et al. 1986), a hypothesis that is still a matter of dispute. Infections with gut-associated bacteria but with no infectious focus at autopsy have been reported in patients undergoing intraoperative stress, victims of trauma, and patients developing MOF syndrome (CARRICO et al. 1986; GARRISON et al. 1982).

The term "translocation" was introduced in 1966 to describe the entry of endogenous micro-organisms into tissues and the circulation (WOLOCHOW et al. 1966). This concept has been refined to include the escape of bacteria and/or bacterial products from the gastrointestinal tract (ALEXANDER et al. 1990). Diagnostic approaches often fail to document the phenomenon of translocation. Alternative approaches, such as therapeutic measures, have therefore been employed to investigate the presence or absence of bacteria and bacterial products and their possible role in the development of systemic sepsis and the related pathophysiological alterations. Data obtained from experimental studies performed under standardized and controlled conditions provide useful information to help us better understand the entity and the phenomenon of bacterial/endotoxin translocation.

In this review we emphasize evidence and/or the significance of bacteria/endotoxin translocation in pathophysiological alterations and the development of multiple organ injury during various clinical conditions and the possible intervention strategies for them.4

2 Hypovolemic/Traumatic Insults

In general, impaired gut barrier permeability can be caused either during the shock period by decreased intestinal blood flow and reduced oxygen delivery (HAGLUND 1993), during reperfusion resulting in a stage of increased intestinal blood flow, or at a later stage again by reduced flow. Both experimental and clinical data suggest increasingly that one of the important initiating events in the sepsis syndrome that follows hypovolemic shock (HS) is the entry of bacteria and endotoxin into the blood of traumatized, hypotensive patients (BAKER et al. 1988; KOZIOL et al. 1988; RUSH et al. 1988; DEITCH 1990b; SCHLAG et al. 1993b;

MARSHALL et al. 1993). Dispute continues, however, over a possible causal relationship between gut-origin endotoxemia/bacteremia and septic complications, on the one hand, and the clinical outcome of hemorrhage and trauma, on the other (MOORE et al. 1991; PEITZMAN et al. 1991; ROUMEN et al. 1993; BRATHWAITE et al. 1993).

We became interested in determining both the time course of bacterial translocation and the kinetics of lipopolysaccharide (LPS) and cytokine appearance in the circulation. Moreover, to evaluate the role of endogenous endotoxin and cytokines in HS-related pathophysiological alterations and mortality we employed anti-LPS and anti-tumor necrosis factor (TNF) measures in rats subjected to prolonged hemorrhagic insult.

2.1 Bacteremia/Endotoxemia and Cytokines

Histological examinations reveal bacterial translocation into the small intestinal wall in rats subjected to HS for 90 min as early as the end of a 30-min resuscitation period (JIANG et al. 1995). Similarly, translocation of bacteria into the small vessels of the colon has been observed in baboons subjected to a hemorrhagic traumatic shock (HTS) consisting of bleeding the animals to maintain a mean arterial pressure (MAP) of 40 mmHg for 3 h, femur fracture, and soft tissue trauma followed by a 3-h resuscitation period (SCHLAG et al. 1991). We noted positive blood cultures in HTS baboons even during the shock and/or reperfusion period (Fig. 1a). At the end of a 6-h acute experiment in baboons the highest bacterial counts were noted in the mesenteric lymph nodes (MLN) followed by the liver and spleen (SCHLAG et al. 1991). These results obtained in a subhuman primate model are in agreement with reports of bacterial translocation, mainly in rodents (DEITCH et al. 1987a; RUSH et al. 1989) and provide further evidence that the phenomenon of translocation may not be limited to lower animals.

Our experiments also document the significance of the early appearance of endotoxin in the systemic circulation following hemorrhage and/or trauma in both rodents and primates (SCHLAG et al. 1993a,b; JIANG et al. 1995; BAHRAMI et al. 1995a). Elevated plasma LPS levels were found in HTS baboons at the end of a 3-h shock period and 1 h after the beginning of reinfusion (Fig. 1b). Similarly, in a subchronic model in baboons consisting of oxygen debt controlled hemorrhage together with an infusion of zymosan-activated plasma (ZAP), to activate the complement system without trauma, the highest plasma endotoxin levels were detected at the end of the 3 to 4-h shock period and at 1 h after the start of reinfusion (SCHLAG et al. 1993b). Our rat studies reveal a marked, albeit transient increase in plasma LPS concentrations, in both the portal and the systemic circulation following HS (JIANG et al. 1995). By comparing portal and systemic endotoxin levels we found that the significant rise in the portal circulation took place 30 min before that in the systemic circulation (Fig. 2). Throughout the experiment LPS levels were higher in the portal than in the systemic circulation.

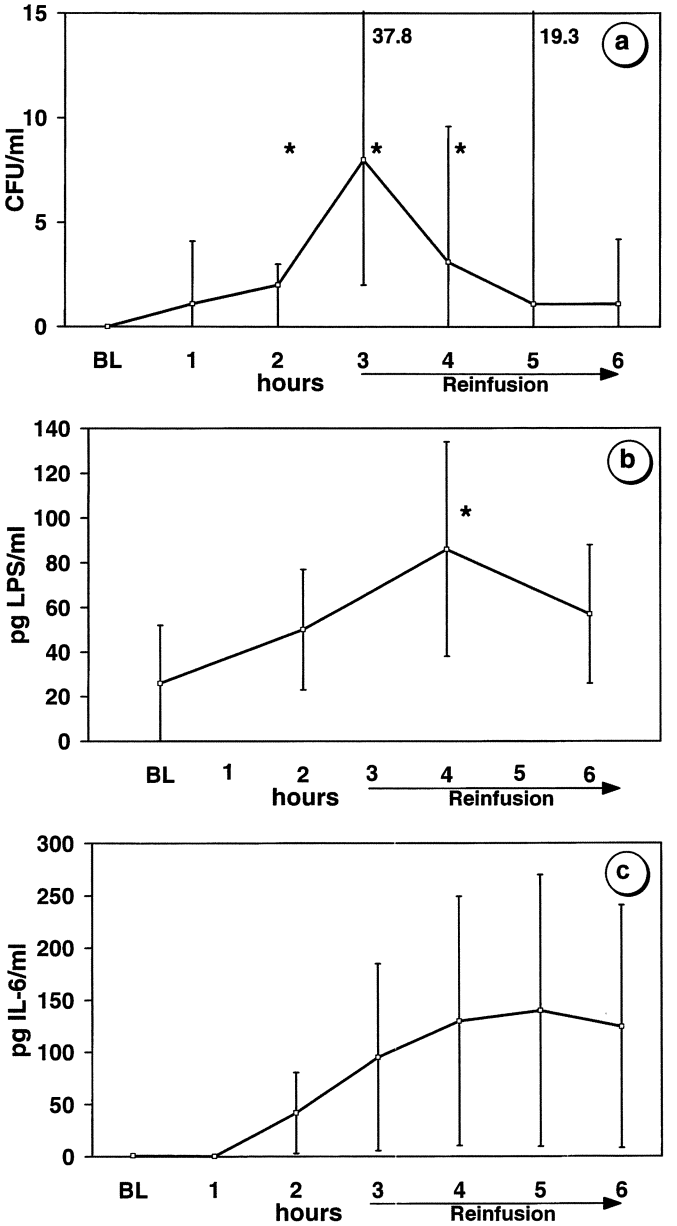

Fig. 1a-c. Measurements in baboons subjected to hypovolemic-traumatic shock. **a,** Bacterial counts in the systemic circulation **b**, Plasma endotoxin levels (mean ± SE). **c**, Plasma levels of interleukin-6 in the systemic circulation (mean ± SE). (Adapted from REDL et al. 1991)

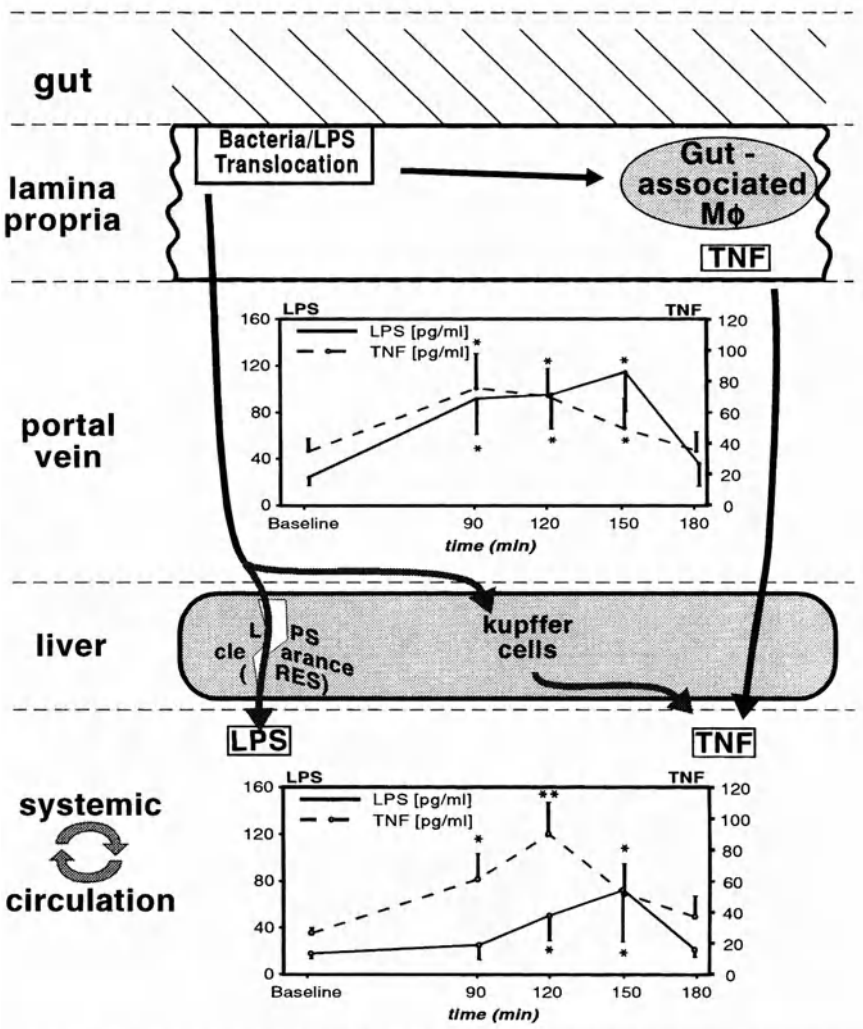

Fig. 2. Possible sequence of events following hemorrhage/trauma-induced translocation leading to cytokine formation in the presence or absence of detectable plasma endotoxin. Data of portal and systemic endotoxin and TNF plasma levels in rats subjected to 90-min hemorrhage (followed by resuscitation; mean ± SD). (Adapted from JIANG et al. 1995)

These results suggest that the development of systemic endotoxemia parallel to the endotoxin appearance in the portal vein represents spillover from elevated portal LPS levels overwhelming the liver reticuloendothelial system. In contrast to our findings, the increase in plasma endotoxin concentration has not been detected by others following hemorrhage in mice (AYALA et al. 1990) or rats (THIEMERMANN et al. 1993). Although the phenomenon of endotoxemia after

shock remains controversial, our experiments provide further evidence confirming the earlier observations of Rush et al. (1988) and Baker et al. (1988), who demonstrated that there is a relationship between HS and bacterial/endotoxin translocation.

Our current data provide further evidence that HS leads to bacterial/endotoxin translocation with concomitant cytokine formation. A number of recent studies have implicated inflammatory mediators following hemorrhagic shock (Chaudry and Ayala 1993). TNF is a cytokine produced by macrophages and monocytes in response to stimulation with endotoxin/bacteria. It has been postulated that translocated endotoxin/bacteria can activate macrophages to produce various cytokines, including TNF, which may lead to the development of sepsis and MOF associated with posttraumatic hypotension (Deitch 1990b; Caty et al. 1990). The mechanism(s) of TNF formation, however, and its role in the pathophysiology of hemorrhagic shock are not clearly understood (Ayala et al. 1991; Rhee et al. 1993; Stylianos et al. 1991). According to a report by Ayala et al. (1991) using a mouse hemorrhage model, TNF was significantly increased during and up to 2 h after blood loss, despite a lack of detectable plasma endotoxin levels. Similarly, studies by Rhee et al. (1993) indicated an early appearance of TNF in rats with a fixed-volume blood loss.

Although only in a few cases have we detected plasma TNF in the HS model in baboons, the levels of soluble TNF receptor (55 kDa), however, peaked between 1 and 2 h (Bahrami et al. 1995a). It may therefore be that the lack of detectable plasma TNF levels is due to the binding of TNF to the cell or soluble receptors. Plasma IL-6 levels, however, were found to be increased in the HTS baboons and peaked after 4–5 h (Fig. 1c). The TNF concentration in the portal vein of rats subjected to HS for 90 min peaked at the end of shock (90 min) and remained at this level until 150 min after shock (Jiang et al. 1995). In the systemic circulation the TNF levels were also markedly elevated after 90 min. In contrast to portal vein TNF levels, however, systemic TNF levels reached a peak at 120 min after shock.

The significant rise in TNF concentrations in the portal blood that occurs prior to the increased concentrations in the systemic circulation indicates a gut-derived TNF formation, for which the gut-associated macrophages in the lamina propria of the intestine can be considered a potential source (Fig. 2). The parallel increase in portal and systemic TNF concentrations until the end of the shock period was followed by a continuous increase in systemic but not in portal levels. This, together with other reports of portal cytokines (Deitch et al. 1994) and studies showing that proinflammatory gene expression occurs in Peyer's patches as early as 1 h following hemorrhage (Shenkar and Abraham 1993) led us to speculate that gut-associated macrophages constitute the major source for TNF formation at the early stage of shock, and that the Kupffer cells stimulated by endotoxin transported to the liver via portal blood serve as the major cellular source for the circulating TNF at a later stage (Fig. 2). In addition, there are also several lines of evidence suggesting that changes in organ blood flow induced by hemorrhage leads to regional hypoxia, which may also contribute directly or

indirectly to the induction of TNF (Ayala et al. 1990; Chaudry and Ayala 1993; Stylianos et al. 1991). Similar results have recently been found in an in vitro study (Scannell et al. 1993), showing that hypoxia may cause the release of TNF-α and its soluble receptors by human macrophages. Thus, both hypoxia and/or translocated LPS acting on local macrophages can be considered inducers of TNF formation that would not necessarily be influenced by anti-LPS treatment at the early stage of HS.

3 Burn Injury

It has been shown that burn injury can lead to acute derangements of multiple host systems, including alterations of the intestinal barrier function, which may predispose the burn victim to bacterial/endotoxin translocation from the gastrointestinal tract into the systemic circulation and to distal organs (Deitch 1990b; Maejima et al. 1984; Deitch and Berg 1987b; Alexander et al. 1990). Although the exact relationship of bacterial/endotoxin translocation to clinical sepsis and MOF is not known, extensive work performed in animal models has clearly demonstrated the importance of translocation in the development of systemic responses and, ultimately, of MOF resulting from major burns.

Using a nonlethal burn injury model, Deitch and Berg (1987b) found that 44% of the rats experiencing 40% total body surface area burn had viable, gram-negative enteric bacilli in MLN on the second postburn day. The combination of trauma and intestinal overgrowth with gram-negative enteric bacilli or *Candida* can result in the synergistic spread of bacteria from the gut to systemic organs, such as the spleen and liver (Deitch and Berg 1987b; Alexander et al. 1990). It was also shown that burn injury followed by wound infection with *Pseudomonas* causes prolonged and enhanced bacterial translocation (Jones et al. 1990b). On the other hand, using a radionuclide probe of a specific organism or endotoxin, Alexander et al. (1991) reported that both organism and endotoxin translocate very soon (within 1 h) after the burn injury. Translocation of endotoxin may have biological significance that is independent of and additional to translocation of intact bacteria. Furthermore, experimental results document that a nonlethal dose of endotoxin promotes bacterial translocation from the gut to MLN and to systemic organs, and increased mortality to 100% in burned mice, while the mortality of mice receiving only endotoxin or burn was less than 10% (Deitch and Berg 1987a). It is therefore possible that a self-sustaining cycle can be established with endotoxin itself under some circumstances because of its effects toward weakening mucosal integrity, increasing gut permeability, and promoting the translocation process (Gans and Matsumoto 1974; Garidis et al. 1973; Deitch et al. 1987b). In this way LPS can potentiate the development or progression of sepsis and result in further injury to distant organs.

In addition to small animals, bacterial translocation has been shown to take place in large animals subjected to thermal injury. Morris et al. (1990), using a 40% total body surface area burn in an ovine model, found significant increases in the number of bacteria cultured from the MLN, liver, and lung compared to sham animals. Decreased blood flow to the gastrointestinal tract after burn certainly plays a critical role in the pathogenesis of bacterial translocation. This phenomenon has also been seen in burned pigs (Tokyay et al. 1990), whose gastrointestinal flora is similar to that in humans, and whose vasculature supply to the gut is nearly identical to that in humans. By nonspecifically preventing the vasoconstriction of the mesenteric artery with a smooth muscle relaxant (Navaratnam et al. 1989) or selectively inhibiting thromboxane A_2 induction (Tokyay et al. 1990), gastrointestinal blood flow was maintained and translocation was reduced. Gut bacteria translocated into the MLN, spleen, and burn wound is thus probably due to gut mucosal failure after burn trauma and sepsis, thereby enhancing and generalizing the inflammatory response to thermal injury alone.

4 The Role of LPS/TNF in the Development of MOF

If invading enteric bacteria and endotoxin, however, were indeed the reason for the development of sepsis and the irreversibility of shock, animals treated with agents to eliminate bacterial endotoxin should be more resistant to hemorrhagic insult. To evaluate the role of endogenous endotoxin in pathophysiological alterations following hemorrhagic shock by neutralizing circulating LPS concentrations, we employed two different anti-LPS measures in a prolonged HS model (MAP at 30–35 mmHg for 180 min, followed by reinfusion and resuscitation over 50 min) in rats. At the onset of shock the animals were treated with an improved recombinant preparation of bactericidal/permeability increasing protein ($rBPI_{21}$), an antibiotic protein isolated from human neutrophils, which has been reported to inactivate endotoxin (Yao et al. 1995b). Furthermore, to check the origin of hemorrhage-induced endotoxemia we employed in a similar manner an anti-core LPS monoclonal antibody (WNI 222–5) which was shown to bind to all Escherichia *coli* clinical isolates and to some *Citrobacter, Enterobacter,* and *Klebsiella* isolates with a common inner core structure (Di Padova et al. 1993). In fact, the administration of $rBPI_{21}$ or WNI 222–5 almost neutralized the plasma endotoxin levels seen in control animals and provided noticeable protection of vital organs, particularly the lung and intestine, against hemorrhage induced injury (Bahrami et al. 1995a; Yao et al. 1995b). Furthermore, treatment with WNI 222–5 led to a survival rate of 71.4%, compared to 21.4% in the control group. Similarly, $rBPI_{21}$ improved 48-h survival to 68.8%, compared to 37.5% in the control group. These results suggest that systemic endotoxemia not only occurs early in the ischemic episode but also plays an important role regarding morbidity and mortality due to severe hemorrhage. Our findings are in accordance with

previous studies in which Re-LPS antiserum was found to increase the survival rate in animals subjected to HS (YAO et al. 1992a,b).

The results from both the $rBPI_{21}$ and WNI 222–5 treated groups showed nevertheless that some animals still died after hemorrhage. This could be due either to our treatment protocol itself (insufficient amount of $rBPI_{21}$/WNI 222–5 and/or limited time points of treatment) or to the fact that mediators other than endotoxin may cause, at least in part, the effects on organ system dysfunction and the resulting mortality observed in this experiment.

It is interesting to note that treatment with $rBPI_{21}$ or WNI 222–5 not only neutralized systemic endotoxemia but also significantly reduced the total incidence of bacterial translocation in the intestinal wall of different segments, which supports the assumption that the vicious circle of endotoxemia-bacterial translocation can be broken by means of the inhibition of LPS activities, for example, by the action of $rBPI_{21}$ or WNI 222–5.

In our anti-LPS treatment experiments the systemic plasma TNF levels were also markedly elevated at the end of reinfusion in control animals, but surprisingly they were not significantly influenced by $rBPI_{21}$ or WNI 222–5 treatment. With regard to the potential inducers responsible for the release of TNF during hemorrhage, it would appear that the elevation in TNF found here is not due to the endotoxin derived from the gut, as neutralization of systemic endotoxemia with $rBPI_{21}$ or WNI 222–5 did not markedly influence plasma TNF concentrations. Nevertheless, the failure of anti-LPS treatment to influence the plasma TNF concentrations in our experiments, despite neutralization of circulating LPS levels, could be a result of the initial TNF formation by the LPS-stimulated gut-associated macrophages prior to the entry of LPS into the circulation and its inactivation by anti-LPS measures.

Measures that control or prohibit translocation have been evaluated in some experimental studies to assess the potential role of bacterial/endotoxin in the pathogenesis of sepsis and MOF associated with large burns. DIJKSTRA et al. (1992) reported that the incidence of bacterial translocation and endotoxemia in *P. aeruginosa* burn wound contaminated mice was significantly reduced by low-dose treatment with polymyxin B, a cationic polypeptide antimicrobial agent known to bind endotoxin via its lipid A moiety both in vitro and in vivo. This study supports the hypothesis that the vicious circle of endotoxemia-bacterial translocation can be broken by blocking the activity of endotoxin. A recent series of studies in burned rats determined that pretreatment with selective decontamination of the digestive tract (SDD) can significantly reduce the incidence of bacterial translocation and attenuate the increase of plasma endotoxin concentrations in both portalvein and systemic blood (YAO et al. 1994). Moreover, the 5-day survival rate was markedly higher with 90.0% in the SDD-treated group than the 63.3% in the control group.

To investigate the role of TNF in HS-related multiple organ injury and mortality we employed a monoclonal antibody (mab) to TNF in rats subjected to HS (MAP of 30–35 mmHg for 180 min followed by resuscitation over 50 min). Treatment of rats with a bolus dose (20 mg/kg) of TNF mab (Celltech, Berkshire,

UK) at 65 min after HS resulted in a reduction in systemic vascular resistance, increased cardiac index, significant reduction in the increase in lung wet weight 3 h after shock, and improved 48-h survival (73.3% compared to 26.7% in the control group; Bahrami et al. 1994, 1995b). Macropathological and histological evaluations also revealed a significantly lower frequency of organ injuries in the TNF mab treated animals than in the control group. These findings, taken together with observations in previous studies, lead us to suggest that hemorrhage can induce a marked release of TNF into the circulation, which may at least in part be responsible for end-organ injury in the hemorrhagic setting.

Our data suggest that systemic endotoxemia not only occurs early in the ischemic episode but also plays an important role in the development of multiple organ injury and mortality resulting from severe hemorrhage, trauma, and burns. The efficacy of mab to LPS indicates that the Enterobacteriaceae, in particular *E. coli* as the largest population among them, may serve as the main source of endotoxin following hemorrhage, at least in rats. The cytokines, in particular TNF, induced following hypovolemic insult may play a key role in the initiation of pathophysiological alterations of HS.

5 Clinical Relevance of Translocation

Although translocation of bacteria and endotoxin after hemorrhage , trauma, and burns has been well demonstrated in the above experimental models, it is difficult to extrapolate these results to humans. As a result, their clinical significance remains controversial. Rush et al. (1988) reported a significant correlation between positive blood cultures and the degree of hemorrhagic shock in patients. Patients with admission blood pressure below 80 mmHg had a 56% rate of positive blood cultures, compared to less than 10% in patients with an initial blood pressure over 110 mmHg. Among patients undergoing elective surgery, Deitch (1989) found that in only 4% did the MLN contain bacteria, compared to 59% in patients undergoing surgery for small bowel obstruction, despite the absence of necrotic bowel or positive peritoneal fluid culture. In a previous study by Cabie et al. (1993) which measured endotoxin and cytokines in systemic and portal blood of patients undergoing abdominal aortic surgery, portal LPS was detected even after bowel manipulation (36%) but was elevated especially during the reperfusion phase (71%). In contrast to endotoxin, positive blood cultures were not found in either the portal or in the systemic circulation. Endotoxin was also detected in humans in the portal vein following aortic clamping. In a previous study performed together with Borst's group we were able to detect endotoxin in the systemic circulation during cardiopulmonary bypass (Schlag et al. 1993a). We also reported the release of cytokines, especially soluble cytokine receptors such as TNF receptors (Schlag et al. 1993a).

In extensive burns early increases in plasma endotoxin have been reported by WINCHURCH et al. (1987) and DOBKE et al. (1989), indicating endotoxemia with the peak serial levels 7–12 h and 3–4 days after the burn. As the burn wound is initially "sterile", and there are no apparent infectious foci in the body, this early endotoxemia cannot be attributed to the burn wound itself. This phenomenon is thus more likely to be due to bacterial translocation and/or the passage of endotoxin across the gut barrier. Recently in a group of extensively burned patients (more than 70% TBSA) it was documented that endotoxemia was not only common in the immediate postinjury period but was also strongly associated with the development of MOF and with an adverse outcome (YAO et al. 1995c). Plasma TNF was already elevated on postburn day 1, and significantly higher values were found in the MOF group than in those without MOF throughout the observation period (SHENG and YANG 1994). In view of the above evidence it ,might be reasonable to assume that, as a result of hypoperfusion and reperfusion injury, translocated bacteria/endotoxin can act as the primary trigger in initiating an exuberant inflammatory response, leading to the clinical manifestations of the systemic inflammatory response syndrome (SIRS) and sepsis and further damage to various organs.

In addition to these studies, there have been some clinical reports that intestinal permeability may be increased shortly after injury, which correspond well to the timetable of early endotoxemia induced by burns in both animals (CARTER et al. 1990) and humans (ZIEGLER et al. 1988; DEITCH 1990a; ROUMEN et al. 1993; RYAN et al. 1992). ZIEGLER et al. (1988), using lactulose and mannitol as permeability probes, demonstrated increased bowel permeability in burned patients with infections, but most patients were not studied in the immediate post burn period (average of 15–18 days after injury). Subsequently DEITCH et al. (1991) observed increased gut permeability early after burn injury in the absence of infection. More recently, using a macromolecule as a permeability probe for the intestine, gut permeability was found to be increased at least within 72 h after the injury and in the absence of infection or other morbid complications. Furthermore, this elevated gut permeability to polythylene glycol 3350 was correlated with the extent of the injury (RYAN et al. 1992). Although limited, the existing indirect clinical evidence supports the hypothesis that bacterial/endotoxin translocation does indeed occur in humans.

In contrast to these data, a prospective clinical study did not confirm portal or systemic endotoxemia and/or bacteremia after severe trauma, despite a subsequent 30% incidence of MOF (MOORE et al. 1991). Recently ROUMEN et al. (1993) reported that intestinal permeability after severe trauma and HS was increased without any correlation to septic complications. ENDO et al. (1992) reported that in the early period after injury when no infection was present very few patients had an endotoxin level above the normal range, and that endotoxin levels were not correlated with the extent of the burn or with the prognosis. Their findings suggest that there is no marked translocation in the first week after the accident. On the other hand, in a clinical study the administration of polymyxin B successfully reduced the plasma endotoxin level during the early

postburn period but did not influence the cytokine cascade, clinically manifested sepsis, or mortality rate. Therefore it has been concluded that the host inflammatory response in the early postburn state is due to mediators of injury rather than early translocated endotoxemia (Munster et al. 1993). Additionally, no convincing reduction in morbidity or mortality has been shown in most clinical trials despite a consistent decrease in the infection rate as a consequence of the use of SDD (Hammond et al. 1992). In this respect, it seems that the clinical significance of bacterial/endotoxin translocation remains an open question. It is still unknown whether septic complications can be prevented in critically ill patients by maintaining the gut mucosal barrier and limiting the translocation process.

6 Intervention Strategies Against Translocation

When the gut is suspected of being the source of bacteria/endotoxin causing systemic infections, the first logical step is to reduce the amount of bacteria and/or endotoxin in the gut lumen and support the mucosal barrier function to minimize the chances of bacterial translocation. Next in importance, if translocation cannot be prevented, is to kill bacteria, neutralize endotoxin, and eliminate bacteria/bacterial products that enter the extraluminal compartments.

6.1 Intraluminal Approaches

Since it is almost impossible to sterilize the gut, the regimen of SDD of the gut was developed using antibiotics (Stoutenbeek et al. 1984). This selective approach is important since one should not suppress the nonpathogenic anaerobes which are protective against bacterial translocation. Several clinical trials with SDD of the gut have been performed, with the rather consistent finding of decreased nosocomial infections; however, only one study also demonstrated clinical benefits in terms of survival (Sanderson 1989). There are several arguments why such therapy might not be successful. Especially with regard to the trauma there is the possibility that the drugs are administered too late or are not effective because they are not transported through the gut due to decreased peristaltic movement. Another important arguement is that the host response is induced not only by bacteria but especially by bacterial toxins such as endotoxin; such toxins may in fact be more important that the bacteria, and SDD is usually not designed to neutralize endotoxin.

Endotoxin is probably one of the reasons for patients developing SIRS,but there is no clinical evidence of infection leading to MOF. Intraluminal endotoxin neutralization has been considered an important approach to minimize endotoxin

translocation and induction of proinflammatory cascades in the host. Although data are available from only a few investigators, there is some evidence that bile salts are an important factor in binding and eliminating endotoxin from the gut by forming detergent like complexes which are poorly absorbed and well eliminated (Bertok 1977; Cahill et al. 1987). The antibacterial and anti-inflammatory properties of lactoferrin are usually explained by its ability to chelate iron. Lactoferrin was therefore used in a rat hemorrhagic shock model (Nitsche et al. 1993). In the therapy group receiving lactoferrin a dose-dependent reduction in endotoxin transfer from the intestinal tract was measured (Nitsche et al. 1993). Moreover, LPS binding of lactoferrin was found to decrease cytokine production (Machnicki et al. 1993). Another substance with antiendotoxin properties is lactulose (Liehr et al. 1980). which could also be beneficial as an intraluminal therapeutic agent. Lactulose was administered in a rat model of hepatic failure after liver resection, and lactulose treatment reduced the level of endotoxemia and enhanced survival (van Leeuwen et al. 1991).

A very simple noninvasive procedure for inhibiting translocation is to maintain peristalsis in order to prevent close contact of the bacteria and the intestinal mucosa and therefore reduce the time available for bacteria to penetrate the mucosal layer. It has been shown that in situations such as bowel obstructions bacteria adhere directly to the mucosa (Plaut et al. 1967). Spaeth et al. (1990) demonstrated that feeding rats dietary fibers in addition to either enteral or parenteral nutrition results in a significant reduction of bacterial translocation. In addition to the direct mechanical effect, the dietary fibers were described as being important in maintaining the normal ecological balance of the micro flora; it was furthermore shown that the degradation products of fibers are trophic for enterocytes. (Topping and Illman 1986; Savage 1978)

Since results indicate that enterocytes (e.g., Caco-2 cells as in vitro models) are actively involved in bacterial translocation, receptor mechanisms have been proposed for binding of bacteria (Cruz et al. 1994). Interfering with binding could thus pose another useful intraluminal therapeutic approach, for example, using mucin (Cruz et al., unpublished manuscript). Furthermore, secretory IgA produced by B-cells lining the mucosal surfaces binds to bacteria, preventing mucosal invasion by blocking their attachment to epithelial cells (Tomasi 1983). Therefore one might speculate that supplementary IgA may be another means to influence bacterial adherence.

6.2 "Maintenance" of Gut Wall

Probably the best strategy for maintaining the integrity of the gut wall and thus low gut permeability is immediate treatment in the posttraumatic period with both sufficient fluid support and operative treatment when necessary. In addition, a thromboxane A_2 receptor blocker could abolish the increase in mesenteric vascular resistance and subsequent bacterial translocation (Tokyay et al. 1990).

Similar to thromboxane, angiotensin II, another potent vasoconstrictor (also of the splanchnic circulation), could be important. An angiotensin-converting enzyme inhibitor was found to reduce burn induced bacterial translocation in rodents (Jones et al. 1990a). In addition, the fact that maintenance of gastrointestinal integrity with epidermal growth factor (Zapata Sirvent et al. 1993) and prevention of atrophy of the intestinal mucosa with enteral nutrition (Alexander 1993) decrease the translocation of both micro-organisms and endotoxin also suggests that there is a clear link between thermal injury and septic responses.

Hemorrhage-induced mucosal damage was found to be mediated by xanthine oxidase-derived oxygen free radicals, which cause lipid peroxidation and mucosal injury (Deitch et al. 1988). In this study the inhibition of xanthine oxidase activity by allopurinol or some other means to block xanthine oxidase significantly reduced mucosal injury and the incidence of bacterial translocation.

An excess of nitric oxide (NO) has been hypothesized as causing the hyporeactivity and decompensation following HS (Thiemermann et al. 1933) and contributing to peripheral vascular failure in septic shock (Petros et al. 1991). In an intestinal ischemia/reperfusion model, Payne and Kubes (1993) studied the effect of exogenous NO on mucosal barrier function. NO donors such as nitroprusside, SIN-1, and L-arginine all reduced mucosal barrier dysfunction without improving intestinal flow, while NO synthase inhibitors further augmented the rise in mucosal permeability. More recently have we found that NO induced by HS in rats is an important mediator for the pathophysiological alterations associated with cardiovascular abnormalities, multiple organ injury, and lethality (Bahrami et al. 1995b). Our experiments demonstrate that administration of *N*-monomethyl-L-arginine, an inhibitor of both constitutive and inducible NO synthase, may be either deleterious or salutary in a dose-dependent manner. While a high dose markedly increases the organ injury seen in the control group, an intermediate dose reduces the acute injuries without affecting the histological incidence of translocation (Yao et al. 1995a). Thus the regulation of NO and the use of inhibitors may provide new advances for the treatment of HS.

Studies have suggested that the parenteral nutrition regimens currently in use do not support the gut (van Leeuwen et al. 1991; Wilmore et al. 1988). There have therefore been several attempts to apply specific substrates to maintain gut mucosa integrity. Glutamine, for example, has been shown to protect intestinal mucosa even when supplied in a total parenteral nutrition regimen (Burke et al. 1989). Others have attempted to use specific growth factors, such as insulin-like growth factor 1 (Zeigler et al. 1993), which may have trophic effects on the intestinal mucosa. Studies have also been performed in burn models in which specific factors were used to prevent mucosal atrophy and translocation (e.g., bombesin; Haskel et al. 1994). Epidermal growth factor has also been shown to maintain mucosal integrity (Zeigler et al. 1993).

6.3 Intravasal Therapeutic Measures

If translocation cannot be prevented by the above procedures, one must deal with the bacteria/endotoxin within the vascular compartment to prevent an exaggerated host response leading to sepsis-related organ dysfunction. DONOHOE et al. (1986) reported that late mortality (72 h) in their rat hemorrhagic shock model was significantly improved when antibiotics (cefoxitin) were given to the animals in the postshock period. In a thermal injury model the increase in bacterial translocation from the gut and endotoxemia in *P. aeruginsa* burn wound colonized mice was significantly reduced by polymyxin B treatment (DIJKSTRA et al. 1992).

Anti-LPS measures such as BPI and monoclonal antibodies to LPS (WNI 222-5) prevent the elevation of systemic endotoxin levels resulting in reduction of intestinal injury and bacterial translocation, attenuation of damage to vital organs, and improvement in 48-h survival following prolonged hemorrhage (YAO et al. 1995b; BAHRAMI et al. 1995a). In experiments in which rabbits were subjected to HS after having been fed *E. coli*, bacterial and endotoxin translocation was detected. Pretreatment of these animals with an endotoxin antiserum significantly reduced the incidence of multiorgan failure and increased survival rate (YAO et al. 1992b). These results indicate that anti-LPS measures may be useful new therapeutic agents against endogenous bacteria/endotoxin-related disorders in severe HS.

There have been attempts to study the role of proinflammatory mediators in bacterial translocation. In studies by DEITCH's group blocking either platelet-activating factor with two different antagonists (DEITCH et al. 1989) or with an anti-TNF antibody (DEITCH et al. 1991) did not attenuate endotoxin-induced bacterial translocation. In contrast, other studies confirm the important role of TNF in the hemorrhagic setting. For example, it has been shown that anti-TNF antibody even with posttreatment significantly improves survival (BAHRAMI et al. 1994) over 48 h. Also in a pretreatment study anti-TNF antibody improved rat condition in an experiment lasting only 2 h (ZINGARELLI et al. 1994). Further positive effects of TNF blockade have been seen on Kupffer cell function (ERTEL et al. 1991b) and positive effects on cecal ligation and puncture sepsis after a previous hemorrhagic shock (CHAUDRY et al. 1993). Similar to anti-TNF antibodies, agents such as chloroquine which reduce TNF formation have been demonstrated to be beneficial when administered during resuscitation following shock (ERTEL et al. 1991a). Furthermore, studies by MARZI et al. (1993, 1995) have indicated that anti-TNF antibody reduces leukocyte adhesion and improves microcirculatory conditions, as seen in significantly improved negative base excess. In rabbit hemorrhagic shock anti-TNF antibody reduces postshock organ damage (FOULKES et al. 1994).

When comparing different therapeutical regimens in experimental models, it should be kept in mind that these models differ, for example, in severity of hemorrhagic shock, with a short hemorrhagic period in most of the experiments by DEITCH et al. (1987a) or CHAUDRY et al. (1993) and very long experiments (up to

250 min) in the shock models of KOZIOL et al. (1988), RUSH (1989), and SCHLAG et al. (1991); our rat model (BAHRAMI et al. 1995a) is in between these (either for 90 or 180 min). Although bacterial translocation occurs in most of these models, the mechanisms may not be identical. The ischemia dependent tissue hypoxia is probably much more prominent in the prolonged shock models (RUSH et al. 1988; BAHRAMI et al. 1995a) than in the 30-min model (DEITCH et al. 1987a).

7 Conclusion

In all recent experimental studies the common theme is that bacterial translocation is clearly evident, not only by less reliable diagnostic techniques but especially in results of therapeutic studies. The therapeutic approach also proves the importance of the translocation event for organ dysfunction and survival, at least experimentally. The lack of convincing clinical data is due to the fact that only studies with diagnostic approaches have been performed so far. In addition to bacterial translocation, current data indicate the important role of secondary mediators such as TNF and NO in the pathogenesis of postshock/postburn sepsis.

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The Mechanisms of Host Defense Dysfunction Following Shock and Trauma

E. FAIST

1 Introduction

Major injury is a profound threat to the individual. Both the extent of injuries and the preinjury health of the patient greatly influence the outcome. In our experience with individuals having an injury severity score (ISS) of 30 points or higher who survive the immediate injury phase, only 50% begin convalescence without inflammatory or infectious complications (FAIST et al. 1993). Thus in every second patient the metabolic and neuroendocrine stress reactions do not shut down and return to normal regulation and homeostasis. The causes of this phenomenon in multisystem injuries are extensive tissue necrosis, prolonged hemorrhagic shock, and perhaps immediate invasive endotoxemia by way of gut translocation.

Severe injury results in a profound dysfunction of host defense mechanisms. This we see as the dire consequence of a systemic, nondiscriminant, and excessive inflammatory response, together with a dramatic paralysis of cell-mediated immune function. We have learned that parts of the immune system are stimulated while others are depressed in a complex order of events that is as yet not well understood. On the one hand, the nonspecific immune system is

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activated as white blood cells and macrophages (Mϕ) mobilize to the site of injury, with the activation of complement and opsonins. Interleukin (IL)-1 should stimulate IL-2, which activates T-helper cells. Acute-phase proteins are produced as a part of the immune response with IL-6 production. On the other hand, shock depresses the activity of white blood cells and lung and liver Mϕ). Catabolism with protein-calorie malnutrition alters immunoglobulin (Ig) production and depresses both T- and B-cell function. Cortisol decreases immune responses, as do some anesthetic agents. Circulating immunosuppressive factors, including suppressor T-cells, appear because of tissue necrosis. With tissue injury, protein denaturation may occur, which could be interpreted as the presence of nonself protein. With a burn injury neutrophils (PMN) are activated systematically, and chemotaxis is depressed. All these factors contribute to increased susceptibility to infection. The immune system can cope with a modest injury. However, when the injury is severe and overwhelming, with extensive tissue necrosis, the immune system is activated systematically and may become self-destructive (Baue 1990). A response which should be local in the area of the wound and critical for survival may become generalized and produce remote organ damage (Baue 1992).

Infection following an operation may result in further insult to the delicate balance of cytokine regulation. Since trauma induces immunosuppression as well as hyperactivation, immunomodulation should not be limited to restoration of depressed immune responses but should also include the regulation of exaggerated responses where appropriate. Support of host resistance is becoming very important (Fig. 1). An incentive for the development of therapeutic regimens comes from the recognition that infection is a major contributor to late morbidity and mortality after injury (second-phase multiple-organ failure, MOF; Faist et al. 1983). Such therapy is based on studies of the downregulatory mechanisms responsible for injury-related immune problems.

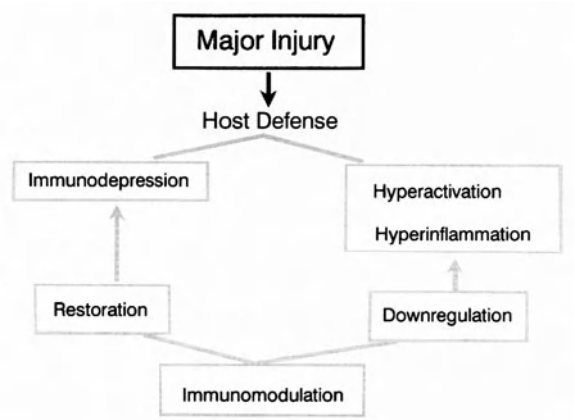

Fig. 1. Components of posttraumatic host defense dysfunction and counterregulatory immunomodulation

2 Immunological Derangements of Mononuclear Leukocytes Following Injury

There is a wide array of immunological derangements which occur after injury, shock, trauma, burns, and extensive surgical procedures. These are induced mainly by Mϕ activation, a significant inhibition of the bone marrow, and changes in the specific and nonspecific immune system (CHAUDRY et al. 1990; MUNSTER 1984; NINNEMANN 1987; MANNICK 1993). The specific immune functions which are altered following hemorrhage and tissue trauma are:

Myelodepression
Lymphopenia
CD4/CD8 ratio <1
T- and B-cell proliferation ↓
Release of lymphokines (IL-2, IL-3, γ-IFN) ↓
IL-2 receptor expression ↓
NK cell activity ↓
Alteration of Mϕ/T-cell interaction
HLA-DR antigen expression ↓
DTH skin test reactivity ↓
Inhibition of B-lymphocyte differentiation
Plasma cells: IgM-synthesis and secretion ↓
Shift from IgM ⟶ IgG Synthesis

2.1 T-Cells

Following major injury one often observes absolute lymphopenia ($CD3^+$ lymphocytes) and simultaneous monocytosis ($CD14^+$ cells). Analysis of T-lymphocyte subpopulations demonstrates a massive decline in $CD4^+$ T-helper cells while IL-2 receptor expression on the surface of lymphocytes is also reduced (ANTONACCI et al. 1984, O'MAHONEY et al. 1984, FAIST et al. 1987, ERTEL and FAIST 1989). The $CD8^+$ cytotoxic and suppressor-active T-cells that play a central role within the posttraumatic immunosuppression are found to be unchanged or elevated. This leads to a conversion of the CD4/CD8 ratio to a value below 1. These alterations have been observed until day 21 after injury (FAIST et al. 1986). Shock, trauma, and burns also produce massive inhibition of lymphocyte proliferation from mitogenic stimulation. Immunomodulatory in vitro studies clearly indicate that excessive release of immunoreactive prostaglandin E_2(PGE_2) is a major lymphoproliferative inhibitory factor (ERTEL et al. 1992; RODRICK et al. 1992). FAIST et al. (1986) demonstrated in 11 patients with major trauma that lymphoblastogenesis was impaired in more than 70%, and 8 patients had a major infectious episode. KEANE et al. (1983) reported a correlation between the degree and duration of inhibition of lymphoproliferation following trauma and the in-

and IL-6 from peritoneal Mϕ as well as from Kupffer cells Takayama et al. (1990) described an accelerated release of these mediators in Mϕ cultures from patients following major surgery. The amount of TNFα secretion (>30 ng/10^6 Mϕ) was correlated with the onset of septic complications. IL-6 plasma levels in patients with major burns were shown by Guo et al. (1989) to be correlated with posttraumatic infections and outcome. In contrast to many of these studies, we found different results by careful study of Mϕ behavior in patients following major trauma and during the sepsis syndrome. In many patients there is an initially marked Mϕ suppression to synthesize and secrete proinflammatory cytokine to endotoxin stimuli. There ia a profound deficit of IL-1 and IL-8 production for several days after injury (Faist et al. 1992; Zimmer et al. 1994). This deficit may well result in the immunodeficiency of traumatized and septic patients since these cytokines in low concentrations are involved in the upregulation of all essential humoral and cellular immune functions. Inadequate IL-1 synthesis appears to be a substantial component of defective IL-2 synthesis following trauma (Faist et al. 1987; Miller-Graziano et al. 1988). A crucial immunomechanistic consequence of Mϕ PGE_2 release after injury is its downregulation of IL-2 synthesis (Rappaport and Dodge 1982).

3 Downregulation of the Monocytic Inflammatory Potential

Within the complex regulatory mechanisms of cytokine synthesis, as far as they are presently understood, a number of naturally occurring negative feedback mechanisms exist that appear able to downregulate an activated Mϕ system. Modified mimicking of these regulatory loops via exogenous delivery of such a broad spectrum mediator might represent a new avenue to regain early control over injury-induced host defense catastrophes. While endotoxin, TNFα, and γIFN are most potent inducers of Mϕ activation with subsequent release of TNFα, IL-1, and PGE_2; PGE_2 turn is capable of reducing TNFα secretion via the activated Mϕ so as to form a counter-regulatory system (Renz et al. 1988). Transforming growth factors-beta 1 and 2 are interesting molecules capable of deactivating Mϕ, mainly by inhibiting its H_2O_2 production; however, it has been found that the crucial characteristics of two other cytokines. IL-4 and IL-10, are their ability to inhibit the Mϕ production of numerous inflammatory mediators (Tsunawaki et al. 1988; Ertel et al. 1993, de Waal Malefyt et al. 1991; Howard and O'garra 1992; Spits and de Waal Halefyt et al. 1992; Oswald et al. 1992). Another lymphokine, IL-13, has been described to have potential anti-inflammatory activity, as judged by its capacity to suppress the production of proinflammatory mediators such as TNFα and IL-6 and to upregulate granulocyte and Mf IL-1ra production (Minty et al. 1993; de Waal Malefyt et al. 1993; Muzio et al. 1994). IL-13 is produced

patients either fail to control their infections, have dissemination of the infection and death, or "overreact" to their infection or the signals triggered by their infection and develop multiple-organ failure and die.

2.2 B-Cells

The effects of trauma on the B-lymphocyte system have also been investigated extensively. Nohr et al. (1983, 1984) studied the in vitro and in vivo antibody response to tetanus toxoid following major surgery and found a deficit of IgG production which was correlated with DTH skin test responses. In contrast, Shorr et al. (1984) found a normal in vivo antibody response to a rechallenge with tetanus toxoid in burned patients. However, lymphocyte in vitro cultures showed that burned patients had an elevated spontaneous Ig production with depressed antigen response. Wood et al. (1986) and Teodorczyk-Injeyan et al. (1986) demonstrated in burn patients a reduced in vivo IgG production following tetanus immunization and increased levels of in vitro IgG production following pokeweed mitogen stimulation. In patients with major trauma we found massive suppression of B-cell differentiation and IgM synthesis in peripheral blood mononuclear cell cultures following pokeweed mitogen stimulation (Ertel and Faist 1989; Faist et al..1989). The degree of suppression was highly correlated with the amount of Mϕ suppression in the peripheral blood mononuclear cell cultures while IgG and IgA synthesis were within the normal range or elevated. Further studies in purified B-cell cultures confirmed these data, demonstrating a persistent IgM/IgG shift. Investigations of the influence of recombinant lymphokines on Ig synthesis showed only a partial restoration of depressed B-cell function by IL-2. Very recently we showed that an anti-μ-induced B-cell proliferation per se is not suppressed by mechanical trauma after elimination of suppressor active extracellular factors (Ertel et al. 1990). Trauma caused a highly elevated antigen-induced proliferation, but the capacity of IL-2, IL-4, or T-cell supernatants to increase proliferation was depressed. We concluded that mechanical trauma causes a high preactivation of B-lymphocytes but impairs the responsiveness to most lymphokines except γIFN.

2.3 Monocytes

There is compelling evidence that altered behavior patterns of activated Mϕ play a key role in the abnormal inflammatory and cell-mediated immune responses. Shock, trauma, and burns similar to endotoxemia and bacteremia induce profound activation of monocyte/Mϕ with elevated synthesis and secretion of inflammatory mediators. Hemorrhagic shock without significant tissue trauma (Ertel et al. 1991) results in a steep elevation of tumor necrosis factor-α (TNFα) plasma levels similar to those detected during septic states (Marks et al. 1990; Michie et al. 1988). Ertel et al. (1991) demonstrated enhanced secretion of TNFα

and IL-6 from peritoneal Mϕ as well as from Kupffer cells Takayama et al. (1990) described an accelerated release of these mediators in Mϕ cultures from patients following major surgery. The amount of TNFα secretion (>30 ng/10^6 Mϕ) was correlated with the onset of septic complications. IL-6 plasma levels in patients with major burns were shown by Guo et al. (1989) to be correlated with posttraumatic infections and outcome. In contrast to many of these studies, we found different results by careful study of Mϕ behavior in patients following major trauma and during the sepsis syndrome. In many patients there is an initially marked Mϕ suppression to synthesize and secrete proinflammatory cytokine to endotoxin stimuli. There ia a profound deficit of IL-1 and IL-8 production for several days after injury (Faist et al. 1992; Zimmer et al. 1994). This deficit may well result in the immunodeficiency of traumatized and septic patients since these cytokines in low concentrations are involved in the upregulation of all essential humoral and cellular immune functions. Inadequate IL-1 synthesis appears to be a substantial component of defective IL-2 synthesis following trauma (Faist et al. 1987; Miller-Graziano et al. 1988). A crucial immunomechanistic consequence of Mϕ PGE_2 release after injury is its downregulation of IL-2 synthesis (Rappaport and Dodge 1982).

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from the activated T helper 2 cell subset which is also able to produce IL-4, IL-5, and IL-6 cytokines which enable T-cells to provide help to B lymphocytes for immunoglobulin synthesis in humoral immune responses (DEFRANCE et al. 1994; MCKENZIE et al. 1992). IL-13 has several biological activities in common with IL-4, and there is accumulating evidence that IL-13 and IL-4 receptors share components which are important for signal transduction (ZURAWSKI and DE VRIES 1994).

In a most recent study we examined the potential of IL-13 to modify in vitro LPS induced Mϕ activity in human cells from individuals that had sustained either major mechanical or burn injury, and whether there is a difference in IL-13 impact on Mϕ that have been preactivated under traumatic stress versus monocytic cells from a more homeostatic environment, as exists in healthy volunteers. Peripheral Mϕ from 20 controls (6 women, 14 men 29 ± 2 years) and 16 patients (2 women, 14 men; 43 ± 4 years) following major burn and mechanical trauma (average ISS 47 ± 3) were separated on postinjury days 1, 3, 5, and 7 and incubated with LPS (1 μg/ml) in the presence or absence of IL-13 (10 ng/ml) over a period of 4 h and for 20 h. Thereafter we examined from the culture supernatants the concentration of inducible NO and its metabolites NO_2^- NO_3^-, a most important mediator of inflammation, and the production of the immunoregulatory proteins TNFα, IL-1, IL-6, and IL-8. Ex vivo LPS activated Mϕ, compared to control cells, displayed a considerably enhanced inflammatory activity during the immediate posttraumatic course with a substantial and consistent elevation in TNFα and IL-6 (peak values 9- and 16-fold above controls, respectively). The addition of hrIL-13 to the Mϕ cultures resulted in an effective downregulation of the synthesis of TNFα (–33%), IL-1β (–35%), IL-6(–35%), and IL-8 (–28%), showing an average reduction in mediator production to two-thirds of the value found in corresponding, solely LPS-stimulated cultures (Fig. 2). The impact of hrIL-13 with respect to inflammatory cytokine production on control monocytes was more or less identical for IL-6 (–35%) and IL-1β (–40%), slightly lower for IL-8 (–20%), and nonexistent for TNFα compared to the values obtained for in vivo traumatic stress preactivated monocytes. While we observed no consistent elevation in monocytic NO release during the posttraumatic course, in a number of individual determinations the addition of hrIL-13 resulted in a striking reduction in peak NO output toward baseline levels.

We observed in coexperiments (results not shown here), when comparing the impact of hrIL-13 on IL-1β and IL-8 synthesis with that achieved by the addition of hrIL-10 to Mϕ cultures, that IL-10 is significantly more potent in terms of its downregulatory capacity for the Mϕ inflammatory potential. In LPS-stimulated Mϕ cultures from controls, burned and polytrauma patients, and patients undergoing cardiac surgery, the administration of hrIL-10 (10 ng/ml) resulted in an average of 80% suppression of inflammatory cytokine release compared to solely LPS-stimulated cultures. These data together with preexisting reports about the biological properties of IL-10 indicate that this cytokine, also unlike IL-13, acts as a major suppressor factor for T-cell performance (proliferation, IL-2 synthesis; DE WAAL MALEFYT et al. 1992; TAGA and TOSATO 1992; AYALA et al. 1994), downregulates Mϕ antigen presenting capacity (FIORENTINO et al.

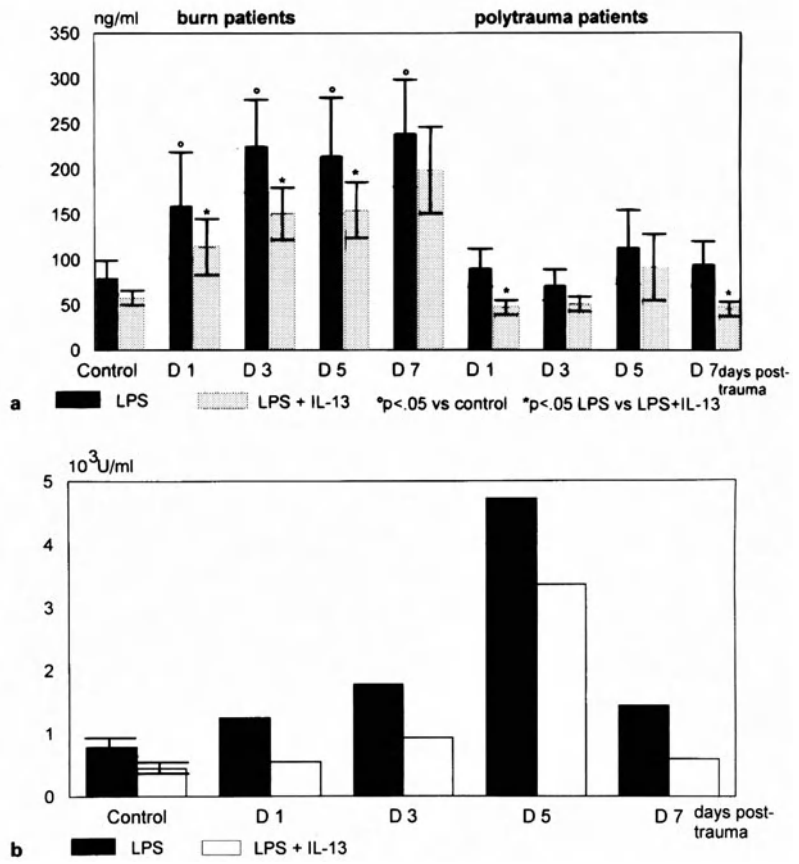

Fig. 2. a Changes in the in vitro capacity of adherent Mϕ to release IL-8 in response to LPS (1 μg/ml) over a period of 20 h in the presence or absence of hrIL-13 (10 ng/ml) after they were harvested on consecutive days (*D*) after trauma from patients who had sustained major burn (n=12) or mechanical (n=4) injuries and from 20 healthy controls. °p 0.05 vs control; *p 0.05 vs. LPS+IL-13. **b** Changes in the in vitro capacity of adherent Mϕ to release IL-6 in response to LPS (1 μg/ml) over a period of 20 h in the presence or absence of hrIL-13 (10 ng/ml) after they were harvested on consecutive days (*D*) after trauma from an individual patient (man, 24 years old, ISS 66)

1991), prompts suppression of γIFN synthesis (D'Andrea et al. 1993) and B-cell function, resulting in an overall immunosuppressive capacity. These facts created doubts about the utility of IL-10 as a biological response modifier for states of posttraumatic SIRS and sepsis, as suggested by others (Napolitano et al. 1994; Vincent 1995).

It is our conviction that the trauma-induced coexistence of two immunomechanistic entities – hyperinflammation and depression of cell-mediated immune responses – requires an integrated and concerted response in terms of a target-seeking counterregulation. Therefore we suggest that hrIL-13, rather than hrIL-10, possesses the essential characteristics which warrant testing this agent

as a modifier of posttraumatic states of deficient host defenses. We observed that hrIL-13 promises moderate reduction rather than complete shut-off of the inflammatory component of immunity; it supports, to a certain degree, cell-mediated immune responses rather than inducing overall immunosuppression; and it possesses a forward regulatory functionality towards a variety of important cellular targets. From this study and other findings we concluded that its biological properties recommend hrIL-13 for testing as a biological response modifier for acute states of trauma-induced host defense deficiency. It is our hypothesis that two recently popular agents granulocyte colony-stimulating factor (Hartung et al. 1995) and pentoxifylline (Hoffmann et al. 1995; Schade and Zabel 1994) which have been found to possess enormous potential for the deactivation of Mϕ predominantly via the downregulation of TNFα synthesis may very well prove useful agents together with hrIL-13 in a combined-drug broad-spectrum therapeutic intervention to control SIRS.

4 Trauma-Induced Impairment of Nonspecific Immune Functions

Nonspecific immune functions which are altered following hemorrhage and tissue injury include:

Monocytosis
Plasma levels of TNF-α and IL-6 ↑
Mϕ TNF-α and IL-6 secretion accelerated
Mϕ IL-1 secretion ↓
Mϕ PGE_2 synthesis ↑
PGE_2 plasma levels ↑
PMN Chemotaxis ↓
  Phagocytic capacity ↓
  β-integrin expression ↓
  Leukotriene B4 synthesis ↓
  Release of O_2 radicals ↓↑
  Release of elastate ↑
Acute-phase protein synthesis ↑
C3a plasma levels ↑
Cathepsin, lactoferrin, myeloperoxidase levels ↑
Neopterin plasma levels ↑
Depletion of fibronectin, opsonins and anglotensin III plasma levels ↑

Abnormalities in PMN function after serious injury have been interpreted by some as signs of unresponsiveness, whereas others have demonstrated evidence for PMN hyperactivation. Several laboratories have found a significant correlation between the degree of PMN dysfunction and morbidity of the patient

(Babcock et al. 1993). A decrease in PMN phagocytic activity (Grogan 1976; Heck et al. 1980), reduction in intracellular bacterial killing (Bjerknes 1989), and large number of cellular parameters were found to be altered. This included a decrease in glucose oxidation and oxygen consumption, reduced H_2O_2 production, alterations in phagolysosomal acidification, and a loss of or defect in lysosomal enzymes (Heck et al. 1975; Duque et al. 1985; Gadd and Hansbrough 1989; Bjerknes et al. 1989; Gallin 1985). Koller et al. (1989) reported lower leukotriene, including B_4, generation from PMNs of severely burn patients, which could account for some of the chemotactic and chemokinetic alterations reported by others. Rothe et al. (1990) used flow cytometry to distinguish the effect on PMN phagocytosis of trauma from that of trauma plus sepsis. They found that severe trauma causes hypoeregic phagocytosis while sepsis following trauma causes hypoergic phagocytosis. Recently, Babcock et al (1994) and Faist and Babbock (1994) found PMN dysfunction in circulating blood within 72 h after trauma; 70% of all PMNs had downregulated or nonexistent β-integrins. These cells did not phagocytose, had reduced respiratory burst, and appeared to be completely degranulated. Other reports, however, stress increased expression of $CR3^+$ receptors (CD11, CD18 complex) on circulating PMNs, increasing the ability of these cells to adhere to intercellular adhesion molecules and thus contributing to pulmonary capillary leakage.

Repair and healing or perpetuation of acute inflammation is characterized by a complex network of interacting cellular and humoral defense mechanisms. Of the numerous inflammatory mediators, the proteolytic lysosomal enzyme PMN elastase is highly destructive when released extracellularly, thus contributing to organ dysfunction after injury. Dittmer et al. (1986) and Nast-Kolb et al. (1992) have described significant increases in plasma elastase following major trauma and demonstrated a correlation between elevated elastase levels and the onset of organ failure. Pacher et al. (1989) predicted the onset of organ failure with a sensitivity of 88% and a specificity of 83% by elevated elastase levels after injury. When neopterin levels were elevated reflecting Mϕ activation, sensitivity was increased to 91% and specificity to 99% (Huber et al. 1987) Brandel et al. (1989) and Strohmaier et al. (1987) observed that increased neopterin in injured patients correlated with lethal outcome, compared to patients with an uneventful clinical course and low neopterin levels. Recently Gaitzsch et al. (1994) studied 40 patients with multiple trauma (mean age, 42 years; average ISS, 28) and found the acute-phase protein C-reactive protein together with IL-6 and TNFα, when elevated, to be most sensitive predictors of septic complications 24 h before the onset of sepsis. Persisting decreases in plasma anglotensin III were also correlated with the onset of multiple-organ failure (Risberg et al. 1986).

5 Is There a Need for Posttraumatic Therapeutic Immunointervention?

It is our understanding that the endogenous provisions of the organism to survive following overwhelming trauma are insufficient and require major exogenous support. It must be the principal clinical goal of modern immunotherapy to prevent a state of systemic inflammatory response in an immunocompromised host from converting into a state of bacterial sepsis. Several strategic approaches to prevent the development of late multiple-organ dysfunction syndrome/multiple-organ failure appear to be feasible (Fig. 3). As of today no clinical trials in septic patients with gram-negative bacterial infections employing therapeutic tools such as anti-LPS monoclonal antibodies, anti-TNF antibodies, soluble TNF receptors, and IL-1 receptor antagonists have shown an overall valid, clinically important, reproducible, and statistically significant treatment benefit. This bitter lesson has convinced us that the earliest possible blockade of the initiation of host responses following tissue destruction with or without a bacterial component may prove to be the most efficacious or perhaps even the only approach for the prevention of life-threatening bacterial infections.

Ideally, the immunoaugmentative intervention should prevent the posttraumatic systemic inflammatory response from turning into a nonreversible autodestructive inflammation with or without infection. This intervention must be employed in a calculated preventive fashion as early as possible following trauma. It should protect multiple cellular targets (e.g., lymphocytes, Mϕ, granulocytes, and endothelial cells), and its mode of action should protect the host from cell hyperactivation and cell exhaustion. It is also most likely from our present immunomechanistic understanding that only a combination of several

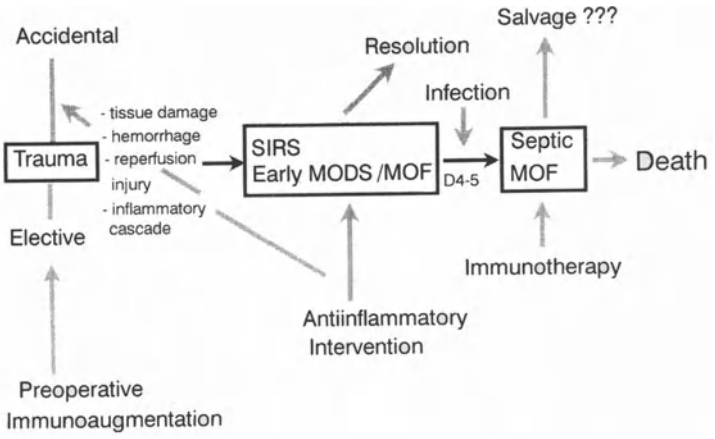

Fig. 3. Potential strategies for a therapeutic anti-inflammatory approach to intercept the development of nonreversible bacterial sepsis

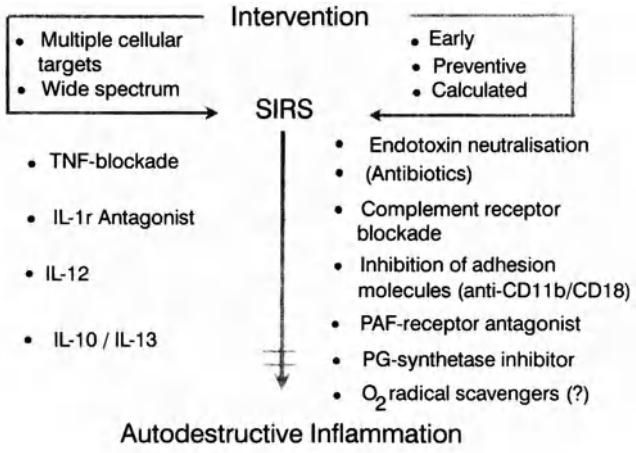

Fig. 4. Schematic depiction of the possibilities for dierentiated therapeutic intervention to prevent or alleviate posttraumatic multiple-organ dysfunction syndrome/multiple-organ failure

drugs will be effective in controlling the posttraumatic dyshomeostasis of the various cell systems. Figure 4 presents a variety of potentially useful pharmacological regimens for preventing autodestructive inflammation. Crucial issues within the complex field of preventive immunomodulation for the control of SIRS include patient selection, timing of administration, the difficulty in avoiding a complete shut-off of inflammatory responses, and cost of therapy.

It is clear that the high susceptibility to infection following trauma is correlated with injury severity and thus with the degree of uncontrolled inflammation. We do know, however, that the onset of infection most frequently occurs in a delayed fashion and appears to be based on a secondary phase of dyshomeostasis rather than on a primary event (Fig. 3). Nevertheless it appears that only primary states of dyshomeostasis can be corrected via immediate cell-directed intervention to prevent a malignant nonreversible systemic inflammatory response. It is our hypothesis that a combined therapeutic strategy should include (a) a global short-term (≤72 h) downregulation of inflammatory Mϕ and PMN activity as previously outlined, (b) the prevention of excessive Mϕ stimulation via neutralization of circulating endo-/exotoxins with high doses of polyvalent immunoglobulins and soluble complement receptors, and (c) cell-mediated specific immune performance upregulation to overcome posttraumatic paralysis via the administration of thymomimetic hormones, γIFN, and gramulocyte colony-stimulating factor.

Inspite of all the progress that has been made in understanding globally the mechanisms of host defense dysfunction in trauma, shock, and sepsis the precondition to employ immunotherapeutic interventions effectively in surgical patients in the near future will depend on (a) our ability to measure the activation state of host defenses accurately, (b) a clear comprehension of the interactions among the various components of the immune system during health and dis-

ease, and (c) a much more rapid and precise identification of pathogens and microbial toxins (DUNN 1944).

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Endotoxin-Based Molecular Strategies for the Prevention and Treatment of Gram-Negative Sepsis and Septic Shock

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1 Introduction

As documented in the other chapters of this volume, there is a rapidly growing appreciation that gram-negative sepsis is both an infectious and a systemic inflammatory disease. Extraordinary progress has been achieved in describing at least some of the molecular mechanisms which produce sepsis, especially as they relate to the causative role of bacterial lipopolysaccharides (LPS) or endotoxin.

Recent research has elucidated structure-function relationships of LPS and has begun to unravel some of the mysteries surrounding molecular events involved in mammalian responses to this complex macromolecule (Rietschel et al. 1993, 1994). Our expanding understanding of LPS-induced biological responses has been stimulated by the characterization of CD14, a putative endotoxin receptor (Lee et al. 1993; Pugin et al. 1993a,b; Tobias and Ulevitch 1993; Ulevitch and Tobias 1994), and LPS-binding protein (LBP), a serum protein that catalyzes

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the CD14-mediated recognition of LPS-LBP complexes by eukaryotic cells (Schumann et al. 1990; Tobias et al. 1992; Mathison et al. 1993; Hailman et al. 1994).

There is accumulating evidence that the recognition of LPS by target cells of the infected host, a process mediated through membrane-bound or soluble forms of CD14 (Frey et al. 1992; Pugin et al. 1993a,b) and facilitated by LBP, is a major triggering event in endotoxin-induced inflammation associated with the development of sepsis. Current research seeks to discover how CD14 and other putative LPS receptors are functionally linked, through signal transduction and transcriptional regulation, to the initiation of intracellular events associated with LPS-induced cell activation (Weinstein et al. 1993; Ulevitch and Tobias 1994).

Meanwhile, much has been learned about host-derived proteins which, through their interactions with LPS, attenuate its proinflammatory activities. These include granulocyte-derived cationic antimicrobial proteins (CAP) such as CAP18 (Larrick et al. 1995) and bactericidal/permeability-increasing protein (BPI; Tobias et al. 1988; Heumann et al. 1993; Wilde et al. 1994), the latter a natural homolog of LBP; serum transport proteins such as low-density lipoprotein (LDL), high-density lipoprotein (HDL), and very low-density lipoprotein (VLDL)/chylomicrons (Harris et al. 1990; Flegel et al. 1993); and antibodies directed against various structural elements of the LPS macromolecule, including the O polysaccharide, core oligosaccharide, and biologically active lipid A moiety (Pollack 1992a; Pollack et al. 1995).

This chapter evaluates therapeutic strategies that target the LPS molecule itself or host-derived factors through which LPS acts to produce a pathogenic proinflammatory response. The former include mammalian proteins with endotoxin-neutralizing activity such as leukocyte-derived BPI and CAP proteins, serum transport proteins, anti-LPS antibodies, nonmammalian proteins such as anti-LPS factors from horseshoe crabs (Warren et al. 1992b; Roth and Tobias 1993), and polymyxin B (PmB) or PmB-related synthetic peptides (Morrison and Jacobs 1976; Rustici et al. 1993). The latter include antibodies to CD14 and LBP, soluble CD14, and natural or synthetic lipid A analogs which act as lipid A antagonists or agonists with anti-inflammatory or tolerizing activities. Also discussed are therapeutic measures which involve the replacement or extracorporal purification of a septic patient's blood or plasma to remove endotoxin (and inflammatory mediators).

2 Lipopolysaccharide Antibodies

LPS antibodies mediate natural and acquired immunity to gram-negative bacteria. They are ubiquitous in human serum and other extracellular fluids, appearing as a function of age and cumulative antigenic exposure related to clinical and subclinical infection or simple colonization.

Naturally acquired antibodies are directed toward epitopes in all three structural domains of LPS (Pollack 1992a). In general, antibodies to the phylogenetically heterogeneous O polysaccharide are species and serotype specific, while antibodies which recognize more conserved epitopes on the core oligosaccharide or lipid A tend to react more broadly among phylogenetically diverse LPS. Differences in the submolecular specificity of LPS antibodies may be associated with other functional disparities as well. For example, antibodies that recognize relatively exposed epitopes associated with repeating oligosaccharide subunits of the LPS O-side chain may exhibit more extensive binding and express higher functional affinity toward whole LPS compared with antibodies directed toward less well exposed and less numerous epitopes associated with the relatively cryptic, nonrepeating LPS core structure (Pollack et al. 1990; Pollack 1992a).

LPS antibodies mediate antibacterial as well as antiendotoxic functions. Antibodies specific for the O-polysaccharide of smooth LPS or core component of phenotypically rough LPS (so-called lipooligosaccharide) may deposit complement on the bacterial cell surface, leading to production of membrane attack complexes, pore formation, and subsequent cell lysis (Oishi et al. 1992). Alternatively, such antibodies may mediate complement-dependent opsonophagocytic bacterial killing in conjunction with professional phagocytes (Pier et al. 1994).

LPS antibodies may also promote the complement-dependent phagocytic uptake of free LPS (Krieger et al. 1993), while inhibiting its uptake by the more proinflammatory CD14-mediated pathway (Pollack et al. 1995). Moreover, in humans LPS antibodies may mediate the clearance of LPS-containing immune complexes through CR1 receptors on erythrocytes which are thought to transport and deliver the immune complexes to fixed tissue macrophages of the reticuloendothelial system (Katsikis et al. 1993; Krieger et al. 1993; Tonoli et al. 1993).

LPS antibodies may thus mediate bacterial killing, facilitate the sequestration or removal of LPS from sites of production, participate in the clearance of circulating LPS, and prevent its inflammation-provoking recognition by monocytes/macrophages.

There is little question that LPS antibodies serve an important protective function against gram-negative bacterial disease. This has been more easy to document experimentally; however, in the case of O-side chain-specific antibodies expressing antibacterial activities (Kirkland and Ziegler 1984; Stoll et al. 1986; Oishi et al. 1992; Hoffman et al. 1994; Pier et al. 1994) than in the case of core- or lipid-A specific antibodies exhibiting antiendotoxic properties (Baumgartner et al. 1990; Appelmelk and Cohen 1992; Pollack 1992a). However, more therapeutic interest has focused in recent years on the core region of LPS, and upon the core hypothesis which predicts that antibodies directed toward phylogenetically conserved, biologically active epitopes in the core/lipid A region express cross-reactive, endotoxin-neutralizing, and cross-protective functions (Appelmelk and Cohen 1992; Pollack 1992a). The popularity of the core hy-

pothesis has been driven by its clear theoretical rationale, a substantial body of suggestive if not definitive experimental evidence, and the widely perceived practical need for a single antiendotoxin reagent with broad therapeutic application in gram-negative sepsis of diverse etiology.

Two putative lipid A-specific IgM monoclonal antibodies (MAbs), one murine, E5 (Wood et al. 1992), and the other human, HA-1A (Teng et al. 1985; Bogard et al. 1993), have undergone extensive preclinical and clinical evaluation. Some evidence suggests that under appropriate conditions both MAbs are able to react with phenotypically smooth as well as rough LPS and both are capable of cross-reacting with LPS produced by gram-negative organisms representing different bacterial species, genera, and families (Parent et al. 1992; Wood et al. 1992; Bogard et al. 1993; Fujihara et al. 1993a,b; Mascelli et al. 1993; Siegel et al. 1993). The apparent cross-reactivity of the MAbs may be attributable in part to their low intrinsic but higher functional affinity, characteristics which often typify polyvalent antibodies of the IgM class and render them more cross-reactive than IgG antibodies of similar specificity (Pollack 1992a).

The putative endotoxin-neutralizing properties of the E5 and HA-1A MAbs have been supported by certain in vitro and in vivo experiments (Teng et al. 1985; Chen et al. 1993; Katsikis et al. 1993; Paleolog et al. 1993; Ono et al. 1994) but not by others (Baumgartner et al. 1990; Warren et al. 1993). Similarly conflicting preclinical data have been produced with regard to in vivo protection against live challenge and cross-protection against heterologous challenge (Teng et al. 1985; Baumgartner et al. 1990; Quezado et al. 1993; Romulo et al. 1993; Rogy et al. 1994). Some of the negative or nonconfirmatory data collected in these experiments have been explained or rationalized on the basis of inadequate animal models of septic shock.

Clinical trial results obtained with the E5 and HA-1A MAbs have also been controversial. In each case initial phase III trials in gram-negative sepsis demonstrated significant MAb-associated reductions in mortality (Greenman et al. 1991; Ziegler et al. 1991; Wortel et al. 1992), albeit only in subgroups of the entire study population (i.e., patients *not* in shock in the case of E5 and patients with documented gram-negative bacteremia with or without shock in the case of HA-1A). Unfortunately, neither of these results was reproduced in second trials designed to confirm the results of the earlier trials (Wenzel et al. 1991; McCloskey et al. 1994). It is plausible that the lack of reproducibility of the E5 and HA-1A clinical trials reflects more the complexity of the disease process, the heterogeneity of the patient populations studied, and the intricacies of study design than the inherent biological limitations of the MAbs themselves. Further phase III trials of both MAbs are still in progress.

It has been argued that the E5 and HA-1A clinical trials were premature in the absence of definitive preclinical data and an adequate understanding of the MAbs' mechanism of action (Warren et al. 1992a). The main counterargument has been that inadequate in vitro and in vivo models existed for testing the core hypothesis prior to human trials of core or lipid A reactive antibodies, and that the decision to conduct such trials was therefore a necessary and courageous one

(Wenzel 1992; Ziegler and Smith 1992). In any case the E5 and HA-1A MAbs were among the earlier examples of such reagents available for testing but not necessarily optimal therapeutic candidates. It is theoretically possible that genetic reengineering of HA-1A or E5 or the development of new MAbs with improved binding and endotoxin-neutralizing characteristics might yield therapeutic reagents superior to the original E5 and HA-1A MAbs.

It has been suggested on the basis of animal data that core-specific MAbs may offer greater therapeutic potential than lipid A-specific antibodies. Moreover, core-reactive MAbs with a narrower spectrum of activity against a single bacterial species, genus, or closely related group of genera (Terashima et al. 1991; Di Padova et al. 1993) may prove more therapeutically active against these more restricted targets than more broadly cross-reactive antibodies. Presumably, these more narrowly targeted core-reactive MAbs could be administered in combination, if necessary, to achieve broader coverage.

There has been considerable recent interest in the exploitation of possible therapeutic synergy between antibiotics which disrupt the bacterial cell wall, releasing LPS into the surrounding environment, and LPS antibodies which neutralize or facilitate the clearance of LPS thus released (Collins et al. 1989; Krieger et al. 1993; Romulo et al. 1993; Siegel et al. 1993). There is also keen interest in combining LPS antibodies with anti-inflammatory agents, such as anticytokine antibodies (Cross et al. 1993; Cross and Opal 1994), in order to interrupt the proinflammatory cascade at its point of origin as well as further downstream in the hope that such a multifocal approach will provide additive or synergistic therapeutic benefits.

Thus, although there is currently a general disaffection with antiendotoxin MAbs as therapeutics in gram-negative sepsis based, in part, upon their uneven performance in clinical trials, the core hypothesis survives intact if somewhat tarnished and a new generation of therapeutically active MAbs directed toward core- or lipid-A associated epitopes appears to be at least a theoretical possibility (Cross 1994; Cross and Opal 1994). LPS antibodies remain an attractive alternative to other adjunctive therapies in gram-negative sepsis because of their potentially beneficial activities against both LPS and bacteria and the fact that they target a critical virulence factor produced by the invading pathogen rather than the delicate homeostasis-maintaining apparatus of the infected host.

3 BPI and Other Granulocyte-Derived (CAP) Proteins

Another therapeutic strategy utilizes a variety of native or recombinant proteins that bind LPS and block its biological functions. Mammalian polymorphonuclear leukocyte granules contain several cationic proteins and peptides with bactericidal and LPS-neutralizing properties (Tobias et al. 1988; Elsbach et al. 1994).

One such protein, BPI (CAP57), is a 55- to 60-kDa azurophilic granule protein with 45% sequence homology with LBP (Tobias et al. 1988). BPI and LBP both bind with high affinity through the 25-kDa N-terminal portion of their respective molecules to lipid A, smooth and rough LPS, and intact gram-negative bacteria (Gazzano-Santoro et al. 1992; Appelmelk et al. 1994b; Theofan et al. 1994). Although LBP binds to LPS and enhances various LPS-induced cellular responses, BPI binds with greater avidity and antagonizes the biological activities of LPS (Heumann et al. 1993; Gazzano-Santoro et al. 1994). In vitro, BPI inhibits LPS uptake by monocytes and neutrophils, LPS-induced cytokine and nitric oxide release by mononuclear cells, LPS priming of neutrophils, and LPS-mediated endothelial cell activation and injury (Ooi et al. 1991; Dentener et al. 1993; Appelmelk et al. 1994b; Arditi et al. 1994; Kohn and Kung 1995). BPI also inhibits bacterial proliferation and exhibits extracellular cytotoxic activity against a broad range of gram-negative bacteria related to its high affinity for outer membrane bound LPS (Weiss et al. 1992; Elsbach et al. 1994).

It is suspected but unproven that granulocyte-derived, extracellular forms of BPI play a protective role in gram-negative infections distinct from the strictly bactericidal functions of BPI. BPI is found in higher concentrations than LBP in certain closed space infections, while LBP predominates in plasma from septic patients. This suggests that measured levels of endogenous BPI are sufficient to counteract the proinflammatory effects of LBP at local tissue sites but not in plasma (Calvano et al. 1994; Opal et al. 1994).

Recombinant BPI (rBPI) and its 23-kDa N-terminal portion (rBPI-23) retain the biological activities of the natural protein or exceed it in potency (Ooi et al. 1991; Gazzano-Santoro et al. 1992, 1994; Weiss et al. 1992; Dentener et al. 1993; Theofan et al. 1994). The evaluation of rBPI-23 in animals revealed a reduction in LPS-induced proinflammatory cytokine release; normalization of LPS-associated hemodynamic, pulmonary and metabolic dysfunction; and protection against lethal endotoxemia (Kohn et al. 1993; Fisher et al. 1994; Kung et al. 1994; Parent 1994; Evans et al. 1995). In recent experiments rBPI-23 reduced mortality in mice and rabbits subjected to otherwise lethal intravenous, intraperitoneal, or intratracheal challenge with a variety of gram-negative bacteria and in most cases reduced levels of bacteremia (Kelly et al. 1993; Ammons et al. 1994; Parent 1994; Evans et al. 1995). The administration of rBPI-23 to human volunteers subsequently challenged with intravenous endotoxin reduced fever and leukopenia, blocked cytokine release, and prevented endotoxin-induced fibrinolysis (van Deventer et al. 1994).

Although BPI and its recombinant products have demonstrated therapeutic promise in preclinical studies, reduced efficacy has been documented in association with delays in treatment. Moreover, intravenously administered rBPI-23 failed to achieve therapeutic concentrations in the cerebrospinal fluid or to reduce endotoxin-induced meningeal inflammation in a rabbit model of bacterial meningitis (Kartalija et al. 1995). These studies suggest that the clinical utility of BPI may be limited by a stringent requirement for early administration relative to the onset of infection and suboptimal pharmacokinetic properties.

Both rBPI and rBPI-23 have been given safely to animals and human volunteers. Since these proteins have a short serum half-life, they must be administered by repeated bolus injection or continuous intravenous infusion. Fusion chimeras comprised of amino acid sequences derived from both BPI and LBP have been constructed and exhibit a longer circulating half-life while retaining the ability to neutralize endotoxin and protect animals against endotoxin-induced lethality (Marra et al. 1994).

The recombinant product rBPI-21 has undergone human phase I safety and pharmacokinetic evaluation, and separate safety and clinical efficacy trials have recently begun in patients with meningococcemia and hemorrhagic trauma (the latter assessing prevention of secondary infections, organ damage, or death).

Several other cationic proteins and peptides with bactericidal and endotoxin-neutralizing properties have been isolated from neutrophil granules. The 18-kDa protein designated CAP18, originally isolated from rabbit granulocytes, was recently identified in normal human granulocytes and cloned (Larrick et al. 1995). Rabbit and human CAP18, and the active C-terminal fragment derived from the parent molecules (CAP7 and $CAP18^{104-135}$, respectively), bind with high affinity to LPS, exhibit LPS-neutralizing properties, inhibit LPS-related proinflammatory activities, and protect mice from LPS-induced lethality (Hirata et al. 1994a; Larrick et al. 1995).

A number of natural and synthetic peptides have been identified with molecular and biological characteristics similar to those of CAP18. Most share homology with the N-terminal region of cathelin, a potent cysteine protease inhibitor (Levy et al. 1993; Gennaro et al. 1994; Hirata et al. 1994b; Larrick et al. 1995). Other granulocyte-derived antimicrobial proteins with antiendotoxin activities include the defensins (Lehrer et al. 1993), lactoferrin (Appelmelk et al. 1994a), and azurocidin, also known as CAP37 (Pereira et al. 1993, 1994).

4 Anti-LPS Factors from Horseshoe Crabs

Two distinct basic proteins which share partial sequence homology and inhibit LPS-induced amebocyte lysate coagulation have been isolated from the horseshoe crabs *Tachypleus tridentatus* and *Limulus polyphemus* (Tanaka et al. 1982). These anti-LPS factors of *T. tridentatus* (TALF) and *L. polyphemus* (LALF), inhibit the growth of gram-negative bacteria, bind phylogenetically diverse endotoxins with high affinity, and inhibit a variety of LPS-associated biological activities in vitro and in vivo (Morita et al. 1985; Desch et al. 1989; Alpert et al. 1992; Warren et al. 1992b). Recombinant LALF (also known as endotoxin-neutralizing protein or ENP) and synthetic fragments of TALF exhibit endotoxin-neutralizing properties similar to those of the native proteins, including the ability to protect animals against experimental endotoxemia and sepsis (Fletcher et al. 1993; Kloczewiak et al. 1994; Kuppermann et al. 1994). The administration of ENP to rats or rabbits

up to 60 min after *Escherichia coli* challenge appeared to lower circulating endotoxin and tumor necrosis factor (TNF) levels, and reduce mortality (Saladino et al. 1994; Nelson et al. 1995).

5 Polymyxin B and PmB-Related Synthetic Peptides

PmB is a cationic cyclic decapeptide that binds avidly to hydrophilic and hydrophobic elements of LPS and lipid A and has the capacity to disorganize the outer membrane of gram-negative bacteria (Morrison and Jacobs 1976; Storm et al. 1977). In addition to its bactericidal activity, PmB inhibits many of the in vitro and in vivo biological activities of endotoxin and prevents LPS-induced lethality (Corrigan and Bell 1971; Walterspiel et al. 1986; Flynn et al. 1987; Baldwin et al. 1991; Coyne and Fenwick 1993; Durando et al. 1994). The endotoxin-neutralizing activity of PmB has been attributed to ionic interactions between diaminobutyric acid residues of the cationic antibiotic and mono- or diphosphate groups of lipid A. The resulting LPS-PmB complex has a reduced capacity for association with LBP and/or recognition by membrane-bound CD14 (Coyne and Fenwick 1993; Gallay et al. 1993b; Helander et al. 1994). Recently designed synthetic peptides mimic primary and secondary structural motifs of PmB and further define molecular structures required for LPS binding and neutralization (Danner et al. 1989; Rustici et al. 1993). Although devoid of antibiotic activity, some of these synthetic peptides exhibit endotoxin-neutralizing properties equivalent or superior to those of PmB (Rustici et al. 1993). Toxicity limits the use of PmB as a systemic antibiotic, but endotoxin-neutralizing activity is expressed at relatively nontoxic serum concentrations (Durando et al. 1994). The low toxicity and intact anti-endotoxic activity of PmB-related synthetic peptides make them particularly attractive therapeutic candidates.

6 Interruption of LPS-Induced, LBP- and CD14-Mediated Cellular Activation Pathways

Cell-bound and soluble forms of the 50- to 55-kDa glycoprotein CD14 and the 58- to 60-kDa serum glycoprotein LBP are the major mammalian host factors implicated in the cellular recognition of LPS leading to transmembrane signaling and cell activation (Ulevitch and Tobias 1994). Accordingly, these proteins are prime targets for interventional therapy in gram-negative sepsis.

The cell-associated form of CD14 (mCD14) is a glycosylphosphatidyl inositol-anchored membrane glycoprotein expressed on monocytes, macrophages, and

polymorphonuclear leukocytes (PMNs). Recent studies indicate that mCD14 is probably one part of multicomponent LPS receptor functionally linked to the initiation of intracellular events associated with LPS-induced cell activation (Ulevitch and Tobias 1994).

The soluble form of CD14 (sCD14), which lacks the glycosylphosphatidyl inositol anchor of mCD14, is found in normal human serum. High circulating levels of sCD14 are associated with increased mortality in gram-negative septic shock (Landmann et al. 1995). Endothelial and epithelial cells, which lack mCD14, are able to recognize and respond to LPS/sCD14 complexes through an as yet unidentified membrane-bound element(s) (Frey et al. 1992; Arditi et al. 1993; Pugin et al. 1993a,b; Goldblum et al. 1994; Ulevitch and Tobias 1994).

A normal constituent of plasma, LBP increases in concentration in association with the acute-phase response and appears to mediate the lethal effects of endotoxemia (Gallay et al. 1993a). LBP controls cellular responses to LPS through the formation of high-affinity LPS-LBP complexes. These complexes are recognized by mCD14 on myeloid cells, facilitating LPS-induced cell activation at LPS concentrations encountered in sepsis (Schumann et al. 1990; Wright et al. 1990; Mathison et al. 1992; Tobias and Ulevitch 1994). Recent evidence (Hailman et al. 1994) suggests that LBP functions catalytically as a lipid transfer protein, accelerating the binding of LPS to both mCD14 and sCD14. LBP may also transfer LPS to serum lipoproteins, thus acting as a cofactor for an activity which results in the neutralization of LPS (Wurfel et al. 1994).

The recognition of LPS-LBP complexes by mCD14 on monocytes of macrophages initiates LPS-induced protein tyrosine phosphorylation (Weinstein et al. 1993), NF-κB activation (Lee et al. 1993), the release of proinflammatory cytokines such as TNF-α (Wright et al. 1990), and enhanced tissue factor expression which is causally related to sepsis-induced coagulopathy (Steinemann et al. 1994; Yang et al. 1994). These events are all important in the pathogenesis of gram-negative sepsis and septic shock, and all are inhibited by antibodies to LBP or CD14 (Wright et al. 1990; Tobias et al. 1992; Gallay et al. 1993b; Lee et al. 1993; Tobias and Ulevitch 1993; Gallay et al. 1994; Martin et al. 1994; Steinemann et al. 1994; Yang et al. 1994). Similarly, LPS-induced, serum-dependent adherence of PMNs to endothelium (Worthen et al. 1992), LPS priming of PMNs for enhanced release of superoxide in response to stimulation with fMet-Leu-Phe (Yasui et al. 1994), and LPS-mediated upregulation of CR1 receptors on PMNs (Weingarten et al. 1993) are all blocked by anti-CD14 or anti-LBP antibodies. LPS-induced endothelial cell activation and injury are also inhibited by CD14-specific antibodies, whether these sepsis-related events are produced directly through the action of LPS-sCD14 complexes in plasma or indirectly through LPS-mCD14 complexes on monocytes in whole blood, (Frey et al. 1992; Arditi et al. 1993; Pugin et al. 1993a,b; Goldblum et al. 1994).

Despite extensive in vitro evaluation of the LPS-neutralizing properties of LBP-and CD14-specific antibodies there are few published data concerning the in vivo effects of these reagents in animal models. One study (Gallay et al. 1993a) documented that an anti-LBP antibody reduced circulating TNF levels and

protected mice against the lethal effect of LPS when given at the same time as LPS challenge, but failed to protect mice when delayed 15 min after LPS challenge. Another more recent study (Leturcq et al. 1994) evaluated two CD14-specific MAbs in a primate model of endotoxin-induced shock, and found that these MAbs reduced systemic hypotension, abnormalities in lung permeability, and elevations in circulating proinflammatory cytokines. The authors concluded that "strategies targeting the CD14 receptor will have utility in diseases such as septic shock or ARDS [adult respiratory distress syndrome] where LPS plays a central role in initiating injury."

One hypothesis holds that sCD14, which is shed from myeloid cells in tissue culture and is found in normal plasma, might be a natural inhibitor of endotoxin (Wright 1991). This hypothesis predicts that sCD14 would "neutralize" LPS-LBP complexes by binding the complexes in competition with mCD14 receptors on macrophages, thereby preventing endotoxin-induced cell activation and TNF release. Several lines of evidence support the endotoxin-neutralizing capacity of sCD14. One study, for example, demonstrated that the addition of human sCD14 to calf serum abrogated the binding of fluorescein isothiocyanate-conjugated LPS to bovine monocytes and protected the cells from LPS-induced activation (Grunwald et al. 1993). The dose-dependent reduction by sCD14 of LPS-inducible reactive oxygen species in human monocytes was documented in a second study (Schütt et al. 1992). Confirmatory data came from another group which found that recombinant sCD14 (rsCD14) antagonized the response of human and murine mononuclear cells to LPS and inhibited the release of TNF-α by monocytes/macrophages (Haziot and Goyert 1994). It was further discovered that administration of rsCD14 following an injection of LPS protected mice from LPS-induced lethality. The authors concluded than even though rsCD14 enhances endothelial (and epithelial) cell responses to LPS in vitro, these effects are overshadowed in vivo by the inhibitory effect of rsCD14 on monocyte/macrophage responses to LPS which are apparently more relevant with respect to LPS-induced mortality. On this basis, the authors predicted that rsCD14 "might provide a new therapeutic for endotoxin shock in man" (Haziot and Goyert 1994).

In order to characterize structure-function relationships within LBP investigators have created and expressed truncated forms of human LBP (Han et al. 1994; Theofan et al. 1994). One group has characterized a truncated form of the molecule (NH-LBP) comprising amino acid residues 1–197 of the parent molecule (Han et al. 1994). NH-LBP was found to bind LPS efficiently but not transfer the LPS to either mCD14 or sCD14. The LBP partial structure inhibited LPS binding to LBP, LBP-enhanced binding of LPS to CD14, and LBP-dependent activation of rabbit peritoneal exudate macrophages. On the basis of these findings the authors concluded that the LPS-binding site of LBP resides in the amino-terminal half of LBP, and that the CD14 interaction site resides in the carboxyl-terminal half of the parent molecule. It was further concluded that "appropriate modifications of the primary sequence of LBP might provide a novel protein that would bind LPS with high affinity, but

prevent its interaction with either mCD14 or sCD14 . . . [and that] such entities may have therapeutic potential in diseases where LPS plays a prominent role'' (Han et al. 1994).

Despite their apparent therapeutic potential, no LBP- or CD14-related therapeutic products are presently in clinical development.

7 Serum Lipoproteins

In contrast with LBP, a serum glycoprotein which facilitates the CD14-mediated, proinflammatory recognition and uptake of LPS by myeloid cells, serum lipoproteins bind and neutralize LPS, suggesting a possible anti-inflammatory role for these complex macromolecules in gram-negative sepsis (Ulevitch and Johnston 1978; Ulevitch et al. 1979; Cavaillon et al. 1990; Emancipator et al. 1992; Parker et al. 1995). In addition, serum lipoproteins appear to enhance the clearance of circulating endotoxins through hepatobiliary excretion (Read et al. 1993).

Recent data (Wurfel et al. 1994) actually suggest that serum LBP, previously characterized as a lipid transport enzyme, is capable of transferring LPS to HDL as well as to soluble or membrane-bound forms of CD14. Moreover, much of the LBP found in human blood is closely associated with the apolipoprotein A-I (ApoA-I) component of native HDL. It thus appears that ApoA-I and LBP-containing HDL particles are naturally equipped to either bind and neutralize LPS or participate in its transfer to CD14-mediated proinflammatory pathways.

Incubation of LPS with plasma, but not delipidated plasma, reduces the proinflammatory activity of endotoxin in animals (Ulevitch and Johnston 1978; Ulevitch et al. 1979). Chylomicrons, VLDL, LDL, HDL, ApoA-I, reconstituted HDL (R-HDL), and synthetic lipid emulsions may all bind and inactivate LPS, as documented experimentally by decrements in LPS-induced *Limulus* amebocyte lysate-gelating activity, cytokine release, physiologic responses, or lethality (Harris et al. 1990; Emancipator et al. 1992; Flegel et al. 1993; Harris et al. 1993; Hubsch et al. 1993; Levine et al. 1993; Parker et al. 1995; Read et al. 1995).

A single published study (Quezado et al. 1995) employed a canine model of *E. coli* peritonitis and septic shock to evaluate the ability of an infused lipoprotein to protect against a live bacterial challenge. Although the intravenous administration of R-HDL reduced endotoxemia, associated leukopenia, and cytokine release, treated dogs showed signs of liver toxicity and some developed seizures, contributing to decreased survival in the treated group compared with controls. The apparent toxicity of R-HDL exhibited in this canine experiment had not been noted in previous studies using small animals.

8 Natural and Synthetic LPS and Lipid A Analogs or Partial Structures That Antagonize or Induce Tolerance to LPS

With detailed descriptions of LPS and lipid A structures, biosynthetic pathways, and structure-function relationships has come the discovery of relatively nontoxic natural forms of LPS and lipid A or lipid A precursors. These discoveries, in turn, have facilitated the laboratory preparation or synthesis of a variety of nontoxic lipid A analogs or partial structures capable of competing with LPS for binding sites on LPS target cells, inducing endotoxin tolerance, stimulating anti-inflammatory pathways, and recruiting nonspecific mechanisms of host resistance. Such lipid A-like molecules may thus have prophylactic or therapeutic applications in gram-negative sepsis and septic shock, and some are currently undergoing preclinical and early clinical evaluation.

Natural lipid A precursors like lipid X and lipid IV_A, synthetic analogs of these molecules such as 3-aza-lipid X-4-phosphate (SDZ880.431) and phosphonooxyethyl derivatives of lipid IV_A, and the non-toxic *Rhodobacter sphaeroides* diphosphoryl lipid A (rsDPLA) all bind to LPS receptors but express markedly reduced endotoxinlike (agonist) activities. These lipid A-like molecules function as LPS antagonists. They compete in a dose-dependent manner with native LPS for occupancy of LPS receptors, prevent the cellular uptake of LPS, block LPS-induced cell activation, and protect against pathophysiological responses to LPS in vivo (Takayama et al. 1989; Kovach et al. 1990; Golenbock et al. 1991, 1994; Lynn et al. 1991; Qureshi et al. 1991; Wang et al. 1991, 1992; Kitchens et al. 1992; Ulmer et al. 1992, 1994; Lei et al. 1993, Manthey et al. 1993; Heine et al. 1994).

A particularly promising LPS receptor antagonist is so-called E5531, a novel synthetic derivative of non-toxic *Rhodobacter capsulatus* lipid A (Christ et al. 1995). In vitro, E5531 demonstrated potent antagonism of cellular activation induced by phylogenetically diverse LPS in a variety of systems. It was reported to be 260 000 times more effective than lipid X, the monosaccharide lipid A precursor, at inhibiting TNF release while displaying no agonistic activity (Kawata et al. 1992). In vivo, E5531 protected mice from LPS-induced lethality, and, in combination with an antibiotic, protected mice against lethal *E. coli* infections (Christ et al. 1995). In healthy volunteers E5531 produced a dose-dependent decrease in LPS-induced fever, chills, headache, nausea, and myalgias (Bunnell et al. 1995a); reduced TNF and interleukin (IL)-6 responses; and blocked the hyperdynamic vascular and myocardial depressant effects of LPS (Bunnel et al. 1995b).

The ability of certain lipid A analogs to induce tolerance is distinct from the ability to competitively inhibit or antagonize LPS-induced cellular activation and corresponding LPS-induced in vivo events (Carpati et al. 1993). Endotoxin tolerance represents a temporary endotoxin-specific hyporesponsive state induced

by prior LPS exposure in relation to subsequent reexposure. It is both an in vitro and in vivo phenomenon. In vitro exposure of macrophages to LPS or certain less toxic lipid A analogs results in a refractoriness to subsequent LPS stimulation marked by a reduction in LPS-induced TNF synthesis and release (MATHISON et al. 1990). Repeated administration of LPS, or lipid A analogs, to experimental animals is associated with a corresponding attenuation of LPS-induced fever and cytokine release, supporting the hypothesis that a critical component of endotoxin tolerance is the reduced production and release of cytokines by monocytes and macrophages upon reexposure to LPS (ROTH et al. 1994). Moreover, recent clinical evidence suggests that a tolerance-like state occurs in septic patients wherein peripheral blood monocytes exhibit markedly diminished proinflammatory cytokine responses to LPS stimulation ex vivo (MUNOZ et al. 1991; VAN DEUREN et al. 1994).

Monophosphoryl lipid A (MPL), a weak LPS agonist, induces tolerance to a subsequent endotoxin challenge in both normal and D-galactosamine sensitized mice. A 4300-fold MPL-induced reduction in the sensitivity of galactosamine-treated mice to endotoxin appears to be mediated by MPL itself as well as by TNF released in response to MPL (RUDBACH et al. 1994). MPL-induced tolerance, as that induced by LPS, entails reduced production of proinflammatory cytokines, including TNF, γ-interferon, colony-stimulating factor, and IL-6 (VOGEL and HENRICSON 1990). However, more MPL than LPS is required to induce the same degree of early endotoxin tolerance, which may be due to the relative inefficiency or low affinity with which MPL binds to LPS cell receptors (VOGEL and HENRICSON 1990). While the results of one study suggested that LPS and MPL are significantly less effective in protecting mice against lethality from peritonitis than against endotoxemia (ASTIZ et al. 1993), another report indicated that MPL given 1 or 2 days, or as little as 6 h, before live *E. coli* challenge produced complete protection (RUDBACH et al. 1994).

In studies reminiscent of those carried out with MPL, rsDPLA induced tolerance to endotoxic shock in rats (ZUCKERMAN and QURESHI 1992). The administration of rsDPLA 48 h before endotoxin challenge reduced the severity of hemodynamic sequelae and mortality associated with endotoxin challenge. This observed tolerance appeared at least in part related to macrophage hyporesponsiveness, as suggested by a decrease in circulating TNF levels in rsDPLA pretreated rats (ZUCKERMAN and QURESHI 1992).

Lipid A analogs may mediate antiendotoxic, anti-inflammatory, or protective activities by mechanisms other than classical LPS antagonism or tolerance. The monosaccharide lipid A analog, SDZ MRL 953, for example, induces resistance to bacterial and fungal infections in part because of its ability to stimulate high levels of secretion of granulocyte colony-stimulating factor and IL-6, and low levels of proinflammatory cytokines such as TNF (ENGELHARDT et al. 1994). This lipid A analog is also capable of inducing high circulating levels of IL-1 receptor antagonist in rhesus monkeys (ENGELHARDT et al. 1994). This activity, together with the development of hyporesponsiveness to endotoxin, may provide a basis for observed protection against infection.

It has been postulated that the in vivo protective effect of rsDPLA against endotoxin challenge in mice actually involves three major mechanisms: antagonism of LPS receptors, induction of tolerance, and ability to elicit an anti-inflammatory response (Zuckerman and Qureshi 1992; Hofman et al. 1994). The latter is supported by an observed stimulatory effect of rsDPLA on the hypothalamic-pituitary-adrenal axis, resulting in enhanced secretion of adrenocorticotrophic hormone and corticosteroids. Elevated corticosterone levels, in turn, inhibit TNF and IL-1 biosynthesis at the pretranslational level, providing a basis for the anti-inflammatory effect of rsDPLA. The inability of rsDPLA to inhibit LPS-induced increases of serum TNF in adrenalectomized mice suggests that the in vivo protective effect of rsDPLA is mediated largely through corticosterone induction and not LPS antagonism or tolerance (Zuckerman and Qureshi 1992).

In addition to its tolerance-inducing activity, MPL induces nonspecific resistance to a variety of infectious agents, including gram-positive bacteria, and is capable of modulating the inflammatory response to host-generated proinflammatory mediators as well as to microbial products (Rudbach et al. 1994).

MPL has been administered safely to healthy volunteers and tolerized them to subsequently administered LPS (Astiz et al. 1995). Plans are underway to evaluate the ability of MPL to prevent infectious and febrile episodes in neutropenic leukemia patients, and consideration is being given to the possible use of MPL as an immunómodulator in human immunodeficiency virus infections and tuberculosis. The intended clinical uses of MPL are thus far broader than those suggested by its endotoxin-tolerizing activities alone.

9 Plasmapheresis, Exchange Transfusion, and Extracorporal LPS Adsorption

An alternative to the systemic administration of specific endotoxin-neutralizing agents in the treatment of gram-negative sepsis is the replacement or purification of a septic patient's blood or plasma in order to remove endotoxin and inflammatory mediators (Pollack 1992b). A number of reports have suggested clinical responses to plasmapheresis or exchange transfusion in septic patients. Most of these studies, however, have been anecdotal, uncontrolled, and/or nonrandomized, and the majority have been limited to patients with meningococcemia (Togari et al. 1983; Drapkin et al. 1989; Westendorp et al. 1990, van Deuren et al. 1992). In contrast, a controlled trial of plasmapheresis in an antibiotic-treated canine septic shock model revealed increased mortality and hemodynamic instability in animals undergoing this treatment (Natanson et al. 1989).

A more specific therapeutic approach to endotoxemia involves extracorporal removal of circulating endotoxin by plasma filtration or selective adsorption (COHEN et al. 1987; HANASAWA et al. 1988; BYSANI et al. 1990). An evaluation, in animal models, of extracorporal adsorption of endotoxin-containing plasma with PmB immobilized on insoluble fibers demonstrated effective endotoxin removal, reduced LPS-induced toxicity, and enhanced survival (HANASAWA et al. 1988). This method of extracorporal adsorption has undergone evaluation in septic patients, and is currently in clinical use in Japan.

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Anticytokine Strategies for the Treatment of Septic Shock: Relevance of Animal Models

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1 Introduction

For many years septic shock has been described as a complication of gram-negative bacteremia, and initial efforts at devising therapeutic approaches focused on developing antibodies directed against endotoxin (LPS), the major toxic component of gram-negative bacteria. Because recent advances in immunology have greatly contributed to our understanding of the pathophysiology of septic shock and acute inflammatory response initiated not only by LPS but also by other microbial products, many novel approaches for the treatment of septic shock have been developed in addition to anti-LPS antibodies.

Invading bacteria and bacterial products trigger within the host the release of a complex array of cytokines. Normally cytokines are believed to be autocrine

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and paracrine (cell to cell) molecules that act locally at their site of production to control the host response to invading organisms. In fact, by influencing coagulation and leukocyte transmigration and by activating professional phagocytes cytokines assist the host to contain a local infection. It is generally recognized that in septic shock an overproduction of cytokines generates a systemic activation which affects vascular permeability and resistance, cardiac function, and induces many metabolic derangements that may result in tissue necrosis, leading eventually to multiple-organ failure and death.

The concept that multiple-organ failure is related to an uncontrolled systemic inflammatory state originated mainly from animal studies. Central to the process was the discovery that macrophages activated by bacterial products release proinflammatory molecules, especially tumor necrosis factor (TNF) and interleukin (IL)-1. In fact the sole macrophage activation with its subsequent cytokine release or the injection of recombinant cytokines can produce experimentally in animals a syndrome indistinguishable from the response to severe infections. In animal models of infection the cascade of events starting from the initial infectious stimulus and usually ending with the death of the animal follows a very predictible time course. In many animal models the course of septic shock usually lasts hours or a few days. The cytokine production that results from infectious challenges also follow a predictible time course, so that experimental

Table 1. Protection over time by anti-TNF antibodies in various models of endotoxemia and intravenous injections of live bacteria

Reference	Model	Time of administration of anti-TNF antibodies (minutes) relative to challenge				
		<120	−60 to −30	0	+30 to +60	>120
Endotoxemia						
Beutler et al. (1985)	Mouse, i.p. LPS, 16 mg/kg	+		±		−
Mathison (1988)	Rabbit, i.v. LPS, 10 mg/kg		+			
Fiedler et al. (1991)	Rhesus, i.v. LPS/GAL				+	
Bagby et al. (1991)	Rat, i.v. LPS, 1 mg/kg	+	+			
Eskandari et al. (1992)	Mouse, i.p. LPS, 100 mg/kg	−		−		
Zanetti et al. (1992)	Mouse, i.p. LPS/GAL			+		
Emerson et al. (1992)	Baboon, i.v. LPS, 2mg/kg		+			
Jin et al. (1994)	Rat, i.v. LPS, 7.5 mg/kg					
IV injection of live bacteria						
Tracey et al. (1987)	Baboon, i.v. *E. coli*	+	±			
Hinshaw et al. (1990)	Baboon, i.v. *E. coli*				+	
Silva et al (1990)	Mouse, i.v. *E. coli*	+			+	−
Jesmok et al. (1992)	Pig, i.v. *E. coli*			+		
Schlag et al. (1994)	Baboon, i.v. *E. coli*					+
Silva et al. (1990)	Mouse, i.v. *Klebsiella*	−				
Opal et al. (1991)	Rat, oral *P. aeruginosa*			±		
Hinshaw et al. (1992)	Baboon, i.v. *S. aureus*				+	
Martin et al. (1993)	Mouse, i.v. *S. pyogenes*		−			

GAL, Mice sensitized with D-galactosammine; +, efficacy; ±, relative efficacy; −, lack of efficacy.

approaches at blocking one cytokine or another are relatively straightforward. In contrast, the sequence of events leading to septic shock in humans is more complex. The micro-organisms and the sites of infections are very diverse. The course of the illness in patients generally takes days rather than hours, as seen in most animal models. Moreover, most patients with septic shock have a large variety of underlying diseases, which are not present in animal studies. For all these reasons it is difficult to extrapolate from observations in straightforward animal models to the very complex situations in patients.

The current strategy to attenuate the detrimental effect of cytokines in human sepsis is based essentially on observations from animal studies. These studies have led to hypotheses that should now be validated in clinical studies planned or currently under way. The present review considers animal studies that have led to the concept of immunotherapy to limit overproduction of proinflammatory cytokines (TNF, IL-1) and focuses particularly on the experimental conditions. We suggest that conclusions drawn from observations in animal models may have somewhat limited applicability to the human sepsis/septic syndrome, thus stressing the need for the careful performance and analysis of the results of clinical trials.

2 Experimental Models

2.1 TNF Blockade in Animal Models

TNF blockade has been investigated in numerous animal models. Table 1 summarizes the studies that have evaluated the protective efficacy of the anti-TNF antibodies in various animals challenged parenterally with either LPS or gram-negative bacteria. In a few studies gram-positive bacteria were also evaluated and are included in this review.

2.1.1 Anti-TNF Antibodies and Parenteral (or Intraperitoneal) LPS Challenge

When LPS was the challenging agent, administered either intravenously (i.v.) or intraperitoneally (i.p.) (Beutler et al. 1985; Mathison et al. 1988; Fiedler et al. 1991; Bagby et al. 1991; Zanetti et al. 1992; Emerson et al. 1992), protection occurred in most experiments, provided the anti-TNF antibodies were given before or during infusion of LPS. Failure to protect the animals by simultaneous injection of LPS and anti-TNF antibodies was reported only once (Eskandari et al. 1992). However, this failure of the anti-TNF antibody may be related to the very high concentration of LPS used in that experiment, i.e., 100 mg/kg (Eskandari et al. 1992). A poor increase of survival was observed in another report (Jin et al. 1994). With regard to delaying TNF blockade with anti-TNF antibodies after LPS

challenge, it is inefficient in endotoxic models. Only one observation of relative success is reported (BEUTLER et al. 1985), while most failures are probably not reported.

2.1.2 Anti-TNF Antibodies and Parenteral Bacterial Challenge

Several studies reported in Table 1 have investigated TNF blockade after i.v. injections of bacteria. Following i.v. challenge of *Escherichia coli* (TRACEY et al. 1987; HINSHAW et al. 1990; SILVA et al. 1990; JESMOK et al. 1992), anti-TNF antibody administration was shown to be protective up to 30 min after the infusion of bacteria in various animal species. A delayed administration given 2 h after challenge did not increase survival in mice (SILVA et al. 1990), whereas in a recent study conducted in baboons a modest protective effect of the anti-TNF antibody was observed when given up to 4 h after bacterial challenge (50% protection over 100% death in controls) (SCHLAG et al. 1994). Upon challenge with gram-negative species other than *E. coli* treatment with anti-TNF antibodies partially protected neutropenic rats from an oral challenge with *Pseudomonas aeruginosa* (OPAL et al. 1991) but did not protect mice from *Klebsiella* infections (SILVA et al. 1990). Finally, after i.v. challenge with gram-positive organisms one study reported protection (HINSHAW et al. 1992) and the other not (MARTIN et al. 1993).

Taken together, the studies of anti-TNF antibody administration after parenteral live bacterial challenge have shown that protection was not uniformly present, and that early treatment was generally associated with a better protection. Information on delayed therapy for many models is lacking, but it is generally recognized that delayed treatment, as after i.v. LPS challenge, is less efficient than early treatment or treatment given before bacterial challenge.

2.1.3 TNF Blockade by Means of Soluble TNF Receptors

A more recent approach for TNF blockade is based on soluble TNF receptors. Two distinct forms of TNF receptors (a p55 form, and a p75 form) are shed from cell surfaces during inflammatory reactions. The TNF soluble receptor shedding is believed to be a regulatory mechanism that prevents circulating TNF to interact with cellular TNF receptors, thus attenuating the effects of excessive TNF production. Fusion proteins have been developed in which each of the two receptors was linked with hinge regions of the human IgG. This dimeric construction extends the half-life of the molecule in vivo and increases the affinity for TNF. Four variants have been constructed using IgG1 of IgG3 as the partner: sTNFR-IgG1 p75, sTNFR-IgG3 p75, sTNFR-IgG1 p55, and sTNFR-IgG3 p55.

These variants have been investigated in several animals (Table 2). All published experiments but one were conducted in mice, most models being parenteral LPS infections. With respect to the sTNFR p55 construct, protection was very efficient when the construct was given prophylactically, and a variable effect was observed depending on the Ig fusion partner (LESSLAUER et al. 1991; ASHKENAZI et al. 1991; LOETSCHER et al. 1993; EVANS et al. 1994; JIN et al. 1994).

Table 2. Protection over time by soluble TNF receptors in various models of endotoxemia and of iv injections of bacteria

Reference	Model	Time of administration of constructs (minutes) relative to challenge				
		<120	−60 to −30	0	+30 to +60	>120
p55						
Lesslauer et al. (1991)	Mouse i.p. LPS, IgG3, p55	+			+	±
Ashkenazi et al. (1991)	Mouse, i.v. LPS, IGI, p55		+		−	
Loetscher et al.(1993)	Mouse LPS, IgG3, p55	+			+	±
Jin et al. (1994)	Rat, i.v. LPS, IgG1, p55		+			
Evans et al. (1994)	Mouse, i.v. *E. coli*, IgG1, p55		+			−
p75						
Mohler et al. (1993)	Mouse, i.v. LPS, IgG1, p75			+	+	±
Loetscher et al. (1993)	Mouse, i.p. LPS, IgG3, p75	−				
Evans et al. (1994)	Mouse, i.v. *E. coli*, IgG1, p75		(±)			

+, Efficacy; ±, relative efficacy; −, lack of efficacy.

Similarly, the sTNFR p55 construct appeared effective in a model of i.v. bacterial challenge (Evans et al. 1994). With respect to the sTNFR75 the IgG1 construct was found protective in a model of bolus LPS injection (Mohler et al. 1993). After a lethal challenge of live *E. coli* it delayed lethality without influencing overall death (Evans et al. 1994). The sTNFR-IgG3 p75 construct was not protective in parenteral LPS injection model (Loetscher et al. 1993) and was not tested after live bacterial challenge.

Taken together, these experiments showed a promising effect of the sTNFR 55 constructs in limiting TNF toxicity, with a superior efficacy of the construct over conventional anti-TNF antibody in one study (Jin et al. 1994). In some models treatment with the sTNFR 55 could be delayed several hours, but this was not true for all models. In contrast, the potential use of the sTNFR 75 constructs appeared more limited. This difference in efficacy may be related to different functions and TNF-binding affinities of the two receptors that are not yet fully understood.

2.2 Blockade of IL-1 by Means of IL-1 Receptor Antagonist

An alternative strategy aimed at attenuating the effects of overproduction of cytokine release investigated blocking of IL-1 by means of the IL-1 receptor antagonist (IL-1ra). Indeed, the IL-1ra inhibits IL-1 by competing with IL-1 for cell receptor sites. Experimentally, IL-1ra has been shown to improve various hemodynamic parameters in models of parenteral LPS or bacterial challenges and to improve survival (Fischer et al. 1992; Wakabayashi et al. 1991; Alexander et al. 1991; Ohlsson et al. 1990; Aiura et al. 1993; Table 3). Due to the short half-life of

Table 3. Effect of interleukin 1 receptor antagonist in various parenteral models of endotoxemia and of i.v. or s.c. injection of bacteria

Reference	Model	IL-1ra (injections)	Effect
Ohlsson et al. (1990)	Rabbit, i.v. LPS	T_0, then every 2 h for 24 h	Increased survival
Alexander et al. (1991)	Mouse, i.p. LPS	$T_{+20\ min}$, then every 4 h for 24 h	Increased survival
Fischer et al. (1992)	Baboon, i.v. LPS	T_0, then continuous	No effect
Wakabayashi et al. (1991)	Rabbit, i.v. *E. coli*	$T_{-15\ min}$, then continuous	Increased survival and hemodynamics
Fischer et al. (1992)	Baboon, i.v. *E. coli*	T_0, then continuous	Increased survival and hemodynamics
Mancilla et al. (1993)	Rat, s.c. *K. pneumoniae*	at T_0 at T_0 and T_{24}	increased survival Decreased survival
Aiura et al. (1993)	Rabbit, i.v. *S. epidermidis*	$T_{-5\ min}$, then continuous	Improved hemodynamics

IL-1ra it must be given in multiple repeated injections or in continuous infusion to be efficacious. Importantly, one report suggested a potential deleterious effect of IL-1 blockade. While a single injection of IL-1ra reduced the lethality of *Klebsiella pneumoniae* sepsis in newborn rabbits, and a second repeated injection 24 h after challenge enhanced lethality (Mancilla et al. 1993).

2.3 Cytokine Blockade in Models of Focal Tissue Infections

While TNF and IL1 blockade have demonstrated efficacy in increasing survival following parenteral LPS or bacterial challenges, several studies have shown no effect or even a deleterious effect in models of local infections with gram-negative bacteria. In murine or rat models of peritonitis, including cecal/ligature puncture (CLP; Echtenacher et al. 1990; Eskandari et al. 1992; Evans et al. 1989; Zanetti et al. 1992; Bagby et al. 1991), anti-TNF antibodies failed to afford protection, even when given prophylactically (Table 4). Similarly, soluble TNFR p55 construct was ineffective in murine of peritonitis (Loetscher et al. 1993). Only one study reported a protective effect in a rat model of peritonitis caused by *Neisseria meningitidis* (Nassif et al. 1992). One study reported a transient early survival in rat pups pretreated with anti-TNF antibodies and infected i.p. with group B streptococci (Teti et al. 1993). However, by 96 h this protection was no longer apparent. No study reported the effect of delayed treatment with anti-TNF antibodies in these models. The effect of IL-1 blockade has not been reported in peritonitis models or in the CLP model.

Thus studies aimed at investigating the effect of TNF blockade in focal infections have suggested the blocking TNF is not or is only marginally effective in preventing death and is even deleterious in some experiments.

Table 4. Effect of anti-TNF antibodies and of soluble TNF receptor constructs in various models of focal infections

Reference	Model	Observations
Anti-TNF antibodies		
Evans et al. (1989)	Mouse, CLP	No effect
Echtenacher et al. (1990)	Mouse, CLP	Deleterious
Bagby et al. (1991)	Rat, peritonitis *E. coli*	No effect
Eskandari et al. (1992)	Mouse, CLP	No effect (deleterious)
Zanetti et al. (1992)	Mouse, peritonitis *E. coli*	No effect
Nassif et al. (1992)	Rat, peritonitis *N. meningitidis*	Protective
Teti et al. (1993)	Rat, peritonitis Group B *streptococcus*	delay in lethality
TNFR-IgG3, p55		
Loetscher et al. (1993)	Mouse, i.p. *E. coli*	No effect

3 Comparison of Parenteral and Tissue Models of Infection

When evaluating the potential efficacy of anticytokine strategies, a clear distinction emerges between models of parenteral LPS or live bacterial challenge and models of focal infection.

On the one hand, parenteral models of LPS challenge are to be considered as models of intoxication rather than true models of infections. With regard to live bacterial parenteral challenge model inocula used in most studies have ranged from between 10^9 and 10^{12} CFU/kg. Both the parenteral LPS challenge and the high bacterial numbers induce extremely high concentrations of cytokine (usually 20–150 ng/ml TNF). Such high concentrations of TNF are probably toxic for the host, as suggested by the observations after parenteral challenge which have shown that prophylactic TNF blockade protects against death. TNF response peaks 1 and 2 h following LPS or bacterial challenge, and is followed by a rapid decline despite the persistance of circulating LPS or of live bacteria (Fig. 1a). This rapid decline may explain the failure of delayed TNF blockade after bacterial challenge when TNF is no longer present in the circulation.

On the other hand, models of tissue infection in which bacterial inocula are lower (usually less than 10^6 CFU) are perhaps better resemble true infectious models because bacteria must multiply to invade tissues and eventually bloodstream. The time course of cytokine production is different than that after parenteral challenge. Figure 1b illustrates this in mice challenged i.p. with *E. coli.* TNF levels are at least ten times lower than after parenteral challenge (usually less than 1 ng/ml) and in strong contrast to parenteral challenge models are sustained during the whole observation period. In fact, cytokine profiles in these models of tissue infection are very reminiscent of profiles observed in patients with shock. Indeed, TNF levels in these patients are of low magnitude (usually less than 500 pg/ml) and sustained (up to 10 days after onset of shock (Calandra et al. 1990; Damas et al. 1989).

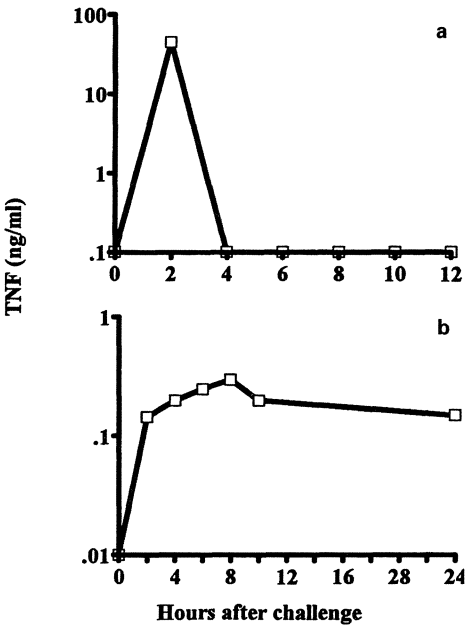

Fig. 1. TNF measured in plasma at various times following challenge. **a** Mice were injected i.v. with 20 mg/kg LPS. **b** Mice were injected i.p. with 10^4 CFU of *E. coli* O111 mixed with mucin/hemoglobin (this inoculum represents the LD_{100} for this strain). (Adapted from ZANETTI et al. 1992)

Given the fact that TNF blockade failed in the models of focal infection or even worsened the prognosis, this might be interpreted as a suggestion that TNF does not play a toxic role in these conditions but rather helps fight infection. Alternatively, one could also postulate that TNF blockade is not efficacious in tissues due to the low penetration of blocking antibodies, allowing TNF to play a detrimental role locally. Indeed, while in parenteral models it has generally been well documented that the anti-TNF antibodies used were administered in sufficiently large does to neutralize circulating TNF levels, similar observations have usually not been made in models of focal infections.

4 Clinical Studies

4.1 Anti-TNF Antibodies

4.1.1 The CB0006 Trial

Results of an open-label, prospective, phase II multicenter trial with escalating doses of an anti-TNF murine mAb (CB0006) have been published (FISHER et al. 1993). A total of 80 patients with severe sepsis or septic shock were enrolled to receive four dosing regimens of CB0006: 0.1, 1, 10 mg/kg, and 1 mg/kg initially and a second dose of 1 mg/kg 24 h later. No placebo group was investigated.

While survival estimates of all patients failed to demonstrate a survival advantage with increasing doses of CB0006, subgroup analysis of those patients with high TNF levels (>50 pg/ml) did suggest a benefit of the intervention.

4.1.2 The Bay X1351 Trials (NORASEPT Trial)

Patients with sepsis syndrome were enrolled, prospectively stratified into shock and nonshock groups, and randomly assigned to receive either 15 or 7.5 mg/kg or a murine anti-TNF mAb, or placebo. The final enrollment was 971 patients, with 49% in shock and 51% in nonshock at randomization (Abraham et al. 1995). Among all infused patients there was no difference in all-cause mortality at 28 days in patients receiving placebo or antibody. Among septic shock patients there was no reduction of mortality in patients receiving anti-TNF antibody. However, a trend toward reduction of mortality at 3 days was observed (15 mg/kg: 44% reduction vs. placebo, p=0.01; 7.5 mg/kg: 49% reduction *vs.* placebo, p=0.004), which was no longer present at 28 days. In contrast, there was a nonstatistically trend toward increased mortality in nonshock patients who received the 15 mg/kg anti-TNF mAb dose regimen.

The Bay X1351 monoclonal antibody has been studied in a European–south African study, the INTERSEPT trial. This study, which randomized only half as many patients as was previously planned, demonstrated no overall effect on mortality but a significant recovery from shock in those patients who survived. The Bay X 1351 monoclonal antibody will be tested in a further trial, NORASEPT II.

4.1.3 The MAK 195F Antibody

The MAK 195F antibody, developed by Knoll, is a Fab fragment of a monoclonal antibody directed against TNF. The results of a phase II study involving fewer than 100 patients showed no effect on overall mortality but apparently a dose-dependent effect on patients with IL-6 levels at the time of randomization of more than 1000 ng/ml. This post hoc finding will be prospectively studied in a phase III trial now underway.

4.2 Soluble TNF Receptor Constructs

A randomized, double-blind trial in patients with sepsis syndrome and hypotension was conducted with escalating doses of sTNFR p75 construct (Agosti et al. 1994). A total of 141 patients were enrolled to receive either placebo or one of three doses of the fusion protein. Inhibition of TNF with the construct was not effective in preventing death in patients at 28 days and increasing doses of the construct were apparently associated with increased mortality. A second trial with the sTNFR p55 construct is under investigation.

4.3 IL-1 Receptor Antagonist

Three trials aimed at blocking IL-1 by means of IL-1ra have been completed. In a preliminary phase II trial 99 patients with sepsis syndrome or septic shock were treated with placebo or escalating doses of IL-1ra (Fisher et al. 1994b). A dose-dependent, 28 day survival benefit was suggested with increasing doses of the drug, but the size of each tratment group was relatively small. A double-blind, phase III confirmatory trial of Il-1ra was undertaken in 893 patients with sepsis or septic shock. While the prospectively defined endpoints did not confirm the preliminary conclusions (Fisher et al. 1994a), since the overall mortality rate in patients receiving placebo was 34% and 29% in patients receiving high-dose IL-1ra, a post hoc analysis suggested a possible survival advantage in patients with increased severity of illness (Fisher et al. 1994ab). A second large phase III study aimed at confirming prospectively this observation was stopped after interim analysis because this effect was not confirmed.

In conclusion, retrospective analyses of the studies aimed at blocking TNF or IL-1 suggest that the interventions benefit only those patients more severely ill (as suggested by the IL-1ra study) or in shock (Intersept study) and/or that the benefit is present early after intervention but vanishes over time and is no longer apparent after 28 days (Norasept study).

5 Differences Between Animal Models and Clinical Trials

Despite great hopes, clinical trials conducted so far in human sepsis have not been as encouraging as animal studies. Several causes may explain this relative and hopefully temporary disappointment.

1. In animal models, the cascade of events starting from the initial stimulus and usually ending with the death of the animal generally follows a predictable time course. The resulting cytokine production also follows a predictible time course, and experimental protocols to block one cytokine cascade or another are relatively straightforward. In many animal models the course of septic shock is usually hours or a few days. In contrast, the sequence of events leading to septic shock in humans is much more complex, and the course of the illness generally lasts days rather than hours, as seen in most animal models.
2. The rationale of clinical trials was based essentially on observations performed in animals receiving parenteral injections of LPS/bacteria, in which cytokine blockade was beneficial, not on observations performed in animal with focal infections, in which such therapy was usually not effcient.
3. The organisms and site of infections are very diverse, and importantly the patients have a large variety of underlying diseases, which is not the case in animal studies.

4. Interventions in animal models have been successful only when applied very early. Indeed, cytokine blockade was efficient in parenteral models, provided blockade was performed prophylactically or very early after challenge.

6 Conclusion

There is one major problem in trying to delineate the best potential interventions of cytokine blockade for the treatment of septic shock, which is the uncertainties about which experimental model best mimics the series of events which lead to septic shock in patients. Simply showing that an antibody can protect mice from a LPS-induced shock is only a crude hint, which wrongly leads one to conclude that the same antibody will be useful in the clinical setting. Notwithstanding, clinical trials based on anticytokine strategies have been based essentially on the lessons learned from models of parenteral injections of LPS or bacteria, not from models of focal infections. With the exception of meningococcal purpura fuliminans, most cases of septic shock in human are due to focal infections, a type of infection in which TNF appears to play a protective role in experimental models.

Given the uncertainties of the relevance of animal models in mimicking and predicting outcome in the clinical setting, our understanding of the sole cytokine blockade as a mean of reducing mortality in septic shock relies only on well-planned, well-performed, and well-interpreted clinical studies.

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