Chapter 1

INTRODUCTION

&

REVIEW OF LITERATURE
1.0 Introduction and Review of Literature

1.1 Background

Sepsis is a spectrum of clinical conditions caused by immune responses of patient to infection that is characterized by systemic inflammation and coagulation. Sepsis and septic shock are most common cause of death in a variety of human patients, including those with cancer and receiving chemotherapy, AIDS patients, individuals with burns or mechanically ventilated (Bone, 1993). Septic shock is a possible consequence of microorganism like viruses, fungi, parasite and bacteria in blood stream. However sepsis caused by gram negative bacteria is most prevalent (50-70%), which involves a complex interaction between bacterial factors like lipopolysaccharide (LPS) and the host immune system producing a systemic inflammatory state that may progress to multiple organ failure and death (Smith, 1998).

Despite adequate antibiotic treatment and optimal intensive care efforts, severe infection caused by gram negative bacteria are still often proceed to shock and multiple organ failure. Thus, in studies performed on patients with sepsis and septic shock the mortality rate has ranged from 30 to 80%. Since the beginning of antibiotic era, experimental studies and research have been pursued to find reasons behind therapeutic failure in cases of sepsis. Many authors have suggested antibiotic treatment to be responsible for the aggregating conditions mainly by virtue of its endotoxin releasing property with out circumventing its lethal effects (Lepper et al., 2002).

1.2 Sepsis Terminology

There is a continuum of clinical manifestations from SIRS to sepsis to severe sepsis to septic shock to Multiple Organ Dysfunction Syndromes (MODS) (Bone et al., 1992).

Systemic Inflammatory Response Syndrome (SIRS): Patient presents with two or more of the following criteria.

1. **temperature** > 38°C or < 36°C
2. **heart rate** > 90 beats/minute
3. **respiration** > 20/min or PaCO₂ < 32mm Hg
4. leukocyte count > 12,000/mm$^3$, < 4,000/mm$^3$ or > 10% immature (band) cells

1.2.1 Sepsis: SIRS plus a documented infection site (documented by positive culture for organisms from that site). Blood cultures do not need to be positive. While SIRS, sepsis, and septic shock are associated commonly with bacterial infection, bacteremia may not be present. Bacteremia is the presence of viable bacteria within the liquid component of blood. Bacteremia may be transient, as is seen commonly after injury to a mucosal surface, primary (without an identifiable focus of infection), or more commonly secondary, to an intravascular or extravascular focus of infection.

1.2.2 Severe Sepsis: Sepsis associated with organ dysfunction, hypoperfusion abnormalities, or hypotension. Hypoperfusion abnormalities include but are not limited to:

1. lactic acidosis,
2. oliguria,
3. Acute alteration in mental status.

1.2.3 Septic Shock: Sepsis-induced hypotension despite fluid resuscitation plus hypoperfusion abnormalities.

1.2.4 Multiple Organ Dysfunction Syndrome (MODS): Presence of altered organ function in an acutely ill patient such that homeostasis cannot be maintained without intervention. Mortality increases with increase in number of SIRS symptoms and in severity of the disease process.

1.3 Pathophysiology of Sepsis

The exposure of LPS or Endotoxin activates the host effectors cells through macrophage or monocyte with initiation of several cascading pathways: complement, coagulation, fibrinolytic and kallikrein-kinin systems (Bochud et al., 2003). These target cells secrete large quantities of inflammatory cytokines, such as tumor necrosis factor (TNFα), interleukin-1 (IL-1), IL-6, and IL-8 and above all leading to activation of neutrophil granulocytes, T-lymphocytes and endothelial cells (Akashi et al., 2003). As a result of this process, activated granulocytes adhere to endothelium that is followed by increase endothelium permeability and capillary leakage, which are characteristic phenomenon in
patients with sepsis and septic shock. Furthermore, activated coagulation system along with activated endothelium with its increased expression of tissue factor and activated platelets together, from microthrombosis (Parrillo, 1993). During the early phases of immune system there is activation of anti-inflammatory system and mediators like IL-1 receptor antagonist, soluble TNF-receptors and prostaglandin E2 are released to modulate the inflammatory responses. Furthermore, a type 2 T-helper cell secretes anti-inflammatory cytokine (eg. IL-10 and IL-4) and also increase the level of corticosteroids and catecholamines (Calandra, 2001).

Further, there is a stimulation of inducible form of nitric oxide (NO) synthase which leads to increased release of nitrite from endothelial cells, vascular smooth muscle cells and macrophages. NO is considered to be a major mediator of vasodilation and hypotension in septic shock. The vasodilation, in conjunction with tissue edema and microthrombosis, results in hypoperfusion with reduced oxygen uptake in tissue and organ, which is manifested by elevated blood lactate concentration. Oxygen extraction is further impaired with hypovolaemia that is caused by combination of capillary leakage, vasodilation and dehydration (Kilbourn et al., 1990). Other changes brought on by immune response may cause coagulation of the blood in the extremities, which can further decrease circulation through the organs. The initial haemodynamic alterations in septic patients are characterized by tachycardia, high cardiac output and low vascular resistance, resulting in hyperdynamic circulation. Despite the high cardiac output, there is often a myocardial dysfunction that is caused by circulatory myocardial depressant such as NO, TNFα and IL-1. If the hypovolaemia is not corrected, a reduced venous return, cardiac output and lowered blood pressure will causes further tissue hypoxia and the eventual development of hypodynamic septic shock. Further, if the inflammation reaction leads to acute respiratory distress syndrome (ARDS), oxygen delivery to the organs will be additionally impaired. The decreased oxygen delivery and uptake causes an anaerobic metabolism and metabolic acidosis which, together with the inflammation process may result in multiple organ failure (Bone, 1991).

High levels of LPS cause a constellation of symptom followed by multiple system organ failure that leads to death in 60 % of cases. Its detoxification is therefore an
interesting and urgent target (Angus et al., 2001). Therefore, the therapy should not only kill the bacteria but also be capable of preventing other derived toxic effects (Frieling et al., 1997; Arditi et al., 1993).

Figure 1.1: Schematic representation of Mode of action of LPS in pathophysiology of sepsis

1.4 Review of Literature

1.4.1 Lipopolysaccharide (LPS)

The bacterial LPS is the major antigen of the outer membrane (OM) of Gram-negative bacteria. Although the term "endotoxin" is occasionally used to refer to any cell associated bacterial toxin, it is properly reserved to refer to the lipopolysaccharide complex associated with the outer membrane of Gram-negative bacteria such as E. coli, Salmonella, Shigella, Pseudomonas, Neisseria, Haemophilus, and other leading pathogens (Sriskandan and Cohen, 1999).

1.4.1.1 The Role of LPS in the Outer Membrane of Gram-negative Bacteria

Endotoxin (LPS) is located on the outer surface of the membrane, where it mediates contact with the environment. LPS is essential for functioning of the outer membrane, and as a structural component of the cell, it may play several roles in the pathogenesis of gram-
negative bacterial infections. First, it is a permeability barrier that is permeable only to low molecular weight, hydrophilic molecules. In the *E. coli* the omp F and omp C porins exclude passage of all hydrophobic molecules and any hydrophilic molecules greater than a molecular weight of about 700 daltons. This prevents penetration of the bacteria by bile salts and other toxic molecules from GI tract. It is also a barrier to lysozyme and many antimicrobial agents. Second, it impedes destruction of bacterial cells by serum components and phagocytic cells. Third, LPS plays an important role as a surface structure during interaction of the pathogen with its host. For example, LPS may be involved in adherence (colonization), or resistance to phagocytosis, or antigenic shifts that determine the course and outcome of an infection (Roberts, 1996).

### 1.4.1.2 Chemical Nature of Endotoxin

Lipopolysaccharide are complex amphiphilic molecules with an MW of about 10 kDa that vary widely in chemical composition both between and among bacterial species. LPS consists of three components or regions, Lipid A, an R polysaccharide and an O polysaccharide. The lipid A harbors the endotoxic principle of LPS and immunogenicity is associated with the polysaccharide components (Rietschel et al., 1996). The general architecture of LPS is shown in Figure 1.2. The general structure of LPS is shown in Figure 1.3 and the complete structure of lipid A is illustrated in Figure 1.4.

![Figure 1.2: General architecture of Lipopolysaccharide](image)
Figure 1.3. General Structure of Lipopolysaccharide

Glc = glucose; GlcNac = N-acetyl-glucosamine; Gal = galactose; Hep = heptose; P = phosphate; Etn = ethanolamine; R1 and R2 = phosphoethanolamine or aminoarabinose. Ra to Re indicate incomplete forms of LPS.

Figure 1.4: Complete structure of the Lipid A Moiety of LPS of *E. coli*
1.4.1.2.1 Region I. Lipid A is the lipid component of LPS. It contains the hydrophobic, membrane-anchoring region of LPS. Lipid A consists of a phosphorylated N-acetylglucosamine (NAG) dimer with 6 or 7 fatty acids (FA) attached. Usually 6 FA are found. All FA in Lipid A are saturated. Some FA are attached directly to the NAG dimer and others are esterified to the 3-hydroxy fatty acids that are characteristically present. The structure of Lipid A is highly conserved among gram-negative bacteria. Among Enterobacteriaceae Lipid A is virtually constant.

1.4.1.2.2 Region II. Core (R) antigen or R polysaccharide is attached to the 6 position of one NAG. The R antigen consists of a short chain of sugars. For example: KDO - Hep - Hep - Glu - Gal - Glu - GluNAc. Two unusual sugars heptose and 2-keto-3-deoxyoctonoic acid (KDO) are usually present in the core polysaccharide. KDO is unique and invariably present in LPS and so has been an indicator in assays for LPS (endotoxin). With minor variations, the core polysaccharide is common to all members of a bacterial genus (e.g. Salmonella), but it is structurally distinct in other genera of Gram-negative bacteria. Salmonella, Shigella and Escherichia have similar but not identical cores.

1.4.1.2.3 Region III. Somatic (O) antigen or O polysaccharide is attached to the core polysaccharide. It consists of repeating oligosaccharide subunits made up of 3-5 sugars. The individual chains vary in length ranging up to 40 repeating units. The O polysaccharide is much longer than the core polysaccharide, and it maintains the hydrophilic domain of LPS molecule. A major antigenic determinant (antibody-combining site) of the Gram-negative cell wall resides in the O polysaccharide (Raetz, 1990; Lugtenberg and Alphen, 1983).

1.4.2 Lipid A and virulence

Endotoxins are toxic to most mammals, and regardless of the bacterial source, all endotoxins produce the same range of biological effects in the animal host. Most of our knowledge of the biological activities of endotoxins derives not from the study of natural disease but by challenge of experimental animals. The injection of living or killed Gram-negative cells, or purified LPS, into experimental animals causes a wide spectrum of nonspecific pathophysiological reactions such as: fever, changes in white blood cell counts, disseminated intravascular coagulation, hypotension, shock and death. Injection of
fairly small doses of endotoxin results in death of most mammals. The sequence of events follows a regular pattern: latent period, physiological distress (diarrhea, prostration, shock) and death (Kotani et al., 1985). How soon death occurs varies on the dose of the endotoxin, route of administration and species of animal. Animals vary in their susceptibility to endotoxin. The physiological effects of endotoxin are thought to be mediated by Lipid A. Since Lipid A is embedded in the outer membrane of bacterial cells, it probably exerts its toxic effects when released from multiplying cells in a soluble form, or when the bacteria are lysed as a result of autolysis, complement and the membrane attack complex (MAC), ingestion and killing by phagocytes, or killing with certain types of antibiotics (Hoffman and Natanson, 1993).

1.4.3 Mechanism of activation of inflammatory cascade

LPS or Endotoxin activates the host effector cells through macrophage stimulation of receptors on their surface. The mechanisms by which LPS activates macrophages are now understood in some detail. LPS-binding, acute-phase proteins present in the blood bind to LPS in the bacteria and transfer it to CD14. CD14 is a protein anchored in the outer leaflet of the plasma membrane cannot induce cellular activation without a transmembrane signal transducing co-receptor (Alexander and Rietschell, 2001). Recent studies have led to the identification of Toll-like receptor 4 (TLR4) as the co receptor for LPS. Besides, binding to CD 14 receptor endotoxin may also activate the macrophages by other receptor such as interglin CD11/CD 18 receptor. Membrane bound CD14 may be detached from the cell surface and this soluble CD14 (sCD14) may bind with LPS-LBP binding complex. LBP is freed and LPS-sCD14 complexes activate cells (which do-not express CD14 receptor, such as endothelial cell (Nagai et al., 2002). These activation by stimulation of intracellular signaling transcription factors that mediate the production of inflammatory cytokines such as tumor necrosis factor (TNF), interleukin (IL)-1, IL-6 and IL-8, platelet-activating factor, arachidonic acid metabolites, erythropoitin and endothelin as demonstrated in Figure 1.5. These pathophysiologic phenomena can lead to endothelial damage (Fujihara et al., 2003) and ultimately to multiple organ failure.
1.5. Treatment Strategies for Sepsis

Current treatments of Gram-negative sepsis in critically ill patients are based on prompt administration of adequate antimicrobial, removal of the infection nidus, and support of organ dysfunction.

1.5.1 Immediate Stabilization of the Patient.

The immediate concern for patients with severe sepsis is reversal of life-threatening abnormalities (ABCs: airway, breathing, circulation). Altered mental status or depressed level of consciousness secondary to sepsis may require immediate protection of the patient's airway. Intubation may also be necessary to deliver higher oxygen concentrations. Mechanical ventilation may help lower oxygen consumption by the respiratory muscles and increase oxygen availability for other tissues. Circulation may be compromised and significant decrease in blood pressure may require aggressive combined empirical therapy with fluids (with crystalloids or colloids) and inotropes/vasopressors (dopamine, dobutamine, phenylephrine, epinephrine, or
norepinephrine). In severe sepsis monitoring of the circulation may be necessary. Normal CVP (central venous pressure) is 10-15 cm of 0.9% NaCl; normal PAW (pulmonary arterial wedge pressure) is 14-18 mm Hg; maintain adequate plasma volume with fluid infusion. Patients with severe sepsis should be in ICU. Their vital signs (blood pressure, heart rate, respiratory rate, and temperature) should be monitored. The frequency of administration depends on severity of infection. Cardiac output and ventilation are maintained adequately with drugs and dialysis is done to assist kidney function. Arterial blood pressure in hypotensive patients is usually maintained using with vasoactive drugs, e.g., dopamine, dobutamine, or norepinephrine (Sharma and Dellinger, 2003).

1.5.2. The original focus of infection must be treated.

The primary cause of infections like foreign bodies, purulent exudates particularly for anaerobic infections, infected organs, debride or gangrenous tissues should be removed.

1.5.3. Antimicrobial or Antibiotic therapy

The blood must be rapidly cleared of microorganisms. There fore prompt institution of empiric treatment with antimicrobials is essential. The early institution of antimicrobials has been shown to decrease the development of shock and to lower the mortality rate. After the appropriate samples are obtained from the patient a regimen of antimicrobials with broad spectrum of activity is needed. This is because antimicrobial therapy is almost always instituted before the organisms causing the sepsis are identified. The specific antibiotic can be given, once source of infection is identified as depicted below (Hardaway, 2000; Wheeler AP and Bernard GR, 1999).

- Community acquired pneumonia a 2 drug regimen is usually utilized. Usually a third (ceftriaxone) or fourth (cefepime) generation cephalosporin is given with an aminoglycoside (usually gentamicin).

- Nosocomial pneumonia: Cefipime or Imipenem-cilastatin and an aminoglycoside.

- Abdominal infection: Imipenem-cilastatin or Pipercillin-tazobactam, aminoglycoside, fluoroquinolones.
Nosocomial abdominal infection: Imipenem-cilastatin and aminoglycoside or Pipercillin-tazobactam and Amphotericin B.

Skin/soft tissue: Vancomycin and Imipenem-cilastatin or Piperacillin-tazobactam

Nosocomial skin/soft tissue: Vancomycin and Cefipime

Urinary tract infection: Ciprofloxacin and aminoglycoside

Nosocomial urinary tract infection: Vancomycin and Cefipime

CNS infection: Vancomycin and third generation cephalosporin or Meropenem.

Nosocomial CNS infection: Meropenem and Vancomycin.

1.5.4 Problem with Antibiotic therapy

The main constraints of antibiotic therapy in sepsis are

1. Antibiotic-induced release of bacterial cell wall components.

2. Poor penetration and accumulation at targeted site.

1.5.4.1. Antibiotic-induced release of bacterial cell wall components.

When conventional antibiotic or antimicrobials are administered during infection bacteria it results in endotoxin release and this phenomenon could have deleterious effects. The effect of antibiotics is always bacterostatic or bactericidal, but there are some effects which may be important for treatment of infection. One of them is impact on liberation of bacterial endotoxin after exposure of different antibiotics (Bucklin et al., 1994). A direct link between endotoxin release and TNFα-liberation from monocytes has also been demonstrated in different in vitro systems (Dofferhoff et al., 1993). In animal model, there is strong evidence that soluble endotoxin release from gram negative bacteria by antibiotics contribute to the pathogenesis and mortality in experimental sepsis (Shenep et al., 1985).

Three mechanisms can account for this increase:

(i) Accumulation of bacterial biomass following antimicrobial treatment;

(ii) An increase in the accessibility of LPS that remains bound to the bacteria; and finally

(iii) Release of unbound LPS (Giacomettir et al., 2005).
Endotoxin may be liberated spontaneously during multiplication of microorganisms, however, its release has been shown to be precipitated and intensified by the antibiotic-induced disintegration of bacteria. When endotoxin is released and appeared as free endotoxin, its biological activity increase considerably compared to bound endotoxin. The first observation that antimicrobial drug treatment could have immediate and adverse effects on patients was made independently by Jarisch and Herxheimer. They noted a sudden rise in body temperature and fall in blood pressure in syphilitic patients treated with arsenic based spirocheticidal drugs (Farmer, 1948). This effect nowadays referred to as the Jarisch-Herxheimer reaction (JHR).

1.5.4.1.1 Differences in the endotoxin-liberating potential of antibiotics

An important step in understanding treatment-related endotoxin release was the finding that different classes of antimicrobial substances led to different levels of circulating endotoxin. The amount of LPS is obviously dependent on the type and number of invading bacteria. However, it also depends on the antibiotic agent used for treatment. This difference may be explained by specific binding sites and various modes of action of the different antibiotics (Dofferhoff et al., 1991). In general bactericidal antibiotics liberate initially more endotoxin then bacterostatic antibiotic and antibiotic active on cell wall such as penicillin and cephalosporin release more than antibiotics active on other mode of action, such as protein synthesis inhibitor. However there are large variation between different antibiotics and even among the β-lactam antibiotics, there are great difference in propensity to free endotoxin (Hurley, 1991).

1.5.4.1.1.1 β-lactam-antibiotics, carbapenems, and penicillin-binding proteins (PBP)

β-lactam mainly shows there activity by binding with penicillin binding protein (PBP). PBP are enzymes that are located in bacterial cell wall and responsible for cell wall synthesis. Depending on affinity of β-lactam antibiotics to PBP varying amount of endotoxin is released (Jackson and Kropp, 1992). β-lactam antibiotic with affinity to PBP-1, leads to rapid killing and without additional release of endotoxin, where as antibiotics has specific binding to PBP-2, leads to conversion of the bacteria to round shape spheroblasts, with loss of viability but without cell wall destruction and excessive endotoxin release. Binding to PBP-3, causes selective inhibition of sepatation and
continuing bacterial elongation with formation of long filaments and subsequent increase endotoxin release (Horiia et al., 1998). Thus, release of high amount of endotoxin is mainly associated with PBP-3 binding. Cefuroxime, cefotaxime, piperacillin, aztreonam antibiotic bind to PBP-3 and are associated with endotoxin release (Giacometti et al., 2005). Ceftazadime, at high concentration binds to PBP-1 and the carbapenams to PBP-2. At, lower concentration ceftazadime and meropenem binds to predominantly PBP-3 resulting in higher endotoxin release at lower than at higher concentration. Simultaneous inhibition of PBP-1 and PBP-3 by ceftazadime at mid concentration level concentration has also resulted in formation of spheroplasts (Trautmann et al., 1998).

1.5.4.1.1.2 Non-β-lactam antibiotics

Among the bactericidal antibiotics, aminoglycosides inhibit protein synthesis by binding with 16S rRNA, which result in excessive killing without excessive endotoxin release. The quinolones inhibit protein synthesis via inhibition of bacterial DNA synthesis. Despite this mode of action, quinolones have in some studies associated with a substantial endotoxin release, whereas in other studies it has not been observed. Some studies report low endotoxin-releasing properties of ciprofloxacin (McConnell and Cohen, 1986), while some studies suggest that treatment with ciprofloxacin exerts similar effects as the PBP-3-specific antibiotics (Prins et al., 1994, Nitsche et al., 1993), including filamentation and release of TNFα as well as IL-6. Data on the glycopeptide antibiotics, vancomycin and teicoplanin e.g., suggest that glycopeptides down-regulate the biological effect of endotoxin. Polymyxins, for instance, have an endotoxin-binding effect (Boman, 1995) and are capable of inhibiting several biological effects of endotoxin (Cooperstock, 1974).

However clinical significance of antibiotic induced endotoxin release has not been completely established. However with regard to antibiotic treatment; data from published studies suggest that differences in the rate and degree of endotoxin release may actually have clinical relevance (Mock et al., 1995; Prince et al., 1994; Weinstein et al., 1983, Kirikae et al., 1997).
1.5.4.1.2 Immunomodulatory action of Antibiotics

In a considerable number of in-vitro and ex-vivo experiments, antibiotics have also been modulating host inflammatory mechanism. Graded doses of various antibiotics have been tested for their ability to effect functions such as chemotaxis, phagocytosis as well as bacterial killing with generation of ROS. These oxidative stress induced by polymorphonuclear cells activation during septic shock are known to trigger inflammatory response and later release of proinflammatory cytokines. Several studies have demonstrated that certain β lactam and aminoglycosides antibiotics provide protection from oxidative stress and subsequent reduction of epithelial damage (Mascini et al., 2001). Immunomodulatory activity of fluoroquinolone in different system is also reported (Dalhoff, 2005). While immunomodulation in vitro and ex-vivo system appears to be market, in-vivo data are limited and clinical decisive effect has not been demonstrated (Hamilton, 2000).

1.5.4.2 Poor penetration and accumulation at targeted site

Successful treatment of infection (sepsis) depends on high sustained drug concentrations in tissues and intracellular sites where the bacteria reside and multiply. Since severe sepsis (especially intra-abdominal sepsis) is often characterized by the presence of intraphagocytic bacteria in hepatic and splenic reticuloendothelial systems. The i.v administration of antibiotic rapidly generate therapeutic drug label within blood and peritoneal fluid. However because of short half-life and fast elimination sustained therapeutic drug level within organs such as liver and spleen are not attained (Martineau and Shek, 1999). Antibiotic treatment of these types of infections has been associated with high failure and/or relapse rates (Shek et al., 1998). Intracellular pathogens, whether obligate or facultative, can hide, reside and multiply within the phagocytic cells of the reticuloendothelial system (RES), and by virtue to their intracellular location, are protected from the actions of the immunological defense cells and antimicrobial agents (Donowitz, 1994). The ineffectiveness of conventional antibiotics against intracellular infections may also be attributable to poor drug penetration, limited drug accumulation in subcellular compartments and/or drug inactivation by acidity in subcellular compartments (Raoult, 1996). These factors may explain why some antibiotics are bactericidal against extracellular bacteria in vitro, but are ineffective in killing intracellular forms of the bacteria (Broek et
Further, indiscriminate use of antibiotics creates conditions of overgrowth, colonization, and subsequent infection by aggressive, antimicrobial-resistant organisms.

1.5.5 Ideal requirement of sepsis therapy

The cornerstone in the treatment of severe sepsis and septic shock include antibiotics and if necessary surgery for killing and removal of causative bacteria, fluid replacement for reversal of hypovolemic, oxygen therapy for treatment of hypoxia and support of deteriorating vital organ. Despite this, mortality is still high and therefore, an effective treatment of patients infected by Gram-negative bacteria must comprise bacterial killing the neutralization of endotoxin.

1.5.6 Recent strategies in the treatment of sepsis

Development of new drugs to treat sepsis has been based in part on the premise that neutralizing bacterial toxins and potentially harmful host mediators could stop or slow this syndrome. Numerous attempts have been made to interfere with proinflammatory cascades in severe sepsis and septic shock as illustrated in Table 1.1.

There are several therapies that are directed at different elements of the inflammatory cascade, including a bacterial toxin (endotoxin), host proteins that mediate the inflammatory response (TNF and IL-1), an inflammatory cell (the neutrophil), and a low-molecular-weight messenger (nitric oxide) that causes hypotension. Other targets (eicosinoids, platelet-activating factor, bradykinin, and so on) are being evaluated to treat this syndrome. Recently, some novel target like MIF and apoptosis inhibitor has been explored (Chapy, 1999).
### Table 1.1: Targets and Therapy for treatment of Sepsis

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<tr>
<th>Therapy</th>
<th>Targets</th>
<th>Agents</th>
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<td><strong>Neutralization of Microbial toxin</strong></td>
<td>Endotoxin</td>
<td>Antilipid A antibody</td>
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<td>By large macromolecules</td>
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<td>By small molecule</td>
<td>Polymixin, colistin etc</td>
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<td><strong>By imparting Cellular signaling</strong></td>
<td>lipopolysaccharide analogs</td>
<td>Deacylated LPS, Lipid X</td>
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<td>Deacylated lipopolysacharide</td>
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<td><strong>Enhancing clearances</strong></td>
<td>Endotoxin</td>
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<td>Lipoproteins (HDL, LDL, CM)</td>
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<td><strong>Blocking Specific receptor/carrier</strong></td>
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<td><strong>Nonspecific antinflammatory &amp; immunomodulatory drugs</strong></td>
<td>Multiple inflammatory and immune mediators</td>
<td>High dose corticosteroids, low dose corticosteroids, pentoxifylline, immunoglobulins, interferon gamma</td>
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<td><strong>Inhibition of specific mediators</strong></td>
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<td>Bradykinin antagonist</td>
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<td>Bradykinin</td>
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<td>Coagulation cascade</td>
<td>Antithrombin III, tissue factor pathway inhibitor, activated protein</td>
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<td><strong>Novel Targets</strong></td>
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<td>High mobility group protein-1 (HMG)</td>
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<td>VX-799 caspase inhibitor</td>
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<td></td>
<td>Neutrophil activation</td>
<td>GM-CSF, PGG-glucan, IFN-γ</td>
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1.5.6.1 By binding and neutralization of endotoxin

An endotoxin is a major activator in severe gram negative sepsis, several clinical trials have studied the effect of polyclonal and monoclonal antibodies directed against either to O-side chain or core sugar including lipid A. The antibodies to O-side chain produce serotype specific (Heumann et al., 1991) complement-dependent bactericidal activity (Hoffman et al., 1994). However serotype specificity limits the treatment therapy since it would be difficult to have effective dose of antibody for every probable infecting bacteria. The antibodies to core sugar and lipid A was thought to be effective since this is a group which mediates the inflammatory response but unfortunately the function of these antibodies are not known or controversial (Greenman et al., 1991). Positive effect, especially in most severely sick patients, was seen but in confirmatory trials mortality was not significantly affected.

In a recent advancement a new paradigm of non immunologic sequestration of LPS by small molecules has been proposed. It has been found that the LPS have both hydrophobic and electrostatic counterparts which can be exploited either to neutralize or slow down the cellular cascading events after LPS release (David, 2002). The anionic amphiphilic nature of lipid A enables it to interact with a variety of cationic hydrophobic ligands. Polymyxin B, a cationic amphiphilic cyclic decapeptide antibiotic isolated from bacillus polymyxa (Storm and Rosenthal, 1977) has long been recognized to bind lipid A and neutralize its toxicity, in-vitro and in animal models of endotoxemia (Stokes et al, 1989). It has been served as a 'gold standard' for endotoxin sequestering agent. But its application is limited as Polymyxin B is too toxic to be used parenterally. Therefore there is need for the development of nontoxic agents able to neutralize LPS. These data include synthetic peptides, based on endotoxin-binding domains of natural binding proteins such as lactoferrin, Limulus anti-LPS factor, NK-lysin, and cathelicidins or based on LPS sequestering polyamines. Many of these compounds could be shown to act not only in vitro, but also in vivo e.g. in animal models of sepsis (Hoess et al., 1993).

Further, investigators are examining several lipid A derivatives that may diminish toxicity. These compounds either directly antagonize endotoxin or simulate its beneficial immunostimulatory properties, such as induction of tolerance and increased nonspecific
resistance to infection. There are reports that endotoxin may be inhibited by binding to a LBP like, the bacterial permeability increasing proteins (BPI) (Elsbach and Weiss, 1993) which is synthesized by neutrophil granulocytes upon endotoxin stimulation (Schumann et al., 1994). In a clinical study in patients with meningococcemia, known to have high concentration, there was trend towards an improved outcome in patient treated with r-BPI but no statistically significant effect could be demonstrated (Levin et al., 2000).

1.5.6.2 Blocking Specific receptor/carryer

Designing new drugs to neutralize microbial products or block their interaction with specific receptor on immune cells is an attractive concept. Potential targets include lipopolysaccharide binding protein, CD14, TLR4, and MD-2 for Gram negative sepsis. Monoclonal antibodies against CD14 are being evaluated in phase II studies. Several intracellular signaling molecules, such as MyD88 and the mitogen-activated protein kinase are other possible therapeutic targets. However, inactivating molecules that are pivotal to innate immunity can be harmful, as shown by the increased sensitivity to bacterial sepsis in mice with mutations of the TLR4 gene. Careful selection of patients with severe infections associated with a high probability of death will therefore be essential (Bochud and Calandra, 2005).

1.5.6.3 Nonspecific antinflammatory & immunomodulatory drugs

The first attempt to nonspecific blockage of inflammatory response was done in 1970 in the form of high dose corticosteroid (HDC) therapy. Although, HDC therapy resulted in positive outcome in animal models and has shown improved survival in selected groups of patient of typhoid fever (Vincent et al., 2002). This therapy was withdrawn due to increased risk of secondary infection in septic patient. Currently, low dose corticosteroids therapy has shown beneficial effect and reduced mortality in septic patients (Annane et al., 2002). Further, Non-steroidal anti-inflammatory drugs have also shown no survival benefits.

1.5.6.4 Inhibition of specific mediator

Other strategies like Anticytokine therapy (includes targets like TNF-alpha, IL-1 etc. to induce passive immunization) and Nitric oxide as therapeutic target have also been
reported (Cannon et al., 1990). Clinical trials with antibodies or other molecule antagonize the effect of several proinflammatory cytokine such as TNF, IL-1B, platelet activating factor or coagulation has shown positive results in some subgroups but significant effect on all cause mortality and therefore these product are not licensed for clinical use (Fisher et al., 1994). The nitric oxide production as such acts as a neurotransmitter regulates vascular tone and inhibits platelet aggregation (Hibbs et al., 1992). Inhibition of nitric oxide production has been considered as an approach to overcome sepsis induced hypotension. During septic shock several mediators (thromboxane and endothelin) get released which causes vasoconstriction and nitric oxide may play a pivotal role to counter this effect (Hotchkiss et al., 1992). Further its property to inhibit platelet aggregation may facilitate to prevent microvascular stasis (Klabunde and Ritger, 1991). Improved survival has been reported by using partial nitric oxide synthase inhibitors (Kilbourn et al., 1990).

1.5.6.5 Novel targets

The neutrophil is a key component to defense mechanism. Several studies have shown that the neutrophil and its toxic byproducts can produce tissue injury and organ dysfunction in sepsis (Zimmerman and Ringer, 1992). Therapies that augment neutrophil count can function to reduce the risk of infection. These data suggest that therapies that inhibit neutrophil activity during sepsis should not interfere with the protective role of the cell in host defense (Repine and Bechler, 1991). However, anti-inflammatory therapies for septic shock that may directly impair neutrophil function have not shown convincing clinical benefits (Bernard et al., 2001).

Macrophage migration inhibitory factor is a cytokine that has recently been shown to be important in innate immunity and sepsis. This factor thus has the potential to endanger life when expressed in excess during sepsis (Froidevaux et al., 2001). Development of drugs to block the production of macrophage migration inhibitory factor or inhibit its function may help treat severe sepsis and other inflammatory diseases (Calandra et al., 2000). Other potential novel targets in sepsis includes application of high mobility group B1 protein (HMGB1) which is a mediator that stimulates human monocytes to produce TNF-alpha, IL-1β, IL-1α, IL-6 and macrophage inflammatory proteins. Improved survival has been documented by blocking this protein with antibodies
Introduction & Review of literature

at later time after LPS infusion (Wang et al., 1999). The usefulness of apoptosis inhibitors like VX-799 a specific caspase inhibitor has shown potential in animal model and soon to be tested in humans (Riedemann et al., 2003). During sepsis there is a considerable consumption of protein C and S as well as down regulation of thrombomodulin on endothelial cell surfaces reducing the ability to generate APC. Activated protein C (APC) therapy includes treatment with APC to improved survival in septic conditions (Hartman et al., 1998).

Unfortunately, most of these strategies did not improve survival of patients when studied in large, multicenter clinical trials. However, these approaches have limited success in animal model and none of approaches has been successful clinically (Manocha, 2002). Recently beneficial effects have been demonstrated for low dose corticosteroid therapy and activated protein C. However, with this treatment there are always difficulty in establishing the optimal dose because, if doses are too high, the beneficial effect may proceed in to adverse one leading to paralysis of immune system and bleeding.

1.5.6.5 Formulation Based approach

The studies on structural based formulations has suggested that ideal requirement for substance to bind with is Hydrophobicity and cationicity. The structrual requirement for simple cationic amphipaths for binding and neutralizing LPS are properties of (a) biscationicity, (b) protonatable cationic functions separated by a distance of about 15Å, and (c) a hydrophobic substituent placed such that steric interference is minimized, may possess LPS-binding and LPS-neutralizing properties. The presence of two protonatable cationic groups positioned about 14Å apart in a linear molecule was sufficient to ascribe high-affinity binding to LPS, and an additional hydrophobic moiety was necessary for the binding to result in neutralization of endotoxic activity (sequestration) David, 2002, Guo et al., 2005). Many studies have suggested that these formulation components interact manly with Lipid A portion of LPS and neutralize its proinflammtory effects in-vitro as well as in-vivo. One such strategy is Lipoprotein, which are known to bind and neutralize LPS.
1.5.6.5.1 Lipoproteins

Lipoproteins are spherical macromolecular particles in which a hydrophobic core containing triglycerides (TG) and cholesteryl esters is emulsified by a shell composed of phospholipids, unesterified cholesterol and proteins are termed as apolipoproteins (apo). Lipoproteins are involved in transports of lipids in the vascular system, across vascular and sinusoidal endothelial linings and into the cells of tissues via receptor-mediated or other pathways. Lipoprotein components, cholesterol and apolipoproteins usually involves in lipid recruitment, modulation of enzyme activity, induction of receptor-mediated binding and endocytosis (Rensen et al., 2001).

1.5.6.5.2 Classification of lipoproteins

Lipoproteins are classified as chylomicrons, very low density lipoprotein (VLDL), low density lipoprotein (LDL) or high density lipoprotein (HDL). These lipoprotein classes differ with respect to size, lipid composition and apoplipoprotein composition. The targeting potential of lipoproteins to different tissues is highly dependent on their class and subclass, which are heterogeneous in particle size, lipids and apolipoproteins.

Table 1.2: Physical properties and composition of plasma lipoprotein classes

<table>
<thead>
<tr>
<th>Properties</th>
<th>Chylo (g/ml)</th>
<th>VLDL</th>
<th>LDL</th>
<th>HDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Density</td>
<td>&lt;0.96</td>
<td>0.96–1.006</td>
<td>1.019–1.063</td>
<td>1.063–1.21</td>
</tr>
<tr>
<td>Diameter (nm)</td>
<td>75–1200</td>
<td>30–80</td>
<td>19–25</td>
<td>5–12</td>
</tr>
<tr>
<td>Mobility on agarose</td>
<td>pre-β</td>
<td>β</td>
<td>α</td>
<td></td>
</tr>
<tr>
<td>Composition (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triglycerides</td>
<td>80–95</td>
<td>45–65</td>
<td>4–8</td>
<td>2–7</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>3–6</td>
<td>15–20</td>
<td>18–24</td>
<td>26–32</td>
</tr>
<tr>
<td>Cholesteryl ester</td>
<td>2–4</td>
<td>6–10</td>
<td>45–50</td>
<td>15–20</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>1–3</td>
<td>4–8</td>
<td>6–8</td>
<td>3–5</td>
</tr>
<tr>
<td>Protein</td>
<td>1–2</td>
<td>6–10</td>
<td>18–22</td>
<td>45–55</td>
</tr>
<tr>
<td>Apolipoproteins</td>
<td>A-I, II, IV</td>
<td>–</td>
<td>–</td>
<td>A-I, II, IV</td>
</tr>
<tr>
<td></td>
<td>B48</td>
<td>B100</td>
<td>B100</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>C-I, II, III</td>
<td>C-I, II, III</td>
<td>–</td>
<td>C-I, II, III</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>E</td>
<td>–</td>
<td>E</td>
</tr>
</tbody>
</table>

Chylo: chylomicron; VLDL: very low density lipoprotein; LDL: low density lipoprotein; HDL: high density lipoprotein.
1.5.6.5.3 Lipoprotein in sepsis treatment

The lipoprotein isolated form body (mostly recombinant) has shown prominent effect on LPS neutralization and clearance. These systems has been extensively used and well cited in literature. The precise mechanism responsible for LPS neutralization by lipoprotein is unknown. LPS is known to get transferred to HDL directly or via lipoprotein binding protein (LBP). LPS can also be scavenged from CD14 (Murch, 2007; Wu A, 2004). Many studies suggest that, in addition to hydrophobic interactions of fatty acids attached to lipid A with the emulsion surface, electrostatic interactions between negatively-charged choleosphosphophate groups within lipid A and cationic amino acids (e.g. arginine residues) within apo-E play an important role (Harris et al., 1993). Chylomicron helps to reduce proinflammatory response and improved survival in animal model of sepsis (Read et al, 1995; Harris et al, 1990). The pro-inflammatory response can be largely prevented by apoE-emulsions and lipid-free apoE (Rensen et al., 1997) which redirect LPS from KC and endothelial cells (EC) to liver parenchymal cells (PC), where LPS can be deactivated through the bile (Read et al., 1993, Harris et al., 1998).

Figure 1.6 Demonstration of LPS neutralization by HDL lipoprotein and improved clearance
1.5.6.5.4 Phospholipid rich emulsion

Lipoproteins are usually isolated from body by recombinant technology, which is not cost effective and usually contains protein, which are mainly responsible for its LPS neutralization effect. Phospholipid rich emulsion (phospholipids 92.5%, soya triglyceride 7.5%) formulation, when administered as continuous i.v infusion resulted in improved cardiopulmonary function and reduced mortality in pig (Goldfarb et al., 2003). Further, it decreased TNFα and leukocyte count in horse and attenuates the clinical and laboratory effects associated with the administration of LPS in humans, suggesting novel approach for the treatment of endotoxemia. This, strongly suggest hydrophobic interaction plays a crucial role. It is presently in clinical development stage by GlaxoSmithkline (Gordon et al., 2005).

1.5.6.5.5 Other Components

Cyclodextrin derivatives (DM-α-Cyd & DMA-β-Cyd) are known to attenuate NO production in RAW 264.7 cells by inhibiting LPS binding to its receptors and prevent LPS and galactosamine induced lethality in mice (Arima et al., 2001, 2005). Lipopolymamines are another group of compound which interacts with LPS has also shown protective effect. These molecules are of particular interest since they are designed to have low toxicity to mammalian cells, and are approved by the FDA for human use as safe alternatives to viral vectors (David, 1999). Dodecylamine (dendrimers) are known to bind with lipid A portion of LPS by simple hydrophobic and electrostatic interaction (Rehman et al., 1999) constructed PMB mimics from cholic acid scaffolds of neutralizing the deleterious effects of LPS and further bile salt enhance excretion of LPS.

1.5.6.5.6 Recombinant vs synthetic lipoproteins

These are endogenous lipoproteins, used as delivery systems for lipophilic compounds is theoretically possible, however constraints like time and difficulty involved in lipoprotein isolation and subsequent incorporation of useful quantities are considerable. The isolation of lipoproteins is difficult and depends on the donor’s plasma levels and techniques involved to isolate, it may yield only 50-100 mg of lipoproteins. It also takes about 48 hrs to isolate lipoprotein. Once isolated, the material is difficult to manipulate and it degrades due to lipid oxidation or physicochemical instability arising from apoB’s amphipathic
properties that lead to aggregation (Rensen et al., 1995). It has shown the feasibility of creating a synthetic LDL particle as a replacement for serum synthesized recombinant lipoproteins from commercially available natural and synthetic lipids and serum-derived or recombinant apolipoproteins, which closely mimic the metabolic behavior (Uterja et al., 1999). However, lipoproteins stabilized by apolipoproteins (ApoE) are difficult to prepare and there are problems related to reproducibility and stability of Apo E emulsions.

1.5.7 Chylomicron mimicking emulsion as drug delivery system

The chylomicron mimicking TG-rich emulsion has been used nowadays as drug delivery vehicle. These emulsions usually have oil phase (triolein, tristearin, soya oil, olive oil etc, EYPC or soya lecithin, lysophosphatidylcholine, CO, and cholesterol in a weight ratio 70:22.7:2.3:3:2. Many authors have demonstrated that lipophilic drug encapsulated in oil phase (Dierling and Zhengrong, 2005) or complexed with cholesterol (Shawer et al., 2002) in these delivery vehicles can be targeted to hepatic parenchymal cells. Apo E is readily acquired from the serum apolipoproteins has paved for active targeting to liver (Robinson and Quarfordt, 1999). Further active targeting can be achieved by surface modification of lipid emulsion with galactose moiety. Galactose due to its hydrophilic nature is fixed on the emulsion surface and it is taken up by the asialoglycoprotein receptor (ASGPr) on the parenchymal cells (Managit et al, 2005). The ASGPr is a high-capacity receptor uniquely expressed on the surface of hepatocytes to mediate the hepatic uptake and subsequent lysosomal processing of galactose (Gal) and N-acetylgalactosamine (GalNAc)-terminated substrates from the serum. The affinity for GalNAc is approximately 50-fold higher than for Gal (Rensen et al., 2004). The galactosylated emulsions rapidly disappear from the blood and get accumulated with up to about 80% of the dose within 10 minutes and are preferentially taken up by parenchymal cells as compared to non-parenchymal cells in the liver (Ishida et al., 2004).
1.5.7.1 Lipid Emulsion

Protein-free synthetic lipid emulsions (LE) (o/w) designed to resemble endogenous lipoprotein complexes represent a particularly interesting and technically less demanding approach for drug delivery. It can be readily prepared in large quantities and yet mimic the endogenous presentation of lipoprotein particles to the liver following peripheral intravenous injection. LE can be defined as oil-in-water (o/w) emulsions with mean droplet diameters ranging from 50 to 1000 nm. Usually, the average droplet size is between 100 and 500 nm. The particles can exist as water-in-oil and oil-in-water forms, where the core of the particle is either water or oil, respectively. The terms nanoemulsion, sub-micron emulsion and mini-emulsion are used as synonyms. Usually, LE contains 10 to 20 per cent oil stabilized with 0.5 to 2 percent egg or soyabean lecithin. Lipid emulsions are made from surfactants approved for human consumption and common food substances that are 'Generally Recognized as Safe' (GRAS) by the FDA. These emulsions are easily produced in large quantities by mixing a water-immiscible oil phase into an aqueous phase with a high-stress; mechanical extrusion process that is available worldwide (Stevens et al., 2003).

Intralipid became the starting point for using lipid emulsions as a delivery matrix for lipid-soluble drugs. TG-rich lipid emulsions (Intralipid, Intrafat, Lipofundin) have been applied clinically for parenteral nutrition from past 30 years. Intravenous injection of these commercial emulsions mimics chylomicrons with respect to the rapid acquisition of apolipoproteins, lipolysis, and uptake by the liver (Karpe and Hultin, 1995). These LE have been developed to provide sustained release or reduced systemic toxicity for antineoplastic agents, anesthetics vaccine adjuvants, and anti-inflammatory a nonspecific delivery of material primarily to peripheral tissues, to cells of the reticuloendothelial system (RES), or to the lymphatics rather than to a specific organ or tissue type (Lundberg et al., 1997).

1.5.7.2 Classification of lipid emulsions

Based on the emulsifier combinations used in the formation of lipid emulsion droplets, the o/w lipid emulsions can easily be classified into two types: one having emulsifiers with the capacity to produce a negative charge at the o/w interface termed as anionic and other
possessing emulsifiers able to provide a positive charge at the o/w interface called cationic (Tamilvanan et al., 2002).

1.5.7.2.1 Anionic lipid emulsion: - The lipid emulsion formulations were stabilized by various combinations of surface active agents, these being comprised mainly of a mixture of phospholipids that exhibit a high negative zeta potential value. This in turn is able to prevent droplet coalescence upon random collisions.

1.5.7.2.2 Cationic lipid emulsion: - It is clear from lipid emulsion literature that neither triglycerides nor phospholipid emulsifier's components of the conventional or anionic lipid emulsions are able to significantly sustain the incorporated lipophilic drug release in simulated or real physiological environments where full sink conditions prevail. Therefore, in an attempt to prolong and/or optimize the drug release, cationic lipid or polysaccharide emulsifiers are added to the lipid emulsions. Indeed, cationic lipid emulsions prepared on the basis of stearylamine, oleyamine and chitosan can serve this purpose (Yang and Benita, 2000).

![Figure 1.7: Structure of Positive charge submicron emulsion](image)

**Figure 1.7: Structure of Positive charge submicron emulsion**

1.5.7.3 Excipients for the manufacturing of lipid emulsion

The common emulsion excipients and the oils suitable for dissolving or dispersing lipophilic drugs, which are recommended for preparing LE are:
1.5.7.3.1 Oil Phase: Fatty oils commonly used in preparation of lipid emulsion are triglycerides like soya, castor, olive and MCT oil. The final oil phase concentration in lipid emulsions is now widely accepted at or even below 10% (w/w) taking into account that the lipid emulsion must be kept in a low viscosity range, of between 2 and 3 cp, which is considered as adequate viscosity for parenteral preparations. Sometimes, a mixture of oils rather than single oil is employed since drug solubilization in the oil phase is a prerequisite to exploit the lipid emulsion advantages (Jumaa and Muller, 1998).

1.5.7.3.2 Emulsifiers: Lecithin or phospholipids have been the emulsifiers of choice to produce ocular lipid emulsions. A parenteral lipid emulsion, such as intralipid, consists of a water phase with droplets composed of a triglyceride core (dia 250–400 nm) stabilized with a phospholipid monolayer (2–3 nm). The phospholipid monolayer stabilizes the emulsion by long-range repulsive electrostatic forces and short-range repulsive hydration forces. Any excess phospholipid exists as dispersed liposomes, unilamellar closed aggregates with a water core (dia 60–90 nm). However, emulsifiers of this kind are not suitable alone to produce submicron sized emulsion droplets or to withstand the heat during steam sterilization. Therefore, additional emulsifiers preferably dissolved in the aqueous phase are usually included in the lipid emulsion composition. A typical example of the aqueous soluble emulsifiers is non-ionic surfactants (e.g. Tween 20®) after taking into consideration their non-irritant nature when compared to ionic surfactants. The non-ionic block copolymer of polyoxyethylene–polyoxypropylene, Pluronics F68 (poloxamer 188), is included to stabilize the lipid emulsion through strong steric repulsion (Trotta et al., 2002).

1.5.7.3.3 Antioxidants: Oxidation of oil phase and phospholipids are major concern while preparing lipid based emulsion. Therefore care must be taken to minimize or eliminate oxidation. Antioxidant like α-Tocopherol is used to obtain a desired stabilized lipid emulsion under prolonged storage conditions. Therefore, α-tocopherol (0.001–0.002%, w/w) should be included in a typical lipid emulsion formulation.

1.5.7.3.4 Additives: In order to impart stability to emulsion excipients like benzalkonium chloride, chlorocresol, parabens etc. are added as preservative in lipid emulsions to prevent microbial spoilage of multi-dose lipid emulsions. The presence of components of natural
origin like lecithin or oils with high calorific potential render the lipid emulsion a good medium to promote microbial growth when it is packed in multi-dose containers (Tamilvanan, 2004).

1.5.7.3.5 Surface Active agents

1.5.7.3.5.1 Chitosan is an interesting natural material occurring in abundance in the environment. Its excellent biocompatibility and several advantages due to its unique polymer cationic character render it highly useful for pharmaceutical application (Illum, 1998). A polysaccharide comparable to cellulose, comprising copolymers of glucosamine and N-acetyl glucosamine linked by (1-4) linkages, chitosan can be derived by partial deacetylation of chitin from crustacean shells. The primary amino groups lead to special properties that render chitosan very interesting for pharmaceutical applications. In contrast to most other natural polymers, it has a positive charge (pKa+6.3) and possesses excellent mucoadhesive property (Kumar, 2000). Besides other applications chitosan has been extensively examined for its potential in the development of controlled release drug delivery systems (Felt et al., 2004).

Schulz et al., 1998 demonstrated emulsifying properties of chitosan and has shown it possess high HLB value of 37. Faldt et al., 1993 studied the interaction between chitosan and soybean oil emulsions coated with phospholipid and glycocholic acid as a function of pH. A structure consisting of adsorbed cationic polysaccharides on the anionic emulsifier surface is probably formed. Many authors have prepared cationic emulsions which were stabilized by lecithin-chitosan membrane at interface (Ogawa et al., 2003; Mun et al., 2005). Calvo et al., (1997) made chitosan-coated polyester nanocapsules and submicron-emulsions which stabilize the oil/water interface and particles displayed a high positive surface potential (+30 up to +60 mV). Chitosan coated colloidal carriers have been revealed as interesting systems for the administration of hydrophobic drugs by various route (Janes et al., 2001). Furthermore, several authors have reported that there are no signs of toxicity upon oral and nasal administration and there low toxicity following intravenous administration of chitosan (Aspden et al., 1993).

1.5.7.3.5.2 Protamine: Protamine is a cationic antimicrobial peptide (CAP) derived from the spermatic cells of vertebrates and cephalopods that is active against a range of Gram-
positive and Gram-negative bacteria, and fungi. Herring protamine has the amino acid sequence `arr rvs ss rpr rrr trrr rrrr rrr rrr`. Being highly charged (+20 mV), it strongly binds to negatively charged food particles, including many proteins and small-molecule surfactants (e.g., lecithins, polysorbates (Hansen and Gill, 2000). Since protamine acts as emulsifying agent stable emulsion can be developed. Some authors have demonstrated that protamine incorporation destabilize the emulsion system and results in phase separation by flocculation (David et al., 2003).

1.5.7.3.5 Stearylamine or Octadecylamine ($\text{CH}_3(\text{CH}_2)_{17}\text{NH}_2$): Stearyl amine is most common charge inducer used in lipid based formulation. Stearylamine, a cationic lipid with a pKa of 10.6 which contributes the overall positive charge to the oil droplet interfaces over a wide pH range owing to its primary amine group.

Table-1.3: List of Common excipients for lipid based submicron emulsion

<table>
<thead>
<tr>
<th>Oils</th>
<th>Emulsifiers</th>
<th>Cationic lipids and Miscellaneous polysaccharide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sesame oil</td>
<td>Cholesterol</td>
<td>Stearylamine</td>
</tr>
<tr>
<td>Castor oil</td>
<td>Phospholipids (Lipoid)</td>
<td>Oleylamine</td>
</tr>
<tr>
<td>Soya oil</td>
<td>Polysorbate 80 (Tween 80)</td>
<td>Chitosan</td>
</tr>
<tr>
<td>Paraffin oil</td>
<td>Transcutol P</td>
<td></td>
</tr>
<tr>
<td>Paraffin light</td>
<td>Cremophor RH</td>
<td></td>
</tr>
<tr>
<td>Lanolin</td>
<td>Poloxamer 407</td>
<td></td>
</tr>
<tr>
<td>Vaseline</td>
<td>Poloxamer 188</td>
<td></td>
</tr>
<tr>
<td>Corn oil</td>
<td>Miranol C$_2$M and MHT</td>
<td></td>
</tr>
<tr>
<td>Glycerin monostearate</td>
<td>Tyloxapol</td>
<td></td>
</tr>
<tr>
<td>Medium chain monoglycerides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medium chain triglycerides</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1.5.7.3.6 Advantages of Lipid based emulsions

The merits of lipid emulsion formulation are:

1. Natural biodegradability.
2. Nanometer droplet size range.
3. Minimizing potential local and systemic side-effects.
4. Substantial drug solubilization either at the innermost oil phase or at the o/w interface.
5. Controlled/sustain release of drug can be achieved.

6. Excipients used in preparation are relatively biocompatible and nontoxic.

7. Sterilizability and less pain on injection site as compared to solvent-based or solubilized formulations.

8. Minimum losses of lipophilic compounds during infusion into plastic tubings and infusion sets.

Lipid emulsions are considered to be superior to vesicular delivery system like liposome as it can be produced on an industrial scale, highly biocompatible, stable during storage and possess high solubilizing capacity for lipophilic drugs (Gold et al., 2000).

1.5.7.3.7 Antimicrobial Nanoemulsions

Antimicrobial nanoemulsions are oil-in-water droplets that range from 200-600 nm. They are composed of oil and water and are stabilized by surfactants and alcohol. The nanoemulsion has a broad spectrum activity against bacteria (e.g., E. coli, Salmonella, S. aureus), enveloped viruses (e.g., HIV, Herpes simplex), fungi (e.g., Candida, Dermatophytes), and spores (e.g., anthrax). The nanoemulsion particles are thermodynamically driven to fuse with lipid-containing organisms. This fusion is enhanced by the electrostatic attraction between the cationic charge of the emulsion and the anionic charge on the pathogen. When enough nanoparticles fuse with the pathogens, they release part of the energy trapped within the emulsion. Both the active ingredient and the energy released destabilize the pathogen lipid membrane, resulting in cell lysis and death (Wright, 1996).

1.5.7.3.8 Methods of Preparation of lipid based emulsion

1.5.7.3.8.1 Low energy emulsification

The emulsification methods making use of chemical energy stored in the components also termed as condensation, low energy or "spontaneous" emulsification methods are extensively used nowadays for the formulation of emulsions with small and uniform size. In these methods emulsions are obtained as a result of phase transitions produced during the emulsification process which is carried out at a constant temperature changing
composition or at constant changing temperature. The latter is better known as Phase inversion temperature.

Basically these methods make use of changing the spontaneous curvature of the surfactant. For non-ionic surfactants, this can be achieved by changing the temperature of the system, forcing a transition of an oil-in-water (O/W) emulsion at low temperatures to a water-in-oil (W/O) emulsion at higher temperatures system (transitional phase inversion). During cooling, the system crosses a point of zero spontaneous curvature and minimal surface tension, promoting the formation of finely dispersed oil droplets. This method is referred to as phase inversion temperature (PIT) method (Fernandez et al., 2004).

1.5.7.3.8.2 High energy emulsification

As emulsions are non-equilibrium systems, the energy input from mechanical devices or from the chemical potential of the components is required for their formation. The emulsification methods such as high pressure homogenization are used to obtain emulsion with small and uniform droplet size. The size of the droplet depends on the type of device used for the rupture of oil phase. Mostly an ultrasonifier (or ultrasonic dismembrator) is used to rupture the oil phase. High shear homogenizer or microfluidiser can also be used for larger volumes of emulsion. The average droplet size obtained after homogenization is a function of the stabilization system (surfactant and hydrophobic agent) and the energy input (Abismail et al., 1995).

An ultra-sonifier produces ultrasonic waves that cause the break up of droplets by cavitations. Only a small region near the probe head is affected by the sonic waves and the droplets present in the mixture do not experience the same energy input. However this problem can be overcome to some extent by stirring the emulsion at the time of sonication. Droplet size decreases with sonication time up to a certain limit. Initially the droplet size decreases rapidly as function of time and finally the average droplet diameter reaches a constant value which depends on the surfactant and co-stabilizer. In high pressure homogenizers (e.g. microfluidizer) the dispersion is pressurized using a pump and forced to flow through a narrow gap at high velocity. Homogenization mainly occurs due to shear forces but cavitation and impact forces also has some role to play. The droplet size and the width of the droplet size distribution decreases as the number of passes through the
homogenizer increases. High shear mixers like Ultra Turrax produce turbulent flow that causes the rupture of the droplets. The smallest droplet size that can be obtained is directly related to the geometry of the mixing head, the receptacle in which mixing is carried out and the number of passes through the mixing zone (Ouzineb et al., 2006).

1.5.8 Hydrophobic ion-pair complexes in drug delivery

Ion-pairs may be defined as neutral species formed by electrostatic attraction between oppositely charged ions in solution, which are often sufficiently lipophilic to dissolve in non-aqueous solvents. Ion-pair product (A⁺B⁻) exists as stable, thermodynamically distinct species and not as a transient, continuously exchanging species. The formation of ion-pair is due to, so called outer sphere interaction and involves no chemical bond formation. Pharmaceutical substance solubility in organic solvent can be enhanced in the form of a hydrophobic ion-pair complex with an amphiphilic material (Gurrero et al., 1997). Preferably, the amphiphilic material and the pharmaceutical substance have oppositely charged ionic portions which associate to form an ion-pair complex. Pharmaceutical substance having a cationic portion associates with an anionic portion of the amphiphilic material or vice-versa. Some examples of anionic amphiphilic materials include sulfates, sulfonates, phosphates (including phospholipids), carboxylates, and sulfosuccinates. Some specific anionic amphiphilic materials useful are: sodium dodecyl sulfate (SDS), bis-(2-ethylhexyl) sodium sulfosuccinate (AOT), and cholesterol sulfate and sodium laurate. Some examples of cationic amphiphilic materials include those having an ammonium group or a guadinium group, including substituted variations of those groups. Specific cationic amphiphilic materials include cetyltrimethylammonium bromide and cetyltrimethylammonium chloride. Particularly preferred anionic amphiphilic materials are SDS and AOT as they possess little or substantially no toxicological problem for the human or animal host (Mark et al., 2004).

Many studies have shown that ion pairing effectively increases the lipophilicity of charged drug molecule, but in-vitro and in-vivo. Ion pair formation has been known to increase the lipophilicity of ionizable drugs by shielding their charge with counter ions. Ion pair formation also has the problem of reducing the solubility of drugs in aqueous solution by increase lipophilicity of the drug compound. Therefore balance between lipophilicity
and water solubility is necessary for optimum delivery of drugs (Schoenwald and Wart, 1981). This problem of low solubility can be overcome by disperse ion-pair complex in colloidal lipospheres, submicron emulsion, liposome, chylomicron, microemulsion etc. Therefore, ion pair formation results in improved retention of drug in these formulations by lyophilization of hydrophilic drugs and ions (Gasco et al., 1989). Further, improved retention and distribution of drug in core sustain the release of drug from lipidic core. Many authors have demonstrated these ion pair can improve permeability and bioavailability by oral, ocular and nasal route. Therefore ion pair formation can be utilized for improved delivery of drug as demonstrated in Table 1.4. However, Physicochemical properties of these ion-pairs and there interaction with biological system ultimately decide the fate of these developed system (Meyer, 1998).
Table 1.4: Application of ion pair formation in Drug delivery

<table>
<thead>
<tr>
<th>Drug</th>
<th>Ion pair/formulation</th>
<th>Interference</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carteolol</td>
<td>Crotonic acid, Sorbic acid, Butylic acid, Caproic acid</td>
<td>Enhance bioavailability</td>
<td>Higashiyama et al., 2006</td>
</tr>
<tr>
<td>Timolol</td>
<td>Fatty acids</td>
<td>Enhance bioavailability</td>
<td>Higashiyama et al., 2004</td>
</tr>
<tr>
<td>Bunazosin</td>
<td>Fatty acids</td>
<td>Improved permeability</td>
<td>Kato and Iwata, 1998</td>
</tr>
<tr>
<td>Pilocarpine</td>
<td>Mono(octyl,hexyl,decyl)phosphate/liposphere</td>
<td>Sustain release</td>
<td>Cavalli, 1995</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>Aluminium</td>
<td>Physical, chemical, microbial stability.</td>
<td>Allemandi et al., 1995</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>Hexadecyphosphate/solid lipid nanoparticle</td>
<td>Improved bioavailability</td>
<td>Cavalli, 2002</td>
</tr>
<tr>
<td>Pilocarpine</td>
<td>mono-dodecylphosphoric acid/submicron emulsion</td>
<td>No improvement in ocular bioavailability</td>
<td>Sznitowska et al., 2000</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>Arylsulphonates/liposome</td>
<td>Improved retention</td>
<td>Zhigaltsev et al., 2006</td>
</tr>
<tr>
<td>Phosphonyomet</td>
<td>hexadecytrimethylammonium bromide</td>
<td>Enhance oral bioavailability</td>
<td>Van Gelder et al., 1999</td>
</tr>
<tr>
<td>(PMPA) &amp; AMD3100</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>salicylate</td>
<td>Enhanced absorption</td>
<td>Nishihata et al., 1984</td>
</tr>
<tr>
<td>Insulin</td>
<td>surfactants</td>
<td>Nasal absorption</td>
<td>Hirari S et al., 1981</td>
</tr>
<tr>
<td>Neurotensin</td>
<td>Fatty acid salt/microsphere</td>
<td>Microencapsulation</td>
<td>Yamakaka I et al., 1992</td>
</tr>
<tr>
<td>(hexapeptide)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leuroprolite</td>
<td>Sodium oleate/microsphere</td>
<td>Sustain release</td>
<td>Choi, 2000</td>
</tr>
<tr>
<td>aceate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Retinoic acid</td>
<td>Phenylalanine esters/microemulsion</td>
<td>Enhanced topical absorption</td>
<td>Trotta, 2003</td>
</tr>
</tbody>
</table>
1.6 Research Envisaged

The present proposal originated from the perception that current treatments of Gram-negative sepsis in critically ill patients are based on prompt administration of adequate antibiotics, removal of the infection nidus, and support of organ dysfunction. Nevertheless, several studies have shown that exposure of gram negative organisms to conventional antibiotics can result in endotoxin (LPS) release which could have deleterious effects. Many studies have also shown that endotoxin-releasing properties of antibiotics affect the clinical outcome of sepsis. Therefore neutralization and detoxification of LPS is by and large mandatory during septic shock.

Therefore, there is a need that antibiotic therapy should not only kill the bacteria but also be capable of preventing other derived toxic effects mediated by endotoxin (LPS). These are many ways to circumvent toxic effects of LPS i.e neutralizing LPS, blocking their interaction with specific receptor on immune cells and/or modifying various inflammatory cascading events and mediators as discussed previously. The development of drugs for septic patient has proved to be graveyard for pharmaceutical companies as none of the approaches has been proved clinically fruitful to till date. Therefore based on the concept that polycationic substances and lipoproteins have ability to bind with lipid A portion of LPS by simple hydrophobic and electrostatic interaction. Therefore considering structural and chemical aspects of LPS, it has been assumed that a rationalized delivery system could be devised so as to facilitate its binding with LPS and subsequently neutralize it, so that toxic implications of LPS could be reduced to a greater extent.

Lipoprotein mimicking cationic and anionic lipid emulsion can be good delivery vehicle for administration of antibiotics as it is anticipated to improve antimicrobial efficacy and at the same time it will neutralize LPS and/or alleviate its fatal implications. The efficacy of developed delivery systems can be assessed for its ability to interact with LPS and further result in decreased microbial load coupled with reduction in LPS release. The LPS is known to stimulate macrophages which results in release of pro-inflammatory mediators like cytokines and nitrites which initiate further inflammatory cascade. Therefore it is plausible to study the effects of developed formulation (with or without antibiotics) on LPS induced secretion of inflammatory mediators using J774 macrophage cells in-vitro.
However, it is extremely important to ascertain, the efficacy of optimized formulation in-vivo using well established animal models of abdominal sepsis. In the proposed study two animal models have been taken up (i) LPS induced sepsis (ii) E-coli induced abdominal sepsis (peritonitis).

(i) It has been considered to establish, if the lipid based formulation (blank) by itself possess capability to mop up LPS due to electrostatic and/or hydrophobic interaction to circumvent the toxic effects of LPS and moreover if ciprofloxacin loaded lipid based formulations could have additional efficacy to prevent/suppress levels of LPS in plasma, degree of mortality, hepatic inflammation and plasma levels of pro-inflammatory mediators on intraperitoneal administration of LPS.

(ii) However, the commercial success of formulation depends on its efficacy in actual infection model. Therefore it has also been taken up to induce abdominal sepsis using clinical isolates of E-coli (ATCC 25922) and to ascertain the efficacy of ciprofloxacin loaded based emulsion in terms of survival, level of microbial load, free LPS and inflammatory mediators.

It has been reported that in experimental intra-abdominal sepsis the liver and spleen are major bacterial reservoirs and it is anticipated that the proposed lipid based formulations will improve animal survival due to selective drug targeting to reticuloendothelial system (RES) compared to free drug. Therefore, it becomes important to study the kinetics of formulation administered parentrally in septic animal.

Therefore objective of this work is to develop cationic and anionic lipid based emulsion formulations incorporating a fluoroquinolone antibiotic (ciprofloxacin) for parenteral delivery with an attempt to alleviate the lethal cascading events subsequent to release of LPS after severe infection. We, Hypothesize that these lipid based emulsion system could serve the following purpose:

- Decrease microbial load.
- Control release of antibiotics and improved therapeutic index due to selective targeting.
- Either neutralizes and/or reduces endotoxin level and its subsequent biological implications.
Redirect LPS through parenchyma cells i.e. increase bile excretion.

1.7 Plan of work

1. Drugs, lipids, surface modifiers and vehicles shall be procured and preformulation studies shall be conducted in terms of, identification of drugs, % purity, solubility profile, partition coefficient, Drug-excipients interactions and characterization of other additives.

2. Development of suitable and reproducible analytical technique for the estimation of LPS and ciprofloxacin.

3. Development of ciprofloxacin laden lipid based emulsions using various lipid/phospholipids, fatty acid, and charge inducers so as to facilitate hydrophobic-hydrophobic and/or electrostatic interaction of formulation with LPS. The system will be further modified by incorporation of charge inducer like stearylamine, chitosan and protamine. An attempt shall be made to modify the surface by appropriate ligand for parenchymal targeting.

4. The developed formulation shall be characterized for shape and surface morphology, average vesicle/particle size and size distribution, drug entrapment, in-vitro drug release and storage stability.

5. In-vitro effectiveness of formulation against LPS shall be studied by assessing:
   (a) Effect of formulation on microbial load and consequent interaction with released LPS.
   (b) Effect of formulation on LPS induced cytokines release in appropriate macrophage cell lines.

6. The efficacy of developed formulation shall be studied in suitable animal model (approved by institutional animal ethics committee) in terms of plasma drug concentration, LPS and cytokine levels.