SEPSIS

Sepsis and the accompanying systemic inflammatory response syndrome (SIRS) represent a spectrum of clinical symptoms. Only recently, the standardized definitions of sepsis have been adopted, which allow us more accurate estimate of its morbidity and mortality (Muckart and Bhagwanjee, 1997). The term sepsis implies a clinical response arising from infection, but a similar, or even identical, response may also develop in the absence of infection (Giudici et al., 1999). This SIRS can occur following a wide variety of insults, infectious or non-infectious; the latter including pancreatitis, ischemia, multiple trauma and tissue injury. When the systemic inflammatory response syndrome is the result of a confirmed infectious process, it is termed as sepsis (Giudici et al., 1999). Severe sepsis commonly results in shock and potentially multiorgan failure (Cruz and Hollenberg, 2003). Septic shock is considered as a sub-set of sepsis (and therefore requires a documented infection) and is defined as sepsis-induced hypotension, persisting despite adequate fluid resuscitation, along with the presence of hypoperfusion abnormalities or organ dysfunction (Bone et al., 1992) (Fig. 1). Unless the cycle is interrupted, irreversible cell damage ensues and ultimately cell death occurs (Sharma et al., 2003).

Despite increasingly sophisticated critical care, the mortality associated with septic shock remains elevated (Martin et al., 2003). Septic shock is the most common cause of death in intensive care units and it is one of the 10 leading causes of both infant and adult mortality in United States (Parrillo et al., 1990; Jones et al., 2002). Approximately 750,000 cases of sepsis (Nasraway, 2003) and 200,000 episodes of septic shock are estimated to occur annually resulting in more than 100,000 deaths (Parrillo et al., 1996). The Italian SEPSIS study, carried out in 99 intensive care units in 1994, reported mortality rates of 52% and 82% for severe sepsis and septic shock respectively (Giudici et al., 1999). Since
1995, the mortality from sepsis has risen by 1.5% yearly (Angus et al., 2001). The incidence of sepsis is expected to rise during the next decade owing to the aging population, a growing immunosuppressed population, the increased use of invasive catheters and prosthetic materials and the growing problem of antimicrobial resistance (Stevens, 2002). In the year 2010, it is estimated that there will be 934,000 new sepsis cases in the United States and in 2020, 1,100,000 (Augus et al., 2001). The manifestations of sepsis include those related to the systemic response to infection (tachycardia, tachypnea, alteration in temperature and leukocytosis) and those related to organ dysfunction cardiovascular, respiratory, renal, hepatic and hematologic abnormalities (Hoffman and Cooper, 1995). The organ system affected commonly include the heart, kidney, lungs, liver, central nervous system and coagulation system (Sharma et al., 2003).

Fig. 1: Proposed interrelationship between systemic inflammatory response syndrome (SIRS), sepsis, and infection

Organisms causing sepsis

An overwhelming infection that leads to sepsis may be caused by different microorganisms such as bacteria, viruses, parasites, protozoa or fungi, but bacterial infections are the most common cause of septic shock (Southwick,
2003). Gram-positive infections account for up to 50% of cases of severe sepsis in the modern intensive care units (Martin and Cohen, 2001). The most common causative gram-positive organisms associated with sepsis are *Staphylococcus aureus* and *Streptococcus pneumoniae* and the most common gram-negative organisms are *Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas* and *Enterobacter* species (Bernard et al., 2001). Bacteria such as *K. pneumoniae*, *E. coli*, *Listeria monocytogenes*, *Neisseria meningitidis* and *Salmonella* are commonly involved in the development of sepsis in newborns and infants younger than 3 months (Balk and Casey, 2000). *Bacteroides fragilis* is the most common cause of anaerobic septicemia (Southwick, 2003). Recently, the researchers have discovered a shift in the organisms responsible for causing septicemia from primarily gram-positive bacteria to gram-negative organisms (Roy et al., 2002; Mahapatra et al., 2002). Reported mortality after gram-negative sepsis is 3 to 4 fold higher than after gram-positive sepsis (Imad et al., 2002). Among these *Klebsiella pneumoniae* has emerged as a predominant organism responsible for neonatal septicemia (Agarwal, 2001; Basu et al., 2001; Kumar and Neelagund, 2004). It is also listed among the microorganisms responsible for many cases of severe infection in the intensive care units (Malik et al., 2003; Karabinis et al., 2004). Therefore, *K. pneumoniae* continues to be a nightmare for neonatologists, microbiologists and hospital administrators (Bhutta et al., 1991).

The wide spread and indiscriminate use of antimicrobial therapy has been held responsible for the occurrence of multiple antibiotic resistant strains in hospitals (Chhibber and Vadhera, 1988). Such strains with resistance to many broadspectrum antibiotics, including carbapenems, pose a severe problem, especially for critically ill patients (Yan et al., 2001). High isolation rates of extended spectrum beta lactamase producing *K. pneumoniae* have been reported from hospitals located in North America, Europe and Latin America (Monnet et al., 1997; Liu et al., 1998). These world wide findings are in accordance with that of National Neonatal Perinatal Network Database (Singh, 1999; Agarwal, 2001). Roilides et al. (2000) have reported an unusual high
incidence of septicemia in neonates due to multi-drug resistant *K. pneumoniae*. In developing countries, nosocomial sepsis in new born units is very common (Ahmad, 2003). The reported incidence of nosocomial sepsis in India ranges from 1.5 to 37% (Malik et al., 2003). Although, any pathogen may be acquired by the neonates in hospital, *Klebsiella* is lately emerging an important cause of neonatal nosocomial infections (Gupta et al., 1993). Ahmed (2003) reported forty-five episodes of sepsis among which *Klebsiella* species was the predominant organism.

The vast majority of *Klebsiella* infections are associated with hospitalization (Lepper et al., 2003). The principle reservoirs for transmission of *Klebsiella* in the hospital setting are the gastrointestinal tract of patients and the hands of hospital personnel (Montogomerie, 1979; Jarnis et al., 1985). According to the statistics of the centers for disease control and prevention, *Klebsiella* species account for 8% of endemic hospital infection and 3% of epidemic outbreaks (Stamm et al., 1981). The carriage of *Klebsiella pneumoniae* strains may be the result of bacterial adhesion to the intestinal epithelial cells of the host (Favre-Bonte et al., 1995). Apart from medical equipments, the invasive life support procedures in hospitalized patients e.g. intravenous catheters used for fluid administration, catheters placed in bladder for urine drainage and breathing tubes for people on a breathing machine can all increase the susceptibility of patients to infection (Stevens, 2002). While these devices may be needed in certain patients, these allow bacteria to bypass the natural barriers to infection and get into the person’s body. The result may be an infection if the person’s immune system cannot fight the bacteria. As opportunistic pathogen, *Klebsiella* species primarily attack immunocompromised individuals who are hospitalized and suffer from severe underlying diseases such as diabetes mellitus or chronic pulmonary obstruction (Shellito et al., 2001). Patients at risk also include neonates, elderly and alcoholics (Shellito et al., 2001; Mostafa et al., 2002).
Pathogenetic sequence in sepsis

The pathogenesis of sepsis and septic shock are not clearly understood. Recent advances in our understanding of the pathogenesis of sepsis have made it clear that uncontrolled infections, whether clinically manifest or occult, are not the only cause of systemic inflammation and organ dysfunction (Martin et al., 2003). Other stimuli such as major trauma, burns etc can also trigger an excessive inflammatory response and lead to multiple organ dysfunction (MODS), however when it is a result of confirmed infectious process, it is termed as sepsis (Saffle et al., 1993; Steinberg and Tenner, 1994). The various events leading to the pathogenic cascade are depicted in Fig.2 (Parrillo, 1993). Gram-positive organisms releasing exotoxins, gram-negative organisms containing endotoxins and fungi can initiate this pathogenic cascade (Giudici et al., 1999). The process begins with the proliferation of microorganisms at a nidus of infection (Cryz and Hollenberg, 2003). Beside these, there are many important pathogen-associated molecular patterns (PAMPs) unique to bacteria, which are released from their surface during infection and lead to a lethal inflammatory response (Medzhitov and Janeway, 2002). Examples of PAMPs include flagellin of bacterial flagella, peptidoglycan of gram-positive bacteria, endotoxin of gram-negative bacteria, lipoteichoic acid as well as internal structures like bacterial DNA or double stranded RNA in some viruses (Martha and Kathy, 2004). Endotoxin is one of the several products, which is now believed to be the primary trigger of gram-negative septic shock (Raetz et al., 1991). Possible source of endotoxin is infection by exogenous gram-negative bacteria or gut translocation and failure of hepatic uptake. Large amount of endotoxin, that is not neutralized by natural cellular components such as bactericidal permeability increasing protein (BPI) in polymorphonuclear leukocytes (PMNs), interacts with lipopolysaccharide binding protein (LBP). This LPS / LBP complex which binds selectively to soluble and membrane-bound CD14 trigger host cells (monocytes, macrophages, endothelial cells, granulocytes and lymphocytes) to release a variety of humoral endogenous mediators such as tumor necrosis factor (TNF-\(\alpha\)), interleukins and arachidonic acid metabolites (Raetz et al., 1991; Morrison et
Among these TNF-α and interleukins IL-1, IL-6 appear to be major determinants of sepsis and have been implicated in the pathophysiologic derangements associated with sepsis (Baykal et al., 2000; Cryz and Hollenberg, 2003). TNF, IL-1 and IL-6 induce fever and cause liver to release acute phase proteins, which may lead to release of additional TNF-α (Baumann and Gauldie, 1994). The complement system is activated to produce additional cytomediators which, in conjunction with TNF and leukocyte aggregation, can lead to leukopenia, blood vessel injury and capillary leakage. Endotoxin is the most potent known activator of coagulation system which converts, this normally beneficial system into a pathological cascade. Factor XII (Hageman factor) of the coagulation cascade plays an important role in the pathogenesis of shock. It activates the coagulation pathway, which in turn activates extrinsic coagulation pathway and leads to disseminated intravascular coagulation (DIC) (Glauser and Zanetti, 1991). Endothelial cells are stimulated to produce nitric oxide, which causes vasodilation, hypotension and increased cardiac output. T cells release colony-stimulating factor and interferon-γ, causing leukocytosis and stimulating macrophages to produce humoral mediators and further perpetuate the inflammatory response (Glauser and Zanetti, 1991; Rietschel et al., 1996). Modulation of this inflammatory response includes the release of anti-inflammatory mediators such as corticosteroids and catecholamins (Astiz et al., 1996). IL-10, IL-14 and prostaglandin, as well as soluble inhibitors for IL-1, TNF and IL-8 receptors, have anti-inflammatory action. These substances promote a period of immune depression that follows the initial shock episode (Cryz and Hollenberg, 2003). However, if the normal host response is unable to control the overwhelming systemic inflammation, it leads to DIC and hemorrhage, vascular leakage, pulmonary edema progressing to acute respiratory distress syndrome (ARDS). Refractory hypotension, progresses to life-threatening decrease in organ perfusion, manifested as metabolic acidosis, renal insufficiency, abdurtion and coma, and ultimately, terminal respiratory failure and/or cardiovascular collapse (Glauser and Zanetti, 1991; Parrillo, 1993; Rietschel et al., 1996).
Fig. 2: Pathogenetic sequence of the events in septic shock

Nidus of Infection
Abscess
Pneumonia
Peritonitis
Pyelonephritis
Cellulitis

Gram-positive and gram-negative organisms
Exotoxin
TSST-1
Toxin A
PAMPs
Endotoxin
LBP

Plasma (complement system)
Monocytes or macrophages
Endothelial cells
Neutrophils

Endogenous mediators
Cytokines
Tumor necrosis factor
Interleukin-1, 2, 6, 8...
Platelet-activating factor
Endorphins
Endothelium-derived relaxing factor

Arachidonic acid metabolites
Cyclooxygenase Prostaglandins
Lipoxygenase Leukotrienes
Complement "C5a"
Kinin
Coagulation
Myocardial depressant substance

Myocardium
Depression
Dilatation

Vasculature
Vasodilatation
Vasoconstriction
Leukocyte aggregation
Endothelial-cell dysfunction

Organs (Kidney, Liver, Lung, Brain)
Dysfunction
?? Metabolic defect

Shock
Refractory hypotension
Multiple-organ-system failure

Recovery
Death

TSST-1 denotes toxic shock syndrome toxin 1; Toxin A is Pseudomonas aeruginosa toxin A; LBP- lipopolysaccharide binding protein; PAMPs-Pathogen associated molecular patterns

A decade ago, it was believed that once endotoxin initiates events, the pathogenic cascade is self-perpetuating and unstoppable. However, recent investigation have added new dimension to the picture of the sepsis cascade. Now, workers are of the view that the continuous presence of endotoxin is necessary to maintain the systemic inflammatory response (SIRS) in addition to simply initiating it (Dedrick and Conlon, 1995; Huang et al., 1995). This finding suggests that even late in the course of this entity, specific endotoxin-neutralization therapy may be able to abort established but still reversible SIRS, sepsis and septic shock (Giroir et al., 1997).

**Endotoxin**

Bacterial endotoxins were first described at the end of the last century after the recognition that, in addition to secreted toxin (exotoxins), certain types of bacteria also produce biologically active, heat stable molecules associated with bacterial cell wall itself (Rietschel et al., 1996). Endotoxin, the lipopolysaccharide (LPS) is now known to be a component of the outer cell wall of all gram-negative bacteria (Prins et al., 1994; Remick, 1995; Carcillo and Cunnion, 1997). As depicted in Fig.3, LPS comprises of three parts: the outer most O-specific side chain, whose composition varies with serotype of a bacterial genus; the core polysaccharide which is less variable and the inner most toxic lipid A (Salles et al., 1989; DiPadova et al., 1993).

**Fig. 3: Bacterial lipopolysaccharide (LPS)**

![Diagram of Bacterial Lipopolysaccharide (LPS)](image-url)
The O-specific chain in enzymatically constructed by the sequential addition of oligosaccharides, therefore, the endotoxin of a given bacterium at a given point of time is heterogenous mixture of molecules with short, intermediate and long O-specific chains. Thus, there is no precise or standard way to measure molecular composition or molecular weight of endotoxin (Nikaido, 1970). The lipid A component of endotoxin is highly conserved from one gram-negative family to another and gives the endotoxin molecule its toxicity (Rietschel et al., 1996).

During episode of rapid logarithmic growth, substantial quantities of LPS are liberated from multiplying population of gram-negative bacteria. When this occurs in an in vivo environment, LPS is absorbed into the systemic intravascular compartment and is thus referred to as endotoxemia (Coyne et al., 1993). LPS is released in blood in a variety of forms such as membrane fragments, blebs, vesicles and combination with bacterial phospholipids (Parker et al., 1995). Once in circulation endotoxin exerts its highly complex array of pathophysiologic effects by interacting with naturally occurring cellular and humoral elements (Bone et al., 1992). These elements routinely mediate the normal host response against infectious insults. However, in sepsis, the host's normal homeostatic mechanisms break down and the inflammatory response manifests as fever, vascular leakage, myocardial depression and shock (Suffredini et al., 1989). In patients with septic shock, detectable levels of circulating endotoxins are correlated with positive blood cultures, lactic academia, low systemic vascular resistance and a depressed ventricular ejection fraction and increased mortality (39 percent, as compared with 7 percent for patients without endotoxemia (Danner et al., 1991). Injection of LPS into the blood stream of experimental animals resulted in pathophysiological changes that are similar to those seen in sepsis (Mozes et al., 1990; Parrillo, 1993). Similar changes were seen in human volunteers as well (Martich et al., 1993; Michie, 1998). The relation between endotoxin and the typical cardiovascular manifestations of sepsis has been studied in normal subjects with the use of a very small intravenous dose of highly purified endotoxin (Suffredini et al., 1989). The small dose of LPS (4 mg/kg) induced hemodynamic, hematological and
metabolic changes that were qualitatively similar to those observed in septic patients, including fever, tachycardia, elevated cardiac output, hypotension, decreased systemic vascular resistance, leukocytosis, lymphopenia, elevated circulating concentration of stress hormones and catecholamines, elevated oxygen consumption ($V_O^2$) and widened alveolar arterial oxygen tension gradient (Revhaug et al., 1988).

**Role of cytokines in sepsis**

Although LPS (lipid A) is capable of causing direct cytotoxic influences, most of the physiologic sequelae precipitated is due to induced synthesis of patent endogenous mediators (Remick, 1995). Although other humoral mediators are induced by LPS, the overwhelming systemic production of pro-inflammatory cytokines such as tumor necrosis factor, interleukins (IL-1, IL-6, IL-8) and interferon gamma (IFN-$\gamma$) lead to continued activation of polymorphonuclear leukocytes, macrophages and lymphocytes. All these processes create a state of destructive immunologic dissonance (Shapira et al., 1996; Zhang et al., 1999; Sharma et al., 2003).

The pro-inflammatory cytokines especially TNF-$\alpha$, play an important role in sepsis and is instrumental in the development of septic shock and death (Gardlund et al., 1994; Son et al., 2002). Several studies have implicated tumor necrosis factor-$\alpha$ (TNF-$\alpha$), also called cachetin in the deleterious effects of gram-negative bacteria and their toxic products (Tracey et al., 1987). Beutler and Cerami (1988) first called TNF-$\alpha$ as cachetin because they believed it was responsible for the cachectic characteristics of chronic infections. Later it was determined that cachectin and TNF were identical and it is a 17 kDa polypeptide produced by monocytes, macrophages and other cells exposed to endotoxin or microorganisms (Parrillo, 1993). TNF-$\alpha$ has received much attention because it is elevated in most patients with sepsis. TNF is considered to be the prototype of a host damaging cytokine (Beutler and Cerami, 1989; Vassali, 1992). It has been shown in many systems to induce immunopathology in vivo, tissue necrosis and cachexia or wasting (Beutler and Cerami, 1988; Tracey and Cerami, 1992). In this regard, serum TNF levels have been positively correlated with the severity of
shock and organ failure and the highest levels of the mediator was detected in fatal cases (Waage et al., 1989; Kirkeboen et al., 1999). TNF-α when present in large amounts systemically, results in inflammatory cascade causing leaky capillaries, leukocyte infiltration, neutrophil mediated endothelial damage and inhibition of pulmonary surfactant (Vassalli, 1992).

The evidence implicating TNF-α as a central mediator of the lethal consequences of bacterial infections was first provided by Beutler et al. (1985) who demonstrated that passive immunization with anti-TNF serum increase the dose of LPS required to kill mice. Similar findings have been reported in rabbits pretreated with antibody prepared against human recombinant TNF (Mathison et al., 1988). Subsequently, several studies have demonstrated the efficacy of anti-TNF antibodies in preventing death in animal models (Tracey et al., 1987) as well as in patients with septic shock (Gardlund et al., 1995). Kirikae et al. (1998) reported a 18 kDa cationic antimicrobial protein which could bind to LPS and neutralizes its ability to induce TNF production by mouse cells. The suppression of TNF production results in protection of the mice from death by endotoxic shock.

TNF-α is reported to have both beneficial and detrimental effects in host during infections (Beutler et al., 1985; Tracey et al., 1987; Blanchard et al., 1988; Rydberg et al., 1995). In addition to its essential protective effects in generation of immunity against gram-negative bacteria, high levels of TNF-α at the site of infection induces an excessive inflammatory response that overwhelms the beneficial effects of this cytokine (Bekker et al., 2000). Previous studies have reported tissue necrosis and cachexia or wasting associated with elevated TNF-α levels (Beutler and Cerami, 1988; Taub et al., 1996). The overproduction of TNF-α in response to LPS is believed to be one of the major contributing factors of morbidity and mortality in gram-negative sepsis (Arditi et al., 1993).

Since, sepsis involves complicated pathophysiological processes, it has been suggested by Walley et al. (1996) that outcome of septic shock is dependent upon balance between pro and anti-inflammatory mediators. Further time course of cytokine production determines the severity of sepsis and
survival. These mechanisms are more important than single inflammatory cytokine concentration taken alone (Shapira, 1996; Oberholzer et al., 2000). Pro-inflammatory cytokines (TNF-\(\alpha\), IL-1\(\beta\), IL-6, IL-8 and IL-12) are necessary for initiating an effective inflammatory process against infection by pathogens and tissue damage, whereas excessive or inappropriate production has a deleterious effect, resulting in defective tissue microcirculation and hypoxia with essential systemic circulatory malfunction, multiple organ system dysfunction and death (Stuber et al., 2000). In contrast, anti-inflammatory cytokines (IL-4, IL-10 and IL-13) appear important in controlling and down-regulating the inflammatory response through depression of the host immune system (Boontham et al., 2003). Adequate modulation of excessive expression of inflammatory cytokines by anti-inflammatory mediators such as glucocorticoids, prostaglandin E\(_2\), IL-1 receptor antagonist and anti-inflammatory cytokines is necessary for maintaining homeostasis in the host (Sharma, 2003; Souza et al., 2003).

**ANIMAL MODELS OF SEPSIS**

Two related but distinct mechanisms of dysregulation of immune system have been considered to cause the fatal process of sepsis (Zantl et al., 1998). On one hand, it is assumed that an exuberant infection results in decreased ability of the immune system to mount an antimicrobial defense, finally leading to immune paralysis (Volk et al., 1996; Warren, 1997). The other hypothesis put forward suggests the role of microbial components which activate a strong immune response resulting in an over production of harmful immune mediators (Beutler and Cerami, 1989; Bone, 1991). Thus, sepsis as a clinical entity is very heterogenous and is characterized by dynamic nature of the pathophysiologic events and generation of complex array of mediators, precluding accurate prediction of the efficacy of novel therapeutic agents from *in vitro* studies alone (Cross et al., 1993). Insight into these complicated pathophysiological processes have, at least in part, been gained from animal studies. While new approaches for therapy ultimately require validation using well controlled clinical trials, it is virtually always necessary to obtain preliminary data in animals before using experimental drugs or devices in humans (Fink and Heard, 1990). Furthermore,
many studies designed to acquire information about pathophysiology can only be performed in animals because they require invasive monitoring (e.g. to measure pulmonary lymph flow) or utilization of pharmacological interventions of uncertain benefit. Thus, progress in sepsis research continues to depend upon studies using clinically relevant animal models. Several models for sepsis have been used in the last decades. On the basis of literature on sepsis models these can be classified as intravenous infusion models, endotoxicosis models and infection models (Wichterman et al., 1980; Fink and Heard, 1990; Deitch, 1998).

**Intravenous infusion of bacteria in animals**

Number of workers used bolus or short term continuous infusion of large doses of bacteria for establishing sepsis (Cryan et al., 1988; Hinshaw et al., 1988). In these models, infusion of bacteria causes a rapid and profound decrease in cardiovascular function and cardiac output and animals die within hours. Intravenous infusion model remains very useful as these avoid the confounding effects of anesthesia and surgical manipulation and adequately reproduce many features of severe septic shock in humans (Shaw and Wolfe, 1984). However, several inherent problems limit the usefulness of these models. Firstly, large doses of bacteria are necessary for establishing infection as bacterial strains used in such models are avirulent and serum sensitive. Such strains are therefore rapidly killed after administration and as a consequence large quantities must be infused to overwhelm or intoxicate the host (Porat et al., 1992). Further, these models do not resemble the clinical disease since most septic patients have an infectious focus from where bacteria continuously disseminate over time (Wichterman et al., 1980; Deitch, 1998). In addition, survival after establishment of infection is short and therefore there is limited time for progression of disease (Fink and Heard, 1990). Finally, the serum cytokine responses produced are transient and of much greater magnitude than seen in patients with sepsis, making it hard to extrapolate results from these studies to situation in patients (Cross et al., 1993).
Intravenous infusion of LPS in animals

Sepsis can be established in animals by intravenous administration of LPS. Fink and Heard (1990) recognized four categories of LPS-infusion models that reproduce more accurately the situation of either sepsis or septic shock in patients:

- Models that utilize small sub-lethal doses of LPS. These doses generally induce a hyperdynamic cardiovascular response (Fink et al., 1984)
- Models that provide aggressive resuscitation of intravascular volume (Breslow et al., 1987; Fink et al., 1989)
- Models that utilize continuous infusion of LPS (Kurtz and Quast, 1982)
- Models that use LPS injected intraperitoneally (Fink et al., 1987)

These models utilizing intravenous LPS infusion in the absence of fluid resuscitations and other supportive therapy (intubation, mechanical ventilation and use of inotropic agents) typically employ mice and rats and mortality as the primary outcome (Hinshaw et al., 1981). Some of the advantages of these models over intravenous infusion of bacteria include the stability of the LPS, which can be easily stored and accurate doses of LPS can be administered in a reproducible manner. A major disadvantage of these models is the lack of an infectious focus.

Infection models of sepsis

These models have an infectious focus, from where bacteria disseminate during the course of the disease. Commonly used infection models are the cecal-ligation-puncture model (CLP), in which fecal peritonitis is induced by a straightforward surgical procedure (Wichterman et al., 1980) and the fibrin-clot model in which bacteria are suspended in a fibrin clot that is implanted in the abdominal cavity (Ahrenholz et al., 1980; Fink et al., 1984; Natanson et al., 1986; Demarsh et al., 1996). In these models, animals develop a hyperdynamic circulation with an increased cardiac output and a decreased systemic vascular resistance similar to human sepsis. Therefore, these animals develop
bacteremia and signs of sepsis (Schultz and Vander Poll, 2002). There are several advantages of CLP which include: the simplicity of the procedure as there is no need to grow or quantify bacteria; development of sepsis due to peritoneal contamination with mixed flora in the presence of devitalized tissue bears an obvious resemblance to clinical problems like perforated appendicitis and diverticulitis. In addition the cytokine response is similar to that observed in septic patients. However, it is difficult to control the magnitude of septic challenge in this model. This problem can be overcome by inoculating the peritoneal cavity with faeces directly or by implanting a fibrin clot in which bacteria are suspended (Fink et al., 1984; Demarsh et al., 1996). The fibrin—clot model allows the investigators to have complete control over the dose of bacteria (Fink et al., 1984; Natanson et al., 1986) and type of bacteria (Natanson et al., 1989). Fibrin delays the systemic absorption of the entrapped bacteria and promotes the development of a more local septic focus (Demarsh et al., 1996).

The choice of animal species in pre-clinical studies is an important variable. Since, animal species differ considerably in their cardiovascular physiology and susceptibility to bacterial endotoxin, investigators should carefully consider the relative merits and limitations of each animal model before extrapolating animal data to clinical efficacy in septic patients (Cross et al., 1993). For example, mice are advantageous as they are relatively cheap, genetically similar and usually pathogen free (Baker et al., 1985; 1988). In studies using small animals (mice and rats), the problem of variability is easily overcome by increasing sample size. However, variability remains a problem in larger species. Recent reports highlighting the genetic similarity between mice and humans (Kondo et al., 2001; Mural et al., 2002) draw attention to the fact that these small affordable animals can serve useful experimental models. A more realistic animal model of sepsis necessitates carefully choosing an experimental host capable of being efficiently infected at relatively low doses with an appropriate pathogen and demonstrating that such initial colonization progresses to sepsis (Cross et al., 1993).
Chemotherapeutic approach to treat sepsis

Effective therapeutic strategy for severe sepsis is aimed at controlling and eradicating the source of infection, which can be achieved by administering antibiotics, surgical drainage or both (Nasraway, 2003). In this relation, it has been emphasized that appropriate empiric antimicrobial therapy must be guided by the knowledge of most common site of infection and the most common infecting organisms (Bernard et al., 2001). For *Klebsiella pneumoniae*, agents with high intrinsic activity are selected for severely ill patients. Examples of such agents include third generation cephalosporins (ceftazidime, cefotaxime, ceftriaxone), carbapenems (imipenem / citastatin), aminoglycosides (e.g. gentamicin and amikacin) and fluoroquinolones (Bouza and Cercenado, 2002). Aztreonam and quinolones are also effective treatment options for susceptible isolates, in patients with carbapenem allergy or major beta-lactam allergy (Wong, 2001). Other antibiotics used to treat susceptible *Klebsiella* isolates include ampicillin / sulbactam, piperacillin / tazobactam, ticarcillin / clavulanate, amoxicillin / clavulanate, cefepime, levofloxacin, norfloxacin, gatifloxacin, moxifloxacin and meropenem (Paterson, 1999; Obiamime and Berkowitz, 2002).

All these antimicrobial agents may be used as monotherapy or combination therapy. During 1970s, all the evidences accumulated indicated that adequate therapy could only be achieved by administering more than one antibiotic, leading to the widespread practice of combination therapy using beta-lactam with an aminoglycoside (Klastersky and Zinner, 1982; Giamarellou, 1986) or two beta-lactams (Middleman et al., 1972; Fainstein et al., 1984). Empirical combination therapy has been often used in clinical practice due to emerging problem of drug resistance and the need to a “get it right first time”. Moreover, better clinical outcome has been demonstrated with combination therapy (Chastre and Fagon, 2002). Superiority of the treatment with combination therapy over the monotherapy for sepsis has been documented in several other studies (Mouton, 1999; Wu et al., 1999; Chastre and Fagon, 2002). Some experts disagree with the use of combination therapy and recommended monotherapy (Hoepelmann et al., 1988; Korvich et al., 1992). Paul et al. (2004)
on the basis of meta-analysis have recommended β-lactam monotherapy for the
treatment of sepsis. According to these workers addition of an aminoglycoside to
β-lactam should be discouraged as the fatality remains unchanged, while the risk
for adverse events is increased when compared to β-lactam monotherapy.
However, observational studies have shown that 50% of those with Klebsiella
bacteraemia (Korvick et al., 1992) and 56% of patients with septic shock in the
intensive care units (Leone et al., 2003) are given β-lactam aminoglycoside
combination therapy. Keeping in view the increasing burden of the bacterial
resistance to β-lactams, hospital clinicians tend to use combination therapy
(Barlow, 2004).

Aminoglycosides

All aminoglycosides are polycations and their polarity in part is
responsible for their pharmacokinetic properties (Acar et al., 1976). Though
these compounds are toxic as compared to other classes of antibiotics, yet are
very useful primarily in infections caused by K. pneumoniae (Craig et al., 1991).
Traditional intermittent therapy of using aminoglycoside is based upon the
premise that higher serum concentrations of these drugs had been proven to be
toxic in experimental animals. However, in late 1980s and early 1990s several
studies were published that demonstrated the advantages (reduced toxicity) of a
single daily dose of an aminoglycoside compared to the conventional divided
doses administered twice or thrice per day (Sturm, 1989; Ter Braak et al., 1990;
Nordstron et al., 1990; DeVries et al., 1990; Prins et al., 1993; Nicolau et al.,
1995; Freeman et al., 1997). Dosing schedules of aminoglycosides and their in
vivo efficacy on gram-negative bacteria like K. pneumoniae depends upon the
ratio of their peak concentration achievable in serum to the minimum inhibitory
concentration of that bacteria and it is also affected by their density, growth rate,
metabolic activity as well as the location of the site of infection (Gerber et al.,
1990). These workers further proved that the activity of these drugs was mainly
dependent upon the amount of the total antibiotic present in the serum either as
short lasting relatively higher concentration or long lasting relatively lower
concentration of the drug.
The most important aminoglycosides include amikacin, streptomycin, kanamycin and gentamicin. Amikacin, which is a derivative of Kanamycin A, has a broader range of antimicrobial activity than any other aminoglycosides (Kawaguchi and Naito, 1972). This drug has been found to be effective against nearly all strains of *Klebsiella, Escherichiae* and *Enterobacter* that are resistant to many other antibiotics like gentamicin, tobramycin and penicillins (Vigano et al., 1995). Amikacin has good *in vitro* activity against extended spectrum beta-lactamase (ESβL) producing *K. pneumoniae* strains and it has became the antibiotic of choice against such strains (Szabo et al., 2002). Infections by strains producing ESβLs and cephalosporinases can be successfully controlled by using amikacin in combination with cefotaxime and a β-lactamase inhibitor like clavulanic acid (Roussell-Delvallez et al., 1997). Elkhaili et al. (1997) studied the effect of a combination of amikacin with extended spectrum cephalosporins, cefepime and cefpirome by means of time kill curves on ESBL producing strains of *K. pneumoniae*. These workers found that complete bacterial killing could be achieved before 6 hours in case of a combination of amikacin with either of the cephalosporins. Similar studies done by O'Hara et al. (1997) have shown that amikacin could have synergistic effect when combined with other cephalosporins and β-lactams. Amikacin in combination with imipenem is therapy of choice against ESβL producing *K. pneumoniae* (Szabo et al., 2002). The use of amikacin has become effective and safer with the application of improved methods like liposomal entrapment and targeted drug delivery (Xiong et al., 1999). Zhang and Zhu (1999) have used a novel method of preparing amikacin entrapped in liposomes composed of soyabean, lecithin and cholesterol, to control bacterial infections and to improve the efficacy of amikacin entrapped in liposome.

**Third generation cephalosporins**

Because of the proliferation of SHV-1 lactamases which provide resistance against ampicillin and penicillins, new broad spectrum cephalosporins were introduced in clinical practice in 1980s (Paterson, 1999). These antibiotics, commonly referred to as third-generation cephalosporins, include agents such as
ceftazidime, ceftriaxone and cefotaxime which are stable to the effects of both TEM-1 and SHV-1. Because of the real or perceived problems with aminoglycosides, many neonatal units have elected to use third generation cephalosporins for empirical treatment of suspected late onset sepsis (Isaacs, 2001).

Third generation cephalosporins are broad spectrum antibiotics active against most gram-negative and many gram-positive organisms (Isaacs and Moxan, 1999; Mandell, 1988). Cephalosporins inhibit bacterial cell wall synthesis in a manner similar to that of penicillins. Cephalosporin antibiotics containing an oxyimino side chain represent major advance in antibiotic development. The merger of the oxyimino chain and a 2-amino-5-thiazolyl nucleus (in antibiotics such as ceftriaxone, cefotaxime and ceftazidime) resulted in stability to the effects of the common TEM-1 and SHV-1 β-lactamases produced by gram-negative bacilli such as *K. pneumoniae* and *E. coli* (Paterson et al., 2004).

Ceftazidime, a semi-synthetic broad spectrum antibiotic, is currently used as empiric monotherapy for serious infections caused by *Klebsiella pneumoniae* (De Pauw et al., 1983; Pizzo et al., 1986; Freifeld et al., 1995). DePauw et al. (1994) in their study found that in microbiologically documented infections, ceftazidime actually produced more favourable results in bacteremias caused by gram-negative bacteria, accomplishing an eradication rate of 95% compared to 77% with combination therapy. Both, the microbiological outcome and satisfactory response rates for ceftazidime alone corresponded with those reported for the long course of amikacin plus ceftazidime. Similar observations have also been reported in other previous trials (Bodey et al., 1990; Rolston et al., 1992).

*K. pneumoniae* is a pathogen that frequently present resistance to cephalosporins through the production of extended spectrum β-lactamases (Decre et al., 1998; Paterson, 1999). A number of studies have demonstrated a strong correlation between the use of third generation cephalosporins especially ceftazidime and antibiotic resistance in *K. pneumoniae* (Patterson, 2001). Within a few years of the commercial release of ceftazidime, *K. pneumoniae* that
harbored mutated versions of the parent SHV enzyme (which could hydrolyze this antibiotic) was detected (Jacoby, 1991). Transferable plasmids frequently carried determinants of resistance to cephalosporins as well as other classes of antibiotics, particularly the aminoglycosides (Jacoby, 1994; 1997). However, several institutions have reported a decrease in the prevalence of ESβL producing \textit{K. pneumoniae} with a shift in antibiotic utilization from third generation cephalosporins to other broad spectrum drugs (Patterson, 2001). Subha and Ananthan. (2000) have advised to discontinue the use of all third generation cephalosporins against \textit{K. pneumoniae} which appear to be resistant to any drug of this group. It is suggested by Obiawime and Berkowitz (2004) that although third generation cephalosporins provide coverage for community acquired infections caused by non-ESβL—producing \textit{K. pneumoniae} isolates, the widespread use of these drugs may be associated with further selection of antibiotic resistance

**Quinolones**

Gram-negative isolates of enterobacteriacea, the major pathogens causing septicaemia (Das \textit{et al.}, 1999; Kapoor \textit{et al.}, 2000) frequently develop resistance to penicillins as well as to extended spectrum cephalosporins in quite large number, making it clear that these drugs alone may be ineffective (Roy \textit{et al.}, 2002). The high frequency of resistance to β-lactam antibiotics can well be due to their indiscriminate use as first line drugs (Obiamiwe and Berkowitz, 2004). Antimicrobial resistance to other traditional first line agents (e.g. sulphonamethoxole-trimethoprim and aminoglycosides) has shifted the attention of clinicians towards fluoroquinolones as empiric and definitive treatment of sepsis (Hooton and Stamm, 1997). A study done by Khaneja \textit{et al.} (1999) have found quinolones to be effective in treating multi-drug resistant gram-negative infections in patients including premature and extremely low birth weight infants.

The quinolones are a group of synthetic antimicrobials characterized by a broad spectrum of activity, favourable pharmacokinetic profiles, a unique mechanism of action and availability in both oral and parenteral dosage forms (Andriole, 1998). Primary target of fluoroquinolone group of antibiotics appears...
to be DNA gyrase, an enzyme that plays an important role in DNA replication, repair, recombination and transposition (Gooding et al., 1976). First quinolone antibiotics, nalidixic acid, cinoxacin and oxolinic acid, were introduced in the 1960s for treating urinary tract infections caused by gram-negative bacilli (Wright et al., 2000). The addition of a fluorine substituent to the main quinolone ring substantially widened their spectrum of antibacterial activity. More than a decade ago ciprofloxacin, the first fluoro-quinolone with broad indications was released for clinical use in United States (Hooper, 1991). The release of ofloxacin, which also had broad applications, followed thereafter (Hooper, 2000). Extensive use of these two agents, was not only because of their increased activity against gram-negative bacilli with relatively few side effects (Hooper, 1998) but also because, the potential for the development of resistance to these agents was predicted to be very low (Neu, 1987; Roy et al., 2002). Further, lack of anti-anaerobic activity of these compounds was helpful in preserving the anaerobic component of the gut microflora, which is of crucial importance. Apart from these merits, the relative tolerability of fluoroquinolones led to widespread use and misuse of these agents in a variety of settings (Hooper, 1998). Less than a decade of their release in the United States, fluoroquinolones were reported to be the second most commonly used antibiotic class in hospitals and the most common antibiotic class used in long term care facilities (Thomas et al., 1997). Use of other quinolones such as norfloxacin, enoxacin and lomefloxacin is limited to treatment of infections of genitourinary tract. Numerous new fluoroquinolones have been introduced in the past 5 years e.g. moxifloxacin and gatifloxacin and other compounds such as gemifloxacin and sitafloxacin are on the horizon (Wright et al., 2000).

Because of the intrinsically low minimum inhibitory concentrations (MICs) of the fluoroquinolones to E. coli and K. pneumoniae, it was believed that emergence of fluoroquinolone resistance in these organisms was unlikely (Lautenbach et al., 2002). Therefore, quinolones had been widely used to treat infection caused by K. pneumoniae. However, increasing reports of fluoroquinolone resistance in E. coli and K. pneumoniae have appeared (Kern et al., 1994; Commetta et al., 1994; Hooper, 2000). While most K. pneumoniae
isolates remain fluoroquinolone susceptible, recent reports suggest the prevalence of fluoroquinolone resistance is increasing. In a survey of bloodstream isolates, fluoroquinolone resistance rates in *E. coli* and *K. pneumoniae* were from 3% to 4% in North America and from 3% to 8% in Europe as compared with rates of less than 1% seen only several years before (Archibald *et al.*, 1997; Pfaller *et al.*, 1997; Fluit *et al.*, 2000). It has been suggested by Lautenbach *et al.* (2002) that the recent fluoroquinolone overuse, has been the risk factor for fluoroquinolone resistance.

**Effect of antibiotics on bacteria**

The activity of antibiotic against clinically significant bacteria is usually expressed in terms of concentrations that either inhibit growth or kill microorganism *in vitro*. Concentration of antibiotic that are less than the MIC are defined as sub-MIC. Evidence is available that antibiotics may be beneficial to infected hosts even when present at sub-MIC concentration (Zak and Kradolfer, 1979). According to these workers this affect appears to be independent of the bactericidal potential of the antibiotic but is related to structural or metabolic changes, which augment the susceptibility of bacterial pathogens to humoral and cellular defenses. Therefore, antibiotics at sub-MIC that act in concert with host defenses may be clinically significant (Adinolfi and Bonventre, 1988). In human body, the concentration of antimicrobial agents is often below the MIC level (Moellering, 1994). It is questionable, therefore, whether such sub-MICs of antimicrobial agents contribute to therapeutic effects. Although the primary targets of most antibiotics are known, there are few reports on the secondary effects of antibiotics on bacteria (Taylor *et al.*, 1982; Gemmell *et al.*, 1983; Rogers and Thurman, 1983). Such secondary effects are of interest, especially with regard to sub-inhibitory concentrations of antibiotics, which can appear in body fluids and tissue during the course of chemotherapy.

One of the effects of antibiotic usage is the change in morphology of bacteria. Gram-negative bacteria tend to form filaments when β-lactam antibiotics are present in the growth medium at MIC and sub-MIC level (Lorian and Atkinson, 1984). Morphology change induced by antimicrobials relates in
part to the affinity of the drug for penicillin binding proteins (PBPs) (Dofferhoff et al., 1991; Jackson and Kropp, 1992; Prins et al., 1994, 1995; Moore et al., 2004). PBPs are the primary biochemical targets of β-lactam antibiotics in bacteria. These are responsible for the synthesis, shape and integrity of the bacterial cell wall, and the drugs with identical MICs but differing PBP specificities may induce different changes in cell morphology (Horri et al., 1999). Selective affinity for PBP 1a and PBP 1b causes rapid and extensive killing of the bacteria, with the degradation of cell wall material and cellular lysis (Prins et al., 1994). Examples of antibiotics that are selective for PBP1 are cephaloridine and cefsulodin (Neu, 1983, 1985; Tomasz, 1986). Antibiotics having selective affinity for PBP2 cause conversion of the bacilli to round cells (also called spheroplast); this is followed by loss of viability but is not accompanied by extensive cell wall degradation (Prins, 1995). Antibiotics selective for PBP2 are mecillinam, clavulanic acid and imipenem. At higher concentrations, imipenem also inhibits PBP1b (Neu, 1985). Inhibitors of PBP3 cause selective inhibition of bacterial septation, which leads to the formation of long filaments, but only limited bactericidal activity and lysis (Neu, 1983; Spratt et al., 1988; Simon et al., 1991). Antimicrobials selective for PBP3 are aztreonam, piperacillin, and mezlocillin. At low concentrations, ceftazidime, cephalexin, cefotaxime, and cefuroxime inhibit PBP3 whereas, at high concentrations, these antibiotics also have affinities for PBP1a (Tomasz, 1986). Several studies have reported filament formation in presence of sub-MIC and bactericidal concentration of ceftazidime (Ohya et al., 1991; Horri et al., 1998a, 1999; Kishi et al., 1999; Yokochi et al., 2000).

In addition, antimicrobials belonging to quinolone group, which mainly act by inhibiting enzyme DNA gyrase are known to induce filamentation in bacterial cells (Vanden Berg et al., 1992; Sonstein and Burnham, 1993; Crosby et al., 1994). It has been demonstrated by Prins et al. (1994) that treatment with ciprofloxacin on cell morphology was very similar to the effects of treatment with PBP-3 specific antibiotic i.e. filamentation with an increase in non-viable bacterial biomass. On the contrary, aminoglycosides, which inhibit protein synthesis do not bring any morphological change (Simon et al., 1991; Crosby et al., 1994).
Antimicrobials belonging to this group result in stabilization of bacterial numbers, with the loss of viability of bacteria in the absence of lysis (Prins et al., 1994).

Exposure of bacteria to antibiotics not only bring about morphological changes but also affect the physicochemical integrity of bacterial membranes. The polycationic antibiotics permeabilize the outer membrane by binding to LPS and displacing divalent cations, thereby perturbing the normal bacterial surface architecture (Nikaido and Varra, 1985). Among the members of enterobacteriaceae, the OMP expression has been reported to be altered under various environmental stresses (Schmid et al., 1991; Werts et al., 1992; Sahu et al., 1994; Chhibber and Bhardwaj, 2004) including antibiotic pressure (Kadurugamuwa et al., 1985; Lun et al., 1994,1997; Domenech-Sanchez; 1999; Chevalier et al., 1999; Emmanuella De et al., 2001). Changes in the surface structure by antimicrobials influences host parasite relation such as bactericidal activity, opsonophagocytosis and complement consumption (Overbeek and Veringa, 1991; Gholia et al., 2004). Outer membrane proteins (porins) of K. pneumoniae have been implicated in permeability to antimicrobial agents (Nikaido, 1994; Martinez-Martinez et al., 1996). The loss of porins in K. pneumoniae strains producing expanded-spectrum β-lactamases ESβLs has been shown to cause increased resistance to third-generation cephalosporins, monobactams and fluoroquinolones (Martinez-Martinez et al., 1996).

However, it has been demonstrated by Domenech-Sanchez et al. (1999) that other porins may replace the function of the lost porins. These workers have identified and charcterized a new porin (omp K37) of K. pneumoniae, whose expression is stimulated under antibiotic pressure. The functional characteristics (a narrow pore) of this new porin may be advantageous over those of the “classical” ompC or ompF type porins. Similarly, Sengupta et al. (1992) and Deb et al. (1995) detected the expression of new proteins of molecular weights 12 kDa and 25kDa respectively in the outer membrane proteins of the bacterial cells grown in presence of beta-lactam antibiotics. In a relatively recent study Emmanuelle et al. (2001), reported a strain of E. aerogenes in which there was change in porin organization/function resulting in substantial decrease in
cephalosporin penetration. Number of previous studies have demonstrated
difference in recognition of protein epitopes by serum which is raised against the
bacteria pre-exposed and unexposed to sub-MIC or MIC concentration of
antibiotics (Osborn, 1980; Neidhart et al., 1987; Lun et al., 1997). These workers
have suggested that the observed difference in the OMP profile on antibiotic
treatment may be due to the re-arrangement of bacterial proteins revealing new
antigenic determinants. It has been recognized that antibiotic treatment causes
reduced production of capsular polysaccharide (Kadurugamuwa et al., 1985;
Keller et al., 1991; Gholia et al., 2004). This could result in exposure of outer
membrane antigens, which were otherwise occluded (Kadurugamuwa et al.,
1988). Capsular material was reduced considerably after treating K. pneumoniae
with penicillins as compared with untreated control and it was slightly reduced by
quinolones. But, no change in the capsule structure was observed with
aminoglycosides (Nomura et al., 1995). Cephalosporins are known to reduce the
thickness of the capsular material layer to render K. pneumoniae more
susceptible to phagocytic activity as the negative charge on the bacterial surface
gets reduced and it leads to decrease in physical repulsion between the bacteria
and phagocyte (Nomura and Nagayama, 1995).

Apart from bringing morphological changes and alterations in structure
and OMP profile of the organisms, antibiotics have the potential to release
important biologically active molecules from them. One of these is endotoxin
[lipopolysaccharide (LPS)], which is a constitutive component of the outer
membrane of gram-negative bacteria (Holzheimer, 2001; Giamarellos-Bourboulis
et al., 2003). On the basis of results of scanning and transmission electron
microscopy, it was demonstrated by Rosenthal et al. (1976) that antibiotics result
in the formation of numerous protrusions or blebs on the surface of E.coli with
apparent release of membrane residues. This observation was confirmed by
Goodell et al. (1978), who demonstrated increased shedding of lipid and LPS
into the medium on exposure to penicillin. Pathogens for which antibiotic induced
endotoxin release is a clinical problem include K. pneumoniae, E. coli, P.
aeruginosa, Haemophilus influenzae and Neisseria meningitidis (Shenep et al.,
Antibiotics differ in their potential for endotoxin release and this has been related to the type of antibiotic used and bacterial strain studied (Prins et al., 1994).

Significant differences in endotoxin liberating potential have been demonstrated between various β-lactam antibiotics, which are cell wall active and specifically targets penicillin binding proteins (PBPs) (Prins et al., 1994). Among third generation cephalosporins, ceftazidime has been found to release high levels of endotoxin in vitro (Eng et al., 1993; Horri et al., 1998a) as well as in vivo (Horri et al., 1998b, 2000). The reports in literature on the endotoxin liberating ability of PBP-2 and PBP-3 specific antibiotics in clinical situations are conflicting (Luchi et al., 2000, Simpson et al., 2000; Holzheimer et al., 2000). For example, Mock et al. (1995) during the treatment of septic trauma patients, observed high level of endotoxin in plasma with PBP-3 specific antibiotic. Similarly, Simpson et al. (2000) in a study conducted in patients with severe melioidosis have found, high endotoxin release with PBP-3 specific antibiotic (ceftazidime) as compared to PBP-2 specific antibiotic (imipenem). However, in a recent study, Giamarellos-Bourboulis et al. (2003) failed to observe rise in levels of endotoxin in cases of pyelonephritis following treatment with PBP-3 specific drug.

Similarly, quinolones, which do not target the cell wall, have been reported to be relatively potent inducers of endotoxin release (Prins, 1995). With ciprofloxacin and ofloxacin high levels of endotoxin liberation from E. coli cells has been demonstrated (Eng et al., 1993; Lamp et al., 1997). Similar observation has been made in earlier studies as well (McConnell and Cohen, 1986; Vander Berg et al., 1992). On the contrary, aminoglycosides seem to be antibiotics with only modest endotoxin liberating ability (Dofferhoff et al., 1991, 1993). It has been demonstrated by Simon et al. (1991) and Bingen et al. (1992) that exposure of E. coli cells to amikacin resulted in only modest endotoxin release. In another study endotoxin release from K. pneumoniae was low when gentamicin was used in the experiments (Eng et al., 1993). Infact, gentamicin and other aminoglycosides can inhibit LPS synthesis (Kreger et al., 1980).
Few studies in literature have compared the effect of bacterial strain on antibiotic induced endotoxin release. Eng et al. (1993) demonstrated that for E. coli and K. pneumoniae, exposure to gentamicin, ciprofloxacin, ofloxacin and imipenem resulted in less endotoxin release than what was observed with ceftazidime. But for Pseudomonas aeruginosa, ceftazidime and imipenem exposure produced equal amount of endotoxin. Horri et al. (1998b) also compared the endotoxin releasing potential of ceftazidime and carbapenems in different gram-negative bacteria. These workers reported that in all strains (K. pneumoniae, E. coli, Serratia marcescens, Proteus vulgaris and Proteus mirabilis) except Pseudomonas aeruginosa the use of ceftazidime induced release of significant amount of endotoxin than carbapenems.

The varying potential of the antimicrobials to induce endotoxin release is likely due to the differences in their mode of action (Jackson and Kropp, 1992; Crosby et al., 1994). Beta-lactam antibiotics specifically target penicillin-binding proteins (PBPs) with selective affinity for individual PBPs (Prins et al., 1994). Antibiotics, such as ceftazidime and cefotaxime, which are associated with large amount of endotoxin release have selective affinity for PBP-3. This is known to cause disruption in bacterial septation leading to abnormal growth and filamentation. Association between this type of change in morphology and endotoxin release could also be due to increased cell mass from where continuous liberation of LPS takes place (Eng et al. 1993; Horri et al., 1998a). In another study by Yokochi et al. (2000) it has been proposed that higher levels of in vivo endotoxin release following treatment of P. aeruginosa infection with ceftazidime might be partly due to decreased clearance of ceftazidime treated filamentous bacteria by phagocytes. Quinolone group of antibiotics are also associated with filamentation and increase in cell biomass. The explanation offered for the high amount of endotoxin release is the lysis of increased biomass (Crosby et al., 1994). In case of aminoglycosides, there is no increase in cell biomass, which could be one reason for lower levels of endotoxin detected with this group of antibiotics. The ability of aminoglycosides to bind and neutralize the released endotoxin, might be another reason for low endotoxin
levels (Foca et al. 1991). Polymixin B is another antibiotic which also possess the endotoxin neutralizing ability (Coyne et al., 1993).

LPS molecule is known to be toxic to host due to lipid A moiety, which is hidden within the micellar structure of this molecule (Rietschel et al., 1996). However, endotoxin molecule released in response to antibiotic exposure is different, where lipid A is readily available to interact adversely with host cells (Leeson and Morrison, 1994). Although, LPS (lipid A) is capable of causing direct cytotoxic influences, most of the biological action of endotoxin operates through the activation of macrophages, monocytes, endothelial cells and fibroblasts. As a result there is production of potent inflammatory mediators such as TNF-α, IL-1β, IL-6 and nitric oxide (Morrison et al., 1994; Alexander et al., 2001). Thus, antibiotics causing large amount of endotoxin release are associated with increased production of TNF-α levels. Elevated levels of TNF-α have been described in sepsis, where it contributes significantly to multiple organ failure, shock and death (Dekimpe et al., 1995; Cusumano et al., 1997; Kirikae et al., 1998; Yao et al., 1998). There are conflicting reports in literature regarding the amount of TNF-α levels induced on antibiotic exposure. Simon et al. (1991) found that exposure of *E. coli* to ceftazidime (third generation cephalosporin) induced greater amount of TNF-α levels. In another study, administration of the same antibiotic in experimentally induced meningitis, failed to detect elevated levels of TNF-α in CSF (Friedland et al., 1993). The differences observed in TNF-α levels in serum and CSF, could be because of the less degree of inflammation in CSF (a closed space) and hence may differ from response that occur outside the central nervous system. However, third generation cephalosporin in studies related to endocarditis and urosepsis have shown to induce raised levels of TNF-α in serum and urine (Mohler et al., 1994 and Prins et al., 1995). In a recent study Giamarellos-Bourboulis et al. (2003), failed to detect any rise in levels of TNF-α in serum while treating cases of pyelonephritis with antibiotics belonging to three different groups i.e. third generation cephalosporins, aminoglycosides and quinolones.
Adjunct therapies for treatment of sepsis

Antibiotics, although are thought to be the most effective form of therapy against infections caused by microorganisms, these are frequently ineffective due to the innate resistance of microbes to these agents. In sepsis, which is a multi-factorial syndrome, administration of antibiotics alone may not suffice. It has been observed over the last 20 years that new antibiotics and increasingly sophisticated critical care have had little impact on the mortality rate of septic shock (Reidemann et al., 2003). No single intervention alone has been successful in managing this syndrome (Nasraway, 2003). The attention of the scientists has therefore been drawn to adjunct therapies for the purpose. These therapies in combination with antimicrobial therapy are considered equally important strategies. Based on past evidence that in sepsis, hyperactivation of inflammatory system is an important feature, most therapeutic strategies have targeted pro-inflammatory mediators. Fig. 4 shows the various clinical trials carried out in the treatment of sepsis. In this figure various strategies in clinical trials are grouped according to a common target.

The history of clinical trials for treatment of sepsis extends back to 1960s, when preclinical studies reported that high doses of glucocorticoids in models of E.coli and endotoxic shock improved survival. These studies prompted the initiation of human sepsis trials (Hinshaw et al., 1967; 1978). Between 1959 and 1986, investigators administered short courses of high doses of corticosteroids to almost 1300 septic patients in nine clinical trials (Bone, 1991). However, administration of high dose corticosteroids had no benefit in patients with septic shock, rather it increased the secondary infections among patients (Lefering and Neugebauer, 1995; Cronin et al., 1995; Zeni et al., 1997). In recent years, a new rationale for the use of corticosteroids has emerged. The relative adrenal insufficiency has been reported in severe septic shock, which is responsible for many complications (Hatherill et al., 1999). Adrenal insufficiency during sepsis is indicated by high cortisol level and an attenuated response to corticotropin stimulation (Annane et al., 2000). These findings suggested that impaired adrenal reserve occurred in septic shock and implied that administering
**Fig. 4:** Clinical trials in sepsis. Various strategies in clinical trials are grouped according to a common target

<table>
<thead>
<tr>
<th>Intervention</th>
<th>Target</th>
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<tbody>
<tr>
<td>Glucocorticoids (IVIG)</td>
<td>Immune response</td>
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<tr>
<td>IVIG (Improvement of host defenses)</td>
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<td>Anti-endotoxin antibodies (BPI)</td>
<td>LPS</td>
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<td>BPI (neutralizes LPS)</td>
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<tr>
<td>LPS elimination (homofiltration)</td>
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<td>TNF-α antibodies</td>
<td>TNF-α</td>
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<td>Soluble TNF receptor</td>
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<td>IL-1 receptor antagonist</td>
<td>IL-1</td>
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<tr>
<td>Phospholipase A2 antagonist (reducing PAF)</td>
<td>PAF</td>
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<td>PAF antagonist</td>
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<td>PAF-acetylhydrolase (PAF inactivation)</td>
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<tr>
<td>Prostaglandin E1</td>
<td>Arachidonic acid metabolites</td>
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<td>Thromboxane inhibitors</td>
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<tr>
<td>Ketoconazole (thromboxane synthetase inhibitor)</td>
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<td>Ibuprofen (cyclooxygenase inhibitor)</td>
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<tr>
<td>Antioxidants:</td>
<td>Oxygen radicals</td>
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<tr>
<td>N acetylcysteine (restoration of cellular antioxidant (potential)</td>
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<tr>
<td>Selenium (selenium-dependent glutathione peroxidase as O₂⁻ scavenger)</td>
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<tr>
<td>L-NAME (NOS inhibitor)</td>
<td>NO</td>
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<tr>
<td>L-NMMA (iNOS inhibitor)</td>
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<tr>
<td>Methylene blue (guanylyl cyclase inhibition)</td>
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<tr>
<td>PHP (NO scavenger)</td>
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<tr>
<td>AT-III (inhibition of thrombin, Factors IXa, Xa and XIIa)</td>
<td>Coagulation/inflammation</td>
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<td>TFFP (inhibition of Factors X and IX)</td>
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<td>APC (inactivation of Factors Va and Vlla)</td>
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<tr>
<td>IFN-γ (reactivation of neutrophil immune functions)</td>
<td>Neutrophil activation</td>
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<tr>
<td>G-CSF, GM-CSF (increase of immune competent blood cells)</td>
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<tr>
<td>PGG-glucan (increasing phagocytosis and bacterial killing in PMN)</td>
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<tr>
<td>Bradykinin antagonist</td>
<td>Bradykinin</td>
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<tr>
<td>Pentoxyfylline (phosphodiesterase inhibitor, cAMP increase)</td>
<td>Phosphodiesterase</td>
</tr>
<tr>
<td>C1 inhibitor (inhibition of classical and lectin pathway activation)</td>
<td>Complement system</td>
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</table>

IVIG, Intravenous immunoglobulin; BPI, bactericidal/permeability increasing protein; PAF, platelet-activating factor; L-NAME, L-arginine methyl ester; L-NMMA, N-methyl-l-arginine; PHP, pyridoxylated hemoglobin polyethylene; GM-CSF, granulocyte-macrophage colony-stimulating factor; PGG-glucan, poly-β-D-glucopyranosyl-β-D-glucopyranose.
glucocorticoids in dosages similar to the amount produced physiologically during a stressful state might help to resolve this condition (Luce, 2004). In a meta-analysis, Minneci et al. (2004) concluded that high doses of corticosteroids are not beneficial in patients with septic shock whereas low-doses are beneficial for such patients. Further these workers suggested that response to corticotropin stimulation does not predict benefit from corticosteroid administration, therefore they recommended the use of low doses of corticosteroids in all patients with vasopressor-dependent septic shock. These workers, however, could not definitely identify the optimal dose and timing of steroid administration for treatment. In contrast, recommendations given by Annane et al. (2002) and Luce (2004) differed from Minneci and colleagues. These investigators agreed with the conclusion that low doses rather than high doses of corticosteroids are beneficial in patients with septic shock but also stressed that the corticosteroids benefit only the patients with adrenal insufficiency, whereas these may harm the group of patients without adrenal insufficiency.

Other approach in the treatment of sepsis has been directed towards boosting the innate immune system of host and to overcome the effect of released endotoxin. For this purpose, anti-inflammatory cytokines such as IL-10, IL-4 and IL-13 which have potential for immunomodulation have been employed (Morre et al., 1993). IL-10 is a non-covalently linked homodimeric cytokine that is produced by a large variety of cells including monocytes, macrophages, B and T lymphocytes and natural killer cells (Boontham et al., 2003). IL-10 has anti-inflammatory and immunosuppressive activities. This cytokine strongly inhibits CD4+ T cell proliferation and production of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, IL-8 and IL-12 (De Waal Malefyt et al., 1991). In contrast, IL-10 has stimulatory effects on CD8+ T cell and induce their recruitment, cytotoxic activity and proliferation (Moore et al., 2001). Inhibitory effects of IL-10 on TNF-α and IL-1 production are crucial to its anti-inflammatory activities because these two cytokines often have synergistic activities on inflammatory response. In animal studies, IL-10 when given at appropriate time prevents mortality and diminishes TNF-α release (Gerard et al., 1993; Vander Poll et al., 1995; Latifi et
al., 2002). Other anti-inflammatory cytokines, IL-4 and IL-13 have similar function as IL-10 (Marchant et al., 1995). Certain antibiotics are known to have immunomodulatory and anti-inflammatory properties, in addition to their traditional antimicrobial effects. For example, tetracyclines, doxycyclin and gentamicin exhibit anti-inflammatory action (Marie-Therese, 2000). Immunomodulatory effect has also been attributed to macrolide group of antibiotics (Culic et al., 2001). Recently, Giamarellos-Bourboulis et al. (2004) demonstrated the anti-inflammatory as well as immunoregulatory of clarithromycin (macrolide), which considerably prolong the survival of rabbits suffering from sepsis induced by P. aeruginosa. Similar effects observed with macrolide group of antibiotics have been confirmed by Woo et al. (2004). These workers reported that macrolide group of antimicrobials not only modulate production of cytokines but also have the potential to increase the level of innate immune response operative through macrophages, monocytes, neutrophils and lymphocytes. However, results obtained with this group of antibiotics are not conclusive and precise mechanism of immunomodulation is not well known.

The other most widely known target in sepsis is TNF-α, a strong pro-inflammatory cytokine found in septic patients and correlating with clinical outcome (Riedemann et al., 2003). There are two cell surface TNF soluble receptors present in normal human plasma: p55 (sTNFR-p55) and p75 (sTNFR-p75). Under normal condition TNF-α bound to sTNFR exists in equilibrium with the unbound form (Dinarello, 1997). In human sepsis, sTNFRs are increased and correlate with severity and mortality (Gogos et al., 2000). Neutralizing antibodies against TNF have been shown to prevent mortality in baboon models of lethal gram-negative sepsis (Hinshaw et al., 1990). Anti-TNF antibodies also prevent or attenuate most of the cardiovascular, metabolic and coagulation changes resulting from sub-lethal endotoxemia (Opal et al., 1991). However, clinical trials with anti-TNF antibodies or receptor antagonists did not show efficacy in human sepsis (Zeni et al., 1997; Reinhert and Karzai, 2001). IL-1 is another pro-inflammatory cytokine that evokes similar pathophysiological responses as TNF-α. Use of interleukin-1 receptor antagonist that blocks IL-1
activity by competing for binding to IL-1Rs has been found to reduce mortality in an animal model of endotoxic shock (Ohlsson et al., 1990). However, human clinical trials again did not show protective effects (Opal et al., 1997, Zeni et al., 1997).

Other strategies for clinical anti-inflammatory intervention of sepsis, includes inhibition of platelet-activating factor (Dhainaut et al., 1994), arachidonic acid metabolites such as prostaglandin E1 and thromboxane (Bernard et al., 1997; Vincent et al., 2001), oxygen radicals (Spies et al., 1994), nitric oxide (Schilling et al., 1993) and bradykinins (Fein et al., 1997). In addition, researchers have also tried to reverse the state of immunosuppression known to occur in sepsis, particularly involving neutrophils. Interferon (IFN-γ) and granulocyte colony-stimulating factor (G-CSF) have been given intravenously to septic patients (Vincent et al., 2002). All these interventions tried, however have proved to be of little benefit for patients with sepsis (Reidemann et al., 2003).

The coagulation system has been an important target for treatment of sepsis (Giudici et al., 1999). The pro-inflammatory cytokines (TNF-α and IL-1) released during sepsis have direct effect on the endothelial surface (Stevens, 2002). As a result, tissue factor, the first step in the extrinsic pathway of coagulation, is expressed on the surfaces of the endothelium and of monocytes. Tissue factor leads to the production of thrombin (pro-inflammatory substance itself). Thrombin results in fibrin clots in the microvasculature. IL-1 and TNF-α also produce plasminogen activator inhibitor-1 (inhibitor of fibrinolysis) (Vervloet et al., 1998). All these events contribute to the pathogenesis of disseminated intravascular coagulation (DIC), microvasculature injury and ultimately development of multi organ dysfunction syndrome (MODS) (Imberti, 2003). Pro-inflammatory cytokines also disrupt the body’s naturally occurring modulators of coagulation and inflammation. These are activated protein C (APC) and antithrombin. These cytokines shear endothelial surface bound protein thrombomodulin and thus prevent conversion of the inactive form of protein to activated protein C (APC) (Stevens, 2002). Because of the strong association of severe sepsis with a state of activation of blood coagulation and the potential
role of capillary thrombosis in the development of MODS, anti-coagulant agents have been tested in the setting of septic shock (Taylor et al., 1987; Guidici et al., 1999).

Three different anti-coagulation strategies have been used in clinical sepsis trials; tissue factor pathway inhibitor; anti-thrombin (AT)-III and activated protein C (APC) (Riedemann et al., 2003). Preclinical and clinical evidence has been provided by Creasey and Reinhart (2001), that recombinant tissue factor pathway inhibitor significantly reduces thrombin generation and mortality in animals as well as humans with sepsis. The rationale for employing anti-thrombin (AT) concentrates in the treatment of DIC associated to sepsis is based on the consideration that AT plasma levels are always decreased in patients with sepsis or septic shock; further, the degree of the decrease is directly proportional to the severity of the disease and the prognosis (Imberti, 2003). Results presented by Warren et al. (2001), demonstrated no substantial improvement with AT-III in septic patients even though a sub-group of septic patients not receiving concomitant heparin seemed to benefit in terms of survival. However, recently Imberti (2003) have suggested that anti-thrombin has double role, affecting coagulation as well as inflammation. It decreases the plasma level of mediators of inflammation in some cases and prevents organ failure leading to reduced mortality. Another substance that has been tried experimentally as well as in clinical studies is protein C (Bernard et al., 2001; Steven, 2002). These workers reported that administration of protein C, known to be anti-thrombotic and profibrinolytic, decreased mortality. Beside these effects APC has anti-inflammatory properties, including inhibiting the production of pro-inflammatory cytokines by LPS-stimulated monocytes, inhibiting leukocyte adhesion and rolling and inhibiting neutrophil accumulation (Murakami et al., 1997; Mizutani et al., 2000). However, the recognized limitation of using protein C is that it is associated with increased risk for bleeding and this therapy tends to be expensive (Nasraway, 2003; Minneci et al., 2004).
Immunoprophylactic approach

All the strategies employed for the treatment of sepsis, whether these are antibiotics or adjunct therapies, have inherent limitations. Therefore, attention of scientists has been shifted towards more specific means of control of this syndrome. Strategies directed against bacterial components have the potential advantage over anti-inflammatory mediator therapy or antibiotics, as these might not compromise host defenses (Cross et al., 1999). Since, certain patient sub-populations are considered to be at particular risk of developing sepsis, the need for developing effective vaccine that either prevent or ameliorate the course of sepsis following infection with gram-negative bacteria is well recognized (Cross et al., 2004). As, outer surface components of bacterial cells are exposed, these play an important role in establishment and maintenance of infection in humans and thus are obvious targets for vaccine development (Cross et al., 2001).

Most widely used vaccines against Klebsiella mediated infections have relied upon capsular polysaccharide (CPS) because of several reasons; Firstly, almost all K. pneumoniae strains are potent producers of a characteristic heavy polysaccharide capsule. In addition, CPS represent the outermost layer of surface structure and it has been proven to be highly immunogenic and non-toxic (Cryz et al., 1985; Amprebi et al., 1993; Herias et al., 1997). Capsule in K. pneumoniae is known to play a major role in colonization, adhesion, maintenance and proliferation of bacteria in host, contributing to the pathogenicity (Hornick et al., 1992; Hansen et al., 1999; Chhibber et al., 2003). It was observed by Cryz et al. (1986a) that only 25 serotypes made up 70% of all bacteremic strains. Based on their sero-epidemiological findings Cryz et al. (1986b) formulated 24-valent Klebsiella CPS vaccine that has been proven to be safe and immunogenic. To date this vaccine seems to be the most promising approach of preventing sepsis caused by Klebsiella and has already passed phase I human trial (Edelman et al., 1994; Podschen and Ullman, 1998). Though, there are reports of successful testing of CPS vaccine in human trials, however the success has only been partial due to the type specificity of CPS based vaccines (Trautmann et al., 1988; 1994). There are 77 different K-
serotypes of CPS known in this genus with no particular predominance of any serotype (Podschun and Ullmann, 1998; Hansen et al., 1999). These drawbacks limit the potential of K-antigen based vaccines in *K. pneumoniae* and focus has now shifted to O-antigen.

O-antigen in *K. pneumoniae* has been generally considered to be masked by the capsular polysaccharide and thus not exposed on the surface. However, Tomas et al. (1991) reported the surface exposure of the O-antigen together with CPS. Several workers have found that antibodies specific to LPS O-antigen can penetrate through capsule of strains belonging to different capsular serotypes including K2 serotype (Meno and Amano, 1990; Tomas et al.; 1991; Jong et al., 1995). This observation has been confirmed by Held et al. (2000) who demonstrated that monoclonal antibodies against O-antigen of *K. pneumoniae* exert opsonic activity depending on the CPS serotype. The protective potential of these antibodies has been demonstrated by several different investigators (Mandine et al., 1990; Rukavina et al., 1997; Trautmann et al., 2004). Besides, being surface exposed and protective antigen, LPS O-antigen appears promising particularly in *K. pneumoniae* as only nine LPS O-serogroups have been recognized for this genus. Further, among nine serogroups, four of these account for more than 82% of the O-antigen serotype found in clinical isolates (Trautmann et al., 2004). Out of these O1 serogroup is most commonly isolated from clinical samples (Hansen et al., 1999).

However, active immunization with complete molecule of lipopolysaccharide (LPS) is associated with adverse toxic reactions. Most of the toxicity of LPS has been linked to lipid A component, which limits its potential use for vaccine development (Rietschel et al., 1984). Consequently, several attempts have been made to circumvent the toxicity associated with native LPS and to improve the immunogenicity of these antigens. Treatment of LPS with polymyxin B, sodium borohydride or alkali causes its detoxification without destroying antigenic determinants (Morrison and Jacobs, 1976; Von Eschen and Rudback, 1976; Stokes et al., 1989). But, detoxified LPS prepared by these
methods was found to be poor immunogen and at a high dose was found to induce pyrogenicity and schwartzman reaction in animals (Kabir et al., 1987; Rani et al., 1990). Another approach of detoxifying LPS is its treatment with mild acid hydrolysis, which results in separation of O-polysaccharide moiety (O-PS) from lipid A component of LPS (Konadu et al., 1994; Chhibber and Bajaj, 1995). A major drawback, however of using polysaccharide antigens is that, these are T-cell independent antigens and induce mainly IgM antibody response (Mosier and Subharao, 1982). Young children less than 2 years of age respond poorly to these antigens. In addition, with these antigens there is no affinity maturation of the antibody response, no immunologic memory and no enhancement of immune response by adjuvants (Ward and Zangwill, 1999). To circumvent these problems several workers have tried covalent binding of O-specific polysaccharide (O-PS) with protein carriers to develop conjugate vaccines (Cryz et al., 1991; Chhibber and Bajaj, 1995; Passwell et al., 2001; Chernyak et al., 2002).

Conjugate Vaccines

The experiments done by Landsteiner, Avery and Goebel (1929) in the 1920s and 1930s showed that non-immunogenic saccharides (haptens) could be converted into immunogens by covalent attachment to proteins. At that time mechanism involved was not known. Later in 1960s, T helper cells were discovered and their role in helping B cells in antibody response lead to the terminology of T-cell independent (TI) or T-cell dependent (TD) antigens (Lindberg, 1999). With TD antigens, an immune response can occur at, or shortly after birth, affinity maturation of the B cell response takes place, immunologic memory occurs, adjuvants can induce an enhanced response and there is a heterogenous immunoglobulin response (Poolman, 1995). Conjugate vaccines offered a solution to convert the TI antigens to TD antigens (Fig. 5).
Glucoconjugates can be prepared, using a variety of coupling strategies, from surface carbohydrates such as capsular polysaccharides, lipopolysaccharides from bacteria or synthetic saccharides (Ravenscroft and Jones, 2000). The success of *Haemophilus influenzae* type b (Hib) conjugate vaccines has inspired the application of this technology to the most important disease causing organisms worldwide including *Streptococcus pneumoniae*, *Neisseria meningitides*, *Staphylococcus aureus*, *Streptococcus*, gram-negative coliforms (*Klebsiella* and *E. coli*) (Robbins and Schneerson, 1990; Robbins *et al.*, 1999).

There are many advantages of glucoconjugate vaccines: In these vaccines polysaccharide is covalently linked to an appropriate carrier protein which provides saccharide moiety with T-dependent characteristics, so that vaccines are able to produce anamnestic response and are immunogenic in infants (Robbins and Schneerson, 1990). Similarly, polysaccharide enhances immunogenicity of covalently linked protein antigen (Gupta *et al.*, 1994a,b).
addition, conjugate vaccine may eliminate the need for free proteins from combination vaccines, there by reducing the antigenic burden on the immune system and complication due to carrier specific epitope suppression (Herzenberg et al., 1983; Barington et al., 1993). Finally, conjugate vaccines increase immunization coverage and reduce cost of immunization (Gupta et al., 1995).

Basically, three methods have been utilized to construct conjugate vaccines. The approach adopted depends on the chemical structure of the antigen. These three strategies are:

i) Random activation of the native polysaccharide antigen through functional groups (e.g. hydroxyl or carboxyl groups) prior to coupling to a protein carrier which can be native or activated (Vella et al., 1992).

ii) Size reduction of the polysaccharide by hydrolysis, sonication, electron beam fragmentation or chemical treatment followed by activation and conjugation. When depolymerisation of the polysaccharide is carried out by periodate oxidation, then aldehyde (CHO) end groups are generated which can be directly coupled to protein carrier by reductive amination (Anderson et al., 1985).

iii) Chemical synthesis of saccharides with a linker or spacer available for coupling. This method utilizes dihydrazide (ADH), a bifunctional nucleophilic spacer molecule to link two antigens in a conjugate (Schennerson et al., 1986). In this method, cyanogen bromide is used to activate polysaccharide moiety. Then polysaccharide adipic hydrazide derivative is conjugated to protein by carbodiimide mediated condensation.

In a conjugate vaccine, choice of conjugation method is important as it has profound effect on immunogenicity of the final preparation (Chu et al., 1991; Shen et al., 2001). Generally, a method in which spacer is utilized is considered favourable as it reduces steric hinderance and avoids direct contact of the serologically important groups of the antigen (Kossaczka et al., 1997). The demonstration that conjugates made with short chain saccharides are
immunogenic, together with advances in synthetic methodology have made this approach more feasible and this may be the preferred in the future (Pozsgay et al., 1999).

Apart from selection of an appropriate conjugation method, selection of an appropriate carrier is equally important as the immunogenecity of the polysaccharide component of a conjugate is affected by its size, the carrier protein, method of conjugation and the ratio of saccharide to protein (Peeters et al., 1992; Polotsky et al., 1994). Bovine serum albumin was the first protein to gain attention of the workers, to be used as a carrier protein. Since then, it has been utilized by many workers to prepare conjugate vaccines (Konadu et al., 1994; Chernyak et al., 2002). The immunogenicity of Vi capsular polysaccharide of *Salmonella typhi* and structurally related, di-o-acetyl derivative of pectin designated as OAcP was improved by chemically coupling to bovine serum albumin (Kossaczka et al., 1997). Similar findings have been obtained by other workers as well (Fattam et al., 1993; Konadu et al., 1994). However, the use of bovine serum albumin (BSA) is associated with inherent limitation which include: lot to lot variation in BSA preparation, instability of the conjugate, protease related degradation and induction of allergic reactions in small percentage of infused patients (Morales et al., 1994; Fiocchi et al., 1998; Michael et al., 2003). Although in recent past recombinant albumin has been developed, that has appreciably improved its recovery and purity, but the cost and labour involved is the main barrier to acceptance of such improved BSA products (Burnhof, 2000; Michael et al., 2003). Cholera toxin B (CTB), a mucosal adjuvant is another molecule that has attracted attention of the workers as a carrier (Menge et al., 1993). George-Chandy et al. (2001) observed that conjugation of CTB to peptide or protein antigen by chemical coupling or genetic fusion enhanced antigen-presenting capacity of not only dendrite cells and B cells but also of macrophages, which expressed low levels of cell surface MH class II and are normally poor activators of native T cells. Further, these workers observed that conjugation of CTB to peptides or protein dramatically lowers the threshold concentration of antigen required for immune cell activation. However, commercially available CTB preparations are histopathologically toxic as it...
contains trace amount of cholera toxin (Goto et al., 2000). The use of recombinant CTB provides an advantage as it excludes the possibility of contamination with endotoxin, thereby making it safe for humans. Similarly, recombinant exotoxin A of *P. aeruginosa* has been utilized as carrier protein (Kossaczka et al., 1999). Usually the carrier proteins used in licensed vaccines are based on existing protein vaccines, for example, diptheria and tetanus toxin and CRM 197 (a genetically toxoided, single amino acid variant of diptheria toxin) and an outer membrane complex from *Neisseria meningitidis* group B strain (Ravenscroft and Jones, 2000). However, major limitation of toxoid vaccines is that reinforcement or booster doses are required to maintain protective antibody levels. When extra doses are administered earlier than the recommended intervals, there is risk of adverse reactions (Richard, 1999; American Academy of Family Physicians, 2003).

Since, use of all the above mentioned protein antigens have inherent limitations, the attention of the researchers has been drawn towards porin or iron-regulated outer membrane proteins as carriers (Paniague et al., 1992; Singh et al., 1999). Outer membrane of gram-negative bacteria has a set of characteristic protein called outer-membrane proteins (OMPs), which may account for upto 50% of the dry weight of this membrane. One family of OMPs, the porins, are present in large amount in the outer membrane and play a crucial role in the interaction between the environment and bacteria (Chakrabarti et al., 1996). Porins form water filled channels that permit the diffusion of small hydrophilic solutes, amino acids and nutrients across the outer membrane (Nikaido, 1994; Koebnik et al., 2000). The surface exposed outer membrane proteins particularly porins are the important candidates for vaccine development because of several reasons. Firstly, the enterobacterial porins share homology in their primary amino acid sequence (Jeanteur et al., 1994) and cross-reactivity of major OMPs has been previously reported by investigators (Hofstra et al., 1980); these porin proteins can bind C1q leading to activation of the classical complement pathway common to enterobacteria and which acts independently of the presence of a capsular polysaccharide layer (Alberti et al., 1993; 1996). In addition, OMPs have been implicated in iron acquisition (Williams et al., 1987)
and in permeability to antimicrobial agents (Martinez – Martinez et al., 1996). Finally, several studies have indicated the importance of immune response against OMPs in protection against gram-negative bacteria (Nikaido et al., 1987; Morrin and Reen, 1993; Gonzalez et al., 1993 and Goldbatt et al., 1994).

The first significant report of using OMPs as immunogens in in vivo system was published by Cooper and Rowley in 1982. These workers demonstrated that a heat labile antigen could induce antibody mediated protection against infection with encapsulated K. pneumoniae in mice. Investigation done by Serusago and Coworkers (1989) demonstrated that mice immunized with OMPs of K. pneumoniae could overcome lethal challenge with wild strain. In a relatively recent study, Lun et al. (1997) demonstrated the involvement of the bacterial outer membrane protein structures in evoking antibodies protective against lethal challenge with homologous and heterologous strains of E. coli. Vaccines containing iron-regulated proteins (IRPs) have also been shown to be effective immunoprophylactic agents against various gram-negative bacteria (Banerjee et al., 1990). It has been assumed that IRPs might enhance the protective efficacy of vaccine by stimulating the production of antibodies to antigens produced in vivo in response to naturally provoked iron depletion (Gilmour et al., 1991). Consequently, researchers have prepared conjugates by coupling iron-regulated outer membrane proteins (IROMPs) to either polysaccharide moiety of LPS (Chhibber and Bajaj, 1995) or Vi polysaccharide antigen of S. typhi (Chhibber and Bhardwaj, 2004). Among the members of enterobacteriaceae, the OMP expression has been reported to be altered under various environmental stresses (Schmid et al., 1991; Wertz et al., 1992; Sahu et al., 1994) including antibiotic pressure (Lun et al., 1994; 1997; Domenech-Sanchez, 1999; Chevalier et al., 1999, Emmanuela De et al., 2001).

In past, Leyer et al. (1986), reported that Escherichia coli OMPs could be affected after treatment with sub-MICs of antibiotics. Similarly, Kadurugamuwa et al. (1985) demonstrated that, in Klebsiella pneumoniae, some antigens become more accessible to antibodies when bacteria were treated with cephalosporins. Later, Lebrun et al. (1992) obtained similar findings with surface exposed antigens of P. multocida. Earlier different workers have identified a beta-lactam-
induced 25 kDa protein which might be responsible for conferring resistance to beta-lactam antibiotics by interfering with OmpU, through which the beta-lactam antibiotics normally enter the cell (Deb et al., 1995; Chakrabarti et al., 1996). Recently, Emmanuele et al. (2001) isolated an unusual strain of Enterobacter which was able to synthesize a modified porin, resulting in a substantial decrease in cephalosporin penetration.

Many studies have reported that proteins, which are tightly associated with LPS are biologically active (Melchers et al., 1975; Goodmann and Sultza, 1976; Bjornson et al., 1988; Zhang et al., 1997; Giambartolmeri et al., 1999). In recent past, work done by Hellman et al. (1997, 2000) have suggested that release of OMPs takes place during antibiotic exposure as well as during logarithmic growth of bacteria and these OMPs could play role in the pathogenesis of sepsis. If so, antibodies against OMPs might protect by detoxifying and clearing the proteins themselves. During sepsis, since immune system is under stress, therefore OMPs expressed under antibiotic pressure would be more meaningful targets for immunoprophylaxis as well as immunotherapy. Further, with more relevant antigen as carrier, the conjugate would elicit antibody responses towards two protective antigens (Paniagua et al., 1992). This approach is more useful if both the antigen are from the same organism (Chhibber and Bhardwaj, 2004).

**Immunotherapeutic Approach**

Immunoprophylactic approach by preventing infection through active immunization is considered a better strategy as compared to immunotherapeutic approach (treating by passive infusion of antibodies), however in controlling infections like septic shock the former approach has limited use. Although immunoprophylaxis has several potential advantages which include; more sustained levels of circulating antibodies, recruitment of other antibody isotypes and antibody may be subject to recall, yet active immunization neither prevent infection nor the onset of sepsis (Baumgartner et al., 1985). It has been shown that most cases of sepsis occur in immunocompromised patients admitted in ICUs and such a population might not respond well to active vaccination (Brun-
Buisson et al., 1996). Further, since time lapse in the onset of sepsis is very less, this approach may be of limited use due to the insufficient time for the antibody response to develop (Cross et al., 2001). Therefore, passive immunization may be a more practical method of protecting individuals against *K. pneumoniae* induced sepsis and has received more attention during the last decade (Trautmann et al., 2004). It has been suggested by Cross et al. (2001) that therapy directed against bacterial components has the potential advantage over anti-inflammatory mediator therapy, in that, unlike cytokine-directed therapies e.g. anti-tumor necrosis factor, IL-1Ra, anti-LPS therapy might not compromise host defenses and lead to uncontrolled infection.

In 1960s and 70s the importance of the role of gram-negative bacterial lipopolysaccharide (LPS) in the pathogenesis of sepsis was documented (Braude et al., 1960). Later with the elucidation of the structure of LPS, the epitopes within the lipid A and core region of the LPS that were widely shared among heterologous gram-negative bacteria were recognized (Chedid et al., 1968; Braude and Douglas, 1972). As a result, it was hypothesized that antibodies directed against such conserved regions could confer broad, heterogenous protection and therefore have clinical utility in the setting of sepsis. Different workers (McCabe, 1972; Ziegler et al., 1973) developed bacterial strains in which core region of LPS was available to the immune system (i.e. not shielded by O-antigen, for example, *S. Minnesota* Re 595 ([Re chemotype] and *E. coli* 0111: B4, J5 mutant [Rc chemotype]). In 1982, Ziegler raised polyclonal immune sera in healthy volunteers against killed *E. coli* 0111: B4, J5 mutant, which gave therapeutic benefit in patients with suspected gram-negative sepsis. However, there was no evidence that antisera prevented infection. Further, these workers were unable to provide conclusive evidence that antibody fraction of immune sera was responsible for improved survival. Subsequent clinical studies carried out by different investigators (Baumgartner et al., 1985; Calandra et al., 1988; J5 Group, 1992) could not confirm the findings of Ziegler et al. (1982). Earlier studies with passive infusion of polyclonal antibodies directed against lipid A and core-oligosaccharide were also not very encouraging in treating sepsis in animal models as well (Bruins et al., 1977; McCabe et al.,
1977; Johns et al., 1977). Despite this fact, monoclonal antibodies to lipid A and core region were raised in order to gain better specificity (Mandine et al., 1990; Nnalue et al., 1992). Studies carried out using monoclonal antibodies against these regions also failed to show cross-protection in vivo (HA-1A sepsis Group, 1991; Xoma Group, 1991; Warren et al., 1992; Muller-Loennies et al., 2000). Following these disappointing results, the belief that antibodies directed against conserved epitopes in the LPS could have clinical utility in septic patients was openly challenged (Baumgartner, 1991; Morrison et al., 1994; Cross and Opal, 1994).

Donta et al. (1996) tested the ability of intravenous immunoglobulins (IVIGs) enriched in type-specific antibodies to multiple Klebsiella and Pseudomonas serotypes to prevent the development of infection in patients. It was found that 9.8% patients who received an albumen control had Klebsiella infection compared to 3.6% of those who received IVIGs. Further, the patients with higher antibody levels had fewest Klebsiella infections suggesting that the type specific antibodies have been associated with beneficial effect. In another study, Bhattacharjee et al. (1994) demonstrated the dose-related protection by anti-J5 LPS IgG antibodies from lethal Pseudomonas sepsis in neutropenic rats. Thus, it was suggested by Cross et al. (1999) that it is not sufficient to simply provide antibody against the proper epitope, but rather there must be sufficient amount of this antibody. Further, these workers suggested that inspite of lack of cross-reactivity, type specific anti-O and anti-capsular polysaccharide antibodies still hold the potential for immunotherapy. Several advantages of O-side chain antibodies have been recognized which include: better in vitro and in vivo, endotoxin neutralizing ability than anti-core antibodies (Baumgartner et al., 1990; Bailat et al., 1997); mechanism of action of O-specific antibodies is known and these have higher affinity for their target; in addition, these type specific antibodies prevent infection while anti-core antibodies only reduce lethal shock following infection (Baumgartner et al., 1985).

K. pneumoniae, which expresses both lipopolysaccharide (O-antigen) and capsular polysaccharide (K-antigen), there are 77 different K-serotypes known
Type specific protection against experimental *Klebsiella* infections has been obtained in animals with polyclonal and monoclonal antibodies (mAbs) specific to K-antigen (Cryz *et al.*, 1986c; Lang *et al.*, 1991; Donta *et al.*, 1996). However, the fact that there are large number of the K-serotypes of capsular polysaccharide (CPS) known in this genus with no predominance of any serotype, limits the use of antibodies specific to K-antigens. The focus, therefore has been shifted, to O-antigen, since in *Klebsiella* only nine serogroups have been recognized. Several workers have demonstrated the beneficial effect of antibodies specific for O-antigen on both *Klebsiella* septicemia and pneumonia in animal model of lethal infection (Rukavina, 1997; Hellman *et al.*, 1997, Trautmann *et al.*, 2004). However, there are reports of past failure of these antibodies in clinical trails. Nasraway, (2003) in their review article suggested that unlike animal studies, researchers investigating immunotherapy for clinical septic shock had not controlled all variables. Testing for the wrong hypothesis, errant study design, using the wrong agent, focusing on in-appropriate target group and excessive expectations has potentially obscured the real efficacy of immunotherapy. In a recent study, Cross *et al.* (2004) have attributed the previous failure of clinical studies to the insufficient amount of antibodies administered early in the course of sepsis and suggested that these antibodies must be provided in close proximity to infection, in order to have a beneficial effect.

**Combination therapy**

Treatment of clinical infections with antibiotics alone has become complicated, both due to increasing emergence of antibiotic-resistant pathogens and increased patient populations intrinsically at risk for nosocomial infections (Poelstra *et al.*, 1999). Combination therapies comprising multiple intravenous antibiotics alone, or in tandem with either intravenous immunoglobulin or antibiotics, have all been used to improve efficacy against clinical infections (Barekzi *et al.*, 2002). With this unique treatment combination, synergistic improvement in host survival, bacterial burden and sepsis indicators were observed. Poelstra *et al.* (1999) reported that pooled human immunoglobulins
applied locally to sites of infection in vivo improved the anti-microbial benefits of a clinical important intravenous antibiotic, ceftazidime against both E. coli induced peritonitis and Klebsiella induced burn wound infection. Studies done in past have also demonstrated the benefit of such therapy against sepsis in new borns (Givener, 1990; Haque et al., 1995), high risk neonates (Chen, 1996) and ventilated ICU patients (Mohr et al., 1997).

Synergy between antibiotics and antibacterial immunoglobulins has been well accepted (Overbeek and Veringa, 1991). This approach is of particular importance for Klebsiella pneumoniae, in which there is extensive production of capsular polysaccharide (CPS). It has been observed that different growth conditions as well as antimicrobial agents have profound effect on the production and composition of capsular polysaccharide (Kadurugamuwa et al., 1985; Williams et al., 1987; Held et al., 1995). Recently, Gholia et al. (2004) have demonstrated appreciable decrease in the production of CPS on growth of K. pneumoniae in presence of MIC and sub-MIC of antibiotics. Thus, antibiotic in combination therapy has been presumed to increase the effectiveness of antibodies directed against O-antigen. In combination therapy, antibodies supplement host, humoral immunity through both specific and non-specific opsonization and neutralization reactions against clinically relevant pathogens, ultimately leading to microbial phagocytic clearance. Because antibodies facilitate antimicrobial mechanisms distinct from those of antibiotics, these do not endanger development of antibiotic resistance (Barekzi et al., 2002). Moreover, the appearance of pathogen antibiotic resistance does not alter bacterial susceptibility to opsonization and phagocytic neutralization (Gemmell, 1996). Additionally, combination therapy has also been reported to reduce post-operative infection rates leading to sepsis after surgery (Cafiera et al., 1992). Clinical problems with antibiotic – induced release of pathogen toxins in septic patients have been addressed using antibodies administered systematically against these toxins (Lamp et al., 1996; El-Zaim et al., 1998). Thus, combination therapies represent the clinical capability to exploit pathogen susceptibility to multiple antimicrobial agents that individually no longer have acceptable clinical efficacy.