Sepsis is a clinical entity, most commonly encountered in hospitalized patients and has the potential to lead to septic shock (Cross et al., 2004). It is characterized by inflammation and tissue injury, observable systemically. In the absence of any infection, similar systemic inflammatory response (SIRS) may develop, but when it is the result of confirmed infectious process, it is termed as sepsis (Sharma et al., 2003). Septic shock is a sequelae of severe sepsis and is associated with hypotension, hypoperfusion abnormalities and multiple organ dysfunction (MODS) (Astiz et al., 1998). Increasing incidence of sepsis as well as septic shock are seen inspite of new antibiotics for treatment (Angus et al., 2001). The factors like frequent use of immunosuppressive therapy and advancements in organ transplantation have increased the complications arising out of infections. Infact, in patients with underlying metabolic diseases like diabetes mellitus, renal failure or cancer, where life expectancy has increased, have also contributed towards increased incidence of this entity. In hospitalized patients invasive life support procedures are also recognized to play a crucial role in development and progress of sepsis (Stevens, 2002). High mortality rate ranging from 52% to 82% is associated with sepsis and septic shock syndrome respectively (Giudici et al., 1999).

Recent advances in the understanding of the pathogenesis of sepsis have helped the workers to explore different management strategies directed towards prophylactic as well as therapeutic approaches. Effective therapy of the infection responsible for severe sepsis is aimed at controlling and eradicating the source of infection (Nasraway, 2003). This can be achieved by appropriate and timely institution of antibiotics, surgical drainage or both (Parrillo, 1993). Empirical antimicrobial therapy should be given, keeping in mind the site of infection and the commonest known organism responsible for these infections (Bernard et al., 2001). However, a major limitation in using this approach is the emergence of multidrug resistant organisms and the suppressed immunologic defenses of the
patients receiving aggressive antimicrobial regimens (Angus et al., 2001; Obiamwe and Berkowitz, 2004).

Apart from having known side effects in the form of toxicity to the host cells, the antibiotics have been found to have the potential to affect the host following interaction of invading bacteria with the drug. This is mainly based on recognition of the fact that there can be release of endotoxin (LPS) from dyeing gram-negative organisms (Holzheimer, 2001; Giamarellos-Bourboulis et al., 2003). This molecule, however is known to be biologically very active and can contribute to induction of deleterious consequences of bacterial sepsis (Holzheimer, 1998). As soon as endotoxin gains access to the systemic circulation, the inflammatory cascade sets in with activation of different inflammatory cells (Cryz and Hollenberg, 2003). There is an abnormal regulation of inflammation with uncontrolled release of indigenously generated inflammatory cytokines, which are the major determinants of sepsis related cellular damage (Sharma et al., 2003).

Based on above mentioned considerations, it is recognized that timely and appropriate administration of antibiotics has little effect on the overall mortality caused by sepsis (Crosby et al., 1994; Mock et al., 1995; Nasraway, 2003). Lately, attention has been drawn towards novel therapeutic approaches in treating septic shock. These are aimed at neutralizing microbial toxins and modulating host's inflammatory mediators. One of the first interventions to gain attention was direct inhibition of the effects of endotoxin and TNF-α by using antibodies (polyclonal or monoclonal) (Ziegler et al., 1991; Bone et al., 1995). Other approaches include, infusion of IL-1 receptor antagonist, platelet activating factor (PAF) antagonist, blockades of eicosanoid production and inhibition of nitric oxide synthase (Schedel et al., 1991). Use of steroids has also shown promise in some studies but requires extremely careful monitoring (Bollaert et al., 1998; Matot, 1998). Because of the strong association of sepsis with activation of blood coagulation factors, another approach has been directed towards inhibition of this process. To achieve this anti-thrombin, protein C and tissue factor pathway inhibitors have been advocated (Giudici et al., 1999).
Inspite of several new therapeutic approaches which have been tried over the past decade, no single intervention so far has been demonstrated to reduce mortality significantly (Cohen, 1998). Advocated immunotherapy alone is not expected to reverse shock (Nasraway, 2003). Sepsis as a clinical entity is extremely complex both medically and in terms of clinical investigation. Halting the ongoing endotoxin stimulation of the inflammatory response at the source would seem to be more effective than inhibiting any individual component of a cascade (Silverman and Ostra, 1999). A multi-factorial approach including antimicrobial therapy, immunotherapy or supportive therapy is required for the management of this syndrome (Cross et al., 2004).

Management of sepsis, not only requires the precise understanding of the pathophysiology of the process, but it is also important to keep in mind the changing scenario of epidemiological aspects of microbes causing this syndrome. Recently researchers have discovered a shift in the organisms responsible for causing septicemia. This has been primarily from gram-positive bacteria to gram-negative organisms (Roy et al., 2002; Mahapatra et al., 2002). Among these, emergence of multi-drug resistant *Klebsiella pneumoniae* is a severe problem world wide, especially for critically ill patients (Yan et al., 2001). High isolation rates of extended spectrum beta lactamase producing *Klebsiella pneumoniae* have been reported from hospitals located in North America, Europe and Latin America (Liu et al., 1998; Monnet et al., 1997; Sader et al., 1999; Sader, 2000). It has been reported as the most frequent pathogen responsible for causing nosocomial sepsis among patients admitted in the ICUs (Martins-Loureiro et al., 2001; Malik et al., 2003; Karabinis et al., 2004). These world wide findings are in accordance with that of National Neonatal Perinatal Network Database (Singh, 1999; Agarwal, 2001). *Klebsiella pneumoniae* is also reported to be the predominant organism associated with neonatal septicemia from different states of India as well (Gupta et al., 1993; Basu et al., 2001; Roy et al., 2002; Kumar and Neelagund, 2004). The present study has therefore been planned, taking into consideration the commonest organism causing sepsis specially in India.
Apart from area based knowledge of the commonest organism responsible for septicemia, it is essential to know the antibiotic susceptibility pattern of the organism, as it may vary depending on the geographical region (Roy et al., 2002). This helps in selection of appropriate antibiotics to be given empirically, to prevent emergence of antibiotic resistance and misuse / overuse of antibiotics. Previous studies evaluating the antimicrobial resistance patterns of *K. pneumoniae* have shown the prevalence of high rates of multi-drug resistance among clinical isolates (Jacoby, 1996; Roy et al., 2002; Kumar and Neelagund, 2004). However, low levels of antimicrobial resistance among *K. pneumoniae* to cephalosporins, aminoglycosides, imipenem and fluoroquinolones have been described due to relatively restricted use of these drugs (Digranes et al., 1997; Schumacher et al., 1997; Hanberger et al., 1999). It has been demonstrated by Martins-Loureiro et al. (2001) that cephalothin, ceftriaxon, cefuroxime, gentamicin, chloramphenicol and trimethoprim-sulfamethoxazole are ineffective agents for treatment of sepsis induced by *K. pneumoniae*. Bouza and Cercenado (2002) have described the effectiveness of fluoroquinolones, trimethoprim-sulfamethoxazole, aminoglycosides, carbapenems and the combination of β-lactams or fluoroquinolones with aminoglycosides against most *Klebsiella* species. Keeping these observations in mind antibiotics belonging to three different groups (on the basis of their mechanism of action) i.e. third generation cephalosporins, aminoglycosides and quinolones were selected for the study. Majority (88.24%) of wild type isolates showed resistance to three or more antibiotics and were referred as multi-drug resistant strains (MDRS). In recent studies, amikacin has been found to be most effective drug against gram-negative organisms. Resistance to ciprofloxacin (a quinolone) was also found to be infrequent (Yu et al., 2001; Roy et al., 2002). Results of the present study corroborate the findings of these workers as the sensitivity of blood isolates to amikacin (an aminoglycoside) and ofloxacin (a quinolone) was 68.63% to 62.75% respectively in the blood isolates.

Efficacy of the treatment would, not only be dependent on antibiogram of the invading pathogen but also on the virulence attributes which in turn can
affect the host parasite interactions. For this reason it is essential to know the virulence capabilities of the selected organism which requires to be tested in the animals. All wild type strains were therefore checked for their pathogenic potential following intraperitoneal inoculation in mice with relatively high dose of organisms ($10^{10}-10^{11}$ cfu/ml). Interestingly, no mortality was observed with any of the wild type isolates tested. Search of the literature in this context brought out that *K. pneumoniae* strain ATCC 43816 capsular serotype 2 (K2) could successfully establish pneumonia in mice following intratracheal inoculation in doses ranging from $10^2$ cfu/ml to $7 \times 10^2$ cfu/ml (Greenberger *et al*., 1996; Tsai *et al*., 1997). Melisson *et al.* (1994) were able to induce lethal septicemia in normal C57BL/ka mice after intraperitoneal (i.p.) inoculation of $10^4$ cfu of *K. pneumoniae* ATCC 43816. This dose was reduced to $10^3$ cfu when mice were rendered leukopenic by prior treatment with cyclophosphamide. Similarly, Ten Hagen *et al.* (1998) have also reported the induction of septicemia in normal C57 BL/ka mice following i.p. injection of $10^3$ cfu with same serotype of *K. pneumoniae*. This strain, therefore was procured from Dr. David P. Speert from the Department of Paediatrics, University of British Columbia, Vancouver, Canada. It was screened for antibiotic sensitivity and virulence in mice. This strain could establish septicemia in normal LACA mice after i.p. inoculation of $10^5$ cfu of *K. pneumoniae*. It was found to be sensitive to all the antibiotics tested, except ceftazidime with which it gave a MIC of 40 µg/ml. Three drugs representing different group (third generation cephalosporins, aminoglycosides and quinolones) selected in the present study were ceftazidime, amikacin and ofloxacin. Since in the present study, OMP expression was required to be checked in presence of antibiotics, it was necessary for this organism to be able to grow in presence of these antibiotics. It is for this reason resistance in the selected strain, to amikacin and ofloxacin was developed in the laboratory following repeated passages in graded doses of the drug. Finally, the standard strain of *K. pneumoniae* ATCC 43816 selected for sepsis model was able to grow in presence of these antibiotics with a MIC of 40 µg/ml for ceftazidime, 20 µg/ml for amikacin and 0.9 µg/ml for ofloxacin.
For validation and acceptability of new drugs, therapies and devices before use in humans, it is necessary to have a suitable, reproducible and affordable animal model for experiments (Cross et al., 1993). It has been suggested by Michie (1998) that the available sepsis models are not representative of the clinical septic shock syndrome since pathophysiological response they produce are not similar to that seen in septic shock syndrome in humans. In this context, the use of fibrin-clot model, previously reported in large animals like dogs (Natanson et al., 1989) and guinea pigs (Alexander et al., 1989) has been found to be superior to others as this model can be used in studying both the initial and persisting stage of infection. In this model, a slow release of bacteria from the nidus of infection takes place into the blood which finally leads to sepsis and septicemia (Toky et al., 2003). The same model has been successfully employed in rodents like rats by DeMarsh et al. (1996). Recent reports highlighting the genetic similarity between mice and humans (Kondo et al., 2001; Mural et al., 2002) draws attention to the fact that these small affordable experimental animals can be used with better possibility of extrapolation of the data to human situations.

Animal models of sepsis in which lethal challenge with endotoxin or live bacteria are given are invalid as the cytokine and other pathophysiological responses they produce are different to those observed in clinical sepsis (Cross et al., 1993; Michie, 1998) Therefore, an ideal situation of a more realistic animal model would be to use a low dose of an appropriate pathogen for establishment of infection progressing as close as possible to clinical septic shock syndrome. In the present study, we were able to establish sepsis by entrapping in fibrin clot, *Klebsiella pneumoniae* at a dose of 150 cfu. This was implanted into the peritoneal cavity of mice, following which bacteremia was established within 24 hours post infection period. Bacterial counts continued to rise in the blood till the death of the animals. But when an equivalent dose was given to mice without entrapment in fibrin clot, the blood counts became negative in all the mice after 72 hours of infection. Our results corroborate the findings of a previous study done by Demarsh et al. (1996). These workers utilized the same model in rats using *E. coli* and observed that the blood culture became positive within 18-25
hours after establishment of infection. It has been reported that gram-negative facultative bacteria translocate more easily than anaerobes and gram-positive bacteria (Wells, 1990). This may explain the early positive blood culture observed in this study as well as in the study of DeMarsh et al. (1996). At a dose of 150 cfu of *K. pneumoniae* in fibrin clot, 50% mortality spread over a period of one week was observed. Whereas, with a dose of $2 \times 10^3$ cfu, 100% mortality was recorded. However, no mortality was observed with these doses when bacteria were not entrapped in a fibrin clot. In earlier studies in dogs, guinea pigs and rabbits, relatively high doses of bacteria like *E. coli*, *S. aureus* and *Salmonella enteritidis* were employed for inducing sepsis (Fink et al., 1984; Alexander et al., 1989; Asheg et al., 2001). A major draw back of these studies is, the utilization of large bolus of gram-negative bacteria ($10^{11}$-$10^{12}$ cfu) which causes an enormous amount of endotoxin (LPS) release, strongly implicated for its capacity to initiate generalized inflammatory response (Bucklin and Morrison, 1995).

In the model employed by Melissen et al. (1994) although dose of the organism (*K. pneumoniae* ATCC 43816) was reduced to $10^3$ cfu but exposure to injectable cyclophosphamide was given which is not ideal, since it alters the immune status of the host. In the present study, successful establishment of sepsis without immunomodulation therefore is more acceptable. Further in this context the injury type models such as cecal ligation and puncture (CLP), colon ascendens stunt peritonitis (CASP) and fibrin-thrombin clot model of sepsis are also suitable since these resembles more closely to the course of sepsis observed in patients (Walley et al., 1996; Zantl et al., 1998).

The success of septicemia model requires that the infecting organism should become localized in distant organs (Waldon et al., 2002). Study of the different sites and organs in experimental animals showed highest bacterial counts in the peritoneal cavity followed by liver, spleen and blood. For direct comparison matching studies are not available in which quantitative measurement of bacterial load in different organs has been determined. However, DeMarsh et al. (1996) observed a maximum of 4.5 log cycles of bacteria in the blood of mice, 40-45 hours after establishment of infection by
utilizing fibrin-thrombin clot model in rats. These results are in accordance with our findings, as in the present study a peak of 4.04 log cycles of *K. pneumoniae* in the blood was observed just before the death of the animals. Whereas, Melisson *et al.* (1994) in their study of *K. pneumoniae* septicemia could detect $10^3$ to $10^4$ cfu of bacteria per ml of blood within 4 hours and $10^6$ cfu/ml of blood after 48 hours. In another study following implantation of colon ascendants stunt peritonitis (CASP), Zantl *et al.* (1998) demonstrated early invasion of bacteria in blood and counts at 12 hours were $10^6$ cfu of bacteria/ml of blood. The observed variability with the present study may be due to the reason that possibly biofilms formed on intracellular stunt release large number of organisms in the blood as compared to their invasion from peritoneal cavity.

The presence of bacteria in different organs is not sufficient to assess the septicemia and thus there is a need to evaluate the extent of tissue damage following translocation of bacteria. In a relatively recent study, Nabber *et al.* (2000) have found that during the development of sepsis, continuous liberation of endotoxin induces the release of pro-and anti-inflammatory mediators. The suppression of the immune system enables the bacteria present in the organs to cause more pronounced tissue damage seen in late sepsis. In order to demonstrate the extent of tissue damage, the study of pathology induced in the organs is therefore mandatory. In the present study, infection with LD$_{50}$ dose (150 cfu) of *K. pneumoniae* induced moderate inflammatory changes in different tissues studied. However, increase in the dose of bacteria (LD$_{100}$) led to appreciable damage as was observed in terms of severe changes in both liver and spleen on 7th post infection day. These findings proved that there is a direct correlation between the bacterial load and pathological changes observed *in vivo*.

One of the immediate approach of treating patients with presentable sepsis is the administration of appropriate antibiotic which interacts with invading bacteria. Antibiotics, which mainly work by affecting the cell wall of bacteria (penicillins, β-lactam antibiotics and cephalosporins) have been shown to induce morphological changes in bacteria (Dofferhoff *et al.*, 1991; Jackson and Kropp,
In the present study, ceftazidime, a third generation cephalosporin acting on cell wall was found to induce filamentation in *K. pneumoniae* following exposure to MIC as well as sub-MIC concentration of the antibiotic. Similar observations have been made earlier where filament formation in presence of ceftazidime (sub-MIC and bactericidal concentration) has been reported by number of workers (Jackson and Kropp, 1992; Ohya *et al.*, 1991; Nishino *et al.*, 1994; Horri *et al.*, 1998a, 1999; Kishi *et al.*, 1999; Yokochi *et al.*, 2000). In addition the antibiotics belonging to quinolone group, which mainly act by inhibiting enzyme DNA gyrase, are also known to induce filamentation in bacterial cells (Van den Berg *et al.*, 1992; Crosby *et al.*, 1994). The results of this study are in accordance with the earlier findings as ofloxacin (a quinolone) in the present study was also found to induce filamentation in *K. pneumoniae*. On the contrary aminoglycosides, which inhibits bacterial protein synthesis, failed to induce any significant morphological change as reported by Simon *et al.* (1991) and Crosby *et al.* (1994). In the present study amikacin belonging to this group also did not induce any morphological change at sub-MIC concentration.

Exposure of bacteria to antibiotics not only induces morphological changes, but it is also associated with release of biologically active substances like endotoxin (LPS) (Holzheimer *et al.*, 2000; Giamarellos-Bourboulis *et al.*, 2003). Antibiotics differ in their potential for endotoxin release and this has been related to the bacterial strain and the antibiotic studied (Prins *et al.*, 1994). Most studies demonstrating antibiotic induced endotoxin release have focussed on *E. coli*. In few studies *K. pneumoniae* has also been included. Eng *et al.* (1993) have 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.* (1998a) 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. These workers have also correlated the endotoxin release with morphology of the pathogens. On the contrary, Trautmann et al. (1998b) in their studies found no correlation between morphological changes (induced in presence of antibiotics) and their LPS liberating ability. These workers demonstrated low endotoxin release following treatment of *E. coli* with ciprofloxacin, despite of its ability to form filaments. They presumed that this effect could be due to inhibition of the production and/or assembly of cell bound LPS by ciprofloxacin. However, Horri et al. (1999) in a relatively recent study (in vitro) reconfirmed the relationship between morphological changes and endotoxin release in presence of carbapenems in clinical isolates of *Pseudomonas aeruginosa*. The results of the present investigation corroborates the findings of Horri et al. (1998a, 1999) as direct relationship between morphological changes and endotoxin release following exposure to two of the antibiotics was observed. It was found that antibiotic ceftazidime, not only induced filamentation in *K. pneumoniae* but also released significantly high amount of endotoxin.

Similarly, quinolones, which do not target the cell wall have also been reported to be relatively potent inducers of endotoxin release (Prins et al., 1995). With ofloxacin high levels of endotoxin liberation from *E. coli* cells has been demonstrated by Lamp et al. (1997). In the present study also, ofloxacin was found to induce appreciable increase in endotoxin level, which was associated with its ability to induce filamentation. On the contrary, negligible endotoxin release was observed following exposure of the standard strain to amikacin. These results support the observations made by Simon et al. (1991) and by Bingen et al. (1992), who reported less endotoxin release from *E. coli* cells following exposure to amikacin. In another study endotoxin release from *K. pneumoniae* was low when gentamicin was used in the experiments (Eng et al., 1993).

The difference in the mode of action of antibiotics possibly, has a bearing for the varying ability of the drugs to induce endotoxin release (Jackson and Kropp, 1992). Beta-lactam antibiotics specifically target penicillin-binding proteins (PBPs) with selective affinity for individual PBPs (Prins et al., 1994). Based on
the available literature, the explanation offered is that large amount of endotoxin release from *K. pneumoniae* in presence of ceftazidime was possibly due to its 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 a relatively recent 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. The lysis of increased biomass of the cell can offer an alternative explanation for higher amount of endotoxin release, reported by Crosby *et al.* (1994) following interaction of *E. coli* to ciprofloxacin. In the present study, the increased biomass production associated with filamentation, as well as its lysis could have contributed to high level of endotoxin released following exposure of *K. pneumoniae* to ofloxacin. In case of aminoglycosides, the lack of increase in cell biomass could be one reason for lower levels of endotoxin release observed in case of amikacin. An additional reason could be the ability of this group of drugs to bind and neutralize the released endotoxin, which has been reported by Foca *et al.* (1991). Another antibiotic like polymixin B is also known to possess this 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). 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). Therefore, use of antibiotics with endotoxin (LPS) neutralizing activity would be useful to control endotoxin dependent sepsis sequelae induced by different species of gram-negative organisms. Keeping this in mind, the antimicrobial spectrum and bactericidal activity of amikacin makes it a better agent for treatment of sepsis caused by gram-negative bacteria. The lack of propensity to elicit excessive release of endotoxin by amikacin may avoid exacerbation of endotoxin related shock in sepsis. This drug in conjunction with
other antibiotics forms very potent combination for this life threatening situation (Marie et al., 1999). However, aminoglycosides are well recognized to have an inherent disadvantage in the form of nephrotoxicity. This nephrotoxicity is known to be enhanced in presence of endotoxin (Ngeleka et al., 1990). Therefore, combination of amikacin with inhibitors of endotoxin or anti-TNF-α antibodies may provide additional benefit in patients with sepsis / septic shock.

In comparison to in vitro studies, evaluation of endotoxin liberation during treatment of experimental bacteremia have more clinical relevance. This is because antibiotic induced endotoxin release may be different depending on the type of infection, the location of infection, virulence of strain, gram character, mode of application and dosage of antibiotic (Holzheimer et al., 2001). The reports in literature on the endotoxin liberating ability of PBP-3 and PBP-2 specific antibiotics in clinical situations are conflicting (Luchi et al., 2000, Simpson et al., 2000; Holzheimer et al., 2000). For example, 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). Similarly, Mock et al. (1995) during the treatment of septic trauma patients, also observed high level of endotoxin in plasma with PBP-3 specific antibiotic. 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 antimicrobials belonging to three different groups which included third generation cephalosporins (cefuroxime), aminoglycosides (netilmicin) and quinolones (ciprofloxacin). The interaction of these three matching groups of antibiotics with K. pneumoniae in this study showed maximal endotoxin levels in the plasma of experimental mice, treated with ceftazidime, followed by ofloxacin. Horri et al. (1998b) also employed K. pneumoniae and E. coli induced sepsis model in rats. They demonstrated high endotoxin levels in plasma of animals, administered ceftazidime in comparison to carbapenems. For comparison, however with antibiotics like ofloxacin, there is paucity of available information. In relation to aminoglycosides, the third group employed in the present investigation, a study by Shenep et al. (1985) showed 20 fold less endotoxin release in rabbits receiving gentamicin (aminoglycoside).
in comparison to moxalactam (cephalosporin) during treatment of *E. coli* induced sepsis recorded. The results of this study are in line with this earlier observation as no appreciable increase in plasma endotoxin level was observed with amikacin. This finding has significant clinical relevance.

Different studies have emphasized that ultimate outcome in a gram-negative bacterial sepsis is not totally dependent on the amount of endotoxin released. In this regard, the defense system, age and individual responses in host have a bearing on induction of sepsis shock and multiple organ failure (MOF) (Michie, 1998; Southwick *et al.* 2003; Sharma *et al.*, 2003). However, endotoxin which is biologically very active molecule still plays a key role and releases pro-inflammatory peptides and cytokines which includes TNF-α (Dinarello, 1997; Stevens, 2002). TNF-α is reported to have potential for dual role, since conflicting reports related to its effect are available following antimicrobial therapy. These observations are based on the experimental as well as clinical studies (Simon *et al.*, 1991; Friedland *et al.*, 1993; Mohler *et al.*, 1994; Prins *et al.*, 1995; Silverstein *et al.*, 2000). The possibility has been expressed that ceftazidime induced TNF-α liberation in *S. aureus* mediated sepsis plays significant harmful role for the host. However, this response was not observed in case of *E. coli* induced sepsis (Silverstein *et al.*, 2000). In an earlier in vitro study carried out by Simon *et al.* (1991) it was 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 group of antibiotics in experimentally induced meningitis, failed to detect elevated levels of TNF-α in CSF (Friedland *et al.*, 1993). However, third generation cephalosporin in studies related to endocarditis and urosepsis have been shown to induce raised levels of TNF-α in serum and urine (Mohler *et al.*, 1994; Prins *et al.*, 1995). The differences observed in TNF-α levels in serum and CSF, could be because of the degree of inflammation in CSF (a closed space) as compared to response that occurs outside the central nervous system. Elevated levels of TNF-α have been described in sepsis, where it contributes significantly to MOF, shock and

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death (Hinshaw et al., 1990; Dekimpe et al., 1995; Cusumano et al., 1997; Kirikae et al., 1998; Yao et al., 1998).

In the present study the TNF-α levels which were detected in serum only, were found to be maximal in response to treatment with ceftazidime (third generation cephalosporin), followed by ofloxacin (quinolone) and least with amikacin (aminoglycoside). These observations made in vivo could not be compared as studies in relation to sepsis were not available for comparison. However, in a recent study by Giamarellos-Bourboulis et al. (2003) matching group of antibiotics were employed for treating cases of upper urinary tract infection (pyelonephritis) and measurement of TNF-α was done in serum of the treated patients. These workers failed to detect any rise in levels of TNF-α in serum with any of the antibiotics used. Pyelonephritis is a localized inflammatory response of the kidney and therefore compartmentalized rise in the levels of TNF-α should have been addressed to by these workers. Infact the levels of TNF-α if measured in urine would have been meaningful and probably could have explained the difference observed in comparison to present study.

In the management of sepsis especially in critically ill patients, only administration of antibiotics may not suffice. Attention of the scientists has therefore been drawn to adjunct therapies for the purpose. One of the approaches has been directed towards neutralization of released endotoxin. It has been reported by Coyne et al. (1993) that antibiotic like polymyxin B has this property and this could prove useful, but toxicity of this drug poses limitation. Zhang et al. (1999) have demonstrated that human lactoferrin derived synthetic peptide (LF-33) also has an endotoxin neutralizing ability demonstratable in vitro and in vivo. However, a major limitation of this peptide is that serum, where the molecule has to reach, may attenuate it leading to loss of its activity.

In severe septic shock syndrome, adrenal insufficiency reported (Hatherill et al., 1999) is responsible of many complications. Administration of glucocorticoids and mineralocorticoids in these patients is recognized to improve the adrenergic responsiveness resulting in reversal of circulatory failure and increased survival (Annane et al., 2002). Very recently an important study
reported by Minneci et al. (2004) have carefully selected the earlier trials where glucocorticoids were given for treating patients with sepsis. Studies included were categorized in two broad groups i.e. ones which were carried out before 1989 (8 studies) and the ones where medline data was obtained after 1989 (5 studies). They concluded that relatively lower physiological dose of hydrocortisone, if administered relatively early (2 to 72 hours of onset of shock) improves the survival in vasopressor dependent shock. They also advocated that to avoid the harmful effect associated with administration of steroids these drugs should be tapered during 5 to 7 day period.

Inspite of having availability of drugs like corticosteroids, efforts of the researches in this field have been directed towards boosting the innate immune system of host and to overcome the effect of released endotoxin. For this basically two approaches have been adopted. According to the first one, cytokines which have potential for immunomodulation (Moore et al., 1993) have been employed. In this context, IL-10, an anti-inflammatory cytokine when given at appropriate time has been shown to diminish TNF-α release and significantly improve survival in experimental animals (Gerard et al., 1993; Vander Poll et al., 1995; Latifi et al., 2002). Other anti-inflammatory cytokines like IL-4 and IL-13 inhibit the secretion of pro-inflammatory cytokines but there is paucity of literature regarding their activities (Boontham et al., 2003). The second approach has been directed towards administering antibiotics belonging to macrolide group, since they are also recognized to have immunomodulatory role (Culic et al, 2001). This effect has been strengthened recently through an experimental study carried by Giamarellos-Bourboulis et al. (2004) where administration of clarithromycin (macrolide), alone as well as with amikacin has been found to considerably prolong the survival of rabbits suffering from sepsis induced by *P. aeruginosa*. This effect has been attributed to anti-inflammatory as well as immunoregulatory properties of this antibiotic. Woo et al. (2004) reviewed the usefulness of broad range of effects of macrolides on the immune system by various experimental and clinical studies. They demonstrated that these antibiotics 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. The immunomodulatory effect was found to be greater with 14- and 15-membered drugs rather than the 16-membered derivatives. However, results obtained with this group of antibiotics are not conclusive and precise mechanism of immunomodulation is not well known.

Since long, strong association of severe sepsis with a state of activation of blood coagulation and potential role of capillary thrombosis in the development of multiple organ failure is known (Taylor et al., 1987; Guidici et al., 1999). It has been demonstrated by Hoffman and Cooper (1995) that locally generated thrombin enhances the deleterious effects of released endotoxin synergistically. Efforts therefore have been directed to manage the affected coagulopathy in cases of septic shock through administration of specific thrombin inhibitors. Two preparations tried in this regard are anti-thrombin (Dickneite and Paques, 1993) and hiruden (Dickneite and Czech, 1994). Results presented by Warren et al., (2001) demonstrated beneficial effects of anti-thrombin in terms of survival, in one sub-set of patients. Similarly, Creasey and Reinhart (2001) in their review article have provided preclinical and clinical evidence that recombinant tissue factor pathway inhibitors significantly reduce thrombin generation and mortality in animals as well as humans with sepsis. Imberti (2003) employed anti-thrombin and concluded that anti-thrombin affects coagulation as well as inflammation. Another substance that has been tried experimentally as well as in clinical studies is protein C (Taylor et al., 1987; Bernard et al., 2001; Stevens, 2002). These workers reported that administration of protein C, known to be anti-thrombotic and profibrinolytic, decreased mortality. However, therapy with protein C tends to be expensive as well as it leads to increased risk of bleeding (Nasraway, 2003; Minneci et al., 2004).

All the adjunct therapies discussed so far are not without inherent limitations. It is for this reason that attention of the workers has been drawn to immunotherapy in the form of immunoglobulins administered intravenously so as to achieve neutralization of the related endotoxin (LPS) in circulation. Therefore this molecule, a cell surface component is a target for new preventive and therapeutic strategies (Lukasiewicz et al., 2002). LPS consist of three structural
domains. These are long O-specific polysaccharide side chains (O-antigen), the core oligosaccharide and hydrophobic lipid A (Di Padova et al., 1993). The latter two components have been shown to be widely shared among heterologous gram-negative bacteria (Chedid et al., 1968; Braude et al., 1972). These conserved regions were therefore considered for immunotherapeutic approach for neutralizing the endotoxin molecule in cases of sepsis (Ziegler et al., 1973, 1982; Dunn and Ferguson, 1982). Earlier studies, utilizing passive infusion of polyclonal antibodies directed against lipid A and core-oligosaccharide, were not very encouraging in treating sepsis in animal models (McCabe et al., 1977, Johns et al., 1977; 1983) and clinical studies (Baumgartner et al., 1985; Calandra et al., 1988; J5 group, 1992). The alternate approach adopted was to raise monoclonal antibodies against these antigens in order to gain better specificity (Teng et al., 1985; Appelmelk et al., 1988; Salles et al., 1989; Mandine et al., 1990; Nnalue et al., 1992; Kuhn et al., 1992). Studies carried out using monoclonal antibodies against these regions also failed to show cross-protection in vivo (Ziegler et al., 1991; Greenman et al., 1991; Warren et al., 1992; Muller-Loennies et al., 2000).

In a clinical study, while testing the ability of intravenous immunoglobulins (IVIGs) to multiple Klebsiella pneumoniae and Pseudomonas serotypes, the beneficial effect of anti-core and type-specific antibodies was realized (Donta et al., 1996). The development of type-specific antibodies predominantly against O-side chains of endotoxin has been observed following infection with gram-negative bacteria or administration of purified endotoxin (Cross et al., 1989). These type-specific antibodies, which only confer protection against homologous serotype, therefore do not have practical application in treatment of sepsis. However, recently it has been advocated that in spite of lack of cross-reactivity, type-specific anti-O and anti-capsular polysaccharide antibodies still hold the potential for immunotherapy (Cross et al., 1999). Different reasons put forward in favour of antibodies to O-side chains include: Firstly, these antibodies have better ability to neutralize the effects of endotoxin in vitro and protect animals in vivo than anti-core antibodies (Baumgartner et al., 1990; Bailat et al., 1997). Secondly, O-specific antibodies have higher affinity for their target and their
mechanism of action is known. In addition, these type-specific antibodies might prevent infection while anti-core antibodies only reduce lethal shock following infection (Baumgratner et al., 1985). On this basis it was suggested that O-antigen can serve as a target epitope for the production of human monoclonal antibodies for immunotherapeutic approach (Cross et al., 1999; Trautmann et al., 2004).

*Klebsiella pneumoniae* typically expresses both lipopolysaccharide (O-antigen) and capsular polysaccharide (K-antigen) on its surface, which play an important role in its pathogenicity (Hansen et al., 1999; Chhibber et al., 2003). Polyclonal and monoclonal antibodies (mAbs) specific to K-antigen have been shown to enhance phagocytosis of bacteria and provide type-specific protection against experimental *Klebsiella* infections (Cryz, 1983; Trautmann et al., 1988; Lang et al., 1991). However, 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 limitations hamper the potential of K-antigen based vaccines in *K. pneumoniae*, due to which the focus has now been shifted to O-antigen. This 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, 01, 02ab, 03 and 05 account for more than 82% of the O-antigen serotype found in clinical isolates (Trautmann et al., 2004). Out of these, 01 serogroup is most commonly isolated from clinical samples (Hansen et al., 1999). In addition, surface exposure of the O-antigen together with CPS has been reported in *K. pneumoniae* (Tomas et al., 1991). It has also been found that antibodies specific to O-antigen can penetrate through capsule of strains belonging to certain K-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 mAbs against O-antigen of *K. pneumoniae* also exert opsonic activity depending on the CPS serotype. The protective potential of these antibodies has been demonstrated by different investigators (Mandine et al., 1990; Rukavina et al., 1997). Recently, Trautmann et al. (2004) also reported the efficacy of O-antigen specific polyclonal and monoclonal antibodies in *Klebsiella* septicemia and
pneumonia in mouse model of lethal sepsis. These workers concluded that O-antigen antibodies may be suited to supplement K-antigen specific hyperimmune globulins for passive immunoprophylaxis of *Klebsiella* infections.

The present investigation therefore, was carried out with an aim to determine the immunoprotective potential of O-specific polysaccharide (O-PS) moiety of LPS antigen. For this purpose whole LPS molecule was extracted from *K. pneumoniae* ATCC 43816 by phenol – water extraction method. On active immunization, the purified antigen was found to be protective in mice against sepsis. Protection with LPS molecule against gram-negative infections has also been observed in few earlier experimental studies, available in literature (Vuopio-Varkila et al., 1988, Rani et al., 1990; Chatley et al., 1996). This protection has been correlated with the ability of LPS molecule to activate long-living phagocytic cells which provide non-specific immunity against infection (Wright and Jong, 1986; Vuopio-Varkila et al., 1988). Our results corroborate these earlier findings where protection was based on enhanced efficacy of phagocytic cells, indicating that immunity operates through innate immune mechanism. However, the complete LPS molecule was found to be toxic, pyrogenic and induced schwartzman reaction in experimental animals. These side effects were also observed in an earlier reported study from our laboratory (Rani et al., 1990). These drawbacks limit the potential of complete LPS molecule as vaccine candidate.

Most of the toxicity of LPS has been linked to lipid A component (Rietschel et al., 1984). For the detoxification of LPS, multiple approaches have been employed by different workers. Treatment of LPS with polymyxin B, sodium borohydride or alkali causes its detoxification without destroying its antigenic determinants (Morrison and Jacobs, 1976; Von Eschen and Rudbach, 1976; Stokes et al., 1989). However, 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, 1987; Rani et al., 1990). Another approach of detoxifying LPS is its treatment with mild acid hydrolysis. The method of Westphal and Jann (1965) which has been lately used by Konadu et al. (1994) as well as Chhibber and Bajaj (1995) was adopted in the
present study to remove lipid A moiety of purified LPS. O-specific polysaccharide (O-PS) thus obtained failed to provide any protection on intramuscular immunization. This was assessed in terms of mortality, bacteriological load and histopathological evidence in different organs of experimental animals. The failure of polysaccharide alone to elicit any protection can be attributed to its poor immunogenicity in vivo. This property, which is well recognized, is therefore considered a major limitation for using this antigen for vaccination.

The polysaccharide antigens are T-cell independent antigens (Mosier and Subharao, 1982) and induce mainly IgM antibody response. The young children under the age of two years respond poorly to these antigens (Kossaczka et al., 1999). Long back in 1929, Avery and Gobel reported that the immunogenicity of T-independent polysaccharide antigen (pneumococcus type 2 polysaccharide) could be enhanced by binding it to a carrier protein. Several subsequent studies demonstrated that conjugation of non-immunogenic polysaccharide antigen to carrier protein restores the immunogenicity that was similar or better than that of native LPS without restoring the endotoxicity inherent in LPS (Tsay and Collin, 1984; Cryz et al., 1986b; Verhuel et al., 1993). This principle has been recognized in the recent studies as well, where conjugate vaccines have been prepared by utilizing polysaccharide moiety from several other pathogens. Number of workers have reported the covalent binding of O-specific polysaccharide (O-PS) with cholera toxin (Cryz et al., 1991), P. aeruginosa recombinant exotoxin A (Fattam et al., 1993; Passwell et al., 2001), formalin treated exotoxin C of Clostridium welchii (Konadu et al., 1994), iron regulated cell surface proteins (Chhibber and Bajaj 1995), tetanus toxoid (Boutonnier et al., 2001) or bovine serum albumin (Chernyak et al., 2002). These conjugates had low levels of endotoxin and elicited serum antibodies with bactericidal activity against LPS of homologous strains. Haemophilus influenzae type b (Hib) vaccine which is also a polysaccharide–protein (PS-PR) conjugate has already been licensed in United States and its success rate has been considerably high, suggesting the potential of such vaccines (Food and Drug administration, 1993a,b). Various advantages of these types of vaccines are: Firstly, the conjugate vaccines are able to produce anamnestic response as protein bound...
to polysaccharide in such preparations provides T-dependent characteristics to polysaccharide moiety and as a result these are immunogenic in infants (Robbins and Schneerson, 1990). Similarly, polysaccharide enhances immunogenicity of covalently linked protein antigens (Gupta et al., 1994a,b). Larger molecular weight of conjugate may account in part for its improved immunogenicity. In addition, conjugate vaccines may eliminate the need for free proteins from combination vaccines, thereby reducing the antigenic burden on the immune system and complications 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.

In conjugate vaccines, selection of an appropriate carrier protein is important. Bovine serum albumin (BSA) has been utilized by workers as a carrier protein (Konadu et al., 1994; Chernyak et al., 2002). However, commercially available BSA preparations vary from lot to lot. Other limitations associated with the use of BSA as a carrier protein are the conjugate's instability, protease related degradation and induction of allergic reactions in a small percentage of infused patients (Morales et al., 1994; Fiocchi et al., 1998; Michael et al., 2003). In recent past improved technology has been adapted to improve recovery and purity of BSA (Burnhouf, 2000). Recombinant albumin has been developed but the cost and labour involved is the main barrier to acceptance of such improved BSA products (Michael et al., 2003). Another carrier molecule that has been employed by researchers is the cholera toxin b (CTB) (Menge et al., 1993). Conjugation of peptide or protein antigen to CTB by chemical coupling or genetic fusion enhances T-cell activating capacity of different antigen presenting cell subsets (George-Chandy et al., 2001). 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). Protein antigens such as tetanus toxoid (Szu et al., 1994), diptheria toxoid (Gupta et al., 1994b) or pig bel toxoid (Konadu et al., 1994) have also been used to induce T-cell dependency in the
polysaccharide antigen. However, with toxoid vaccines reinforcement or booster doses are required to maintain protective antibody levels. Risk of adverse reactions with such vaccines might increase when extra doses are administered earlier than the recommended intervals (Richard, 1999; American Academy of Family Physicians, 2003).

An alternate approach in the development of conjugate vaccines is the use of porin or iron-regulated outer membrane proteins as carriers (Paniagua et al., 1992; Chhibber and Bajaj, 1995; Singh et al., 1999). Outer membrane proteins are categorized into high molecular and low molecular weight proteins. One family of OMPs, the porins which come in the latter category are present in large amounts in outer membrane and form water filled channels that permit diffusion across the membrane of small polar molecules, amino acids and nutrients (Domenech and Sanchez et al., 1999; Koebnik et al., 2000). 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 (Lun et al., 1994; 1997; Domenech-Sanchez; 1999; Chevalier et al., 1999; Emmanuella De et al., 2001). Since during bacterial sepsis the immune system is already under stress, the OMPs expressed in presence of antibiotic exposure therefore, would be more meaningful targets for immunotherapy so as to avoid extra stress. With a more relevant antigen as a carrier, the conjugate would elicit antibody responses towards two protective antigens (Paniagua et al., 1992). This approach is more useful if both the antigens are from the same organism (Chhibber and Bhardwaj, 2004). In the present investigation, the outer membrane proteins (porins) of K. pneumoniae were conjugated with O-PS moiety of LPS antigen obtained from the same strain. The proteins selected for conjugation were those additional protein bands which were expressed in the OMP profiles of heavily encapsulated K. pneumoniae following exposure to ceftazidime and ofloxacin. Amikacin, however failed to induce any change in the OMP profile of the bacterium. These changes observed in the SDS-PAGE analysis were confirmed by immunoblot study. The results obtained are in agreement with a previous study by Lun et al. (1997) who demonstrated a
difference in recognition of protein epitopes by the serum which is raised against the bacteria pre-exposed and un-exposed to the sub-MIC concentration of aztreonam. The observed differences in the present study in the OMP profiles following exposure to antibiotics (ceftazidime and ofloxacin) may be due to the re-arrangement of bacterial proteins revealing new antigenic determinants (Osborn, 1980; Neidhart et al., 1987; Lun., et al 1994). It is also likely that the exposure of antimicrobial causes reduction in production of capsular polysaccharide, resulting in expression of outer membrane antigens which are otherwise occluded (Kadurugamuwa et al., 1988).

Additional immunoblot experiments were carried out to confirm the surface location of the antibiotic induced newly expressed proteins. It was found that proteins of molecular weight 40 kDa and 30 kDa were expressed on the cell surface following exposure to ceftazidime and ofloxacin respectively. Since, outer surface components of bacteria come in contact with external milieu and constitute the first barrier against host defenses, these OMPs are considered as important epitopes. In the present study, therefore these proteins were purified by the method of Hager and Burgess (1980). Efficient purification of protein could be achieved by utilizing this method, since negligible amount of LPS contamination was detected in purified protein preparation. These were then conjugated separately to the O-PS portion of LPS.

Apart from selection of clinically useful carrier protein in conjugate vaccine, the judicious choice of a conjugation method is equally important as it has profound effect on the immunogenicity of final preparation (Chu et al., 1991; Shen et al., 2001). Not only this, the coupling method should take into account the serologically important groups of the antigens to be conjugated (Vann and Jann, 1979). Depending upon the nature of the polysaccharide and proteins, few modifications of the original method of Avery and Gobel (1929) have been adopted by different workers. Schneerson et al. (1980) coupled Haemophilus influenzae type b polysaccharide to different carrier proteins by a method which couple cyanogen bromide activated polysaccharide to adipic acid dihydrazide derivatized protein. Szu et al., (1987) devised a new method to couple Vi antigen of Salmonella to carrier protein by thiol derivatization, which needs the
participation of carboxylate groups of Vi-polysaccharide. These workers also carried out direct coupling of Vi to diptheria toxoid by 1-ethyl-1-3-(3-dimethylaminopropyl) carbodiimide (EDAC) and found that conjugate prepared by thiolation process was better in its immunogenicity as compared to conjugate prepared by direct coupling. Thus, a method which utilizes a spacer molecule to link two antigens in a conjugate 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 spacer molecule, adipic acid dihydrazide (ADH), a six carbon moiety selected for O-PS – protein conjugate has already been shown to provide consistently high yield of Hib-protein conjugate (Schneerson et al., 1980). Other studies have shown the efficacy of O-PS – protein conjugates prepared by utilizing a similar approach (Kabir, 1987; Konadu et al., 1994; Passwell et al., 2001; Shen et al., 2001). This multipoint attachment method utilizing ADH as a linker was employed in this study to couple O-PS of K. pneumoniae to new proteins expressed by bacteria on exposure to antibiotics. Carbodiimide condensation reaction used to couple K. pneumoniae O-PS with purified proteins gave a high yield of coupling (45.2% for O-PS – PR-CD and 44% for O-PS – PR-OF) and a good polysaccharide/protein ratio (1.58 for O-PS – PR-CD and 1.9 for O-PS – PR-OF) necessary for a strong IgG response in immunized mice. Both the conjugates were found to be non-pyrogenic and non-toxic. The conjugate so prepared fulfilled the following criteria laid down by Kabir (1987): The conjugate was eluted in the void volume fractions following column chromatography and the proteins not subjected to the conjugation reaction, eluted separately and in the inclusion volume of the column. Secondly, when the conjugate was subjected to SDS-PAGE, it did not enter the gel. In addition, O-PS itself was poorly immunogenic, whereas high titre of antibodies to polysaccharide moiety were detected when mice were immunized with the conjugate.

The immunoprotective potential of the conjugates (O-PS – PR-CD and O-PS – PR-OF) was examined against K. pneumoniae induced sepsis in mice following immunization. Both the conjugates were found to be protective, with 75% percentage survival rate with O-PS – PR-CD and 62.5% with O-PS – PR-
OF as compared to no survival in the infected control group. A significant decrease in the bacterial load was observed in the blood as well as different organs in groups immunized with either of the conjugates. Histopathological evaluation of different organs from groups immunized with conjugates further confirmed their efficacy as mild inflammation observed in all tissues by 3rd post infection day resolved by 7th post infection day. Though, matching protection studies with polysaccharide–outer membrane protein conjugate in septicemia model are not available in literature, however Cross et al. (2001) have reported the protective efficacy of detoxified J5 LPS/Neisseria meningitidis group B outer membrane protein (OMP) complex vaccine against sepsis induced by P. aeruginosa and K. pneumoniae in neutropenic rats. Similarly, conjugation of iron regulated outer membrane proteins (IROMPs) either to polysaccharide moiety of LPS of K. pneumoniae or Vi-polysaccharide of Salmonella typhi has been shown to be protective in respective animal models (Chhibber and Bajaj, 1995; Chhibber and Bhardwaj, 2004).

To elucidate the underlying mechanisms of protection operative in an immunized host, the immune responses in terms of non-specific and specific host defenses are important. Previous studies have shown that immunostimulation with bacterial cell wall components can cause non-specific resistance to infection (Nowotny, 1985; Chase et al., 1986). Activation of long living phagocytic cells (macrophages) constitutes the first line of defense against infectious agents (Rani et al., 1990). Activated macrophages are known to produce pro-inflammatory and anti-inflammatory cytokines. TNF-α, which is generated along with many other pro-inflammatory cytokines, plays an important role in septic shock (Bagby et al., 1991; Casey et al., 1993). In the present study, prolonged production of TNF-α was associated with improved bacterial clearance from blood as well as different organs in immunized animals. In fact TNF-α is known to activate T-cells or T-cell subsets, thereby fasciliating the generation of cytokines and/or cytotoxic effector molecules and ensuring their sustained expression by the cells (Ulich et al., 1991; Vowels et al., 1995; Tessier et al., 1997). In an early study carried out by Buret et al. (1994) on the role of TNF-α in pulmonary immunity, it has been suggested that this cytokine improves
bacterial clearance from the lungs indirectly via its immunomodulatory pathway rather than because of a direct bactericidal effect. This cytokine along with gamma interferon has been shown to prime peritoneal macrophages for enhanced antibody dependent cellular cytotoxicity as well (Fan et al., 1991) and both these cytokines potentiate the uptake and intracellular killing of bacteria by peritoneal macrophages (Pierangeli and Sonnenfeld, 1993). The latter observation corroborate the findings of this study as enhanced phagocytic indices of peritoneal macrophages were obtained on immunization with all the antigens except O-PS alone. This and the above mentioned mechanisms possibly in turn could have resulted in reduced mortality as was observed following immunization. Since macrophages constitute the first line of defense in any system, such observations are important as stimulation of macrophages can provide an early resistance to infection (Rani et al., 1990). Besides, TNF-α has been suggested to play a synergistic role with other mediators by initiating the host response at the infected site (Astiz, 1998).

Apart from the above mentioned functions, TNF-α has been shown to play a dual role in vivo. In addition to its essential protective effects in the generation of immunity against gram-negative bacteria, TNF-α has also been implicated in its ability to induce immunopathology in vivo (Bekker et al., 2000). Elevated TNF-α levels is known to contribute to tissue necrosis, cachexia or wasting (Beutler and Cerami, 1988; Taub et al., 1996). However, in the present study no detrimental effects of excess of TNF-α level were observed in different organs. In fact a mild inflammation observed on 3rd post infection day was completely resolved by 7th post infection day in immunized animals. This can be explained on the basis of observation made in an earlier study that outcome of septic shock syndrome is dependent upon balance between pro and anti-inflammatory mediators (Walley et al., 1996). According to these workers the balance and 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 et al., 1996; Stuber et al., 2000; Oberholzer et al., 2000). Thus, the problem with the cytokine biology-sepsis axis is not the
expression of pro-inflammatory cytokines but whether these are adequately modified by anti-inflammatory mediators or not (Walley et al., 1996; Boontham et al., 2003). Modulation of the cytokine responses therefore is necessary for maintaining homeostasis in the host. The various anti-inflammatory mediators include gluco-corticoids, prostaglandin E2, IL-1 receptor antagonist and anti-inflammatory cytokines (Sharma, 2003). IL-10, which is expressed in elevated concentration during sepsis is considered important in determining the outcome of sepsis (Marchant et al., 1995). This cytokine down-regulates the expression of TNF-α and chemokines from macrophages (Morre et al., 1993; Souza et al., 2003). No attempt was made in this study to look for this anti-inflammatory cytokine which has been shown to delay and decrease the pro-inflammatory response, resulting in improved survival. Hence, it is suggested that future studies assessing the role of pro-inflammatory cytokines such as TNF-α also should take into account generation of anti-inflammatory cytokine responses.

Besides innate immune mechanisms, specific humoral immunity is particularly effective in preventing infections by pathogens. Vaccination with microbial antigens is the most effective way of inducing protective humoral immunity which is mediated by the presence of bactericidal, opsonic and haemagglutinating circulating antibodies (Cryz et al., 1984). Different studies carried out in the past support the concept that hyper-immune sera with specificity for bacterial polysaccharide protects animals and humans from consequences of serious infections by gram-negative bacteria (Bougoudogo et al., 1995; Robbins et al., 1995; Passwell et al., 2001). In addition, several studies have reported the importance of immune response against outer membrane proteins (OMPs) in protection against gram-negative bacteria (Nikaido and Vaara, 1985; Morrin et al., 1993; Goldblatt et al., 1994; Lun et al., 1994; 1997). The conjugation of polysaccharide moiety of LPS with tetanus toxoid or protein has been shown to elicit IgG anti-O-PS antibody response which was boosted following re-immunization, as conjugate had typical T-dependent properties (Chu et al., 1991; Konadu et al., 1994; Boutonnier et al., 2001). Protective IgG antibodies specific to IROMPs have also been detected in the serum of animals immunized with polysaccharide IROMP conjugates (Chhibber and Bajaj, 1995;
Chhibber and Bhardwaj, 2004). The results of the present investigation are consistent with these observations as significantly higher systemic IgG immune response specific to both O-PS moiety of LPS as well as OMPs was generated in the immunized animals following intramuscular administration of both the conjugates.

Opsonophagocytic uptake and complement mediated killing constitutes the important mechanisms through which IgG antibodies operate *in vivo*. Complement mediated killing though important for clearance of bacteria, might not be operative in *K. pneumoniae* as strains possessing smooth LPS and CPS inhibit the deposition of lytic complement components (Alberti *et al.*, 1996). Results of the present study suggest that IgG specific to protein, generated following active immunization might have contributed towards protection by acting as opsonins and helping in clearance of bacteria by phagocytic cells. *In vitro* studies carried out by number of workers have demonstrated that antibodies specific to O-antigen were able to opsonize non-encapsulated strains while fully encapsulated were resistant against opsonizing effect under the same assay conditions (Rukavina *et al.*, 1997; Held *et al.*, 2000; Cortes *et al.*, 2002; Lepper *et al.*, 2003). Nevertheless *in vivo* experiment conducted by Rukavina *et al.* (1997) to evaluate the protective capability of such antibodies revealed that high doses of O-antigen specific antibodies were protective against lethal infection due to encapsulated *K. pneumoniae* strains. These and other workers have further suggested that during multiplication *in vivo*, significant subpopulation of encapsulated organisms may have thinner capsule or even lack the capsule (Frasa *et al.*, 1996; Rukavina *et al.*, 1997). In addition to promoting opsononphagocytosis, antibodies also exert protection by neutralizing circulating free LPS (Rukavina *et al.*, 1997; Held *et al.*, 2000). These observations have been recently confirmed by Trautmann *et al.* (2004) who demonstrated that inspite of lack of opsonic activity for encapsulated strains, O-specific antisera and monoclonal antibodies showed prophylactic effect in *Klebsiella* mediated septicemia and pneumonia in mice.

Active immunization is generally considered a better strategy for preventing (as opposed to treating) infections as compared to passive infusion of
antibodies. The former approach has several potential advantages which include more sustained levels of circulating antibodies, recruitment of other antibody isotypes and antibody may be subject to recall (Baumgartner et al., 1985). In addition, an active immunization strategy may be of limited use in sepsis, due to the insufficient time for the antibody response to develop, since time lapse in the onset of septic shock is very less (Cross et al., 2001). Also, 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). Therefore, in the present investigation, protective efficacy of passive immunotherapy with antisera raised against conjugates was also checked. In contrast to the results obtained with active immunization of the conjugates, passive supplementation of the antisera in septic mice was unable to provide any protection. However, complete protection was observed when antibodies against the respective conjugate were given in combination with either ceftazidime or ofloxacin. Studies in the 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). In a relatively recent study, Cross et al. (2001) demonstrated significant higher protection in the group of animals actively immunized with detoxified J5 LPS/group B complex vaccine in combination with ceftazidime treatment as compared to group treated with ceftazidime alone. Since, different growth conditions as well as antimicrobial agents change the production and composition of capsular polysaccharide (Gholia et al., 2004), it has been suggested that combination therapy with antibodies against O-antigen epitopes and antibiotics may be effective in the presence of extensive CPS production (Kadurugamuwa et al., 1985; Williams et al., 1987; Held et al., 1995). Synergy between antibiotics and antibacterial immunoglobulins is well accepted (Overbeek and Verigna, 1991). As antibodies have antimicrobial mechanisms distinct from those of antibiotics, these do not endanger development of antibiotic resistance. In the present study, failure of passive immunotherapy alone to provide protection could be due to the insufficient and non-persistent levels of antibodies. Bhattacharjee et al. (1994) specially addressed the
importance of passive immunotherapy with antisera and demonstrated dose-related protection with antibodies specific to LPS in neutropenic rats. It has been recently suggested by Cross et al. (2004) that if passive immunotherapy alone has to be utilized it is important to maintain meaningful antibody levels and this should be kept in mind by future workers.

The treatment of septic shock even today offers a challenge to the treating physician. The first choice is the administration of antibiotics, likely to be effective in curbing the infection. Based on the results of the present study, while amikacin definitely offers an advantage in terms of negligible release of endotoxin and pro-inflammatory cytokine (TNF-α), its inherent toxicity limits its usage. The choice of the treating physician, usually falls on effective antibiotic with minimal side effects. In case, choice falls on antibiotics which cause excessive release of biologically active endotoxin molecule, as was demonstrated in this study with ceftazidime and ofloxacin, then there is a need to supplement it with an effective adjunct therapy. In this regard, immunotherapy holds promise as shown in the present investigation. Active immunization on the other hand, employing immunogenic antigens, was effective by itself, but it may not be of actual practical utility because of non-availability of time required to built antibodies. Therefore, antisera to two conjugates tried in this study by coupling PS-moiety of LPS to OMPs expressed under antibiotic pressure offers a practical solution in conjunction with antibiotics administered. The results of the present study suggest that there is a potential of therapy with antibodies, which can not be discarded, as it has not been sufficiently tested. Failure of antibodies alone in providing protection, however is a subject that needs to be explained thoroughly to be able to explain the underlying reasons. There is a need to pay special attention to the dose of antibody administered and to ensure persistence of adequate levels after initial infusion as suggested by Cross et al. (2004).