DISCUSSION

Despite the widespread use of antibiotics urinary tract infections (UTI) remain among the most common bacterial infection in the human population. About 5% of the adult females are likely to have UTI at any moment in time, while more than 50% of the women will experience a UTI at some stage in their lives (Asscher, 1980). A great deal of attention has been paid in the last two decades to the identification and characterization of potential bacterial virulence determinants. The ultimate goal of defining the nature of pathogenic mechanisms which operate in the urinary tract is to enable a suitable prophylactic measure, such as specific vaccine to be developed for use in high risk patients (Harber et al, 1986).

*Escherichia coli* is the etiological agent in 80% of the urinary tract infections in the normal host (Lipsky, 1989) and this infection is the most common form of extra intestinal infections caused by this organism. The study of Andriole (1987) have clarified the relationship of bacterial virulence factors and host defence mechanisms in the pathogenesis of urinary tract infections. *Escherichia coli* strains, that produce acute pyelonephritis in healthy young girls or adult women, generally manifest specific virulence factors not found in randomly selected fecal *E. coli* strains (Nowicki *et al*, 1987; Stenqvist *et al*, 1987). These properties include specific fimbrial adhesins that promote attachment to the urinary tract; the ability to produce hemolysin, cytolysin and aerobactins, which helps the organism to acquire iron and to initiate cytonecrotic response; a capsular or K antigen which enables the organisms to resist
phagocytosis and bactericidal action of complement (Johnson, 1991). Uropathogenic strains of *E. coli* belong to only certain O and K serotypes. Strains having five K antigens viz. K₁, K₂, K₃, K₁₂ and K₁₃ account for 70% of the pyelonephritis cases in healthy individuals. Since, these strains belong to a limited number of *E. coli* serotypes, these pyelonephritic strains may belong to small number of clones that express these virulence properties (Stamm, 1989). Recognised mode of bacterial self defence is through camouflage of bacteria with the help of their capsule. Due to structural similarities with host material, the encapsulated *E. coli* cells cannot be properly recognised as foreign by the immune system. (Silver *et al*, 1988). In the present investigation, *Escherichia coli* strain 06:K13:H1 (WHO designation SU 4344/41) with high expression of K13 antigen was employed to induce pyelonephritis and to elucidate the potential of purified K13 antigen in preventing against the disease.

The *Escherichia coli* strain 06:K13:H1 had previously been found to cause pyelonephritis in animal models (Kaijser and Olling, 1973; Kaijser *et al*, 1978; Larsson *et al*, 1980; Kaijser *et al*, 1983a) with renal attack rate of 60-80% in experimental animals. Further, this strain has O, K and H antigen relevant for uropathogenicity and it produces hemolysis which is said to be positively correlated to a capacity for producing pyelonephritis (Fried *et al*, 1971).

Direct assessment of the contribution to pathogenicity of the virulence properties requires the use of animal models, for which selection of appropriate model is crucial. Models involving non physiological manipulation of the urinary tract (renal trauma, ureteral
ligation, direct intra renal injection, etc) or intravenous injection of the bacteria (hematogenous infection) do not faithfully reproduce human UTI (Kaijser and Larsson, 1982). Human infections most commonly occur in patients with anatomically and functionally normal urinary tract and involves spontaneous ascend of bacteria from urethra to the bladder and further to kidneys (Bergstrom et al, 1972; Stamey and Sexton, 1975; Fowler and Stamey, 1977; Kunin et al, 1980).

Several animal species (Rabbit, Rat, Monkey) had been used earlier to develop UTI and most of these models include mechanical manipulation of the urinary tract to ensure high frequency of pyelonephritis (Stanford et al, 1962; Heptinstall, 1964; Kaijser and Olling, 1973; Braude et al, 1959; Miller and Robinson, 1973; Brooks et al, 1974; Summer, 1961; Larsson et al, 1980; Kaijser et al, 1983a; Garg et al, 1985; 1986; 1987*). Experimental pyelonephritis is relatively easy to establish in rats, because the vesicoureteral reflux occurs spontaneously (Kaijser and Larsson, 1982), which allows easy passage of bacteria upto renal pelvis (Sommer, 1961; Heptinstall, 1964; Tuan et al, 1970). However, rat uroepithelium cells do not express receptors for P-fimbriae and the form of globoside isolated from rat kidneys contains a Galα(1-3)Galβ linkage in place of critical Galα(1-4)Galβ linkage present in human urogenital tract, which provides receptors for P-fimbriae (Korhonen et al, 1981). Thus, rat is a species of questionable value for evaluating the pathogenesis of disease. In contrast to rat tissue, mouse renal tissue contains appreciable concentration of Galα(1-4)Galβ containing glycolipids (Lyrela et al, 1986; O’Hanley et al, 1985). In the present investigation, therefore, inbred strain of BALB/c mouse was
employed for inducing ascending pyelonephritis without any manipulation of urinary tract.

To standardize the model of ascending pyelonephritis, it is mandatory to find out the dose, which produces lesions in maximum number of animals with minimum mortality rate. In pilot experiments, therefore, different doses ranging from $10^6$ to $10^{10}$ CFU were administered and dose dependent induction of infection was studied, to standardize the optimal dose producing high infection take rate in our experimental conditions. The doses lower than $10^8$ CFU induced infection in lesser number of animals than the doses higher than $10^8$ CFU. At the higher dose level, significant increase in the mortality rate was seen. Finally, therefore, the dose of $10^8$ CFU/100 μl was selected as the infecting dose of *E. coli* strain. Volume of the inoculum injected intraurethrally is very important in this model of infection. The volume of 500 μl in rats and 200 μl in mice used in earlier studies, is reported to induce vesicoureteral reflux in animals (Hodson and Edwards, 1960; Kincaid-Smith and Becker, 1979; Larsson *et al*, 1980 and O’Hanley *et al*, 1985). In order to minimize the chances of producing any reflux in our model we used only a volume of 100 μl in female BALB/c mice.

Once bacteria were inoculated into bladder, bacteria were able to ascend from bladder upto the kidneys through the ureters and localized themselves in the kidneys in significant numbers to cause infection. Specific number of intrarenal deposition of bacteria is essential for the production of disease, evading all the protective mechanisms of bladder (Stamey *et al*, 1978) and kidneys (Braude, 1973). In the present study, the bacterial counts in renal homogenate were found to be increased
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significantly with increasing post infection period upto 4 days but no significant difference was observed in bacterial counts after 4 to 7 days post infection period. Increased bacterial number in kidneys had also been reported with progression of infection by Miller et al (1978), Miller and Marshall (1980) and Kaur et al, (1988).

The usefulness of anti K-antibodies for protection against infection had been previously described (Kaijser and Ahistedt, 1977; Kaijser et al, 1972; Kaijser et al, 1978 and Mattsby-Balter et al, 1982). With the availability of knowledge of the chemical composition of the capsular polysaccharide K-antigens, it is now possible to find out the role of purified K antigen in protection against ascending pyelonephritis. Certain capsular antigens, like K1 and K13 are recognised to be of special interest for prophylactic immunization against UTI. Since, they are most commonly found among E. coli strains causing UTI (Kaijser et al, 1977).

For isolation and purification of capsular polysaccharide, it is mandatory to know the best culture conditions required to grow the organisms for better expression of the antigen. The K13 antigen of E. coli belongs to a group of acidic polysaccharides with a relatively low molecular weight and high electrophoretic mobility (Orskov et al, 1971 and Orskov et al, 1977) and for the isolation of this group of polysaccharides, it was found best to grow the bacteria in liquid culture rather than agar plates (Gotschlich et al, 1972 and Vann and Jann, 1979). The maximum expression of these polysaccharides is at capsule permissive temperature of 37°C (Jann and Jann, 1983, 1990). The bacteria harvested at late log phase culture had been shown to be

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suitable for isolation of K antigen (Jann, 1985). Out of different liquid media, which had been employed to grow the bacteria for the isolation of capsular polysaccharide by earlier workers (Kajiser and Ahlstedt, 1977, tryptic soya broth; Jann, 1985, ‘DO’ broth). We used ‘Do’ broth and found it to be suitable for the purpose, since, it did not interfere with the isolation procedure. In the present investigation, for purification of K antigen, the organism was therefore, grown in liquid culture in a 20 litre fermenter at 37°C under controlled conditions upto the late exponential phase, which was attained within 5-6 h of growth.

Due to the presence of phosphatidic acid moiety at the reducing end of the group II capsular polysaccharide, these antigens tend to aggregate. The aggregation could be reversed with detergent or by heating of polysaccharide at pH 5-6 (Schmidt et al, 1982). However, there is a possibility that procedures employed could inactivate or may affect the immunogenicity of the desired antigen. Since, we were not sure of the extent of aggregation, we used sepharose 6B column with polysaccharide separation range of 10 KD to 1000 KD. Isolated native K13 antigen, when passed through this column, eluted at Kav (distribution coefficient) of 0.27 in the presence of 0.2M NaCl. The elution of same antigen through the sepharose 4B had shown the molecule to be eluted at relative elution volume of 0.51 (Kaijser and Ahlstedt, 1977). To completely retain the immunogenicity of purified capsular polysaccharide, we collected the antigen in native and aggregated form. Further, the purified antigen was checked for any contamination of LPS protein and nucleic acid. Based on disaccharide repeating units, the molecular weight of K13 polysaccharide chain had been reported to be roughly of 20 KD (Vann and Jann, 1979).
After purification of K antigen, it was coupled to diphtheria toxoid in order to enhance its immunogenicity, which is based upon existing information in the literature. Capsular polysaccharide from pathogenic E. coli strains are recognised to be poorly immunogenic in animals (Kaijser and Olling, 1973 and Kaijser, 1981) and humans (Hanson et al, 1977; Kaijser, 1983 and Salit et al, 1988). The weak immunogenicity of capsular polysaccharide K antigen had been attributed to molecular structure of this antigen, which contribute to its thymus independent nature as well (Silver et al, 1988). Originally, as early as in 1929, Avery and Goebel showed that the immunogenicity of thymus independent polysaccharide antigen (pneumococcus type 2 polysaccharide) could be increased by binding it chemically to a carrier protein. In contrast to poor immunogenicity of purified pneumococcus type 2 polysaccharide in rabbits, these carbohydrate-protein conjugate induced high titre of serum antibody (Goebel, 1939 and Goebel, 1940). The principle has been recognised in recent studies as well and had been applied successfully to increase the immunogenicity of polysaccharides from other pathogens (Jorbeck et al, 1981; Schneerson et al, 1980; Chu et al, 1983; Anderson et al, 1986 and Szu et al, 1986). Attempts had been made to couple K-antigen with bovine serum albumin or tetanus toxoid to enhance its immunogenicity (Kaijser et al, 1983a,b), where a limited success had been achieved in regard to enhancement of immunogenicity of K1 antigen (Kaijser and Ahlstedt, 1977). Similar kind of strategy to couple K13 CPS to thymus dependent protein (diphtheria-toxoid) was adopted in this study, with an aim both to increase the immunogenicity and to confer upon it the property of ‘T’ dependency. The rationale of using diphtheria-
toxoid in place of other carrier protein (like BSA in previous studies) is based on the fact (i) diphtheria-toxoid is ‘T’ dependent protein (ii) synthetically prepared K13-DT conjugate is likely to be better suited for human use.

It seems likely that the selection of a conjugation method and carrier protein has profound effect on the immunogenicity of final conjugate. Vann and Jann (1979) suggested that, while the chemical modification is done to improve the immunogenicity of K13 antigen, the coupling method should take into account the serologically important O-acetyl and carboxylate group on KDO (2-keto-3-deoxyoctulosonic acid), which is the center of serological specificity of this antigen.

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\begin{array}{c}
\text{3 Ribose} \\
\rightarrow
\end{array}
\begin{array}{c}
\text{1,7 KDO} \\
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\begin{array}{c}
\text{2 OAc} \\
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\]

Depending upon the polysaccharide and protein, few modifications of the original method of Avery and Goebel were done. Schneerson et al (1980) coupled Haemophilus influenzae type b polysaccharide to different carrier proteins via a method which couple cyanogen bromide activated polysaccharide to ADH derivatized protein. Szu et al (1987) devised a new method to couple Vi antigen of salmonella to carrier protein by thiol derivatization, which need the participation of carboxylate groups of Vi polysaccharide. They also performed the direct coupling of Vi to diphtheria toxoid by EDAC and found that conjugate prepared by thiolation process were better in their immunogenicity as compared to
conjugate by direct coupling. It is possible that the direct coupling by EDAC through carboxylate and amino groups may produce some steric hindrance and also inactivate the serologically important carboxylate groups. Therefore, need for a method which utilizes a spacer molecule to reduce steric hinderance and does not touch the serologically important carboxylate and O-acetyl groups of K13 antigen is recognisable.

Chu et al (1991) compared three methods of polysaccharide protein conjugation with ADH as linker. The first two methods used adipic dihydrazide (ADH) which bind to the carboxyls of KDO at the reducing end and the third method used ADH which bind to CNBr activated polysaccharide (multipoint attachment method). The conjugates prepared by third method showed superior immunogenicity over the other two methods. Therefore, it is worth considering that the multipoint attachment method employed in our study to couple K13 polysaccharide to diphtheria toxoid is superior over the other methods of conjugations. The spacer molecule adipic acid dihydrazide (ADH), a six carbon moiety selected for K13-DT conjugate had already been known to provide consistently high yield of Hib-protein conjugate (Schneerson et al, 1980). Moreover, ADH derivatized antigens and conjugate containing ADH had not shown any toxicity or mutagenic activity using an in vitro assay system (Ames et al, 1975).

Many workers have tried to enhance the immunogenicity of different K-antigens of E. coli employing different methods and have reported variable success. Low anti-K1 antibody levels have been seen and the possible reason ascribed to it is due to structural similarities of
K1 antigen with sialic acid moiety found on the mammalian cells (Vann et al, 1981). Kaijser and Ahlstedt (1977) initially observed that the immunogenicity of K1 could not be improved by directly conjugating to bovine serum albumin. Later on, Kaijser et al (1983a) showed that coupling of K13 with bovine serum albumin rendered it immunogenic and high titres of both IgG and IgM antibodies were detected after two injections of conjugate at four weeks interval. In another study by Kaijser et al (1983b), attempt was made to couple K1 polysaccharide antigen of E. coli to bovine serum albumin. In all the 10 immunized rats, anti K1 IgG antibodies were detected. However, following immunization with isolated nonconjugated K1 antigen, no anti K1 antibodies were detected in the serum. In the present study, the conjugation of K13 capsular polysaccharide to diphtheria toxoid increased the immunogenicity of the K13 polysaccharide in mice. Three injections of K13-DT conjugate in mice subcutaneously at an interval of two weeks elicited both (i) an increased serum anti K13 antibodies titres and (ii) a booster response with memory. It has been reported that coupling of thymus independent polysaccharide antigens to carrier protein render them thymus dependent and immune response is characterized by high titres of IgG antibody and memory (Aplin et al, 1981). Several studies have confirmed the increased immunogenicity of H. influenzae, meningococcal and pneumococcal polysaccharides by their covalent attachment to immunogenic proteins (Beuvery et al, 1982; Anderson, 1983; Jenning et al, 1984 and Porro et al, 1985).

The selection of adjuvant has great influence on the antibody production to conjugate. The current United States infant vaccine
formula of DPT (diphtheria toxoid-tetanus toxoid-pertussis) vaccine is compounded with aluminum salt (adsorbed). The adsorption on the aluminum salt results in an adjuvant action upon the antibody response to DT and TT components and antibody levels remain elevated longer (Greenberg and Flemming, 1947 and Orenstein et al, 1983). Moreover, aluminum salts have an appreciable safety records as adjuvant for DPT vaccine and other vaccines used for humans. The aluminum hydroxide adsorbed Hib-TT conjugate (Hemophilus influenzae type b) elicited both earlier and higher titres of Hib antibodies in infant rhesus than did the same dose of unadsorbed conjugate (Schneerson et al, 1986). Adsorption of polysaccharide-protein conjugate on adjuvant had been observed to enhance the serum antibody response to polysaccharide antigen of various pathogens (Porro et al, 1985 and Chu et al, 1991). In present study a similar kind of observation was made, since, the adsorption of K13-DT conjugate on aluminum phosphate resulted in an earlier and higher antibody titres to K13 as compared to non-adsorbed conjugate.

Before evaluating the protective potential of K13-DT conjugate against ascending pyelonephritis, it was necessary to find out the suitable dose and route of immunization in mice. Out of the many doses of K13-DT conjugate injected based on corresponding level of K13 polysaccharide into the preparation, no statistically significant increase in anti K13 antibodies titres was observed following the dose level of 2.5 \(\mu g\) of K13 polysaccharide. Kaijser et al (1983a,b) also used the dose of 2.5 \(\mu g\) of K antigen to evoke an immune response to K-BSA conjugate while carrying out the protection studies in rats. In a model of mice, Szu
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*etal (1987) found a dose of 2.5 μg Vi antigen suitable to produce protective response by Vi-protein conjugates against typhoid fever. The *H. influenzae* and *N. meningitidis* polysaccharide vaccine dose in infant trials being used (Gold *et al*, 1977; Parke *et al*, 1977 and Peltola *et al*, 1977), correspond to the dose level of 2.5 μg of polysaccharide, which was used in mice in the present study.

In present investigation, subcutaneous route of immunization and a dose of K13-DT conjugate corresponding to 2.5 μg of K13 antigen was used for the experiments. In the earlier studies of protection against pyelonephritis in experimental animals many routes have been employed to administer the vaccine by different workers. Intravenous immunization with formalin or heat killed bacteria (Kaijser and Olling, 1973 and Brooks *et al*, 1974), intravesical immunization with formalin killed bacteria (Kaijser *et al*, 1978) and peroral immunization with live bacteria (Mattsby-Baltzer *et al*, 1982) had been tried to immunize the experimental animals. However, many reports of immunization with polysaccharide-protein conjugates in human and experimental animals had preferred subcutaneous route of immunization (Schneersen *et al*, 1980; Kaijser *et al*, 1983a,b; Schneersen *et al*, 1986; Chu *et al*, 1991 and Robberts *et al*, 1993).

In general, serum capsular antibodies whether natural or induced by convalescence from disease or by vaccination confer immunity against invasive disease caused by invasive bacteria (Robbins *et al*, 1975; Peltola *et al*, 1984; Gotschlich *et al*, 1984; Austrian, 1984 and Szu *et al*, 1987). In many studies, where whole cell immunization in experimental animals was carried out, the role of anti K antibodies in protection is also
documented (Kaijser and Oiling, 1973; Kaijser et al, 1978 and Mattsby-Baltzer et al, 1982). The degree of protection observed in our study using diphtheria toxoid as carrier protein was higher than previous K-BSA conjugates used in rats (Kaijser et al, 1983a,b). Protected animals in present investigation are also showing higher levels of anti K13 antibodies. Kaijser et al (1983a) had reported a good correlation between anti K13 antibodies and protection against pyelonephritis. The protective effect of antibodies against K1 and K13 had also been reported by passive immunization with monoclonal antibodies against K13 antigen (Kaijser et al, 1983b). We observed that immunization with K13 alone or K13 mixed with DT failed to protect against pyelonephritis. However, immunization with K13-DT conjugate showed significant decrease in the number of animals with pyelonephritis, when animals were sacrificed 7 days postinfection period in comparison to other groups.

To have better insight into the protection provided by K13-DT conjugate, the quantitative load of bacteria was evaluated in the kidneys. The pattern of disease was same in control and in animals immunized with unconjugated K13 antigen. However, K13-DT conjugate immunized groups showed a significantly less infection. The decrease in the bacterial counts could be due to the presence of anti K13 antibodies in the urine. It has been reported that the intraperitoneal, intravascular and intramuscular immunization with whole cells produces antibodies to specific antigens of bacteria in the urine (Kaijser et al, 1978 and Kruze et al, 1989). The transudation of antibodies from the serum is one of the sources of antibodies in the urine. However, it was speculated that transudation depends upon the concentration of antibodies in the serum.
of immunized animals, and when a threshold is exceeded, then the antibodies appeared in the urine (Kruze et al, 1989). In another study, both anti O and anti K antibodies were detected in the urine of the immunized animals. However, with the adsorption experiments it was shown that urinary anti K antibodies were especially important for the protection against pyelonephritis (Kaijser et al, 1978). Recently, immunization with O8 oligosaccharide-BSA conjugate vaccine did not significantly alter the duration of bacteriuria in the monkey model of pyelonephritis, but vaccine was efficient in preventing some renal histopathological damage (Roberts et al, 1993). In contrast, in the present investigation K13-DT conjugate was able to help in early clearance of bacteria from the kidneys, but could not prevent the initial colonization of organisms, since bacterial counts in all the groups are comparable after 2 days postinfection period.

O’Hanley et al (1990) had reported that though colonization proceeds, and is a prerequisite for invasion, the colonization and invasion of the mucosa are distinct pathogenic events mediated by separate molecules. Receptors for the pili are present in the renal tissue, which help in colonization but not in invasion. There are, therefore, some other molecules helping organisms to invade the tissue. The presence and the amount of capsular polysaccharide in general, or of the K1 polysaccharide in particular had been shown to be associated with increased bladder and renal colonization, renal pathology and lethality in experimental infections in mice (Kalmanson et al, 1975; Nicholson and Glynn, 1975; Montgomery, 1978; Keyti, 1981; Verweij-Van Vught et al, 1983 and Domingue et al, 1988). Kaijser et al (1983) demonstrated the
decrease in renal histopathological damage in K13-BSA immunized rats showing high titres of anti K13 antibodies. To confirm the renal invasion by the infecting organism, histopathology of the renal tissue was done at different time intervals in all the groups. K13-DT conjugate immunized groups showed lower pathological changes at all time intervals as compared to nonconjugate immunized groups. However, in these results, no apparent correlation between bacterial load and histopathological damage was observed, as the kidneys which were found to be sterile in the later stages of infection, also showed some degree of damage. Along with the protection, alternate mechanisms of tissue destruction is likely to be contributing to ultimate pathology observed in the experimental animals.

Acute pyelonephritis has been reported to be accompanied by suppurative inflammatory infiltrate (Kalmanson et al, 1975). The depletion of leukocytes in rats reduced the renal tissue destruction during the early phase of *E. coli* induced exudative pyelonephritis in spite of higher bacterial counts (Shimamura, 1981 and Bille and Glauser, 1982). These cells produce various Reactive Oxygen species (ROS) through respiratory burst activity to combat the invading bacteria. A self implicated excessive production of ROS by this inflammatory reaction may be the major underlying cause of tissue damage observed during the progression of disease.

To pinpoint the role of ROS in tissue damage, capacity of various phagocytic cells both from blood and kidneys in experimental animals was studied. In the K13-DT conjugate immunized and nonimmunized groups, the method of chemiluminescence (CL) was used in the study to
measure the generated ROS. This quantitate intra as well as extracellular ROS produced (Sangone et al., 1977). It is preferred in contrast to cytochrome C reduction method, which measures primarily the extracellular O₂ production and NBT reduction method which measures only intracellular O₂ production. The CL was measured at 37°C as recommended by Repine et al. (1979), who stated that PMNLs produce CL at 37°C. In addition, the CL response depends upon various factors including source and number of cells (Anderson and Amirault, 1979), type of gradient separation, medium used (pH, ions, glucose), purity of cells separated (Helstenson et al., 1986). The release of these ROS is influenced by a number of factors both in vitro and in vivo (Berton and Gorden, 1983). Sometimes, the excessive production of ROS (when more than 5% of oxygen converted into ROS) leads to damage of phagocytes themselves which produce them (Hanson, 1971) and contribute to surrounding tissue damage.

In all the phagocytic cells studied in the present study, there was an increase in ROS generation in the infected group (PBS immunized), which showed rise with the increase in infection days. However, study of localized macrophages and neutrophils from the kidneys showed that the major response was inflicted upon by the renal resident macrophages and neutrophils. The production of ROS by circulating monocytes and neutrophils was lesser, yet significantly higher than the cells from the control animals. The difference may be attributed to the direct and continuously localized interaction between the renal resident macrophages and neutrophils to invading pathogen, which leads to an enhanced response in kidneys.
Several investigators have suggested that if the bacteria remain extracellularly attached and continue triggering the release of reactive oxidative metabolites and lysosomal enzymes without being ingested, they may potentiate the inflammatory process and ultimately the tissue damage (Fantone et al., 1982; Ohman et al., 1982 and Ward et al., 1983). The results of Iwahi and Imada (1988) showed that two virulent strains of uropathogenic E. coli expressed an antiphagocytic activity during their growth in bladder and they stimulated continuously the oxidative metabolic burst of PMN without being ingested and killed. The antiphagocytic activity of these two strains was thought to be related to bacterial surface component(s). The capsular polysaccharide K-antigen of E. coli is known to impart antiphagocytic activity to the invasive E. coli strains (Hortwitz and Silverstein, 1980; Aguero and Cabello, 1983; Allen et al., 1987 and Sokolowska et al., 1989). It is now well established that addition of anti K-antibodies efficiently enhance the phagocytosis and PMN mediated killing of K-antigen encapsulated E. coli (Kim et al., 1990). Therefore, the possibility is that the less CL response in the K13-DT immunized-infected group as compared to only infected group (PBS immunized) is due to early and fast clearance of bacteria in the presence of anti K13 antibodies in K13-DT immunized-infected group. This, thus leads to the elimination of cause of continuous stimulus to generate ROS from the phagocytic cells. However, in the K13-DT immunized-infected group, CL-response measured was higher than the control animals even after the elimination of bacteria from the kidneys at 7 days of postinfection period. This finding can be explained on the basis of earlier reports that increased inflammatory response to localised bacterial
antigen even in the absence of bacteria and persistence of inflammation
even in the absence of viable bacteria in the kidneys has been reported

The time for attainment of peak CL response decreased in both the
K13-DT immunized-infected and infected groups (PBS immunized),
following quantitative increase of ROS in the cells of infected animals.
The results are in agreement with the study of Gupta et al (1992) who
studied the mechanism of tissue injury at the cellular level by following
the chemiluminescence response of various phagocytic cells in the E. coli
induced pyelonephritis. Their observation also showed that there was
marked increase in the capacity of phagocytic cells to produce ROS with
increasing days postinfection and peak CL response time was observed
to be decreased with the progression of disease.

We studied the change inflicted upon by ROS mainly through the
initiation of lipid peroxidation and alteration in enzyme activation. The
extent of lipid peroxidation can be quantitated by the formation of
malondialdehyde which was used as an index of lipid peroxidation in
various studies (Laurent and Ardaillou, 1986). Unsaturated fatty acids
which form an essential constituent of the cell membrane are most
vulnerable to oxidative attack by ROS and forms lipid peroxides, which
breakdown to form MDA as a stable product (Barber and Bernheim,
1967). MDA could be estimated by TBA reaction, which is widely used
as it offers speed, simplicity, sensitivity and could be directly applied to
complex biological tissues (Gutteridge and Quinlan, 1983). This
technique was used to estimate MDA in present study.

With the progression of disease, a significant increase in formation
of MDA in renal tissue homogenate was observed in infected group (PBS
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immunized). However, change was also observed at 4 and 7 postinfection days in K13-DT immunized-infected group. These results correspond with the level of ROS (CL response) and histopathological changes observed in these groups at specific interval. The low levels of MDA in K13-DT immunized-infected groups than the infected group (PBS immunized) shows the partial protection provided by K13-DT immunization. Clinically, rise in MDA contents in the chronic renal failure patients as a consequence of pyelonephritis accompanied by increased lipid peroxidation had been reported (Kishore et al., 1983). The similar kind of damage was also observed following ischemia (McCord, 1985). Vascular occlusion leading to ischemia was reported to be one of the mediator of tissue injury in pyelonephritis (Kaack et al., 1986). The tubulointestinal injury had also been found to be directly correlated with the amount of lipids, especially unsaturated fatty acids of membrane phospholipids (Kasiske et al., 1989). It has been suggested by Mathys et al. (1984) that the initial phase of cell damage may be due to accumulation of toxic breakdown products which enhance epithelial necrosis and induce bulbous expansion of the plasma membrane causing cell death ultimately. Lipid peroxides have also been shown to induce toxic effects on the cell structure observable as changes in the membrane fluidity, permeability, loss of membrane integrity, protein degradation and ultimately cell lysis (Halliwell and Gutteridge, 1982).

The activities of various BBM enzymes have been reported to be decreased in the renal BBM during pyelonephritis and could be used as biochemical marker of tissue injury by the disease (Garg et al., 1987 and Kaur et al., 1988). Some enzymes are important in generation of free
radicals and it was suggested by Roberts (1991) that the activity of these enzymes viz. glutathione reductase, glucose-6-P\textsubscript{4} dehydrogenase and lactate dehydrogenase (LDH) can also be used as biochemical markers of tissue injury. So, the activity of these enzymes was estimated in present study in order to assess the extent of tissue damage. It was found that the increase in their activity corresponded to the heightened tissue damage and increase level of MDA, which demonstrates that tissue damage might be a direct consequence of lipid peroxidation.

The MDA level and activity of glutathione reductase, glucose-6-phosphate dehydrogenase and lactate dehydrogenase in K13-DT conjugate immunized-infected group was significantly lesser than the infected group (PBS immunized) at different stages of infection. This difference also reveals the partial protection provided by K13-DT immunization against ascending pyelonephritis assessed by marker enzymes in the renal homogenate. Garg et al (1987b, 1987c) evaluated the protection against ascending pyelonephritis in rats following immunization with pili antigen using BBM enzymes as marker.

Since bacteria survive in spite of marked generation of ROS by phagocytes, it, therefore seems that ROS are unable to participate in the killing of invading organism. Some other oxygen independent mechanism might be involved for bacterial killing. The organism is probably able to survive the acute inflammatory reaction due to protective action of some specific antigens against ROS, which may further prevent their phagocytosis, as it was observed in uremic patients (Wysocka et al, 1984). Welch (1980) was unable to show a complete correlation between chemiluminescence (ROS generation) response by bacteria and their susceptibility to phagocytosis. The ability of various phagocytic cells
from blood and kidneys to kill the bacteria was also estimated by bactericidal assay. The observed significant decrease of percent killing in infected group (PBS immunized) was more pronounced in localized neutrophils and macrophages in the kidneys as compared to blood phagocytes and neutrophils. Wysocka et al (1984) found that phagocytosis in uremic patients was decreased, which might be responsible for the diminished bactericidal activity. Serum bactericidal activity had been reported to be decreased during pyelonephritis (Kalmanson et al, 1964). In addition, lysosomal enzymes of phagocytes, which are important for intracellular killing of bacteria are released in increased amount during experimental pyelonephritis (Vigano et al, 1987). The release of these hydrolases has been reported due to damage of lysosomes caused by lipid peroxidation in response to excessive production of ROS (Allison and Young, 1969). Fussel and Robert (1984) reported that phagocytosis during pyelonephritis was accompanied by mortal damage to both the surrounding tubules and phagocytes themselves, due to excessive inflammatory response.

The presence of capsular polysaccharide enables the bacteria to overcome host defence and protect the cells against bactericidal action of complement and phagocytosis. Allen et al (1987) reported that most of the intraphagocytosed \textit{E. coli} K1+ bacteria were able to remain intact upto 1 h after endocytosed in PMN. Many other studies also showed an antiphagocytic and anti-complementary affect of K antigen of \textit{E. coli} (Vermeulen et al, 1988; Sokolowska et al, 1989 and Kim et al, 1990). It was reported that for phagocytosis and killing of encapsulated \textit{E. coli}, both complement and anti K antibodies were required. In the absence of
Discussion

Antibody, *E. coli* K1 capsule blocked complement fixation to bacterial surface (Horwitz and Silverstein, 1980). The enhancement in the bactericidal activity of serum and PMN mediated killing of K encapsulated *E. coli* by anti K antibodies has also been evidenced (Kim et al., 1990). The difference observed in percent killing of bacteria by K13-DT immunized-infected group and infected group (PBS immunized) in the present work can be explained on the basis of comparatively presence of high titres of anti K13 antibodies in the former group. However, the exact contribution of capsular antibodies to the process are to be defined.

Experimental pyelonephritis is characterized by the presence of numerous mononuclear cells in the interstitial filtrate, most of which appears to be medium or large sized lymphocytes on the morphological ground. Although major protection against the invading bacteria was thought to be provided by the antibody production, role of cells mediated immunity in the process is yet not fully understood (Smith, 1975). To determine the association between cell mediated immunity and renal infection, the lymphoblastogenic responses to nonspecific and specific antigens were studied in the present study. In the infected group (PBS immunized) suppression of T-lymphocyte responses to Con A and PHA was noted both on 4 and 7 days postinfection and maximum degree of suppression was seen on 4 days post infection. The suppression in the lymphoblastogenic response may be attributed to burst of rapid bacterial multiplication on 4 days postinfection. The suppression of T lymphocytes to Con A and PHA in pyelonephritis was also demonstrated by Williams et al (1976); Miller et al (1979) and Ahlstedt et al (1983).
In the K13-DT immunized-infected animals, no marked suppression of the lymphoblastogenic response to Con A, PHA, K13-DT and DT was seen except little suppression on 4 days postinfection. This may be due to rapid clearance of bacteria in these groups. However, the stimulation indices were higher in these groups as compared to infected group (PBS immunized). In one of the studies done on patients of pyelonephritis, increasing lymphoblastogenic responses were observed parallel to the clinical improvement (Ahlstedt et al, 1983).

Miller et al (1978) attributed the suppression of lymphoblastogenic response to a increase in suppressor cell population generated during renal infection. The strong evidence for the generation of suppressor cells was shown by Miller et al (1979), who observed restoration of the immune responsiveness of spleen lymphocytes by removing the suppressor cell population. The results of present study showed a decrease in the CD4+/CD8+ cells ratio in infected group (PBS immunized) on 4 and 7 days postinfection periods. However, higher CD4+/CD8+ ratio was observed in K13-DT immunized-infected group as compared to infected group (PBS immunized). Immune system in K13-DT immunized infected group was less affected by the bacterial infection in the kidneys.

During establishment of pyelonephritis potentially effective CMI may be ablated by cells forming the cellular milieu. This may also explain the persistence of infection in pyelonephritis which remain one of the characteristic feature of the disease, despite seemingly adequate local immune response within infected kidneys. However, immunization with K13-DT prevented suppression of the cell mediated immune system to some extent and cell mediated response along with humoral responses
to K13 antigen helped in rapid clearance of bacteria in K13-DT immunized animals.

The results of present study conclusively indicate that the conjugation of capsular polysaccharide K13-antigen to diphtheria toxoid enhances the immunogenicity of K13-antigen. Further, it produces right type of immune response through activation of humoral and cell-mediated immunity against invading organism. The immunoprotective effect noticed against the disease opens the vistas of its feasibility as subunit vaccine against ascending pyelonephritis, keeping in view the renal protection and effective activation of immune system in the K13-DT immunised animals.