Community as well as hospital acquired respiratory tract infections are a major cause of morbidity and mortality throughout the world, despite therapeutic advances. *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* are two major gram negative bacteria associated with both acute and chronic pulmonary infections in hospitalized and immunocompromised patients (Merchant *et al*., 1998; Gales *et al.*, 2002). Pneumonia caused by these two pathogens is difficult to treat, due to their multifactorial virulence strategy as well as resistance to antibiotics. Formation of biofilms on medical devices, like endotracheal tubes, is a common approach used by these organisms for survival in the host’s respiratory tract (Donlan, 2001). This leads to the occurrence of persistent infections in patients requiring mechanical ventilation leading to further complications specially in hospitalised patients.

In the initial stages, the invading pathogen, in the respiratory tract, encounters the phagocytic cells (alveolar macrophages) of the host. Interaction between the host’s defense system and the pathogen determines the final outcome of the infection (Green *et al*., 1977). Efficacy of the host’s immune system is also dependent on many factors which include the nutritional status (Field *et al*., 2002). In the present study, therefore, initially it was planned to establish reproducible acute and chronic pneumonia in normal animals with planktonic and biofilm cells of *P. aeruginosa* (PA103 strain) and *K. pneumoniae* (B5055 strain). This has
been done in normal mice where the course of infection has been studied upto 14 days in acute form of infection and upto six weeks in chronic model of infection, with both the organisms. Recently, attention has been focussed on dietary n-3 polyunsaturated fatty acids (PUFA), since these fatty acids have proven to be beneficial against many non-infected diseases (Simopoulos, 2002). However, about their role in host’s immunity against infectious diseases, little is known. It is in this context, that, in the later part of this study, it was planned to elucidate the role of dietary supplementation with n-3 PUFA against experimental pneumonia induced by the two gram negative bacterial strains. Majority of the experiments have been carried out with alveolar macrophages and lung tissue homogenates collected from different groups of animals given diet supplementation.

Well characterized animal models, that mimic clinical infections in man, are very useful in providing precise insight into the pathogenesis of infectious diseases as well as evaluation of antibiotic treatment schedules in human infections under similar conditions of intensity and duration of infection (Bakker-Woudenberg, 2003). Rats (Bakker-Woudenberg et al., 1982) and mice (Nishi and Tsuchiya, 1980; Cortes et al., 2002) have been widely used to study acute lung infections. The use of mice as animal models for pneumonia has increased over the last decade due to economic factors and easy availability. In addition, the genetic similarity between mice and humans also allows the extrapolation of results to human situations in a more meaningful way (Kamnasaran, 2000). With the availability and use of immunocompromised (including leukopenic, irradiated etc.), genetically altered and knock-out mice, studies can be carried out in the context of human clinical situations (Aratani, 2000; Ye et al., 2001; Moore et al., 2003, 2005). All these factors form the basis for the use of mice, as experimental animals, in the present study so as to establish acute as well as chronic pneumonia with both the pathogens in two different cell forms (planktonic and biofilm cells).
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*P. aeruginosa* is a frequent cause of life threatening infections in immunocompromised and hospitalised patients but it rarely infects a healthy host. Animal models described in literature have been developed, either in immunodeficient mice or specific gene-deficient mice, to study *P. aeruginosa* induced lung infection. In a pilot experiment, acute pneumonia was induced in BALB/c and LACA (Swiss-Webster) strains of mice with planktonic cells of *P. aeruginosa* PA103. The pathogen was cleared from the lungs of BALB/c mice within 24 h. In LACA mice, the infection persisted till 24 h and lungs became bacteriologically sterile at 48 h. Keeping the importance of suitable animal strain in mind, LACA mice were used throughout the study carried out with *P. aeruginosa* PA103. Earlier, Morissette et al. (1995, 1996) observed that BALB/c mice strain had strong ability to clear *P. aeruginosa* from their lungs and were termed as resistant strains, while susceptible DBA strain developed severe lung infection.

Allewelt *et al.* (2000) in their study, brought out the importance of anaesthesia to be given to mice as a necessary step for successfully inducing infection with *P. aeruginosa*, at an approximate dose of $3 \times 10^8$ CFU per mouse. In contrast, unanesthetised mice were able to clear up to $10^{10}$ CFU per mouse. In a pilot experiment of the current study, mice were anaesthetized, with dizapam and diethyl ether, prior to induction of pneumonia. However, no significant variation in the bacterial colonization of lungs among different groups, was observed when compared to unaesthetized infected mice. Hence, animals were subsequently given infection without anaesthesia. The strain employed by the workers in the above mentioned study was *P. aeruginosa* PA103, which is a cytotoxic and highly virulent strain. It is known to express most of the virulence factors In the present study also, same strain has been employed. Acute pneumonia was induced in LACA (Swiss Webster) mice, with planktonic cells of *P. aeruginosa* PA103, at an optimal dose of $1 \times 10^8$ CFU per mouse. For comparison, a matching study is available where a higher
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inoculum in the range of $1.5 \times 10^9$ CFU per mouse has been instilled in neonatal mice to induce acute pneumonia (Pearson et al., 2000). However, the strain of *P. aeruginosa* employed by these workers was PA01. This brings out that an opportunistic pathogen like *P. aeruginosa* can successfully induce acute lung infection in normal host, at a relatively high dose which also gets cleared within 24 to 48 h.

The three most commonly used routes for instillation of microorganisms to produce pulmonary infections are direct intratracheal route, aerosol inhalation and intranasal inoculation (Bakker–Woudenberg et al., 2003). Out of these, intratracheal inoculation is artificially induced route of infection, since it requires anaesthesia and surgical skills to directly introduce bacteria into the lower respiratory tract of animals (Kooguchi et al., 1998). However, this technique is precise and reproducible, having low variation among the individual animals. Aerosol method of inoculation offers the possibility of infecting a large number of animals simultaneously, but it is time consuming and lacks the exact delivery of inoculation. Also, the stability of some virulent strains is affected by aerosolization (Bakker-Woudenberg, 2003). Intranasal route is closest to the natural portal of entry for pathogens. It is easy to follow and also gives reproducible results (Tang et al., 1995; Pearson et al., 2000; Schultz et al., 2001). Mild anaesthesia prior to inoculation into the nostrils is recommended by Bakker-Woudenberg (2003) to minimize variable lung deposition or swallowing of inoculum. In the present study also, intranasal route of inoculation was adopted to induce pneumonia in experimental mice.

Quantitative cultures from infected organs at different time intervals following infection, are often performed to assess a progressive microbial growth during acute pulmonary infection. In the current study, it was observed that lung bacterial load, with planktonic *P. aeruginosa* PA103, was maximum at 6 h post infection period with a sharp decline at 24 h. There was complete elimination of bacteria from the lungs of infected mice.
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at 48 h. Similar observations were made by George et al. (1993) and Cheung et al. (2000) who also recorded maximum lung bacterial load in mice at 3 h following intranasal instillation of *P. aeruginosa* PA01 which declined on subsequent days. In an earlier study, Tang et al. (1995) also studied the time course of *P. aeruginosa* mediated pneumonia in neonatal mice. They found evidence of pulmonary infection as early as 4 h by organ culture with peak of infection at 24 h. The bacteria persisted till 72 h. Since adult mice were used in the present study, this possibly could explain the difference in peak infection time as well as the persistence of organisms observed in the two studies. Schultz et al. (2001), in their investigation with *P. aeruginosa* PA103, induced acute pneumonia in C57 B1/6 mice. They found significant lung bacterial load at 6 h which further increased at 24 h after intranasal inoculation. The difference in the peak time of infection observed in the current study (6 h) attributed to the different mice strains used in two studies. C57B1/6 is a nude strain used for a wide range of studies which require an immunodeficient host status. On the contrary, LACA mice employed in the current investigation are healthy hosts, providing immunocompetent host environment. This investigation brings out that it is possible to induce acute pulmonary infection in healthy adult mice, provided the strain of animals, pathogen as well as infective dose of the organism is carefully selected.

The evaluation of pathology of lung tissue carried out at different time intervals gives insight in the severity of tissue destruction and inflammatory response. In previous studies, severe acute inflammatory response and tissue destruction has been reported during early phase of *P. aeruginosa* induced acute pneumonia (Sawa et al., 1998; Smith et al., 2004). In the present study also, lung pathology revealed signs of severe bronchopneumonia as early as 6 h post infection with observable acute inflammatory destruction of bronchioles and peribronchiolar alveoli and oedema in alveolar lumina. In addition, bacterial colonies were also demonstrable at certain places. Though, this opportunist organism was
able to colonise and invade the normal lungs of LACA mice, but the severity of inflammation decreased rapidly by 72 h post infection.

The extent of tissue destruction is determined by ability of the host to provide defense within the pulmonary compartment which is regulated by a complex interplay between cellular and humoral effector mechanisms. The recruitment of polymorphonuclear neutrophils (PMNs) and density of alveolar macrophages is essential for the clearance of virulent bacteria which enter the lower airspaces (Ozaki et al., 1989). The acute inflammatory response to the invasion by *P. aeruginosa* consists of immigration of neutrophils from pulmonary capillaries into the alveolar spaces. Therefore, to assess PMN recruitment, whole-lung bronchoalveolar lavage was performed following intranasal instillation of *P. aeruginosa*. The study revealed that maximum PMN influx occurred at 6 h post infection time and was followed by a sharp decline at 24 h. Rapid neutrophil influx in the lungs of mice during *P. aeruginosa* challenge has also been reported in earlier studies (Kooguchi et al., 1998; Daecheux et al., 2001). However, Schultz et al. (2001) observed maximum PMN infiltration in the lungs of infected mice at 24 h post infection. The variance observed in this finding can be attributed to the difference in animal strains employed in the two studies. The results of the present study are in accordance with recent observations made by Ader et al. (2005) who recorded an initial rapid increase in PMN counts in BALF at 4 h followed by a steady decline in their numbers in mice infected with *P. aeruginosa* PA103.

A biochemical method that allows indirect quantification of tissue-associated PMN infiltration is based on the assessment of myeloperoxidase (MPO) activity. This enzyme is present in azurophil granules of the PMNs recruited to the injured tissue and mediate the acute phase of inflammatory response (Graff et al., 1998). MPO is not found in normal, non-inflammed tissue. In the present study, maximal MPO levels were detected in the lungs infected with planktonic cells of *P. aeruginosa*.
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PA103 after 6 h and decreased at subsequent time intervals. This coincided with maximum PMN counts and lung tissue pathology. Tsai et al. (2000) also reported an early rise (observable at 12 h) rise in MPO levels in mice following *P. aeruginosa* induced lung infection. Earlier, Kooguchi et al. (1998) observed an initial increase in MPO levels at 8 h following intratracheal administration of *P. aeruginosa* in mice which correlated with maximum PMN counts in the BALF. In another study, MPO deficient mice showed increased susceptibility to *P. aeruginosa* induced lung infection (Aratani et al., 2000). MPO estimation, thus, provides an indirect but accurate and relatively rapid index of intrapulmonary sequestration of neutrophils.

The damage inflicted upon the host cells by reactive oxygen species (ROS), produced by host phagocytes to combat the invading pathogens, includes initiation of lipid peroxidation and nitric oxide production. Unsaturated fatty acids, which form an essential constituent of the cell membranes are most vulnerable to oxidative ROS. They form lipid peroxides which break down to form malondialdehyde (MDA) as a stable end product (Barber and Berhein, 1967). Therefore, the extent of lipid peroxidation can be quantitated by the formation of MDA. It can be estimated by TBA reaction which is widely used since it offers, simplicity, sensitivity and can be directly applied to complex biological tissues (Gutteridge and Quinlan, 1983). In the present study, maximum lipid peroxidation, as detected by MDA production, was observed at 6 h post infection in the lung tissues of mice infected with planktonic cells of *P. aeruginosa* PA103. Significantly enhanced lipid peroxidation has earlier been reported in *P. aeruginosa* mediated infections (Nakayama et al., 1998; Shmarina et al., 2001).

Nitric oxide is one of the major mediators of innate immune response due to its involvement in the alveolar macrophage bactericidal activity and neutrophil activation ability. It has been shown to possess broad spectrum antimicrobial properties and is synthesized by the
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epithelium of respiratory tract together with inflammatory cells (Bogdan, 2001; Declaux and Azoulay, 2003). Consequently, nitric oxide diffuses in the airway and can, therefore, be measured in the exhaled gas (Kharitonov et al., 2001). During infectious process of respiratory tract, an increase in the exhaled nitric oxide is reported, probably due to increased output from both the epithelium and inflammatory cells. In the current investigation, nitric oxide production in BALF as well as lung tissues of mice induced acute pneumonia was evaluated by measuring nitrite which is a stable end product. Maximum nitrite levels were observed at 6 h post infection followed by a decrease in the lung bacterial load till 48 h post infection. In a matching study, similar observations were recorded with maximum nitric oxide production at 6 h of infection in BALF of mice given intranasal infection with P. aeruginosa PA103 strain (Schultz et al., 2001). Nitric oxide generation, therefore, is important for host defense against pneumonia.

Cytotoxic effects of the bacteria in the lung tissue were studied through the estimation of lactate dehydrogenase (LDH) enzyme activity which acts as a biochemical marker of tissue injury in the host. It is a cytoplasmic enzyme which is released upon cell disruption due to cytotoxic activity of pathogens. Maximum LDH activity was observed at 6 h of infection. The virulence and cytotoxicity of P. aeruginosa PA103 have been associated with the expression of exoenzyme U (Exo U). Disruption of exo U has been reported to cause loss of cytotoxicity and reduced virulence in murine acute lung infection model (Fink-Barbancon et al., 1997; Allewelt et al., 2000). Elevated levels of MDA and LDH correlated with maximum tissue damage in the present investigation, thereby suggesting a positive correlation between the two.

The inflammation during pneumonia is orchestrated by locally produced cytokines. The role of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 in pneumonia has been demonstrated in several experiments (Nelson, 2001). These cytokines and chemokines (such as
MIP-1 and MIP-2), produced by the alveolar macrophages upon invasion of pathogens, lead to vigorous inflammatory response, involving the recruitment and activation of neutrophils, coupled with production of nitric oxide which are critical for the resolution of infection (Jain-Vora et al., 1998). Amongst these, TNF-α is known to play a protective role in resistance to *P. aeruginosa* infection. Gosselin et al. (1995) reported that the levels of TNF-α mRNA expression were up-regulated in different strains of mice during early phase of the *P. aeruginosa* mediated lung infection. The increase was observed to a greater extent in resistant BALB/c mice than in susceptible DBA/2, C57B1/6 and A/J strains of mice. This observation paralleled a higher secretion of TNF-α into alveolar space of BALB/c at 3 and 6 h post infection. These findings corroborate the results of the present investigation where maximum levels of TNF-α were detected in BALF as well as LHS at 6 h post infection followed by a decline during acute pneumonia in LACA (Swiss Webster) mice induced with planktonic cells of *P. aeruginosa* PA103. In another matching study, Schultz et al. (2001) have reported a localized and markedly enhanced lung levels of TNF-α at 6 h post infection period with strain PA103 in C57B1/6 mice. In the earlier study of Gosselin et al. (1995), treatment of mice with anti-murine TNF-α monoclonal antibody or with aminoguanidine significantly increased the number of *P. aeruginosa* detected in the lungs. Treatment of granulocytopenic mice with low doses of TNF-α significantly diminished mortality and enhanced pulmonary clearance of *P. aeruginosa* during severe pneumonia (Amura et al., 1994). All these studies conclude that endogenously produced TNF-α is important for host defense against pneumonia. Enhanced levels of TNF-α, in the BALF as well as LHS, in the early course of infection could be responsible for rapid clearance of the organism from the lungs of experimental mice.

There are reports available, where intramuscular or intraperitoneal administration of recombinant (r)IL-1α or rIL-1β respectively, protected
mice from lethal systemic *P. aeruginosa* infections (Ozaki *et al.*, 1987; van der Meer *et al.*, 1988). In relation to lung infections, besides TNF-α, production of IL-1 (IL-1 alpha and IL-1 beta) has also been reported, in the pulmonary compartment, though its precise role has yet not been defined (Kronberg, *et al.*, 1993; Bonfield *et al.*, 1995). A study reported by Schultz *et al.* (2002) brings out that high levels of IL-1β, in the lungs of C57B1/6 mice following intranasal infection with *P. aeruginosa* PA103, were recorded at 6 h which further increased at 24 h post infection. However, to confirm the role of endogenous IL-1 during pneumonia, these workers employed IL-1R type 1 gene – deficient mice (IL-1R\(^{-}\)). It was observed that absence of pro-inflammatory IL-1 signal was associated with an improved clearance of *P. aeruginosa* from the lungs of infected mice. However, this findings was apparent only after 24 h of infection based on the lung bacterial count as well as number of neutrophils going down in IL-1R\(^{-}\) mice as compared to wild type normal mice. In the present study, acute pneumonia induced by planktonic cells of *P. aeruginosa* PA103 resulted in significant production of IL-1β in BALF as well as lung tissues. Maximum levels of this pro-inflammatory cytokine were detected at 6 h and remained elevated till 24 h post infection period in lung tissues which was followed by a sharp decline. Despite the elevated levels of IL-1β even after 24 h, there was a sharp decrease in the lung bacterial counts and severity of infection which is contrary to the above reported study. This can be attributed to different mice strains employed in the two studies. Normal immunocompetent LACA mice were employed in our study, whereas C57B1/6 mouse strain was used by Schultz *et al.* (2002).

The anti-inflammatory cytokines IL-10, IL-4, IL-11 and IL-13 are a series of immunoregulatory molecules that control the inflammatory response (Opal and DePalo, 2000). Amongst these, IL-10 is a potent inhibitor of pro-inflammatory cytokines like TNF-α, IL-1 and IL-6 (Fiorentino *et al.*, 1991; Casatella *et al.*, 1993; Salez *et al.*, 2001). IL-10 production in the lungs of mice with *P. aeruginosa* induced acute
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Pneumonia has not only been found to improve lung injury but survival as well, (Sawa et al., 1997). Relatively earlier studies in different experimental settings, where infections were induced with different organisms, also bring out the beneficial effects of administration of rIL-10 on the survival: IL-10 administration prevented hypothermia and improved the mortality in BALB/c mice given LPS (Gerard et al., 1993; Howard et al., 1993). On the contrary, IL-10 knock out mice had increased susceptibility to shock induced by LPS injection when administered IL-10 concomitantly which protected these mice from lethal endotoxemia. IL-10 has also been suggested to play a protective role in staphylococcal enterotoxin β-induced shock and against lethal group B streptococci (GBS) induced sepsis (Florquin et al., 1994; Cusumano et al., 1996). In contrast, adverse effects of IL-10 production or administration have been observed against S. pneumoniae induced lung infection in mice (Mohler et al., 1998). The variability in these studies may be due to different bacterial species and different strains of mice employed, coupled with different routes followed for administration of IL-10. Sawa et al. (1997) reported that instillation of cytotoxic P. aeruginosa PA103 strain led to the upregulation of IL-10 mRNA in the lungs and increased the concentration of IL-10 in the blood which was not observed with non-cytotoxic strain (PA01). Also, the systemic administration of rIL-10 significantly decreased the lung injury and the mortality in mice infected with cytotoxic bacterial strain. These workers suggested a potential beneficial role of IL-10 in the treatment of cytotoxic P. aeruginosa pneumonia. Chmiel et al. (1999) have described that IL-10 null mice showed prolonged and excessive pro-inflammatory cytokine production and neutrophil infiltration in the airways after P. aeruginosa infection. In the present investigation also, kinetics of IL-10 production in the BALF and lung homogenates bring out that there is a gradual increase in the production of this anti-inflammatory cytokine with maximum levels observable at 48 h post infection period. The increase in IL-10 level was associated with corresponding decrease in TNF-α, as well
as IL-1\(\beta\). Production of IL-10 also appears to have helped the host to overcome acute lung infection since its production was associated with decreased bacterial load as well as improved lung pathology.

The formation and development of biofilms is considered to be an important stage in the pathogenesis of numerous bacterial species including *P. aeruginosa* and *K. pneumoniae*. The tendency of microorganisms to develop biofilms is also well documented on a number of medical devices like urinary catheters and endotracheal tubes. This is a great cause of concern to the clinicians due to the persistent and chronic infections caused by this form of organisms (O'Toole and Kolter, 1998; Costerton *et al.*, 1999). Biofilm formation is a process whereby microorganisms irreversibly attach to and grow on a surface and produce extracellular polymers and that facilitate attachment and matrix formation, resulting in alteration in the phenotype of the organisms with respect to growth rate and gene transcription (Watnick and Kolter, 1999; Donlan, 2001). The establishment of biofilms by pathogenic bacteria, on the tissues of susceptible hosts, is believed to inhibit the effectiveness of antibiotic treatment. It also protects the organism against host defense mechanisms which facilitate the bacterial colonization and expression of virulence determinants (Lavender, 2004). *P. aeruginosa* is an efficient opportunistic pathogen which is often associated with lung infections in cystic fibrosis patients as well as mechanically ventilated patients where they are exposed to aspiration of stationary phase biofilm-grown bacteria (Sadikot, 2005).

In the present study, 4-day old, preformed biofilm cells of *P. aeruginosa* PA103 have been employed to induce acute as well as chronic pulmonary infection in normal mice. The model serves to compare the basic virulence potential of the two cell forms in a normal host having no predisposing factors. Earlier, Hoiby *et al.* (2001) stated that animal models of biofilm bacteria like *P. aeruginosa* can be established in experimental mice by intratracheal inoculation of bacteria in agar beads or
alginate beads. The histopathological changes, immune response of the host and the efficiency of antibiotic therapy can then be followed. Importance of fresh and old biofilms has been addressed to by Anwar et al. (1992) and Stewart et al. (1994). These workers described that older biofilms (7 day) are more resistant to antibiotics and towards host phagocytic response, whereas young biofilms (1 day) are less resistant. This is due to several factors like slow growth (prolonged generation time), reduced oxygen concentrations at the base of the biofilm, production of β-lactamase by the bacteria and nature of the surface material supporting the biofilm. However, earlier work carried out in our laboratory, in relation to *P. aeruginosa* induced urinary tract infections, which is another example of mucosal infections, brought out that severe and more persistent urinary tract infection can be established with 4 day-old biofilm cells of *P. aeruginosa* in normal mice (Yadav et al., 2003; Mittal et al., 2004). Hence, in the present study also, acute pneumonia was induced with a 4 day-old biofilm cells of *P. aeruginosa* PA103 grown *in vitro* on endotracheal tube. Time course of infection studied over a period of three days revealed that, animals infected with planktonic cells showed clearance of bacteria earlier than the ones given infection with biofilm cells. Similarly, tissue damage and other parameters were significantly higher in biofilm cells infected animals. For comparison, there are no matching studies available. It is possible that biofilm cells, prepared *in vitro*, could have reverted to the original planktonic form, in the host environment *in vivo*. This is unlike the human situations, where *in vivo* biofilm formation takes place on the endotracheal tubes which accounts for longer persistence and severity of infection observed in such situations. To gain a better understanding of the pathogenesis of biofilm cell forms of bacterial infections, future studies should be directed towards generating biofilms *in vivo*, simulating growth conditions that match with different clinical situations.

Chronic lung infections with *P. aeruginosa* and associated airway inflammation are the major causes of morbidity and mortality in patients with cystic fibrosis (Govan and Deretic, 1996). Although complete
understanding of host–pathogen interactions in cystic fibrosis is lacking, it is likely that persistence of *P. aeruginosa*, the most common pathogen in this disease condition, contributes to overall progression of the disease (Welsh *et al.*, 1995; Yu *et al.*, 1998). Several useful models of chronic *P. aeruginosa* lung infections have been developed in mice and other animals (Cash *et al.*, 1979; Winnie *et al.*, 1982; van Heeckeren *et al.*, 1997; 2001; Boyer *et al.*, 2005). The type of tissue damage and host response to *P. aeruginosa* in mouse, are similar to those that occur in cystic fibrosis patients (Day *et al.*, 2004). Most of these workers have employed agar bead model of chronic bronchopulmonary infection, with different strains of *P. aeruginosa* to study the pathogenesis of cystic fibrosis. However, one disadvantage of agarose bead infection model is that it does not simulate the natural mechanisms operative in the evolution of *Pseudomonas* infection, from airway colonization to the stage of lung injury (Starke *et al.*, 1987). In addition, the making of agarose beads is a critical and tedious step in itself. Inoculation of mice and mortality due to technical and infection related factors also needs consideration (van Heeckeren and Schultzer, 2002).

In order to investigate the factors contributing to the bacterial persistence and associated inflammatory processes in the lung, Yu *et al.* (1998; 2000) developed a mouse model of *P. aeruginosa* with repeated aerosol exposures. Based on similar approach, chronic lung infection was established in normal LACA mice in the present study. Animals were given repeated intranasal infections of planktonic cells of *P. aeruginosa* PA103. The usefulness of recurrent lung infections in cystic fibrosis patients can be illustrated in such experimental situations.

In the current study, it was observed that though lung bacterial counts declined sharply following first exposure, but, were significantly raised subsequently with repeated infections. The corresponding lung pathology performed at weekly intervals also revealed mild inflammation after 1st exposure, comprising mainly of neutrophil infiltration in
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peribronchiolar areas. This was followed by progressive increase in pathology, characterized by intense chronic inflammation observable at 4th week post infection. There was evidence of chronic abscess formation, with a focus of necrosis, surrounded by fibroblastic proliferation and lymphomononuclear inflammatory cells. Matching pathological observations have been reported in an earlier study by Starke et al. (1987). These workers had developed a mouse model of chronic pulmonary infection with *P. aeruginosa* impregnated agar beads. Necrotizing and granulomatous changes with fibrosis were noticeable as early as 10 days post infection, extending up to 21 days, in the lung tissues of infected mice. The findings in the present study corroborated the recent observations made by Yu et al. (1998), who also reported maximum lung tissue destruction at 3 weeks post infection with most prominent changes consisting of peribronchial cuffing and bronchial epithelial hyperplasia. This was followed by a subsequent decrease in the lung pathology at 6th week onwards. The similar trend was also observed in the present investigation. In the present study, a new bolus of *P. aeruginosa* PA103 was deposited intranasally in the lungs every 48 h when the previous bacterial load was significantly reduced. On the other hand, Yu et al. (1998) exposed experimental mice to a fresh aerosol of bacterial load every 72 h. This variation in the time of inducing fresh infection, in the two studies can be attributed to the difference in persistence of the bacterial strains as well as different strains of mice employed. The repeated intranasal infection model of chronic lung infection, induced by *P. aeruginosa*, supports the notion that this opportunistic pathogen can cause considerable damage to the normal host if it is exposed to the organism repeatedly.

Before the development of lesions in the lung tissue, in the form of observable pathology, there is an interplay between the host and pathogen leading to certain cellular as well as biochemical changes, resulting in lipid peroxidation and cytotoxicity. Significant levels of lipid
peroxidation and cell cytotoxicity were noticeable during the course of chronic pneumonia induced by *P. aeruginosa* PA103 in mice. The production of iNOS (inducible nitric oxide synthase) has been reported to be constitutive in both human and murine airway epithelia (Hardley and Drumm, 1998). Nitric oxide dependent killing of *P. aeruginosa* in excised murine lungs and reduced control of the pathogens in animals treated with iNOS inhibitor amino-guanidine has been observed earlier (Gosselin et al., 1995). Reduced clearance of *P. aeruginosa* from the respiratory tract of transgenic mice lacking TNF-α and iNOS has also been reported by Yu et al. (2000). This suggests that these mediators of innate immunity may be critical for lung defense against *P. aeruginosa* induced chronic lung infections. The results of the present study also show that consistently elevated levels of nitrite in BALF as well as LHS might have played a protective role towards later time period when there was improved lung pathology.

Pulmonary infections caused by *P. aeruginosa* are associated with increased production of various cytokines that regulate lung host defense and inflammation. TNF-α plays a dual role by upregulating adhesion molecules such as ICAM-1, a vital component of polymorphonuclear leukocyte recruitment and by contributing to the restriction of microbial growth by amplifying the innate clearance mechanisms (Bermudez and Young, 1988; Mulligan et al., 1993). Protective role of TNF-α in innate resistance to *P. aeruginosa* infections has been proposed by several workers in animal models (Buret et al., 1994; Gosselin et al., 1995; Chen et al., 2000; Lee et al., 2003). Diminished clearance of *P. aeruginosa* in transgenic TNF-α deficient mice has been reported by Yu et al. (2000) in a chronic lung infection model. Paradoxically, though TNF-α levels are elevated in the BALF of cystic fibrosis patients, yet they cannot clear *P. aeruginosa* from their lungs. This is because immunoreactive TNF-α in their bronchoalveolar fluid, is biologically inactive and thus not available to stimulate antipseudomonal activities in the respiratory tract (Yu et al., 1995).
The role of pro-inflammatory cytokines is intriguing, as most of the available data suggests that TNF-α is critical for activating phagocytic cells to clear the bacteria (Sadikot, 2005). Although TNF-α appears to be necessary for effective host defense against *P. aeruginosa*, IL-1 family of pro-inflammatory cytokines is reported to have deleterious affect on the host against the bacterial infection in a mouse model of *P. aeruginosa* induced acute pneumonia (Schultz *et al.*, 2002). However, the precise role of IL-1β production during chronic infections still remains to be defined. In the present study, concentrations of TNF-α and IL-1β remained significantly increased during the course of chronic infection with maximum levels observed following 1st infection. How exactly raised levels of IL-1β contribute towards the evolution of chronic lung infection, induced by *P. aeruginosa* PA103, needs to be studied.

IL-10 is an anti-inflammatory cytokine which has been shown to be important in evolution of bacterial pneumonia and in sepsis (Walley *et al.*, 1996; Steinhauser *et al.*, 1999). Anti-inflammatory cytokines are involved in regulating the potentially damaging effects of neutrophil infiltration. Yu *et al.* (1998) highlighted the importance of IL-10 production in their mouse model of chronic lung infection. They employed IL-10T (transgenic) mice to induce chronic infection and found that IL-10 deficient mice displayed an array of differences in contrast to normal mice. This included significant mortality upon 1st exposure of the pathogen and increased pathology in the lungs detectable on repeated exposures. In another well planned study, Chmiel *et al.* (1999) reported that IL-10 deficiency was associated with excessive and prolonged inflammatory response, as well as morbidity, in a mouse model of chronic lung infection induced by *P. aeruginosa*. These effects were ameliorated by exogenous IL-10 administration in infected normal mice. In the present investigation also, the kinetics of IL-10 production revealed maximum production of IL-10 in BALF and LHS of infected mice after the first exposure. This was followed by a fall in its levels. There was again a slow and gradual increase in its
production in the lung tissues with repeated infections observed till 6th week. This sustained rise in IL-10 could have played an important role in the improved pathology observed towards the end of the study period. The current study, thus, brings out that repeated intranasal infections with \textit{P. aeruginosa}, in normal mice, presents an ideal situation to study host-pathogen responses in relation to chronic lung infection.

Persistence of opportunistic pathogens like \textit{P. aeruginosa} has been linked with its ability to convert to mucoid phenotype form characterized by overproduction of exopolysaccharide alginate (Hentzer \textit{et al.}, 2001). This property, in turn, affects the structure and function of biofilm formation by \textit{P. aeruginosa}, leading to establishment of chronic infections, especially in ventilated patients and those suffering from cystic fibrosis or diffuse panbronchiolitis. Biofilm, formed by an alginate overproducing \textit{P. aeruginosa} strain, exhibits a highly structured architecture and is significantly more resistant to antibiotics than a biofilm formed by a non-mucoid strain. Another important mechanism exhibited by \textit{P. aeruginosa} is the coordination of gene expression for adaptation to such situations. This is regulated by quorum-sensing systems, a complex regulatory circuit involving cell-to-cell signalling (Davies \textit{et al.}, 1998; Singh \textit{et al.}, 2000). Quorum sensing signalling molecules, such as acyl homoserine lactones (AHL), activate the cascade which promotes the formation of biofilms (Fuqua \textit{et al.}, 2002; Derenkard \textit{et al.}, 2002; Lesprit \textit{et al.}, 2003). In such conditions, bacterial growth occurs in association with human tissues or indwelling medical devices such as endotracheal tubes and gives rise to biofilm associated chronic infection. The bacteria growing in biofilms often display a variety of phenotypic differences from the same strains growing in planktonic form, which include changes in motility, increased production of extracellular polysaccharide and antibiotic resistance, changes in several genes expression as well as protein profiles. Hence, they pose a serious problem for clinicians who find it difficult to eradicate chronic lung infections particularly in patients with indwelling endotracheal tubes
Yanagihara et al. (1997; 2002) have described an animal model to obtain an insight into the biofilm pathogenesis and host responses which are often not effective against *P. aeruginosa* biofilms. These workers (Yanagihara et al., 1997) established a murine model of chronic *P. aeruginosa* respiratory infection by placement of catheter tube pre-coated with bacteria into the bronchus of anesthetized animals. Viable bacteria were isolated in significant numbers from the lungs of infected animals for more than a year. Pathological examination revealed intense chronic inflammation surrounding the bronchi, its lumen filled with inflammatory cells and secretions along with hyperplasia at 30 days post-infection period. At one year, marked bronchiectasis, proliferation of lymphoid cells and accumulation of foam cells around bronchi could be observed. These findings could be attributed to the direct placement of tube in the bronchus. This model has been successfully used to study various aspects of host–pathogen interactions during chronic lung infections also applicable to human situations (Yanagihara et al., 2000; 2002; 2003). In the present study, a chronic lung infection was established in normal LACA mice with repeated intranasal instillation of pre-formed biofilm cells of *P. aeruginosa* PA103 every 48 h. Progressive pathological changes in the lungs of infected mice at weekly intervals with persistent bacterial counts showed that maximum lung tissue destruction occurred at 4 weeks post infection period. Severe chronic inflammation comprising of lymphocytes and histocytes alongwith chronic abscess formation and fibroblastic proliferation was observed. However, in comparison to chronic lung infection induced with planktonic cells of *P. aeruginosa* PA103, there was only a marginal increase in the severity of infection caused by preformed biofilm cells. No study is available for direct comparison under similar conditions. The possibility of the preformed biofilm cells reverting back to original planktonic form in the *in vivo* environment, provided by the host, cannot be ruled out. In their mouse model of *P. aeruginosa* induced
chronic lung infection, Yu et al. (1998) employed mucoid as well as non-mucoid derivative of the same strain. They found that with repeated infections, the difference in the virulence of the two cell forms (mucoid and non-mucoid) gets abrogated and similar intensity of infection is observed. Similarly, in the present study also, it is possible that the differences in the virulence of planktonic as well as biofilm cells get minimised due to the repeated infections given over a period of six weeks.

The inflammatory response during chronic infection, induced by biofilm cell form of *P. aeruginosa*, can be assessed in terms of proinflammatory and anti-inflammatory cytokine production. Yanagihara et al. (2000) reported that the lung levels of TNF-α and IL-1β were constantly raised over a period of 60 days during chronic lung infection induced by biofilm cells of *P. aeruginosa*. These workers also highlighted the importance of TNF-α and IL-1β in chronic infection. Treatment of experimental mice with anti-TNF-α antibody was found to significantly attenuate both lymphocyte numbers and levels of IL-1β in the lungs. They suggested that TNF-α, in particular, is essential for the accumulation of lymphocytes in the lung (Yanagihara et al., 2000). These observations corroborate the findings of the present investigation, where the levels of TNF-α and IL-1β were elevated throughout the period of six weeks, with maximum production observed after the initial exposure. The kinetics of anti-inflammatory cytokine, IL-10, production showed that although the level of IL-10 was significantly lower than the levels of pro-inflammatory cytokines, yet, it was several fold greater than the amounts present in uninfected tissues. In addition, there was a slow and progressive increase in IL-10 production observed up to 6 weeks observation period. Unfortunately, there are no matching studies available in relation to IL-10 production following biofilm cell induced chronic infection by *P. aeruginosa* PA103.

Besides *P. aeruginosa*, *K. pneumoniae* is an important pathogen
which frequently causes nosocomial and community acquired gram negative bacterial pneumonia. Without therapeutic intervention, it is known to cause severe respiratory tract reaction with high rates of mortality (Moore et al., 2002). In the present study, *K. pneumoniae* B5055 strain was employed to induce acute and chronic pneumonia in the experimental mice. This strain belongs to serotype 01:K2 which is the most commonly isolated serogroup from human clinical situations (Podschun and Ullmann, 1998). Selection of mouse for the model, coupled with the choice of frequently isolated strain from clinical situations, makes it more relevant to be able to extrapolate the results of the present study in relation to patients suffering from pneumonia caused by this organism.

Several animal models have been described in literature to get a better understanding of host–pathogen interactions during *K. pneumoniae* induced pneumonia. In these studies, immunocompetent mice (Nishi and Tsuchiya, 1980; Legget *et al.*, 1989; Yoshida *et al.*, 2000; Cortes *et al.*, 2002) as well as immunocompromised mice (Aratani *et al.*, 2000; Ye *et al.*, 2001; Moore *et al.*, 2002) have been employed. A mouse model of acute pneumonia induced by *K. pneumoniae* B5055, initially developed by Held *et al.* (1998), has been adopted in our laboratory by Yadav *et al.* (2003). This model has been used in the present investigation. An infection inoculum of 10⁴CFU/ml of planktonic cells was introduced intranasally to establish acute pneumonia in BALB/c mice. The course of infection studied over a period of 10 days in the present study revealed that maximum lung bacterial load was observed on 3rd post infection day with a decline thereafter. The lungs became bacteriologically sterile by 10th post infection day. This is in accordance with observations made by Held *et al.* (1998) and Yadav *et al.* (2003) who reported maximal infection on 3rd post infection day with same bacterial strain. On the other hand, Yoshida *et al.* (2000) reported maximum bacterial counts in the lungs of ICR mice infected with *K. pneumoniae* (DT strain) at 48 h post infection period with complete mortality observed within 72 h of inducing pneumonia. This variation in the
peak infection time in comparison to the present study could be due to different bacterial and mouse strains used in the two studies. Examination of pathology in the current investigation, revealed mild pneumonia with low neutrophil infiltration in the lungs on 1st post infection day. This progressed to severe pneumonia on 3rd post infection day, characterized by intense PMN influx and destruction of alveoli. Bronchi showed necrosis with the lumen containing necrotic slough admixed with acute inflammatory cells. There were signs of resolving lobar and broncho pneumonia observed towards 7th post infection day. Yoshida et al. (2000) observed maximum destruction after 48 h of infection, with thickening of alveolar septa, infiltration of inflammatory cells along and ballooning of alveolar spaces. In contrast, matching pathological observations with severe lobar pneumonia, as observed in this study, have been reported earlier by Held et al. (1998) and Yadav et al. (2003).

Effective host defense against lung bacterial infections is dependent primarily upon rapid clearance of the organism from the respiratory tract. This is mediated by influx as well as activation of phagocytic cells which includes neutrophils (PMN) and macrophages (Laichalk et al., 1998). Enumeration of PMNs in bronchoalveolar lavage (BAL) gives an index of infiltration by these cells in the lung tissue. In the present study, maximum lung PMN counts were observed on 3rd post infection day. There was a gradual decrease in the levels of PMN counts from 5th day onwards. A direct correlation between maximum PMN influx, corresponding lung bacterial load as well as lung pathology was observable. Similar results have earlier been obtained by Yoshida et al. (2000), who recorded maximum inflammatory cell counts in BALF at peak infection period in their mouse model of acute pneumonia. Myeloperoxidase (MPO) enzyme activity provided an indirect measurement of PMN infiltration in the lungs. In the present study, maximum levels of myeloperoxidase enzyme activity were observed on 3rd post infection day which coincided with highest PMN counts in the BALF.
Nitric oxide, a crucial and versatile molecule, produced in high amounts during an infectious process contributes to an effective host defense. This increase in nitric oxide synthesis and its role in the control of several microbial, viral, parasitic and fungal infections has been described in detail by several workers (Liew and Cox, 1991; Alaspaugh and Granger, 1991; Bi and Reiss, 1995). In relation to *K. pneumoniae* mediated pneumonia, Tsai *et al.*, (1997) highlighted the critical role played by nitric oxide (NO) in host’s defense. They demonstrated a time dependent increase in NO production in the lungs of mice with maximum production of lung nitrite levels at 48 h post infection followed by a decline at 72 h. To confirm the protective role of NO, they induced pneumonia in mice treated with a nitric oxide inhibitor. Significant increase in lung bacterial counts and mortality in NO–depleted mice was observed. Further, NO-inhibitor adversely affected phagocytic and microbicidal capacity of alveolar macrophages. These workers concluded that augmentation of NO synthesis or local administration of NO may improve the clinical management of patients with severe bacterial pneumonia. Results of the current investigation corroborate the findings of Tsai *et al.* (1997) since in the present study also, there was an increase in the nitric oxide production, in the BALF as well as lung homogenates. Maximal levels were observed on the peak infection day (3rd day) followed by gradual decline upto 10th post infection day.

The recruitment and activation of leucocytes in the setting of bacterial challenge is a complex and dynamic process which involves the coordinated expression of both pro– and anti–inflammatory cytokines (Laichalk *et al.*, 1998). Among various cytokines that have been identified to play an important protective/beneficial role in bacterial pneumonia, TNF–α and IL–1 have received a special attention. TNF–α is expressed in increased amounts in the airspace of humans with bacterial pneumonia (Moussa *et al.*, 1994) and in the lungs of mice challenged with bacterial pathogens including *P. aeruginosa* (Gosselin *et al.*, 1995; Kolls *et al.*, 1998).
TNF–α is a potent activator of both PMNs and macrophage microbicidal activity, which is augmented by both endogenous and exogenous TNF (Oswald, 1992; Tam, 1995).

Localized time dependent production of endogenous TNF–α, in the lungs of mice, during K. pneumoniae induced acute pneumonia was described by Laichalk et al. (1996). Maximum lung levels of TNF–α were reported on 2nd post infection day followed by a decrease and second peak in the cytokine levels on the 6th post infection day. These workers further demonstrated that administration of TNF–α antagonist results in significant reduction of BALF neutrophil influx, coupled with increased lung bacterial counts and shortened survival time of mice. On the other hand, administration of TNF–α agonist reversed these effects (Laichalk et al., 1998). Yoshida et al. (2000) also examined the dynamics of TNF–α production, in mice, during the development of acute pneumonia mediated by K. pneumoniae. These workers, however, noted high levels of TNF–α not only in the lungs, but also in the serum of infected mice on 2nd post infection day. Moore et al. (2003) have reported increased mortality and dysregulated cytokine production in TNF–α receptor–1 deficient mice, following systemic K. pneumoniae infection. The results of the present study, bring out that, intranasal inoculation of mice with planktonic cells of K. pneumoniae B5055 resulted in a time dependent production of TNF–α. Maximum production of TNF–α was observed on 3rd post infection day, in the BALF as well as LHS, followed by a gradual decline up to 10th day of infection. It, thus, shows that the clearance of bacteria from the lung tissue coincides with a fall in the lung level of TNF–α. The results also bring out that the presence of the pathogen plays a direct role in the secretion of this cytokine. Further, the observed differences in the peak time of TNF–α production in comparison to earlier two studies (Laichalk et al., 1996; Yoshida et al., 2000) can be attributed to the different strains of mice, bacteria as well as different routes of infection employed in these studies.
In the immune system, IL-1 (IL-1α and IL-1β), a prototypic multifunctional cytokine, is known as a lymphocyte activating factor. It acts on monocytes and macrophages to induce production of TNF-α, prostaglandin E2 and nitric oxide, enhancing their killing ability against bacterial, protozoa and tumour cells. IL-1 also acts on natural killer cells, in collaboration with IL-2 and interferon -gamma (IFN-γ), to potentiate their cytotoxic activity (Dinarello, 1996; Nakae et al., 2001). Although the precise molecular mechanisms of this activation remain largely unknown, IL-1 seems to play an important role in the host’s defense mechanism against microbes through these effects on immune cells. In relation to bacterial infection, IL-1β is a widely studied pro-inflammatory cytokine (Bonfield, 1995; Mohler et al., 1998; Schultz et al., 2002). Yoshida et al. (2000) described the kinetics of IL-1β production in K. pneumoniae mediated acute pneumonia in experimental mice. They reported that rise in IL-1β production coincided with maximum production of TNF-α, as detected in the BALF of infected animals.

In the present study, the production of IL-1β was monitored, in the BALF as well as LHS of mice, following induction of acute pneumonia by K. pneumoniae B5055. A time dependent production of IL-1β was observed with highest concentration on 3rd post-infection day, followed by a gradual decline upto 10th post infection day. These results showed consistency with the results of Yoshida et al. (2000). This is especially with regard to maximal production of IL-1β coinciding with highest levels of TNF-α production. The exact mechanism behind this observation in K. pneumoniae mediated pneumonia is not clear at present. Further studies are, therefore, warranted to elucidate the role of IL-1β in this context.

While the generation of inflammation is critical for effective clearance of microbial agents, modulation of the inflammatory response is equally important to ensure preservation of immune homeostasis (Greenberger et al., 1995). IL-10 cytokine is known to exert anti-inflammatory role
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(Cassatella et al., 1993). Studies have shown that IL-10 is released in the experimental models of endotoxin – or staphylococcal enterotoxin B–induced shock. In addition, IL-10 (either endogenously produced or exogenously administered) confers a protective effect, through down regulation of pro–inflammatory cytokines, like TNF–α, IL-1, IL-6 and IFN–γ (Smith et al., 1994; Florquin et al., 1994; Merchant et al., 1994; Matsumoto et al., 1998). However, this down regulation by anti–inflammatory actions of IL-10 can interfere with clearance of invasive microbial pathogens. Its expression has been shown to be detrimental in models of bacterial infections. Greenberger et al. (1995) reported that, in some experimental model of murine K. pneumoniae induced acute pneumonia, levels of TNF–α, MIP–1α, MIP–2 and IL–10 were found to be maximum in the lung homogenates at 48 h post–infection. Administration of anti–IL–10 antibody resulted in enhancement of bacterial clearance, elevation of TNF–α, MIP–1α, MIP–2 and prolonged survival of infected mice. In another study, van der Poll et al. (1996) described that in murine model of experimental streptococcal pneumonia, levels of IL–10 in the lungs were maximum at 72 h post–infection period. When recombinant IL–10 was administered intratracheally with bacteria, lung levels of TNF–α and IFN–γ decreased and lung bacterial load as well as mouse mortality increased. More recently, Yoshida et al. (2001) demonstrated that intranasal infection with K. pneumoniae in mice induced IL–10 production during early phase of infection and down regulated the expression of TNF–α, IL–6 and IFN–γ in the lungs. Contrary to these observations, time course study of IL–10 production in the present investigation brings out that, there is a slow rise in the production of IL–10 (both in BALF and LHS) over the first three days of infection which coincides with increase in the severity of infection. Further rise in the levels of IL–10 from 5th day onwards, with maximum concentrations detected on 7th post–infection day, in the lungs is accompanied by a fall in the bacterial counts and decreased lung pathology. This was also associated with a fall in the levels of TNF–α and
IL-1β cytokines. It, therefore, can be concluded that the increase in the levels of IL-10 towards the later stages of acute pneumonia seems to play a beneficial role in resolution of infection. The disparity in results, obtained from the two previous studies with *K. pneumoniae*, might be due to the reason that both the groups of researchers (Greenberger *et al.*, 1995; Yoshida *et al.*, 2001) have studied only the early acute phase of *K. pneumoniae* mediated pneumonia over a span of 48 h. On the other hand, in the present study acute pneumonia was investigated in mice over an extended period where the process of resolution of infection was also studied.

Like *P. aeruginosa*, *K. pneumoniae* is another important gram negative bacterium associated with the ability to form biofilms. Human infections with *K. pneumoniae* are characterized by the ability of bacteria to produce copious amounts of capsular polysaccharide and distinct adherence factors (Tarkkanen *et al.*, 1992). These play a significant role in the biofilm formation which requires different signals operative through production of multiple gene products (O'Toole and Kolter, 1998). Quorum sensing mechanisms are used by numerous bacterial species including *P. aeruginosa* and *K. pneumoniae* to regulate virulence factor expression or phenotypic changes. Bacteria control gene expression on a community wide scale by secreting, detecting and responding to extracellular signalling molecules, called autoinducers. Recently, the role of type 2 quorum-sensing has been highlighted since it plays an important role in the development of *K. pneumoniae* microcolonies, mostly in the early stages of biofilm formation by this organism (Balestrino *et al.*, 2005).

It has been demonstrated by Langstraat *et al.* (2001), that the presence of type 3 fimbriae facilitated the rapid formation of dense *K. pneumoniae* biofilms. These fimbriae have been implicated in binding to eukaryotic cells and matrix proteins. Earlier studies have shown that these fimbriae mediate adherence, *in vitro*, to basement membrane and the submucosa of human lung tissue (Kukkonen *et al.*, 1993; Hornick *et al.*, 2001).
The role of type 3 fimbriae, in the formation of biofilms as well as host defense, has further been elucidated in murine model of K. pneumoniae mediated lung infection (Lavender et al., 2004; 2005). In addition, type I fimbriae of K. pneumoniae have also been suggested to promote initial bacterial adhesion, invasion and biofilm formation. On the other hand, capsular polysaccharide also plays a significant role during the development of mature biofilms (Schembri et al., 2005).

The production of biofilm matrix not only protects the pathogen against host defense mechanisms, but also prevents therapeutic concentrations of antibiotics from reaching the organism. Role of various antibiotics has been well studied against K. pneumoniae biofilms (Anderl et al., 2000; Zahler et al., 2002; Anderl et al., 2003). The formation of these sessile bacterial communities and their inherent resistance, thus, have an enormous impact on medical world due to their ability to persist on medical implants. This is especially relevant in mechanically ventilated patients or patients with endotracheal intubation. The formation of biofilms, during the early stages of K. pneumoniae airway infections, is a complex process.

In the present study, acute pneumonia has been established in normal BALB/c mice using 3 day-old, preformed biofilm cells of K. pneumoniae. Recent work in relation to K. pneumoniae biofilm formation, in vitro by Langstraat et al. (2001) brought out that dense mats of biofilm could be demonstrated after 3 to 5 days of incubation. Growth curve of the biofilm performed on endotracheal tube in the current study, also revealed that maximum growth was observable on 3rd day of incubation. Therefore, 3-day old, preformed biofilm cells were employed to induce acute and chronic pneumonia in mice. More recently, Lavender et al. (2004) investigated the correlation between K. pneumoniae biofilm formation in vitro and its ability to cause infection in vivo. They constructed a series of mutants that were altered in their ability to form biofilm, in vitro, and checked them for their ability to infect animals in vivo. Mutation at a critical site resulted in the inability of the bacterium to form biofilm which rendered
the organism non–virulent, in vivo. Both in vitro and in vivo studies, therefore, are important to investigate the pathogenesis of biofilm bacteria.

In the present study, the course of acute pneumonia induced by preformed biofilm cells of K. pneumoniae B5055 was studied over a period of 14 days. In comparison to planktonic cell induced infection, lung bacterial load as well as tissue destruction were only marginally higher till 3rd day (peak day of infection) but became significantly more from 5th day onwards and this trend continued upto 10th post infection day. The bacteria persisted in the lungs of biofilm cell infected mice till 12th day with lungs becoming bacteriologically sterile on 14th post infection day. On the other hand planktonic cells persisted till 7th day only. Similarly, other biochemical parameters evaluated to assess tissue destruction were significantly higher in case of animals infected with biofilm cells, but only from 5th day onwards. Unfortunately, no studies are available for direct comparison. There is a possibility that the preformed biofilm cells, employed in this study, could have reverted back to planktonic form following introduction in the animals. As a result, although infection persisted for comparatively longer duration in case of acute pneumonia induced with biofilm cells, but the difference in the severity of infection at the peak time period is not significant. It, therefore, brings out that in case of K. pneumoniae also, like P. aeruginosa, there is a need to induce and study the role of biofilm formation in vivo, in the context of lung infections. The model being reported here can serve useful purpose for acute as well as chronic form of lung infection in a normal host.

Nosocomial infections by K. pneumoniae, especially in immunocompromised patients, mostly result in chronic lung infection which is difficult to eradicate. The severity/chronicity of lung infection is primarily controlled by the virulence of the infecting strain. Among the major factors responsible for marked variability in the pathogenicity of different K. pneumoniae strains, capsular polysaccharide (CPS) production by the bacteria is most important. Circulating cell-free CPS has been demonstrated in the blood of patients infected with K. pneumoniae (Pollack
Limited studies are available where animal models have been used to describe chronic pneumonia by *K. pneumoniae*. Initially, Berendt *et al.* (1977) described bronchopneumonia in rats, by intranasal inoculation of bacteria, but they eventually abandoned their model due to high mortality in the early phase of infection. However, the authors emphasized economical, practical and statistical advantages of using a rodent model. Domenico *et al.* (1982) produced chronic lobar pneumonia model by intratracheal inoculation of bacteria in rats. Relatively uniform course of infection was observed for a period of 14 days, after which increase in lung bacterial counts was coupled with the death of animals. These workers highlighted the importance of virulence of infecting strain in inducing chronic lung infection in experimental animals. They demonstrated that, employing appropriate *K. pneumoniae* strain has a strong bearing on the nature and extent of the disease produced. Variants within a single population of same strain show marked difference in pathogenicity which is mainly attributed to CPS production. In the present study, chronic pneumonia was established in normal BALB/c mice with planktonic cells of *K. pneumoniae* B5055, which is a highly virulent encapsulated strain.

Chronic pulmonary infection in mice by *K. pneumoniae*, has so far been reported only by Lizawa *et al.* (1988). Their study also brought out the importance of mouse strain susceptibility towards the infection. A marked strain difference in susceptibility to an experimental respiratory tract infection, induced with aerosol inoculation of *K. pneumoniae*, was observed. The results revealed that, out of eight different mouse strains used in the study, chronic pulmonary infection could be most suitably established in CBA/J mice. BALB/c mice were also found to be sensitive towards the infection, but gross lung lesions were not observable in all the animals. In all these reports, experimental animals were exposed to only one infection dose of *K. pneumoniae*. In the present study, a single exposure of $10^4$ CFU/ml of bacteria resulted in only acute pneumonia with signs of resolution after a week, as described earlier. Therefore, to
successfully establish chronic changes in the lungs of mice, three infection
doses of $10^4$ CFU/ml each were given intranasally, at weekly intervals. This
is in contrast to the chronic pneumonia induced by *P. aeruginosa* PA103,
where repeated intranasal infections were given every 48 h till the end of
experiment in order to maintain chronicity of infection.

Domenico *et al.* (1982) studied the course of chronic pneumonia by
*K. pneumoniae* in rats over a period of 30 days. They reported that
bacterial counts in the lungs remained consistent for the first six days with a
rise on the 7th post-infection day and a decline observed thereafter. Correspon
ding lung pathology revealed the formation of chronic abscess, surrounded by a
wall of collagen fibres, on 9th day of infection. On the contrary, Lizawa *et al.* (1988)
reported that lung bacterial counts in their chronic mouse model changed with
time, showing four different phases: initial decrease, regrowth, steady state and
finally increase leading to the death of animals. Marked hyperplasia of lymphoid
tissue was observed around the bronchi at 3 weeks post-infection time. However,
no fibrosis was reported in their model. The course of chronic pneumonia
induced with planktonic cells of *K. pneumoniae* B5055 in mice, in the present study,
brought out that there was a steady increase in the lung bacterial counts
with peak observed on 18th post-infection day followed by a decline. Pathological
findings in the infected lungs revealed extensive chronic inflammation charac
terized by infiltration of lymphocytes, plasma cells and macrophages on the peak
infection day. Chronic abscess formation with fibroblastic proliferation around the
bronchioles and alveolar destruction at certain places was noted. Maximum tissue destruction was also
demonstrable, through peak production of MDA and LDH, in the lungs of
infected animals. However, maximum nitric oxide production is followed by
a decrease in the lung bacterial load.

In the present study, the inflammatory response generated during
chronic pneumonia induced in mice by planktonic cells of *K. pneumoniae*
B5055 was also assessed through the generation of pro-inflammatory
(TNF-α and IL-1β) and anti-inflammatory (IL-10) cytokines. Maximum production of TNF-α and IL-1β, both in the BALF and LHS, were observed on 18th post-infection day followed by a decrease in their production. No studies are available for direct comparison in such situation. However, the protective role of TNF-α and IL-1β production during \textit{K. pneumoniae} induced acute pneumonia has been investigated by several workers. How far these cytokines are important for the host defense during the course of chronic pneumonia, needs further consideration. The kinetics of anti-inflammatory cytokine, IL-10, produced in the BALF and LHS in the present study showed that the cytokine generation was gradually enhanced after the 3rd infection. This increase in the production of IL-10 coincides with corresponding decline in TNF-α and IL-1β concentrations and also decrease in the severity of infection. This suggests that IL-10 production appears to play an important role in controlling the chronic infection by down regulating the inflammatory process. IL-10 production has been shown to play an important role in ameliorating excessive inflammation in relation to chronic pneumonia induced by \textit{P. aeruginosa}. The mouse model of chronic pneumonia described in this study, thus presents an ideal situation to investigate the pathogenesis of \textit{K. pneumoniae}.

The ability of \textit{K. pneumoniae} to adhere and subsequently form biofilms on medical devices in the hospitalised patients results in persistent chronic infections (Costerton \textit{et al.}, 1999; Donlan, 2001). Such infections, once established, are difficult to eradicate since biofilm cell forms can successfully evade host immune defense mechanisms and are also resistant to antibiotic treatment. In the current study, chronic pneumonia was established in normal BALB/c mice, by three intranasal inoculations at weekly intervals, with preformed biofilm cells of \textit{K. pneumoniae} B5055. Course of infection was studied over a period of 30 days. Lung bacterial counts and the corresponding lung pathology revealed that peak infection was observed on 18th post infection. This was followed by a decrease in the severity of infection at later stages. The biochemical parameters and pro-
inflammatory cytokines (TNF-α and IL-1β) production also confirmed the peak infection induced by biofilm cell form of the bacteria. The course and severity of infection was similar to that observed during chronic infection induced with planktonic cells. It is possible that repeated infections with the two cell forms reduced the differences in their pathogenicity and resulted in matching lung tissue destruction. In this context, the possibility of preformed biofilm cells reverting back to parental planktonic cell form following introduction into the host also cannot be ruled out. It remains to be seen whether in vivo formation of biofilm, on endotracheal tube in experimental mice, has the potential to induce more severe form of lung pathology. As discussed earlier, the chronic infection induced by preformed biofilm cells of P. aeruginosa PA103 in normal healthy mice required continuous exposure to the pathogen. However, matching chronicity was attainable with fewer exposures of mice to K. pneumoniae B5055 strain. Absence of comparable studies with biofilm cells of K. pneumoniae, in vitro or in vivo, hampers the possibility of direct comparisons but draws attention of the future workers in this direction.

During the process of initiation, establishment and evolution of an infectious process which is also applicable to the respiratory tract infections, the immune system comes to the defense of the host against the invading pathogen. Basically, the system has two functional divisions, i.e. the innate and the acquired immune responses. Both components involve blood borne as well as formed elements of blood and tissues. Inflammation is body’s immediate response to the injury, caused by the invading microbe and is a part of protective immune response. Functional immune system is essential for health, since it serves to protect the host from the pathogenic as well as opportunistic organisms. Nutrition is considered to be a critical regulator of immunocompetence (Simopoulos, 2002). Almost all the nutrients in the diet play a crucial role in maintaining an optimal immune response. Their excessive as well as deficient intake can have negative effect making the host susceptible to a variety of pathogens (Field et al.,}
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2002). A growing body of literature demonstrates the immune benefits associated with the optimal intake of specific nutrients like vitamins including A, C, E, iron and zinc (Vojdani et al., 2000; Moriguchi and Muraga, 2000; Stephensen, 2001).

Relatively recently, attention of the workers has been drawn to ‘Eicosanoids’. These are group of chemical messengers that act within the immune system as inflammatory mediators (James, 2000). These compounds provide a link between polyunsaturated fatty acids (PUFA), inflammation and immune function. Eicosanoids are synthesized from PUFA, in particular, dihomo-\(\gamma\)-linolenic acid (DGLA; 20 : 3n-6), arachidonic acid (\(\alpha\), 20:4n-6) and eicosapentaenoic acid (EPA, 20:5n-3). However, EPA results in the generation of less potent inflammatory eicosanoids (Yaqoob, 2004). Eicosanoids include prostaglandins (PG), thromboxanes, leukotrienes, lipoxins, hydroperoxyeicosatetraenoic acids (HPETE) and hydroxyeicosatetraenoic acids (HETE) (Simpoulus, 2002). The fatty acid precursor for eicosanoid synthesis, is released from the cell membrane phospholipids, usually by the action of phospholipase A\(_2\) activated in response to a cellular stimulus. Since the membranes of immune cells contain large amounts of arachidonic acid, it is usually the principle precursor of eicosanoid synthesis (James et al., 2000; Calder, 2001; Yaqoob, 2004).

There are two main families of PUFA : n-3 (Omega-3) PUFA (e.g. Alpha-linolenic acid; 18:3n-3) and n-6 (Omega-6) PUFA (e.g. linoleic acid). These two fatty acids are termed ‘essential’ because they cannot be synthesized by the human body and must be provided in the diet. Upon consumption, alpha-linolenic acid is metabolised to long chain derivatives eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) (Lake, 2004). EPA and DHA are biologically most potent n-3 PUFA. n-3 PUFA (especially EPA and DHA) consumed in the diet compete with arachidonic acid and results in its decrease in the membrane.
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phospholipids of the cells. It, thereby, lowers the production of inflammatory eicosanoids (Schwartz, 2000). In addition, long chain n-3 PUFA appear to inhibit the release of arachidonic acid from membrane phospholipids. This causes suppression of inflammatory response (James et al., 2000; Calder, 2001).

In addition to understanding the role of PUFA in relation to inflammation, last few decades have evoked a keen interest in the immunomodulatory functions exerted by dietary lipids. This is specially due to clinical benefits observed with n-3 PUFA supplementation (Mayes, 2000). Initial epidemiological studies with the Greenland Eskimos brought out that low incidence of heart diseases among this population was attributed to their high n-3 PUFA intake (Kromann and Green, 1980). Thousands of people all over the world are supplementing their diets with n-3 PUFA in the hope to improve the quality and/or length of their lives with these supplements. Numerous studies have documented the protective ant-inflammatory role of n-3 PUFA, particularly in the form of fish oil, against several cardio-vascular (Harris, 1996; Connor, 1997), autoimmune (Harbige, 1998; Wu and Meydani, 1998) and inflammatory disorders (Simopoulos, 1991; 2002). It has been shown that n-3 PUFA alter different functions of the immune system operative through lymphocyte proliferation (Meydani et al., 1991; Yaqoob et al., 1994), phagocytic activity (De Pablo, 1998), NK cell activity and cytokine production (Mayer et al., 2003; Sundrarjun et al., 2004). These essential fatty acids are important not only for optimal health of adults but are also required for normal embryonic development (Smuts et al., 2003). Clinical studies with hospitalised trauma and cancer patients have also revealed beneficial results due to immunomodulatory effects of n-3 PUFA consumption (Zhou et al., 2005).

In relation to lung diseases, only few clinical and experimental studies with n-3 PUFA supplementation are available. Dietary supplementation with n-3 PUFA in children has been associated with a positive impact on recurrent respiratory tract infections (Venuta et al.,
1996). Fish oil consumption has also been shown to improve lung functions in asthma patients (Dry and Vincent, 1991; Jaber, 2000). EPA and DHA supplementation has also been found to reduce inflammation in cystic fibrosis patients (Katz et al., 1996; De Vizia et al., 2003) and protect the smokers against development of chronic obstructive pulmonary disease like chronic bronchitis and emphysema (Shahar et al., 1994; Schwartz and Weiss, 1994; Schwartz, 2000). EPA supplementation has also been reported to reduce pulmonary oedema and leukotriene B4 and B5 production in experimental animals with endotoxin induced acute lung injury (Mancuso et al., 1997; Sane et al., 2000). Regarding the potential of n-3 PUFA to affect host’s resistance against infectious diseases, limited and equivocal studies are available. Both beneficial as well as adverse effects of these fatty acids on host’s survival after an infectious challenge have been reported, based on experimental studies. Existing studies have also been somewhat narrow in scope, with only survival as the primary focus. Anderson and Fritsche (2002) have recently reviewed this topic and have pointed out the shortcomings of the investigations. They have highlighted that to reduce the variability observed in the outcome of different studies, proper planning of experimental investigation is required. They have stressed the need to pay attention to the nature of the pathogen, route of infection as well as selection of the host. From the nutritional perspective, experiments should be planned keeping in mind the dose and duration of administration of different PUFA preparations since it can have a bearing on the outcome of results. All these factors have been addressed to in the present investigation.

The present study was specially planned to observe the effect of n-3 PUFA supplementation on the course of acute pneumonia induced in mice by planktonic and biofilm cells of P. aeruginosa PA103 and K. pneumoniae B5055. Both these gram negative pathogens are frequently involved in nosocomial as well as community acquired pneumonia. Besides planktonic cells, biofilm cell forms of bacteria were employed to induce infection in
experimental mice since these organisms are involved in lung infections amongst the patients put on medical devices like ventilators and endotracheal tubes. In the current study’s protocol, three commonly consumed commercial preparations of n-3 PUFA (cod liver oil, maxigard and flaxseed oil) have been employed, in order to see if there is variability in their effect. Cod liver oil is a rich source of EPA and DHA with micro quantities of Vitamin A and D. Maxigard is also a purified preparation of EPA and DHA. Flaxseed oil is a major source of \( \alpha \)-linolenic acid. Olive oil, a monounsaturated fatty acid, commonly prescribed for human intake and known for health benefits especially against coronary heart disease, served as an oil control (Guigliano et al., 2005; Berbert et al., 2005). Earlier, it has been used as placebo in several experimental studies (Clark, et al., 1994). On the whole, cod liver oil provided best results in terms of protection against experimental pneumonia. Earlier, different workers have employed 3-4 fatty acids to compare the variability in the effects of these supplements on the host’s resistance against infections. However, all these reports have also found fish oil or cod liver oil to be the most beneficial (Blok et al., 1992; Wallace et al., 1999; Sadeghi et al., 1999; Fritsche et al., 2000).

There is a great degree of variability in the duration of n-3 PUFA administration in experimental studies ranging from 1 to 6 weeks. Two as well as six weeks of oral feeding with the selective fatty acids was done in our study. In addition, in the available literature, workers have employed highly variable doses of n-3 PUFA (40 mg/day to 300 mg/day) due to which it is difficult to make direct comparisons with the present investigation. In the light of existing variability, regarding the doses of PUFA administered, the need for standardisation of the dose through initial pilot experiment was realised. For this purpose, graded doses of these fatty acids were given orally, to different groups of mice, to observe their effect on acute pneumonia induced in mice. Final dose of 5% n-3 PUFA providing 350-400 mg/kg body weight was administered to different groups of mice for two and
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six weeks duration. Similar amounts of n-3 PUFA have been used as
dietary supplement by workers and this dose also falls within clinically
achievable levels (Allen, et al., 1998; Collins et al., 2004, Zhang et al.,
2005). The current investigation, thus, confounds the basis of future
experimentation in relation to respiratory tract infections and PUFA
supplementation.

Initial studies by Rubin et al. (1989) and Clouva-Molyvdas et al.
(1992) brought out that supplementation of diet with fish oil did not affect
the survival of mice when challenged intraperitoneally with P. aeruginosa.
Peck et al. (1990), observed a dose dependent effect, with either reduced
survival or no change, in the survival of mice fed either high or low dose of
Max EPA (n-3 PUFA), following bacterial infection induced at the burn site.
In case of pulmonary infections, van Heeckeren et al. (2004) recently
reported that, dietary supplementation with DHA did not improve the clinical
outcome of cftr-knockout or wild-type mice in response to bronchopulmonary infection with P. aeruginosa. On the whole, these
studies have been found to have no advantage of n-3 PUFA feeding
against P. aeruginosa induced localised, systemic or pulmonary infections.

In relation to K. pneumoniae mediated infections, Blok et al. (1992)
and Bjornsson et al. (1997) had investigated the effect of fish oil
supplementation on intramuscular infection in mice. They observed
improved survival of animals following six weeks of diet supplementation,
despite no change in the blood, liver and spleen bacterial counts. More
recently, Thors et al. (2004) showed protective effects of dietary fish oil on
survival of mice with experimental pneumonia induced by K. pneumoniae.
In the present investigation, two weeks of oral feeding, with either of the
three n-3 PUFA preparations, did not prove beneficial in terms of lung
bacterial load or pathology However, in contrast, when animals were given
infection following six weeks of dietary supplementation, a significant
decrease in the lung bacterial load as well as pathology was observable.
This protective effect was better in the cod liver oil fed group, although the
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difference with maxigard or flaxseed oil fed groups was only marginal. Olive oil fed group exhibited no significant change on establishment of infection when compared to normal saline controls. The protective effects of n-3 PUFA, thus, became evident only with relatively longer feeding for a period of 6 weeks. In relation to *P. aeruginosa* mediated infections, workers followed 1-4 weeks of fatty acid feeding while in studies reported with *K. pneumoniae*, six weeks of fatty acids feeding was done which provided protection to the fed animals. Based on all these observations, it appears that a minimum duration of six weeks is required to elicit beneficial effects of n-3 PUFA.

The ability of several pathogenic bacteria or their products to induce apoptosis in different host cells has been found to play a key role in the pathogenesis of infections caused by these organisms. Experimental studies, in both animals and humans, have shown an important role of several fatty acids in the induction or inhibition of apoptosis of different cell types (De Pablo *et al.*, 2002). Dietary n-3 PUFA induces higher apoptosis in mouse splenic lymphocytes by increasing the generation of lipid peroxides and elevating Fas-L expression coupled with decreased Bcl-2 expression (Fernandes *et al.*, 1996; 1998; Avula *et al.*, 1999). EPA and fish oil intake has also been shown to exert protective effect against colon cancer by regulating the apoptotic process both in *in vitro* as well as *in vivo* in experimental mice (Clarke *et al.*, 1999; Hong *et al.*, 2000; Collet *et al.*, 2001). Pathogen induced apoptosis could impair host defenses favouring the bacterial survival and persistence (Hauser, *et al.*, 1999; Usher, *et al.*, 2002; Tateda, *et al.*, 2003).

It is of relevance to assess the induction of apoptosis in macrophages since, these resident as well as activated circulatory monocytes form the first line of defense against respiratory tract infections. In the present study, alveolar macrophages, obtained from different groups of mice given n-3 PUFA supplementation for 6 weeks, exhibited a significant decrease in frequency of apoptosis with both the bacterial
strains. Matching observations have been recently reported by Lee and Wander (2005) who observed reduced apoptosis in U937 cells upon incubation with oxidized low-density lipoprotein obtained from women given fish oil supplementation. In the context of infectious diseases, Puertollano et al. (2004) observed that infection with *L. monocytogenes*, in thymocytes from mice fed with dietary lipids (fish oil), does not promote a synergistic effect on the induction of apoptosis. Enhanced lipid peroxidation, due to PUFA intake has been reported to be of crucial importance in induction of apoptosis (De Pablo et al., 2002. Recently, Sweeny et al. (2005) have brought out that n-3 PUFA supplementation increases the susceptibility of immune cells to lipid peroxidation and products of this process induce apoptosis. However, in the current investigation, there was reduction in the extent of lipid peroxidation, in n-3 PUFA fed mice given infection. This decrease in lipid peroxidation could, therefore, account for the reduced apoptosis observable in alveolar macrophages. In the light of these findings, down regulation of apoptosis in alveolar macrophages of n-3 PUFA fed mice, by the bacteria, can thus, provide benefit to the host in improving the outcome of infection. In addition to the effect mediated through apoptosis, other mechanisms like phagocytosis, which are important defense mechanisms, have also been studied in n-3 PUFA fed mice.

Host’s early response operative through rapid PMN influx was assessed in *P. aeruginosa* (PA103) as well as *K. pneumoniae* (B5055) mediated acute pneumonia. Decrease in the lung bacterial load correlated with significant increase in PMN infiltration. This, in turn, was also reflected by enhanced production of MPO enzyme units in the lung tissue. On the contrary, Son et al. (2000) reported that reduced pulmonary oedema was observed, in endotoxin-induced acute lung injury in rats, following EPA supplementation despite no change in lung PMN counts. However, no studies are available for direct comparison in this context.

Rapid clearance of pathogens, by the resident as well as
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inflammatory phagocytes, thus forms the first line of host’s defense mechanism. In a relatively earlier study, depressed phagocytic functions of PMNs was observed in human volunteers given fish oil supplementation (Virella et al., 1989). Fish oil or flaxseed oil supplementation in healthy human subjects did not alter the percentage or phagocytic activity of neutrophils or monocytes against E. coli (Kew et al., 2003; 2004). In another study, reduced phagocytosis of S. typhimurium by murine kupffer cells after fish oil feeding was demonstrated but this was not associated with reduced ability to kill the bacteria (Eicher et al., 1995). On the other hand, no change in the phagocytic potential of alveolar macrophages was noted despite diminished lung clearance of inspired S. aureus, in fish oil fed rabbits (D’Ambola et al., 1991). Palombo et al. (1999) also reported that irrespective of dietary treatment, the ability of alveolar macrophages, collected from rats fed on EPA and α-linolenic acid, to phagocytose opsonized zymosan and to kill S. aureus was unchanged.

Enhanced phagocytic capacity and microbicidal action of macrophages in vitro, especially with DHA, has been reported by Zheng et al. (1999). Turini et al. (2001) also demonstrated increased phagocytic activity of monocytes obtained from human volunteers fed on fish oil. More recently, enhanced phagocytic potential of macrophages, collected from experimental rats fed on fish oil for long durations, has been reported (Trushina et al., 2003; Bonatto et al., 2004). In the present study, in vitro phagocytic potential of alveolar macrophages, obtained from different fatty acid fed groups against planktonic and biofilm cells of the two bacterial strains showed significant enhancement in the phagocytic uptake and killing. This was noted with the alveolar macrophages of mice fed with all the three n-3 PUFA preparations. Maximal effect was observable in cod liver oil fed group. Planktonic cells were phagocytosed and killed in significant higher numbers as compared to biofilm cells which are known to acquire antiphagocytic ability. The overall increase in phagocytosis of bacterial cells by alveolar macrophages, could be responsible for reduced
severity of infection and extent of tissue damage observed in the lungs of experimental animals.

During inflammatory process, products of tissue destruction play a crucial role in relation to establishment and severity of infection. Nitric oxide (NO) produced by phagocytes, is a non-specific anti-bacterial host defense mechanism which plays an important role in the clearance of invading pathogens. At the same time, NO, when produced in excess reacts with superoxide anion to form a peroxynitrite anion which is highly toxic molecule leading to DNA damage and protein modification (Vliet et al., 2000). Earlier work by Khair-EI-Din et al. (1996) showed that incubation of murine macrophages with DHA resulted in lower levels of mRNA for inducible NO synthase as well as NO production. Jeyarajah et al. (1999) reported that DHA in fish oil inhibited NO production by murine macrophages in response to LPS. Recently, Sarsilmaz et al. (2003) observed that fish oil supplementation resulted in a decreased production of NO. This in turn, decreased the oxidant status in corpus stratum of rats. In the present study, nitric oxide production, in the lungs (BALF and LHS) of infected n-3 PUFA fed groups of mice, showed a marginal decline as compared to normal saline or olive oil fed mice. n-3 PUFA feeding, thus, could have possibly offered the protection against experimental acute pneumonia.

Tissue damage caused by oxidation of unsaturated fatty acids of membrane lipids is measured through estimation of lipid peroxidation in terms of MDA production. Luostarinen et al. (1997) observed that fish oil feeding in rats did not alter the plasma MDA concentration but the myocardial MDA levels were found to increase in normal animals given supplementation. Allard et al. (1997) also demonstrated that supplementing the diet with n-3 PUFA (EPA and DHA), in humans, resulted in increased lipid peroxidation as measured by plasma MDA release. Higdon et al. (2001) found that supplementation of women with fish oil did not alter the levels of in vivo plasma lipid peroxidation. In contrast, Songur et al. (2004)
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reported reduced MDA levels in hypothalamus of rats fed with fish oil, thereby offering an explanation for protective effect of fish oil against neuropsychiatric disorders. More recently, Iraz et al. (2005) determined the oxidant status of erythrocytes in rats given fish oil supplementation and reported lower MDA and NO levels. This could be the reason for beneficial effect of fish oil against oxygen free radical mediated tissue injury in various diseases and can be applicable to the present study also since significant decrease in MDA as well as LDH levels in different groups was observed.

Activated mononuclear phagocytes, which include resident as well as circulating monocytes, are major producers of pro-inflammatory and anti-inflammatory cytokines. The generation of pro-inflammatory cytokines like IL-1, TNF-α and IL-6 is pivotal in mediating the host’s response to infective inflammatory stimuli (Blok et al., 1996). IL-1 and TNF-α act synergistically to induce local inflammation and mediate several systemic acute phase responses. Anti-inflammatory effects of dietary n-3 PUFA (fish oil and flaxseed oil), in healthy individuals as well as in several inflammatory and auto-immune disorders, have been attributed to decreased production of pro-inflammatory cytokines like TNF-α and IL-1β (Meydani et al., 1991; Caughey et al., 1996; Grimble et al., 2002; Watson et al., 2005). Recently, Vaisman et al. (2005), have reported an increase in the production of both pro-inflammatory and anti-inflammatory cytokines by peripheral blood mononuclear cells collected from children given fish oil supplementation.

In contrast, data from most of the animal studies show increased IL-1 and TNF-α production following n-3 PUFA intake. Hardardottir et al. (1992) and Blok et al. (1992) initially observed enhanced TNF-α production by murine resident peritoneal macrophages following fish oil intake. These workers concluded that acute increase in local cytokine levels may be beneficial in enhancing resistance to infections. Similar results were observed by Petursdottir et al. (2002), where resident peritoneal macrophages obtained from fish oil fed mice, exhibited higher levels of
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TNF-α. The results of the present study corroborate these findings since, there was significantly enhanced production of both TNF-α and IL-1β, in the BALF as well as LHS, of n-3 PUFA fed experimental mice. The effect was more pronounced in cod liver oil fed animals. Decrease in the severity of infection observed, thus, could have been contributed by enhanced levels of pro-inflammatory cytokines.

Dietary lipids also modify IL-10, which is an anti-inflammatory cytokine. It also down-regulates immune responses by influencing the production of TNF-α and IL-1 (Nelson, 2001). Fish oil feeding, on one hand, is reported to reduce IL-10 production by murine peritoneal macrophages (Petursdottir et al., 2002). Vaisman et al. (2005), on the other hand, have reported higher levels of IL-10 by monocytes collected from children given fish oil as compared to controls. In the present investigation, marginal increase in the levels of IL-10 was observed in BALF and LHS of n-3 PUFA fed infected mice. Marginally high levels of IL-10, thus, could not have possibly provided direct benefit to the host. However, it remains to be seen as to how it influences the production of pro-inflammatory cytokines, since, it has been reported that, this cytokine has the capacity to down regulate the production of TNF-α and IL-1.

To summarize, the present investigation brings out that diet supplementation with different n-3 PUFA preparations (Cod liver oil, maxigard and flaxseed oil) for 6 weeks, in normal mice, provides benefit against experimental pneumonia by P. aeruginosa and K. pneumoniae. This observation is based on the reduced lung bacterial load and associated lung pathology. It was also accompanied by increase in PMN influx as well as enhanced phagocytic activity of alveolar macrophages. In addition, parameters like reduced MDA and LDH levels along with reduced NO generation, in the BALF and lung tissues, helped to contain the inflammation. Decreased apoptosis in alveolar macrophages achievable by dietary n-3 PUFA could have provided an additional benefit to the host. In
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relation to cytokines, enhanced production of pro-inflammatory cytokines (like TNF-α and IL-1β) coupled with marginal increase in anti-inflammatory cytokine (IL-10) both in the BALF and lung tissues draw attention to the fact, that there is a need to extensively study the effect of n-3 PUFA on these parameters in humans and in animals. Recently, Das (2006) has attributed the beneficial effects of n-3 PUFA on hypertension (described as low grade systemic inflammatory condition) to the down regulation of inflammatory cytokines and biochemical parameters like NO and free oxygen radicals.

This study, thus, forms the basis for further investigations to elucidate the precise role of nutrition on the course of respiratory tract infections where longer duration of supplementation with n-3 PUFA and multiple and different clinical isolates of different pathogenic bacterial species can be employed in the proposed experimental model established in normal mice.