INFLUENCE OF CATHETER AND *E.coli* BIOFILM ON PERIPHERAL BLOOD MONONUCLEAR CELL CULTURES

A DISSERTATION SUBMITTED

BY

SUJALEKSHMY B S

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF

MASTER OF PHILOSOPHY

SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND TECHNOLOGY

TRIVANDRUM – 695 011

DECLARATION

I, **Sujalekshmy B S**, hereby declare that I had personally carried out the work depicted in the dissertation entitled "**Influence of catheter and *E.coli* biofilm on Peripheral Blood Mononuclear Cell culture**" under the direct supervision of "**Dr. A Maya Nandkumar, Scientist E, Division of Microbiology,** Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala, India. External help sought are acknowledged.

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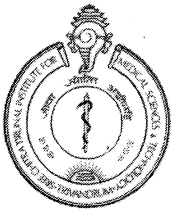

CERTIFICATE

This is to certify that the dissertation entitled " **Influence of catheter and *E.coli* biofilm on Peripheral Blood Mononuclear Cell cultures** " submitted by **Sujalekshmy BS** in partial fulfilment for the Degree of Master of Philosophy in Biomedical Technology to be awarded by this Institute. The entire work was done by her under my supervision and guidance at Division **of Microbiology**, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology (SCTIMST), Thiruvananthapuram-695012.

Thiruvananthapuram

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Date 31.8.2010

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The Dissertation

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Submitted

By

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LIST OF ABBREVATIONS

E.coli	*Escherichia coli*
UPEC	Uropathogenic *Escherichia coli*
UTI	Urinary Tract Infection
CAUTI	Catheter associated urinary tract infection
PBMC	Peripheral Blood Mononuclear Cells
EPS	Extra cellular polysaccharide
IBC	Intracellular bacterial community
qrt-PCR	Quantitative Real Time Polymerase Chain Reaction
RPMI	Roswell Park Memorial Institute
TAE	Tris Acetate EDTA
SEM	Scanning Electron Microscope
CLSM	Confocal Laser Scanning Microscope
EPS	Extracellular Polysaccharide
IL-1β	Interleukin-1 beta
IL-4	Interleukin-4
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-10	Interleukin-10
IL-12	Interleukin-12
MCP-1	Monocyte chemo attractant protein-1
IFN-γ	Interferon gamma
TNF-α	Tumour necrosis factor- α
β-actin	Beta actin

SYNOPSIS

Biofilms are responsible for a large majority of infections in man. Nosocomial infections are the fourth leading cause of death and about 60-70% of them are associated with some type of implanted medical devices. Among them Catheter associated urinary tract infection (CAUTI) is the most common nosocomial infection. The body typically reacts to a biomedical device by coating them with a film consisting of proteins and glycoproteins, such as fibronectin, vitronectin, fibrinogen, albumin and immunoglobulins, many of which serve as binding ligands to receptors on colonizing bacteria or incoming mammalian cells. Biofilm formation and persistence has serious implications for the patient, as microorganisms responsible for this phenomenon are significantly less susceptible to antibiotics and host defense mechanisms.

In CAUTI, the bacteria form biofilm on the surface of urinary catheter. *E.coli* is the major microorganism associated with CAUTI. Biofilm formation on the surface of urinary catheter, elicits host responses by recruiting neutrophils and leukocytes to the site of infection. The interaction between the biofilm and PBMCs result in the release of certain chemical mediators. The most important among them are cytokines, which forms the major components of inflammatory responses. Only very few studies are done on the modulation of cytokine gene expression by *E.coli* biofilm on PBMCs but none by uropathogenic *E.coli*. In the present study we intend to elucidate the modulation of cytokine gene expression in normal human PBMCs upon interaction with uropathogenic *E.coli* biofilm and catheter over a 12h time course.

The *E.coli* 171 strain used in the present study was selected based on Congo Red and *curli* expression agar analysis. The biofilm of the above mentioned strain was constructed on the surface of Foley's latex urinary catheter. PBMCs isolated from human whole blood using Ficoll density gradient centrifugation was cultured in 24 well tissue culture polystyrene plates. The cultured PBMCs were stimulated with both catheter and *E.coli*171 biofilm on catheter and the trigonal interactions between PBMC- catheter-

E.coli biofilm were studied. These studies were conducted to understand effect, on the morphology using different microscopic methods, PBMC proliferation by [^{3}H] thymidine incorporation assay and cytokine gene expression modulation in PBMC by qrt-PCR. Total RNA was isolated from the unstimulated PBMCs and stimulated with both catheter and *E.coli* biofilm. The isolated RNA was used to study the gene expression analysis using qrt-PCR. Confirmation of the PCR amplification was done by agarose gel electrophoresis.

Morphological analysis of PBMC-biofilm interaction showed the persistence of biofilm even in the presence of PBMC and its inability to adhere to biofilm. But in the case of PBMC with catheter alone, the cells adhered forming intricate network of pseudopodia.

Gene expression analysis showed that unstimulated PBMC from normal healthy individuals expressed low levels of all seven cytokine mRNAs analysed except TNF-α and IL-4 through out the study. When PBMCs were stimulated with catheter, an interaction was seen but there was only a slight modulation in the gene expression profile. However, when the PBMCs were stimulated with *E.coli* 171 biofilm, a significant change was seen in the gene expression profile. Proinflammatory cytokines like IL-1β, IL-6, IL-12 and IFN-γ and chemokines like IL-8 showed maximal gene expression, which would lead to recruitment of immune cells and its activation. At twelve hour time point in biofilm stimulated PBMCs all the cytokine genes were highly down regulated. These responses suggest that *E.coli* biofilms are capable of eliciting an immune response at early point, but they either do not prevail, or the biofilm has mechanism for evading the harmful effect of this immune response leading to its persistence which might be one of the cause of recurrent or chronic UTI in catheterized patients.

This diminished immune response may be either due to the resistance of bacterial biofilm to host immune response or may be due to some of the factors released from bacteria. Perhaps, biofilm resistance against immune response may also be due to some of the components present in the EPS which may interfere with host immune response. A new view point on opposition of bacterial biofilm against immune response is the cell-cell communication or quorum sensing.

CHAPTER 1
INTRODUCTION

1.1 Background

The great advancement in technology has found its expression in modern medicine leading to improved and complicated medical devices and equipments like coronary stent, drug-coated vascular stents, cardiac pacemakers, prosthetic heart valves, chronic ambulatory peritoneal dialysis catheters, prosthetic joints, indwelling vascular catheters, urinary catheters etc. These devices are employed for the management of many serious illnesses. Even though they help the patient and alleviate their suffering, they cause various complications. Among them the most serious and devastating one is an infection.

According to Center for Disease Control (CDC) and prevention report (2007) nosocomial infections account for an estimated 1.7 million infection a year and 90,000 associated deaths a year. Of this 32 % is urinary tract infection (UTI). European centre for disease prevention and control (ECDC) June 2007 reports that 3 million people succumb to nosocomial infection each year and an estimated 50,000 die as a result. Nosocomial UTI is associated with an indwelling urethral catheter in the majority of cases. Catheter associated urinary tract infection (CAUTI) has been a leading cause of morbidity and mortality in hospitalized patients. Urinary catheters are tubular latex or silicone devices, which when inserted may readily acquire biofilms on the inner or outer surfaces. The most common urinary catheter in use is the Foley's indwelling urethral catheter. Most episodes of bacteriuria occur when enteric bacteria, colonizing the periurethral area migrate retrogradely into the bladder along both outside and inside of the catheter.

Escherichia coli is the most frequent cause of urinary tract infection (UTI). Uropathogenic *E. coli* (UPEC) strains have a number of virulence factors that increase their ability to colonize and persist in the urogenital tract [Mayon-White *et al*, 1988].

Catheters with high coefficients of friction, such as those of latex rubber, result in mechanical irritation to the urethra and incite an intense, acute and sometimes chronic inflammatory tissue response that can result in the formation of a scar. The major problem associated with the use of urinary catheter is the bacterial colonization and biofilm formation leading to recurrent UTI which is recalcitrant to antibiotics treatment and the host immune response. CAUTI involves a host immune response to microbial pathogens on the urinary catheter. CAUTI normally results in the development of localized tissue inflammation and injury. Peripheral blood mononuclear cells (PBMCs) are clinically important in sustaining local tissue inflammation and injury.

The innate immune response is effective against planktonic, single-celled microbes, and efficiently maintains the host healthy, without dependence on the adaptive immune response. But these defenses are not effective when microbes evade the components of the host defense system and then attach to the surfaces of the materials to form heterogeneous communities. These persistent communities can lead to debilitating chronic infections in humans and sometimes death.

White blood cells (WBCs) or leukocytes are cells of immune system involved in defending the body against both infectious diseases and foreign materials. The PBMCs consist mainly of monocytes, T-cells and B-cells, and smaller amounts of NK cells and dendritic cells. Mononuclear cells (lymphocytes and monocytes) and neutrophils are responsible for phagocytosis and elimination of bacterial pathogens. This is accomplished by direct phagocytosis, antigen presentation for antibody response and also by secretion of cytokines, which fulfill various functions.

Cytokines are proteins which plays fundamental role in the human immune response. The functions of these proteins are varied and include roles in normal T-cell mediated immunity, the inflammatory response, cancer, autoimmunity, and allergy. Cytokines act to induce, or inhibit, other cytokines, creating a population, or network, of

cytokines to which cells respond. In recent years it has become apparent that bacteria produce many molecules which have profound effects on the capacity of leukocytes and tissue cells to produce selected cytokine networks. Polymorpho nuclear cells (PMNCs) increase in urine when ever there is UTI, forming one of the indicators of UTI. Their reaction to presence of Foley's catheter with biofilm will lead to cytokine responses which are poorly characterized.

Here the cytokine production by PBMC is a biological parameter for evaluation of the influence of stimulation by both latex urinary catheter and catheter with biofilm. Also its interactions have been studied in an effort to understand the mechanisms of immune modulation and survival of bacterial biofilms (specifically *E.coli* biofilms) on Foley's latex urinary catheters.

1.2 Review of literature

1.2.1 Urinary Tract Infection

The urinary tract is the body's filtering system for removing liquid waste, or urine. It comprises the kidneys, ureters (tubes that carry urine from kidneys to bladder), bladder (fig: 1.1), and urethra (tube that carries urine from the bladder for excretion). A urinary tract infection is an infection in the urinary system which includes the bladder and the kidneys. Urinary tract infection (UTI) is caused by bacteria that enter the urinary tract.

The urinary tract has two parts: the lower and upper tracts. Most infections occur in the lower urinary tract and can also be called bladder infections. Infections in the lower tract (involving the bladder and urethra) are more common because bacteria can easily enter this area. Infections in the upper urinary tract (involving the kidneys and ureters) can also be called kidney infections and they cause serious illness.

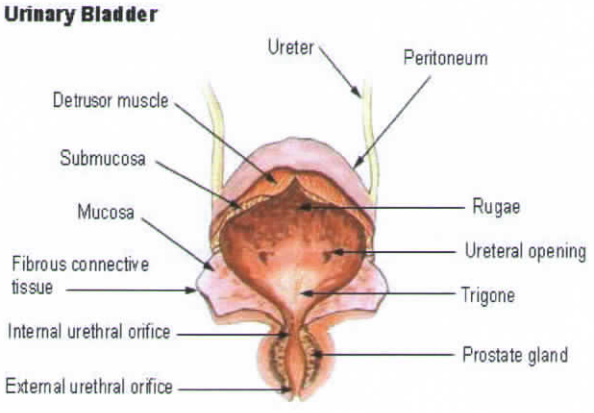


Fig: 1.1 Anatomy of urinary bladder

Types of urinary tract infections

The most common types of urinary tract infections are:

• **Urethritis** – Inflammation of the urethra (the tube-like structure that allows urine to pass from the bladder to be eliminated outside the body)

• **Cystitis** – Inflammation of the bladder (the balloon-like structure that stores urine before elimination through the urethra)

• **Pyelonephritis** – A more serious condition that is characterized by inflammation of the upper urinary tract, which includes the kidneys and the ureters (the 2 tube-like structures that connect each kidney to the bladder)

Acute UTI caused by UPEC can lead to recurrent infection, which is defined as 're-infection' when it involves a strain other than that causing the original infection, or as 'relapse' when it is caused by the same strain as that involved in the original UTI.

A biofilm on an indwelling urinary catheter consists of adherent microorganisms, their extracellular products, and host components deposited on the catheter. Urinary catheter biofilms may initially be composed of single species, but longer exposures inevitably lead to multispecies biofilms (Mayon-White *et al*, 1988).

The urinary bladder is coated with various mannosylated proteins, such as Tamm-Horsfall proteins (THP), which interfere with the binding of bacteria to the uroepithelium. Binding is an important factor in establishing pathogenecity for these organisms. The use of urinary catheters would physically disturb this protective lining, thereby allowing bacteria to invade the exposed epithelium.

During cystitis, uropathogenic *E.coli* (UPEC) subvert innate defenses by invading superficial umbrella cells and rapidly increasing in numbers to form intracellular bacterial communities (IBCs). By working together, bacteria in biofilms, build themselves into structures that are more firmly anchored in infected cells and are more resistant to

immune system and antibiotic treatments. This is often the cause of chronic urinary tract infections specifically when catheterization of the bladder is done.

1.2.2 Causes of UTIs

In women, urinary tract infections usually are caused by bacteria that live on the skin near the rectum or vagina. These bacteria can travel through the urinary tract and cause infections in the bladder or other parts of the urinary tract. UTIs in men are rare and usually indicate an abnormal urinary tract or an enlarged prostate.

The most common causes of urinary tract infections are:

- Sexual intercourse
- Waiting too long to urinate
- kidney stones that may physically block the free flow of urine
- cystocele -- relaxing of the bladder and vaginal area, which causes pools of urine to remain in the bladder
- diverticula -infections that develop on the inside wall of the urethra, allowing urine to collect
- urethral stenosis --a narrowing of the urethra, preventing an easy flow of urine out of the body This can be present at birth or result from a number of conditions or activities
- urinary tract infections in childhood
- diabetes
- pregnancy
- bladder catheter insertion

Some of the common symptoms of a urinary tract infection are:

Burning or pain in the lower abdomen

Fever, chills, nausea, and vomiting

Pus or blood in the urine

Burning during urination or an increase in the frequency of urination after the catheter is removed

1.2.3 Catheter associated urinary tract infection (CAUTI)

Indwelling urinary catheters are standard medical devices utilized in both hospital and nursing home settings to relieve urinary retention and urinary incontinence. The most notable complication associated with indwelling urinary catheters is the development of nosocomial urinary tract infections (UTIs), known as catheter-associated UTIs (CAUTIs). CAUTIs, the most common type of nosocomial infection, account for over 1 million cases annually [Tambyah & Maki, 2000] or over 40% of all nosocomial infections in hospitals and nursing homes [Stamm, 1991; Stamm & Hooton,1993; Warren, 1997] and constitute 80% of all nosocomial UTIs [Hartstein *et al.*,1981].

1.2.4 Urinary catheter

Catheters have been used for centuries; the term catheter is found in Greek writings attributed to Hippocrates, describing an instrument to drain fluid from a body cavity. Bernard and Dr. Frederick Foley were other pioneers in the field of catheter development. Urinary catheters are available in a variety of sizes ranging from 5 to 26 French. The most common urinary catheter in use is the Foley's indwelling urethral catheter, which has an inflatable 5- to 30-cc balloon that keeps the catheter in the bladder. It is a closed sterile system that is comprised of a tube inserted through the urethra and held in place by an inflatable balloon to allow urinary drainage of the bladder (fig: 1.2)

Fig: 1.2 Foley's latex urinary catheter with a balloon (▷) inflated. The catheter is held in place by the inflated balloon

The major development in the history of urinary catheter is the introduction of a closed collecting system i.e., one in which the tube empties into a receptacle in such a way that the urine is always contained within a lumen and protected from the environment. A closed sterile urinary catheter system consists of a tube inserted through the urethra and held in place by an inflatable balloon to allow urinary drainage of the bladder (fig: 1.3).

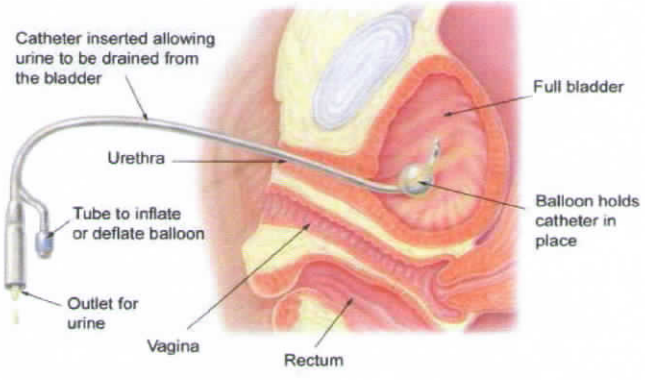


Fig: 1.3 Catheterized bladder

The organisms commonly contaminating these devices and developing biofilms are *Escherichia coli, Staphylococcus epidermidis, Enterococcus faecalis, Proteus mirabilis, Pseudomonas aeruginosa, Klebsiella pneumoniae,* and other gram-negative organisms [Mayon-White et al, 1988]. Some species of bacteria are uniquely capable of adhering to catheter materials and forming encrustations which results in the obstruction of urine flow.

So bacteriuria remains the major complication of the closed type of catheterization. A factor, which regularly appears to precede the development of bacteriuria, is periurethral or meatal colonization. Once organisms colonize the periurethral area, the surface of the catheter, collecting tube, or bag, there are three possible routes of entry for bacteria into the catheter and/or bladder. One route is by direct extension via the mucous sheath, which forms between the catheter and the urethral

mucosa directly into the bladder [Garibaldi *et al.*, 1980; Kass & Schneiderman, 1957; Brehmer & Madsen, 1972]. Another site of bacterial entry is via the disconnectable junction between the catheter and the collection tube. A third route is via the urine contained within the collection bag which may become contaminated.

Despite the many benefits urinary catheters provide, they can also produce complication as such as urinary tract infections, the formation of calculi, and urethral strictures. Several different biomaterials have been used in the manufacture of urethral catheters including uncoated latex, silicone-coated latex, silicone and hydrogel. Each material mentioned is capable of causing clinical problems including stone formation and urethral strictures.

A less common but more troublesome catheter-associated problem is the development of calculi on the catheter or in the bladder. Most of these stones are composed of a mixture of ammonium, magnesium, and phosphate crystals, which precipitates when the urine is alkalinized by urea splitting bacteria, such as *Proteus sp.* These calculi can be a source of significant patient morbidity. In contrast to typical urinary calculi that develop over a period of years, catheter-related calculi can form rapidly because the catheter acts as a polymer substrate that encourages accelerated crystallization.

A major reason for patient's death after catheter placement is the infrequently occurring urethral strictures. Catheters with high coefficients of friction, such as rubber, result in mechanical irritation to the urethra and incite an intense, acute, and chronic inflammatory tissue response that can result in the formation of a scar.

1.2.5 Pathogenesis of CAUTIs

The insertion of a foreign body such as an indwelling catheter into the bladder increases the susceptibility of a patient to UTIs, as these devices serve as the initiation site of infection by introducing opportunistic organisms into the urinary tract. The majority of these uropathogens are fecal contaminants or skin residents from the patient's own native or transitory microflora that colonize the periurethral area [Jacobsen *et al.*, 2008]. At the time of catheter insertion, bacterial entry into the bladder can occur through the catheter lumen, or along the catheter-urethral interface [Warren, 1996]. The preferred mechanism of bladder entry during CAUTIs is extra luminal (66%), where organisms ascend from the urethral meatus along the catheter urethral interface. Organisms can also enter the bladder intraluminally (34%), the migration of bacteria into the bladder occurs as a result of manipulation of the catheter system [Tambyah *et al.*, 1999; Warren, 2001].

Indwelling urinary catheters further favor the colonization of uropathogens by providing a surface for the attachment of host cell binding receptors that are recognized by bacterial adhesins, thus enhancing microbial adhesion. During insertion, urinary catheters may damage the protective uroepithelial mucosa, which results in the exposure of new binding sites for bacterial adhesins [Garibaldi *et al.*, 1980]. The occurrence of the indwelling catheter in the urinary tract interrupts normal host mechanical defenses, resulting in an over distension of the bladder and incomplete voiding that leaves residual urine for microbial growth [Hashmi *et al.*,2003].

Once colonized on the catheter and uroepithelium, uropathogens must adapt to the urinary tract environment and acquire nutrients. The production and secretion of degradative enzymes and toxins into the local environment may lead to a breakdown of tissue, releasing nutrients. Since iron is a limiting nutrient in the human host [Weinberg, 1984], uropathogens have developed complex iron acquisition systems for example heme transporters, ferric and ferrous iron transport systems, and siderophore iron uptake systems to evade host iron-sequestering mechanisms. Due to the expression of urease

some uropathogens are capable of using urea [Breitenbach, 1988; Jones & Mobley, 1987] as a nitrogen source. As a result of urease-mediated hydrolysis of urea to ammonia and carbon dioxide, the local environment becomes alkalinized. This leads to the precipitation of polyvalent ions that become enmeshed in the biofilms on catheters and urinary epithelial surfaces [Griffith *et al.*, 1976].

Gram-negative uropathogens enact a number of mechanisms of host immune evasion, including the production of capsules, immunoglobulin A (IgA) proteases, and lipopolysaccharides (LPSs). Capsules play a role in escaping the immune system by resisting phagocytosis, antimicrobial peptides, and the bactericidal effects of human serum [Jacobsen *et al.*, 2008]. Due to their structural similarities to polysialic acid residues found on human cells [Troy, 1992] capsular structures elicit a poor immunogenic response. Capsule plays a role in late biofilm development [Schembri *et al.*, 2005] and protects against desiccation and phage attack. It also helps in accelerating the urinary stone crystallization process through electrostatic interactions that accumulate urinary ions at the bacterial surface [Jacobsen *et al.*, 2008]. LPS, a requisite constituent of gram-negative bacterial outer membranes, elicits a potent inflammatory response that initiates the development of septic shock in systemic infections.

1.2.6 CAUTIs due to *E.coli*

Catheter-associated urinary tract infections are caused by a variety of pathogens, including *Escherichia coli, Klebsiella, Proteus,* Enterococcus, *Pseudomonas, Enterobacter, Serratia, and Candida*. Many of these microorganisms are part of the patient's endogenous bowel flora, but they can also be acquired by cross-contamination from other patients or hospital personnel or by exposure to contaminated solutions or non-sterile equipment [Selden *et al.*, 1971; McLeod, 1958].

E.coli is a facultative anaerobe that is a member of the family *Enterobacteriaceae.* The primary causative agents for greater than 80%of these infections, are strains of uropathogenic *Escherichia coli* (UPEC). While both commensal and uropathogenic *E. coli* (UPEC) strains colonize the large intestines of humans, only UPEC strains are primarily selected for growth in the urinary tract. Virulence factors that differentiate these avirulent commensals from virulent strains of *E. coli* were acquired on mobile genetic elements by horizontal gene transfer. These virulence factors allows *E. coli* strains to colonize and persist in the human host despite highly effective host defenses [Nataro & Kaper, 1998].

UPEC strains, are the most commonly isolated organisms in community-acquired UTIs (70 to 90%) and among the most commonly isolated in nosocomially acquired UTIs (50%) including CAUTIs [Kucheria, 2005]. *E. coli* has been recognized as the causative agent in 90% of all case of UTI in ambulatory patients [Johnson & Stamm, 1989]. UPEC strains can be classified into four phylogenetic groups, such as A, B1, B2, and D. The strains classified as B2 and D usually causing the most extra intestinal infections including UTIs [Nowrouzian *et al.*, 2006]. UPEC strains possess an arsenal of virulence factors that specifically contribute to their ability to cause disease in the human urinary tract. Genes encoding hemolysin, P fimbriae, S fimbriae, and cytotoxic necrotizing factor 1 (CNF1) have been identified on various pathogenicity islands in different UPEC strains [Hacker & Kaper, 2000].

1.2.7 Biofilm

A biofilm is defined as the accumulation of micro-organisms and their extra-cellular products to form a structured community on a surface [Costerton JW *et al.,* 1987]. Biofilm-associated organisms also differ from their planktonic (freely suspended) counterparts with respect to the genes that are transcribed. Biofilms may form on a wide variety of surfaces, including living tissues, indwelling medical devices, industrial or potable water system piping, or natural aquatic systems. Biofilms are basically comprised

of three layers [Reid G & Busscher HJ, 1998., Busscher HJ *et al.*, 1995]: a linking film, which attaches to the surface of a tissue or biomaterial, a base film of compact micro-organisms and a surface film on the outer side from which planktonic organisms can arise and spread (fig:1.4)

The sequences of events leading to the formation of biofilm are:

- Deposition of a host conditioning film onto a device
- Attachment of micro-organisms
- Microbial adhesion and anchorage to the surface by exopolymer production
- Growth, multiplication and dissemination of the organisms

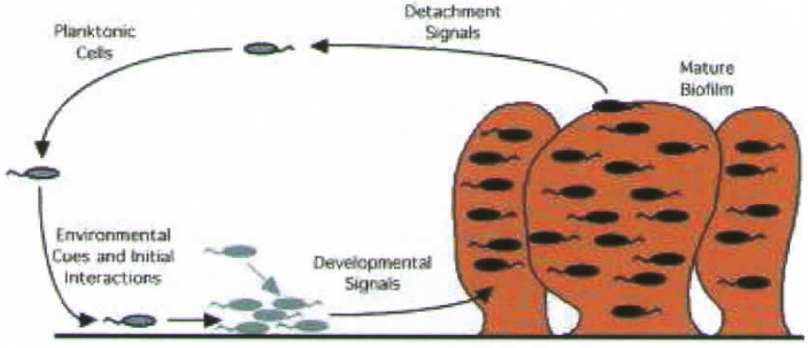


Fig: 1.4 Mechanism of bacterial biofilm formation

1.2.8 Mechanism of bacterial adhesion

The mechanism of bacterial adhesion is very complicated. But it can basically be described as a two-phase phenomenon. Phase I is an initial, instantaneous and reversible physical process and phase II is time dependent irreversible molecular and cellular phase [Marshall, 1985; Marshall, 1997]. Planktonic bacteria are moved towards a material surface by physical forces like Brownian movement, vander Waals forces, gravitational forces, surface electrostatic charges and hydrophobic interactions. This is the phase I which initiates bacterial attachment and makes the molecular and cellular phase of adhesion possible. In the second phase the molecular interactions between the bacterial

surface and substratum become predominant. In this the bacterial surface structures like the capsules, fimbriae, pili and slime are involved.

1.2.9 Biofilm structure

Biofilms are composed primarily of microbial cells and EPS. It may account for 50% to 90% of the total organic carbon of biofilms [Flemming *et al.*, 2000] and can be considered the primary matrix material of the biofilm. EPS may vary in chemical and physical properties, but it is primarily composed of polysaccharides. Some of these polysaccharides are neutral or polyanionic, as is the case for the EPS of gram-negative bacteria. The presence of uronic acids (such as D-glucuronic, D-galacturonic, and mannuronic acids) or ketal-linked pyruvates gives the anionic property [Sutherland, 2001]. This property is important because it allows association of divalent cations such as calcium and magnesium, which have been shown to cross-link with the polymer strands and provide greater binding force in a developed biofilm [Flemming *et al.*, 2000]. Back bone of EPS contains 1, 3 or 1, 4-β linked hexose residues. The amount of EPS increases with the age of biofilm. EPS may associate with metal ions, divalent cations and macromolecules (proteins, DNA, lipids and even humic substances). The production of EPS is known to be influenced by nutrient status of the growth medium, excess available carbon; however, limitation of nitrogen, potassium, and phosphate help the EPS synthesis [Donlan & Costerton, 2002].

1.2.10 Factors favoring biofilm formation

Biofilm may be formed on a wide variety of surfaces including living tissues, indwelling medical devices. Different factors affecting the formation of biofilm are as follows:

1. Substratum effects

The extent of microbial colonization appears to increase as the surface roughness increases due to the diminished shear forces and higher surface area. Maximum attachment depends upon high surface free energy or wettability of surfaces. Surfaces

with high surface energies such as stainless steel and glass are more hydrophilic. These surfaces generally show greater bacterial attachment.

2. Conditioning films

Solid surfaces which have been exposed in an aqueous medium become conditioned or coated with polymers from the medium. The chemical modification of surfaces affects the rate and extent of microbial attachment. A number of host produced conditioning films such as blood, saliva, tears, urine, intravascular fluid and respiratory secretions influence the attachment of bacteria to biomaterials. The surface energy of the suspending medium may affect hydrodynamic interactions of the microbial cells with surfaces by changing the substratum effects [Donlan, 2002; Chmielewski & Frank, 2003].

3. Hydrodynamics

Biofilm response is altered in flow conditions. Biofilms grown under laminar flow are found to be patchy and consist of rough cell aggregates separated by interstitial voids. Biofilms grown under turbulent flow cells are also patchy but are elongated streamers that oscillate in the bulk fluid [Davey & Otoole, 2000]

4. Characteristics of the Aqueous Medium

Physico-chemical characteristics of the aqueous medium, such as pH, nutrient levels, ionic strength, and temperature, may play a role in the rate of microbial attachment to a substratum. In a laboratory study Cowan *et al.* showed that an increase in nutrient concentration correlated with an increase in the number of attached bacterial cells.

5. Properties of the Cell

Cell surface hydrophobicity, presence of fimbriae and flagella, and production of EPS all influence the rate and extent of attachment of microbial cells. Fimbriae play a role in cell surface hydrophobicity and attachment, probably by overcoming the initial electrostatic repulsion barrier that is present between the cell and substratum [Corpe, 1980]. Flagella apparently play an important role in attachment in the early stages by overcoming the repulsive forces associated with the substratum.

6. Horizontal gene transfer

Horizontal gene transfer is important for the evolution and genetic diversity of natural microbial communities. During evolution, an adaptation of bacteria to new environment often results in the acquisition of new genetic traits via horizontal gene transfer. Mobile genetic elements mediate horizontal gene transfer between bacteria. These elements can be conjugative plasmids, transposons or bacteriophages. Bacteria in biofilm express different phenotypic characters from planktonic counterparts. This is due to different genes transcribed in the planktonic and biofilm-associated phases of bacterial life cycle.

7. Quorum sensing

Quorum sensing is the regulation of gene expression in response to fluctuations in cell-population density. Quorum sensing bacteria produce and release chemical signal molecules called autoinducers that increase in concentration as a function of cell density. The detection of a minimal threshold stimulatory concentration of an autoinducer leads to an alteration in gene expression. The signal molecules include bacterial metabolites, acyl HSLs secreted proteins, genetic material such as DNA or RNA etc. This signal may alter distribution of specific bacterial species in the biofilm, alter protein expression on the neighbouring cells, introduce new genetic trait in neighbouring cells and incorporate bacteria in biofilm.

1.2.11 Biofilm on urinary catheter

Prolonged urinary tract catheterization can facilitate the development of catheter biofilms. Urinary tract infections in catheterized patients can occur in several ways. Organisms that colonize the periurethral skin can migrate into the bladder through the mucoid film that forms between the epithelial surface of the urethra and the catheter. Besides, contamination of the urine in the drainage bag can permit organisms to access the bladder through the drainage tube and the catheter lumen [Stamm, 1991; Tambyah *et al.*, 1999]. Urinary infection in the presence of a catheter has a bimodal spread; an ascending spread of adherent bacterial biofilms along the catheter and a rapid turbulent

flow of planktonic bacteria by 'salutatory movement' ahead of the adherent biofilm [Nickel et al., 1992]. The risk of urinary tract infection is related to the length of time the catheter is in place. Most patients catheterized for a week or less should escape infection, but bacteriuria is inevitable in the case of many elderly and disabled patients who are catheterized for several months or years [Warren, 1991; Kunin, 1997]. In patients with long-term indwelling catheters, catheter changes are commonly scheduled at 10–12-week intervals; contaminated urine can, therefore, be flowing through individual catheters for periods of 3 months at a time. Thus, catheters provide attractive sites for bacterial colonization, the biofilm bacteria thrive in their matrix gel and the gentle flow of warm nutritious urine.

A variety of bacterial species colonize catheters, and many of these biofilms can induce serious complications. The most common species present in the mixed-population biofilms were *E. faecalis*, *P. aeruginosa*, *E. coli*, and *P. mirabilis*. Biofilms on urinary catheters may contain organisms that have the ability to hydrolyze urea in the urine to form free ammonia through the action of urease. The ammonia may then elevate the pH at the biofilm liquid interface, resulting in the precipitation of minerals such as calcium phosphate (hydroxyapatite) and magnesium ammonium phosphate (struvite) [Tunney *et al.*, 1999]. These minerals can then become entrapped in the biofilm and cause encrustation of the catheter and became completely blocked by this mineral build-up. This causes the blockage in the flow of urine through the catheter. As a consequence, urine often leaks along the outside of the catheter and patients become incontinent. Blockage of the catheter can lead to retention of urine in the bladder and vesicoureteric reflux of infected urine; if the blockage is not detected and if the catheter is not changed, patients can suffer episodes of pyelonephritis and septicemia [Liedl, 2001; Kunin, 1987].

1.2.12 Intracellular bacterial communities

The first step in the encounter between a host and pathogen is the attachment of the pathogen to the host epithelium by way of an interaction between an adhesion on the pathogen and a receptor on the host tissue. This contact initiates a dynamic molecular cross-talk that ultimately determines the outcome of the infectious process. The interaction of UPEC with bladder epithelial cells, mediated by FimH triggers a signal transduction cascade that results in the uptake of the attached bacteria by superficial umbrella cells [Martinez *et al.*, 2000].Invasion of UPEC into these epithelial cells stimulates a complex genetic cascade resulting in the formation of intracellular bacterial communities (IBCs) [Anderson *et al.*, 2003].IBC maturation progresses via four distinct developmental stages that differ with respect to growth rate, bacterial length, colony organization, motility and dispersal. During IBC development, a loose collection of fast-growing, rod-shaped bacteria mature into slower growing, highly organized biofilm-like communities consisting of coccoid bacteria. These ultimately fill most of the umbrella cell cytoplasm, causing luminal protrusions termed pods. Subsequently, the IBCs change to a motile rod-shaped phenotype, allowing bacteria at the IBC periphery to detach from the community and flux out of the host cell, facilitating their spread within the urinary tract. Ultimately, bacterial replication ceases and a quiescent reservoir is established in the bladder tissue that, in response to unknown signals in a murine model, can re-activate and trigger a recurrent infection [Justice *et al.*, 2004].

1.2.13 Immune system

The immune system is a remarkably adaptive defense system that has evolved in vertebrates to protect them from invading pathogenic microorganisms and cancer. It is able to generate an enormous variety of cells and molecules capable of specifically recognizing and eliminating an apparently limitless variety of foreign invaders.

The components of immunity are innate or nonspecific immunity and acquired or specific immunity. Innate immunity refers to the basic resistance to disease that an

individual is born with. Acquired immunity requires the activity of a functional immune system, involving cells called lymphocytes and their products (fig: 1.5).

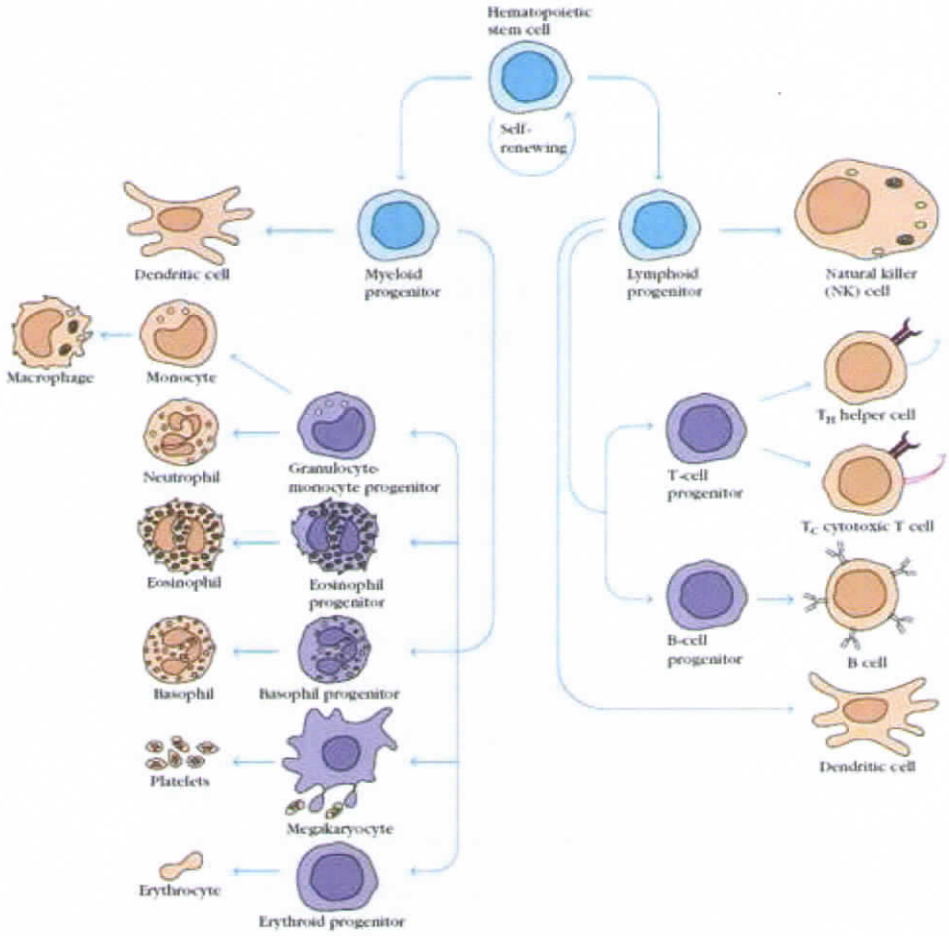


Fig: 1.5 Overview of immune system

1.2.14 Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells (PBMCs) constitute a very important part of our peripheral immune system. A Peripheral Blood Mononuclear Cell (PBMC) is any blood cell having a round nucleus. The PBMCs consist mainly of monocytes, T-cells and B-cells, and smaller amounts of NK cells and dendritic cells of both myeloid and plasmacytoid origin. The PBMCs are suitable for assessment of the general immune

activation of drug candidates or microorganisms, for which an immune stimulation is of interest (e.g. vaccine adjuvants and particles, immune stimulants for cancer treatment or immune stimulating natural extracts or micro organisms). These blood cells are a critical component in the immune system to fight infection and adapt to intruders.

Peripheral blood mononuclear cells (PBMCs) have been a popular model system that serves as a circulatory mirror of the *in vivo* physiological and metabolic activity of cells. Among the plethora of available primary cells, PBMCs and its individual subsets have enabled a broad spectrum of applications from *in vitro* cell-based assays to the monitoring of *ex vivo* changes before and after treatment.

1.2.15 Biofilm: Immune Cell Interactions

Freely suspended microorganisms have evolved a multitude of ways to avoid detection by the immune system. One of these mechanisms is to form biofilms. Biofilm bacteria may subtly manipulate immune responses while reactions of immune cells may exacerbate biofilm formation and reinforce virulence.

The mechanisms that enable bacteria in biofilms to resist host defenses include

(i) limited penetration of leukocytes and their bactericidal products into the biofilm

(ii) global response regulators and quorum sensing activities that increase resistance to leukocytes

(iii) decreased ability of leukocytes to engulf biofilm bacteria

(iv) genetic switches that increase resistance of bacterial cells in biofilms to the immune system and suppression of leukocyte activity through effector regulation

One explanation behind a biofilm's resistance to the immune system was that immune cells could not penetrate into the biofilm. [Leid *et al.*, 2002] documented such penetration in an *in vitro* study of freshly isolated human leukocytes contacting 2-day-old

(early, maturing) and 7-day-old (fully developed) *S. aureus* biofilms, under static and flowing fluid conditions that mimic physiological shear. Leukocytes were able to penetrate biofilms but unable to phagocytose bacteria suggests that other mechanisms may inhibit normal leukocyte function. In another experiment [Leid *et al.*, 2005] it was shown that exopolysaccharide alginate matrix protects *Pseudomonas aeruginosa* biofilm from IFN-γ mediated leukocyte killing. The alginate in *Pseudomonas aeruginosa* biofilm scavenges hypochlorite, reduces polymorphonuclear chemotaxis, inhibits its activation of compliment, and decreases phagocytosis by neutrophils and macrophages.

Walker *et al.* (2005) has reported that human neutrophils serve to enhance the initial development of *P. aeruginosa* biofilms. The mechanism of biofilm enhancement by neutrophils was attributed to neutrophil-generated polymers comprised of actin and DNA. The bacteria bind to F-actin and the free DNA promotes biofilm matrix stability.

Chandra *et al.* (2007) showed that peripheral blood mononuclear cells (PBMCs) enhanced the ability of the yeast, *Candida albicans*, to form biofilms and that the majority of PBMCs were localized to the basal and middle layers of the biofilm. They also found that only viable PBMCs enhanced Candida biofilm formation and that PBMC's cell surface components did not contribute to this biofilm enhancement; the biofilm-enhancing effect is mediated by a soluble factor released into the medium, and that the supernatant collected from co-cultures contained differential levels of pro- and anti-inflammatory cytokines.

In an *in vitro* assay Zimmermann *et al.* (2006) reported that the quorum sensing molecule N-(3-oxododecanoyl)-L-homoserine lactone (3OC12-HSL) synthesized by *P. aeruginosa* induces directed migration (chemotaxis) of neutrophils. They have postulated that it could be possible that these bacterial signals are used by adherent bacteria to stimulate an immune cell response, which in turn enhances biofilm formation and virulence.

Wu *et al.* (2005) demonstrated that IFN-γ binds to an outer membrane protein in *P. aeruginosa*, OprF, resulting in the expression of a quorum-sensing dependent virulence determinant, the PA-I lectin. The results by Wu et al. and others [Smith & Iglewski, 2003] suggest that adherent bacteria not only actively sense but can also manipulate host immune response (Tateda *et al.*, 2003; Smith & Iglewski, 2003].

Mittal *et al.* (2006) reports that strains of *P. aeruginosa* vary in their ability to induce macrophage response, which effects the composition of secreted products (cytokines, chemokines). These different compositions of macrophage-secreted products influenced overall biofilm formation and up-regulated expression of a litany of virulence factors in a cytokine composition-dependent manner.

1.2.16 Bacterial defense against phagocytosis

Microorganisms invading tissues are first and foremost exposed to phagocytes. Most bacteria that are successful as pathogens interfere to some extent with the activities of phagocytes or in some way avoid their attention. Bacterial pathogens have devised numerous and diverse strategies to avoid phagocytic engulfment and killing. Most are aimed at blocking one or more of the steps in phagocytosis, thereby halting the process.

- Avoiding Contact with Phagocytes
- Inhibition of Phagocytic Engulfment
- Survival Inside of Cells
- Products of Bacteria that Kill or Damage Phagocytes

1.2.17 Cytokine induction by bacteria

Cytokines and chemokines are redundant secreted proteins with growth, differentiation, and activation functions that regulate and determine the nature of immune responses and control immune cell trafficking and the cellular arrangement of immune organs. A cascade of responses can be seen in response to cytokines, and often several cytokines are required to synergize to express optimal function. Numerous cytokines have both pro-inflammatory and anti-inflammatory potential; which activity is observed

depends on the immune cells present and their state of responsiveness to the cytokine. Cytokines, the most important class of inflammatory mediators [di Giovine *et al.*,1996], are signaling molecules which can behave as classic endocrine hormones have been subdivided into families such as the interleukins, chemokines, interferons etc.

Interaction between bacteria and host cells invariably results in the release of one or more cytokines; the actual cytokines produced depending mainly on the nature of the bacterium and host cells involved. The resulting cytokine network constitutes an important part of the innate immune response and represents the host's attempt to deal with that particular organism. The ability of bacterial components (or bacterial activities) to induce cytokine release from host cells can be regarded as bacterial virulence.

Lipopolysaccharide (LPS) is a potent inducer of cytokine release from a variety of host cell types. 15 classes of bacterial surface components or secretory products are known to stimulate cytokine release [Henderson & Wilson, 1996; Wilson *etal.*, 1996). This chemically diverse group of compounds, recognized as a separate class of virulence factors are the modulins, so called because of their ability to modulate the behavior of cells due to the induction of cytokine synthesis (Henderson & Wilson, 1995). Such molecules include peptidoglycans, teichoic acids, and fimbrial proteins, etc.

McCormick *et al.* (1993) reported that adhesion of *Salmonella typhimurium* to human intestinal epithelial cells induced the release of IL-8. Subsequently, it was shown that adhesion of this organism to murine macrophages increased the levels of mRNA for IL-1β, IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF), and chemokines, such as MIP-1β and MIP-2, and melanocyte growth stimulatory activity [Yamamoto *et al.*,1996]. Yamamoto *et al.*(1994) have also reported that binding of *Legionella pneumophila* to the surface of murine macrophages (treated with cytochalasin D to prevent phagocytosis) results in increased levels of mRNA for IL-1α, IL-1β, IL-6, tumor necrosis factor alpha (TNF-α), and GM-CSF. Intravesicular inoculation of mice

with P fimbriated strains of *E. coli* has been shown to cause release of IL-6 into the urine [Linder *et al.*, 1991]. Adhesion of an *E. coli* strain with type 1 and P fimbriae to a bladder epithelial cell line was found to induce the release of IL-6, IL-8, and IL-1α. [Agace *et al.*, 1993].

Helicobacter pylori can induce cytokine expression in epithelial cells. Crabtree *et al.* (1994) reported that patients infected with the organism express much greater levels of IL-8 in their gastric epithelia than those patients in which the organism could not be detected. Levels of mRNA for IL-6, IL-7, IL-8, IL-10, and TNF-α has also been reported to be significantly higher in the gastric mucosa of patients infected with *H. pylori* than in those free of the organism [Yamaoka *et al.*, 1996]. Eckmann *et al.* (1993) reported that invasion of epithelial cell lines by *Salmonella dublin*, *Yersinia enterocolitica*, *Shigella dysenteriae*, and *Listeria monocytogenes* induced the release of the chemotactic cytokine IL-8.

The neutrophils, monocytes and macrophages are the front runners in innate immune response to introduction of a medical device and will influence the adhesion and development of biofilms which results in recurrent infections called the implant associated infections. In the urinary tract on catheterization, the specific role of these members of the innate immune response is not well understood. The mechanism of immune modulation, which leads to continued persistence of bacterial biofilms on urinary catheters in chronic CAUTI, is poorly understood, and needs to be delineated for greater understanding of chronic UTI in catheterized patients and development of infection resistant devices.

1.3 Hypo dissertation

Our hypothesis is that the monocytes, macrophages, lymphocytes, which are components of peripheral blood mononuclear cells and neutrophils that are a component of innate immune mechanism, will influence the formation and persistence of bacterial biofilms on urinary catheters. These interactions will lead to modulations of cytokine gene expression in the PBMCs. An effort has been made in this work to delineate these interactions to understand continued persistence of bacterial biofilms on urinary catheters leading to chronic CAUTI.

1.4 Objectives

- Morphological analysis of PBMC-biofilm interaction using microscopy.
- Analysis of basic levels of cytokine mRNA expressions in normal human PBMC *in vitro* during culture.
- Analysis of the interaction of Latex Foley's urinary catheter on PBMC culture
- Modulation of cytokine gene expression in normal human PBMC *in vitro* upon interaction with *E.coli* biofilms.
- Influence of catheter and *E.coli* biofilm on PBMC proliferation *in vitro.*

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials used:

2.1.1 Reagents used for bacterial biofilm preparation

All media and reagents used for the bacterial culture preparation were purchased from Himedia. Latex Foley's urinary catheter (SISCO brand) which was purchased from the market was used for the preparation of biofilm. Glutaraldehyde, a chemical used for fixation of bacterial biofilms was purchased from Spectrochem.

2.1.2 Reagents used for PBMC culture

Ficoll-Paque™ PREMIUM used for PBMC isolation was purchased from GE Health care. All plastic ware used were purchased from BD falcon.

2.1.3 Reagents used for gene expression analysis

Total RNA was extracted from the PBMC using the TRIzol® Reagent (Invitrogen). Chloroform used in RNA isolation was purchased from Merck. The primers used for qrt-PCR were purchased from Metabion International AG. qrt-PCR was done using SYBR Green chemistry and Eurogentec two step kit. Amplification of the genes were confirmed by agarose gel electrophoresis. Agarose gel was purchased from Himedia. The 6X gel loading dye and λ DNA/ *Eco*471 (*Ava*II) Marker, 13 were purchased from Fermentas.

2.2 Selection of *E.coli* strain

E.coli strain used in the experiments was isolated from urinary catheters retrieved from patients in neuro-surgery ward of SCTIMST and maintained as lyophilized culture stock in the Division of Microbiology. The *E.coli* strain had been characterized for biofilm formation by 2 methods, Congo Red agar assay and curli expression analysis.

2.2.1 Congo Red agar assay

The Congo Red agar medium was prepared. The bacterial strains were streaked on the medium and incubated at 37^0C for 24 hrs and then at room temperature for 48 hr. Reaction was recorded at 24, 48 and 72 hours. The colour of the medium changes to black within 72 hours of incubation was recorded as a positive reaction. In the case of negative reaction, the colour of the medium remains red after 72 hours of incubation.

2.2.2 *Curli* expression analysis

The *curli* expression agar medium was prepared. The bacterial strains were streaked on the medium, incubated at 37^0C for 24 hrs and then at room temperature for 48 hr. Bacteria possessing *curli* usually display a red, dry, and rough morphology when grown on plates supplemented with the diazo dye Congo Red (CR). Curli-deficient strains often produce smooth, white, and moist colonies.

The strain selected was *E.coli* 171. *E.coli* 171 was positive for both for Congo Red and *curli* expression (fig: 2.1 & 2.2).

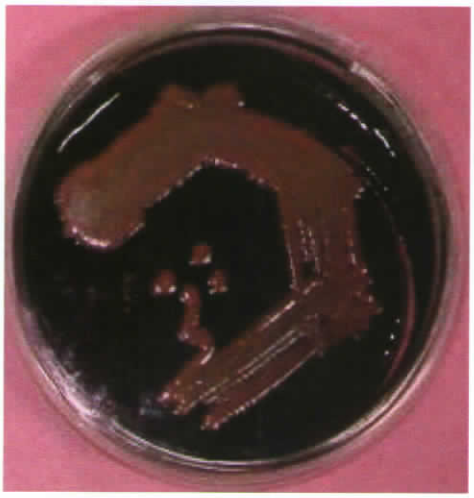


Fig: 2.1. *E.coli* 171 strain on Congo Red agar medium. Biofilm production is seen by the black colouration in the medium subsequent to incubation for 72 hours.

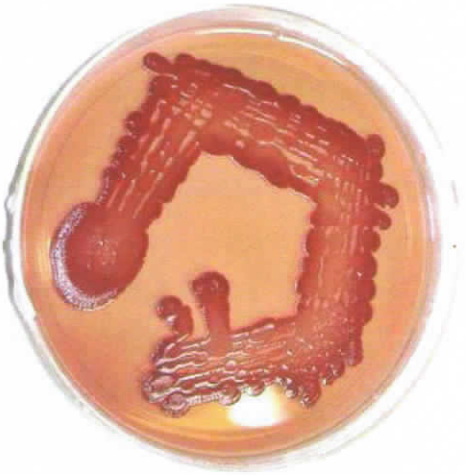


Fig:2.2. *E.coli* 171 strain culture on curli expression agar medium. E.coli 171 strain is Curli positive and produces red, dry, and rough colonies.

2.3 *E.coli* biofilm formation on Foley's latex urinary catheter

2.3.1 Formation of *E.coli* biofilm on catheter pieces

The pre-inoculum of *E.coli*171 strain was prepared in LB medium supplemented with 1% glucose. Ethylene oxide (ETO) sterilized Foley's balloon urinary catheter purchased from the market were used for the study and it was cut into small pieces under sterile conditions. The inoculum density was adjusted to 10^5 cells/mL based on Mc-Farland Standard No: 1 in LB broth and catheter pieces were added to broth and incubated at 37^0C for 3 hr. After 3 hours catheter pieces transferred using a sterile forceps into new LB broth and incubated at 37^0C overnight under static condition.

2.3.2 Morphological study of biofilm

The *E.coli* biofilm formed were analyzed by fluorescent microscopy using acridine orange staining. Acridine orange (Himedia) is a fluorochrome that binds to the nucleic acids of bacteria and stains them orange. *E.coli* biofilm was washed in sterile PBS (Himedia) and fixed with 2.5% gluteraldehyde (Spectrochem) for 15 minutes. Excess glutaraldehyde was washed off with acetate buffer and stained in 0.2 % acridine orange

for 5 minutes at room temperature. Excess acridine orange was washed off with PBS and observed in upright fluorescent microscope (LEICA DMR).

CLSM was used to examine the biofilm formed on Foley's latex urinary catheter after incubation for 72 hours at 37°C in LB broth. Bacterial biofilms on urinary catheter was fixed with 5%Glutaraldehyde and stained with Propidium Iodide. Confocal Laser Scanning Microscopy (Carl Zeiss 510 meta software) was used; laser used was HeNe543, excitation done at 536nm and emission at 617nm.

2.3.3 Viable counting of bacteria in a biofilm

The counting of *E.coli* in a biofilm was done using spread plate method. The method was standardized in the Division of Microbiology. Aseptically transfered the catheter with biofilm from the *E.coli* suspension into a sterile tube and washed repeatedly with sterile physiological saline to remove any loosely bounded bacteria. The biofilm was then transferred into fresh sterile tube containing sterile physiological saline and sonicated for 1 minute. After sonication, tube was vortexed for 1 minute. This step was repeated 3 times. 5µl of undiluted suspension from the tube was mixed with 20µl of sterile physiological saline and plated for viable count. Three dilutions of the initial suspension was made and plated in triplicates for each dilution on TSA for viable count. The plates were incubated at 35^0C to 37^0C for overnight and the number of colonies grown was counted. The viable count was calculated for each dilution, mean determined, and expressed as colony forming units (cfu) per strip or cfu/square centimeter.

2.4 Peripheral blood mononuclear cell isolation and culturing

Heparinised blood samples drawn from healthy human donors were diluted with equal amounts of RPMI-1640 with L-Glutamine and 25mM HEPES buffer (Himedia) medium. Then it was mixed thoroughly and carefully layered 20 mL on top of 14 mL Ficoll-Paque (GE Healthcare) taken in a falcon tube and centrifuged at 3000 rpm for 15 minutes using Biofuge stratos (Heraeus instruments). The interphase was transferred into

the 50 ml sterile falcon tubes containing 20 mL serum free RPMI. Contaminating Ficoll-Paque was removed by centrifugation at 3000 rpm for 10 minutes. This step was repeated twice to completely remove Ficoll-Paque and then resuspended in RPMI with serum containing medium. Cell counting was done using haemocytometer and seeded at a density of $2x10^6$ cells/mL onto a 24 well tissue culture polystyrene plate. Culture was incubated at 37^0C for 24 hrs.

2.5 PBMC- Foley's latex urinary catheter interaction

PBMCs were exposed to Foley's latex urinary catheter under static condition by incubation at 37^0C for various time intervals (2, 4, 6, 8, 10 &12 hr). The morphological changes and cytokine gene modulations were studied by analyzing mRNA expression in PBMC cultures.

2.6 PBMC- *E.coli* Biofilm interaction

PBMCs were stimulated with *E.coli* 171 biofilm on Foley's latex catheters under static condition at 37 ^{0}C. The morphological changes and cytokine gene modulations were studied by analyzing mRNA at various time intervals of 2, 4, 6, 8, 10 &12 hrs.

2.7 Isolation of RNA from PBMC

Total RNA was isolated from PBMC cultures at various time intervals of 2, 4, 6, 8, 10 &12 hrs, were unstimulated cells used to find the base line expression of cytokine genes. This was used as control.

Procedure for Isolation of RNA

Total RNA was extracted from the cells using the TRIzol® Reagent [Invitrogen]. Protocol of the kit was followed strictly. In brief, cells were collected from the dish using TRIzol reagent and kept on ice. For every 1ml of TRIzol 200µl of chloroform (Merck) was added and mixed for 15 seconds. It was then incubated at room temperature for 5-10minutes followed by a spin of 12000 rpm for 15 minutes at 4°C.

Three layers were formed - upper aqueous phase (colourless), interphase and lower phase (pink). The upper aqueous phase was transferred into a fresh tube and 500µl of isopropanol was added. RNA was precipitated by incubation at room temperature for 5-10 minutes and then centrifuged at 12000 g for 8 minutes at 4°C. The pellet was washed with 1ml of 75% ethanol , air dried for 3 minutes and resuspended in 10-15µl of RNase free autoclaved water and stored at -20°C. It was quantified using mass spectroscopy (Eppendorf) before use.

2.8 Gene expression analysis using qrt-PCR

Cytokines genes analyzed in the study were IL-1β, IL-8, IFN-γ, IL-12, IL-6, MCP-1, TNF-α, IL-4 and IL-10. β-actin was the house keeping gene used. The primer sequences of these genes are listed below in Table 2.1

Cytokine gene	Primer sequence	Annealing temperature
IL-1β	FP: 5' ATAAGCCCACTCTACAGCT3' RP:5'ATTGGCCCTGAAAGGAGAGA3'	56.5^0C
IL-8	FP: 5' GCTTTCTGATGGAAGAGAGC3' RP: 5' GGCACAGTGGAACAAGGACT3'	59^0C
IFN-γ	FP: 5' ATGCAGAGCCAAATTGTCTCC3' RP:5'TTACTGGGATGCTCTTCGACC3'	60^0C
IL-12	FP: 5'TCACAAAGGAGGCGAGGTTC3 RP: 5' TGAACGGCATCCACCATGAC 3'	60^0C
IL-6	FP: 5' CAGCCACTCACCTCTTCAGAAC3' RP: 5' TGCAGGAACTGGATCAGGAC3'	62^0C
MCP-1	FP: 5' TCAAACTGAAGCTCGCACTCT3' RP:5'AGCTGCAGATTCTTGGGTTGTGG3'	62^0C
TNF-α	FP: 5' CAGAGGGAAGAGTTCCCCAG3' RP: 5' CCTTGGTCTGGTAGGAGACG3'	63^0C
IL-4	FP: 5' ACTGCTTCCCCCTCTGTTCTTC3' RP:5' GTACTGTGGTTGGCTTCCTTCAC3'	64.5^0C
IL-10	FP:5'ATGCCCCAAGCTGAGAACCAAGAC3' RP:5'TCTCAAGGGGCTGGGTCAGCTATCCCA3'	70^0C
β-actin	FP: 5' CCTGGCACCCAGCACAAT3' RP: 5'GCCGATCCACACGGAGTACT3'	60.5^0C

Table 2.1 Primer sequences of cytokine genes and house keeping gene

qrt-PCR was done using SYBR Green chemistry and Eurogentec two step kits. Kit comprised of a Reverse Transcription core kit and a MESA GREEN qrt-PCR Master mix plus for SYBR assay. The protocol mentioned in the kit was followed. In brief, mRNA extract was primed with random nanomer and reverse transcribed with Euro script RT to produce cDNA. A 10µl reaction was made. Constituents of the reaction mixture are listed below in Table 2.2

Component	Volume (µL)
10X Reaction buffer	1
25mM $MgCl_2$	2
2.5mM dNTP	2
Random nanomer	0.5
RNAase inhibitor	0.2
Euroscript RT	0.25
RNase free water	3.05
Template	1
Total	10

Table 2.2 Constituents of reaction mixture for cDNA synthesis

cDNA amplification was done using Chromo4™ system and the conditions of the experimental setup are listed in Table 2.3

Step	Time	Temperature
Initial step	10 minutes	25°C
Reverse Transcriptase step	30 minutes	48°C
Inactivation of RT enzyme	5 minutes	95°C

Table 2.3 PCR conditions for cDNA synthesis

A 25µl reaction was set. The constituents of reaction mixture and the PCR conditions are given in Table 2.4 and 2.5 respectively.

Component	Volume (µL)
2X Reaction buffer Forward primer Reverse primer Template Water	12.5 2.5 2.5 2.5 5.0
Total	25

Table 2.4 Constituents of reaction mixture for cDNA amplification

Temperature	Time
95^{0}C	10 minutes
95^{0}C	15 seconds
57^{0}C to 70^{0}C	20 seconds
72^{0}C	40 seconds
Plate reader	
Go to line 2 for 39 more times	
Melting curve from 57^{0}C to 94^{0}C	
72°C	5 minutes
10°C	Forever
End	

Table 2.5 PCR conditions for cDNA amplification

It was programmed for 40 cycles. Melting curves were used to establish the purity of the amplified band. The expression level of each mRNA was normalized to that of β-actin [ΔCT], a housekeeping gene, using the below mentioned mathematical expression.

$\Delta CT = C(t)_1 - C(t)_2$, Where [$C(t)_1$ = β-actin] [$C(t)_2$ = gene of interest]

2.9 Agarose gel electrophoresis

PCR products of 10 genes were run on agarose gel based on standard protocol (Sambrook). 1% agarose gel was prepared in 1X TAE buffer and ethidium bromide was added at a final concentration of 0. 5μg/mL to the gel. Sample DNA was mixed with 6X gel loading dye and was run along with λ DNA/ *Eco*471(*Ava*II)Marker,13 (8126 bp)in electrophoresis apparatus (Balaji scientific apparatus). The gel was documented using UVI pro gel documentation system.

2.10 Morphological analysis of PBMC-catheter interaction using Scanning Electron Microscopy

The samples were fixed in 3%glutaraldehyde and excess glutaraldehyde was washed off with phosphate buffer. The samples were dehydrated with increasing concentrations of ethanol and washed off with phosphate buffer. After critical point drying and gold coating the samples were viewed under SEM (FEI, Quanta 200).

2.11 Morphological analysis of PBMC-biofilm interaction using Scanning Electron Microscopy

The samples were fixed in 3%glutaraldehyde and excess glutaraldehyde was washed off with phosphate buffer. The samples were dehydrated with increasing concentrations of ethanol and washed off with phosphate buffer. After critical point drying and gold coating the samples were viewed under SEM (HITACHI, S2400).

2.12 Study of influence of catheter and associated biofilm on PBMC proliferation

PBMCs were isolated and cultured at a density of 10^6 cells/mL on a tissue culture polystyrene plate and incubated overnight. It was then stimulated with *E.coli* 171 biofilm (10^5 cells/catheter) and incubated for 24 hr time period. Gentamicin was added at a concentration of 500μg/mL. After incubation the plates were pulsed with 1μCi/Ml of [^{3}H] Thymidine 18 hr before harvesting. Cells were collected and centrifuged at 3000 rpm for 5 minutes. Supernatant was discarded and then added 0.5ml of 0.1M NaOH containing 0.1% SDS. 0.5 ml of 10% TCA was added to the cell and kept for 1 hr at 4^0C. Pellet was colleted by centrifugation at 10,000g for 15 minutes at 4^0C. The pellet was suspended in 1mL of absolute alcohol and kept in ice bath for 30 minutes. Then it was centrifuged at 10,000g for 15 minutes at 4^0C, pellet was air dried and dissolved in 0.5ml of 0.1 N NaOH and counted using liquid scintillation counter, Triathler Multilabel tester (Hidex, Germany).

CHAPTER 3

RESULTS AND DISCUSSIONS

The immune system has evolved to protect the host from infection by either development of innate immunity or adaptive immunity. Innate immunity is the ability to produce response with in minutes or hours after infection by recognizing certain ligands on pathogens triggering signaling cascades within these cells. The mucosal immune system is the first line of attack in any pathogenic infection. In patients when the urinary bladder is catheterized we have observed that there is development of biofilm by 72 hours and this biofilm is in close proximity to the bladder mucosa. We hypothesized that this would lead to immune responses in the urinary bladder mucosa.

The thesis consists of three parts:

- **Part I** consists of basic understanding of the system components used in the study
- **Part II** the interaction of PBMC to Foley's latex urinary catheter alone
- **Part III** trigonal interactions between PBMC- catheter- *E.coli* biofilm

These interactions are analysed both microscopically and at molecular level by delineating the modulation of mRNA expression of both pro- and anti- inflammatory cytokines in the PBMC. The cultured PBMCs were stimulated with both Foley's latex catheter and *E.coli*171 biofilm on these catheters and trigonal interactions between PBMC- catheter- *E.coli* biofilm were studied. These studies were conducted to understand effect on the morphology, PBMC proliferation and cytokine gene expression modulation in PBMC.

3.1 Part I

E.coli strain for this study was selected from the library of pathogens in the Division of Microbiology. This library of strains consisted of isolates from Foley's catheters retrieved from patients. *E.coli* 171 strain was selected based on Congo Red and *Curli* expression agar analysis for biofilm formation. *E.coli* strain 171 was used to form biofilm on the surface of Foley's latex urinary catheter. Morphological analysis of these biofilms were done by Scanning electron microscopy (fig: 3.1A) and fluorescence

microscopy (fig: 3.1B) using acridine-orange staining and Confocal microscopy (fig: 3.1 C) using Propedium Iodide staining.

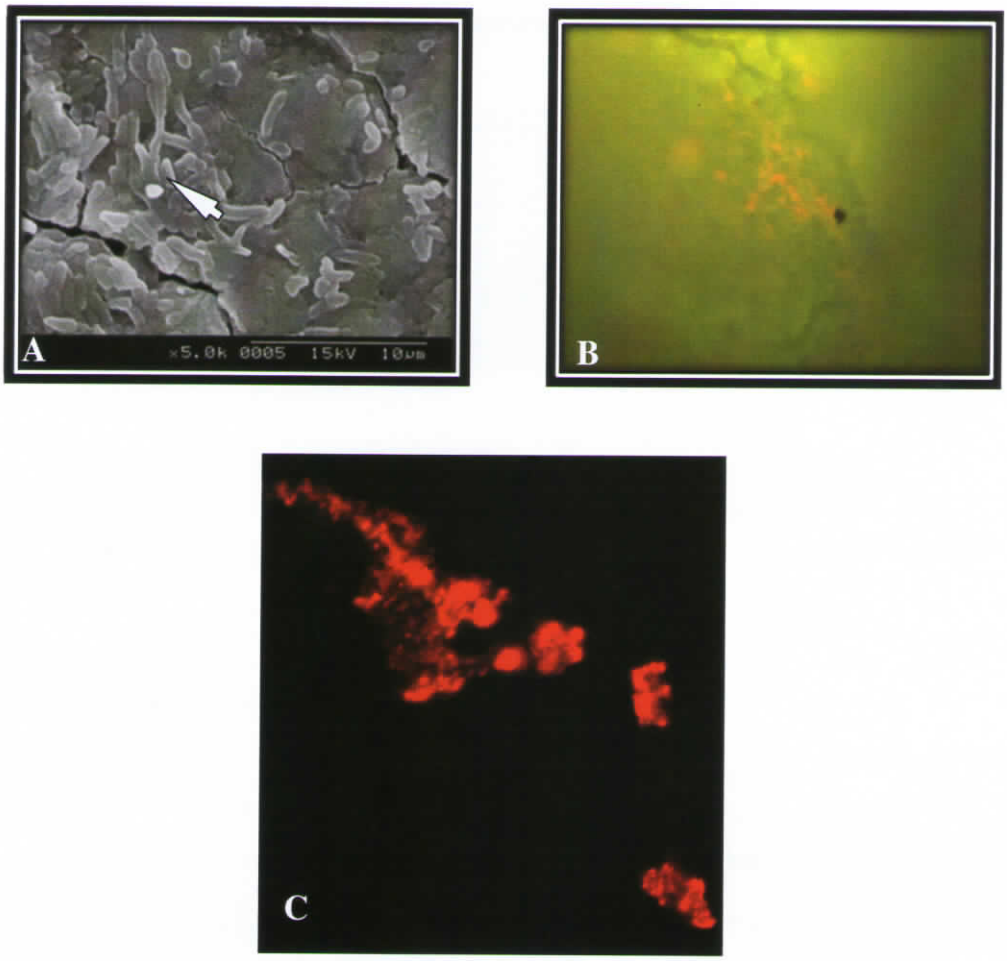


Fig: 3.1 Formation of *E.coli* 171 biofilm on the surface of urinary catheter. (A) SEM micrograph of ( ) *E.coli* embedded in extracellular polysaccharide slime forming a thin film called biofilm on latex Foley's catheters. (B) Fluorescence micrograph of *E.coli* biofilm. *E.coli* is stained with acridiene orange which shows *E.coli* as orange- red and is viewed using 50X oil immersion objective. (C) Confocal microscopic examination of biofilm of *E.coli* 171 on urinary catheter (PI staining) using 40X objective.

The PBMCs were isolated from whole blood of healthy individuals using Ficoll density gradient centrifugation. They were cultured in standard 24 well tissue culture polystyrene plates at a density of $2x10^6$cells/mL (fig: 3.2). Phase contrast microscopy was used to study the morphology of PBMC in culture.

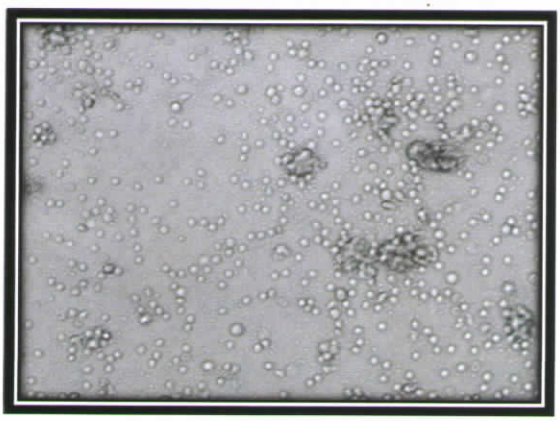


Fig: 3.2 Phase contrast microscopy of PBMCs cultured on tissue culture plate (20X) immediately after seeding.

3.1.1 Cytokine gene expression in unstimulated PBMCs

Since most cytokine genes are expressed transiently and at low levels, qrt-PCR was used, which provides the most sensitive method for quantification of mRNA. A time course experiment was carried out to define and compare the kinetics of IL-1β, IL-8, IFN-γ, IL-12, IL-6, MCP-I, TNF-α, IL-4 and IL-10 gene expression in PBMCs following an *in vitro* stimulation by catheter and *E.coli* biofilm respectively. Semiquantitative RT-PCR analysis was used to determine the level of each cytokine mRNA at each time point for both stimulus. The experimental results from each sample were normalized to the sample's β- actin (a housekeeping gene) expression.

A panel of both pro- and anti-inflammatory cytokines were selected for basal level profiling of PBMCs in culture. The selected cytokine gene expressions in unstimulated PBMCs were analysed using qrt-PCR which gave a clear expression profile for the following genes, at different time points. IL-1β, IL-6, IL-8, IL-12 IFN-γ, MCP 1

and TNF-α were pro-inflammatory cytokines and anti -inflammatory cytokines were IL-4 & IL-10.

TNF-α and IL-4 were not expressed in any of the time points in this study. In Fig (3.3) IL-1β, IL-10, IL-8, IL-12 showed basal level expression at an early time point of two hours in culture. IFN-γ, IL-6 and MCP-1 genes showed no expression at the initial time point of 2h (fig: 3.3 G, C, E). Maximum expression of IL-1β, IL-8, IL-12 and IL-6 occurred at 4h after which down regulation of some of these genes was initiated (fig:3.3 A,B,D,C). The expression for rest of the genes, IFN-γ, MCP-1 and IL-10 in 4^{th} h was quite low. As we move on to the 6^{th} h, slight down regulation was seen in the expression of all the genes. In the 8^{th} h MCP-1 and IL-10 expression was maximum (fig: 3.3 E, F) while the expression pattern for all the genes except for INF-γ showed slight modulations. Fig (3.3 G) shows that at 8^{th} h INF-γ is not amplified but by the 10^{th} h IFN-γ reaches its maximum expression, probably it is delayed expression. The slight increase in the expression of IL-8 seen in (fig: 3.3 B), may be an experimental error, whereas all the other genes showed a slight decrease in the expression. As we reached the 12^{th} h all genes began to get down regulated. These observations indicate that IL-8 was maximally expressed cytokine in unstimulated PBMCS where as IL-12 was expressed minimally.

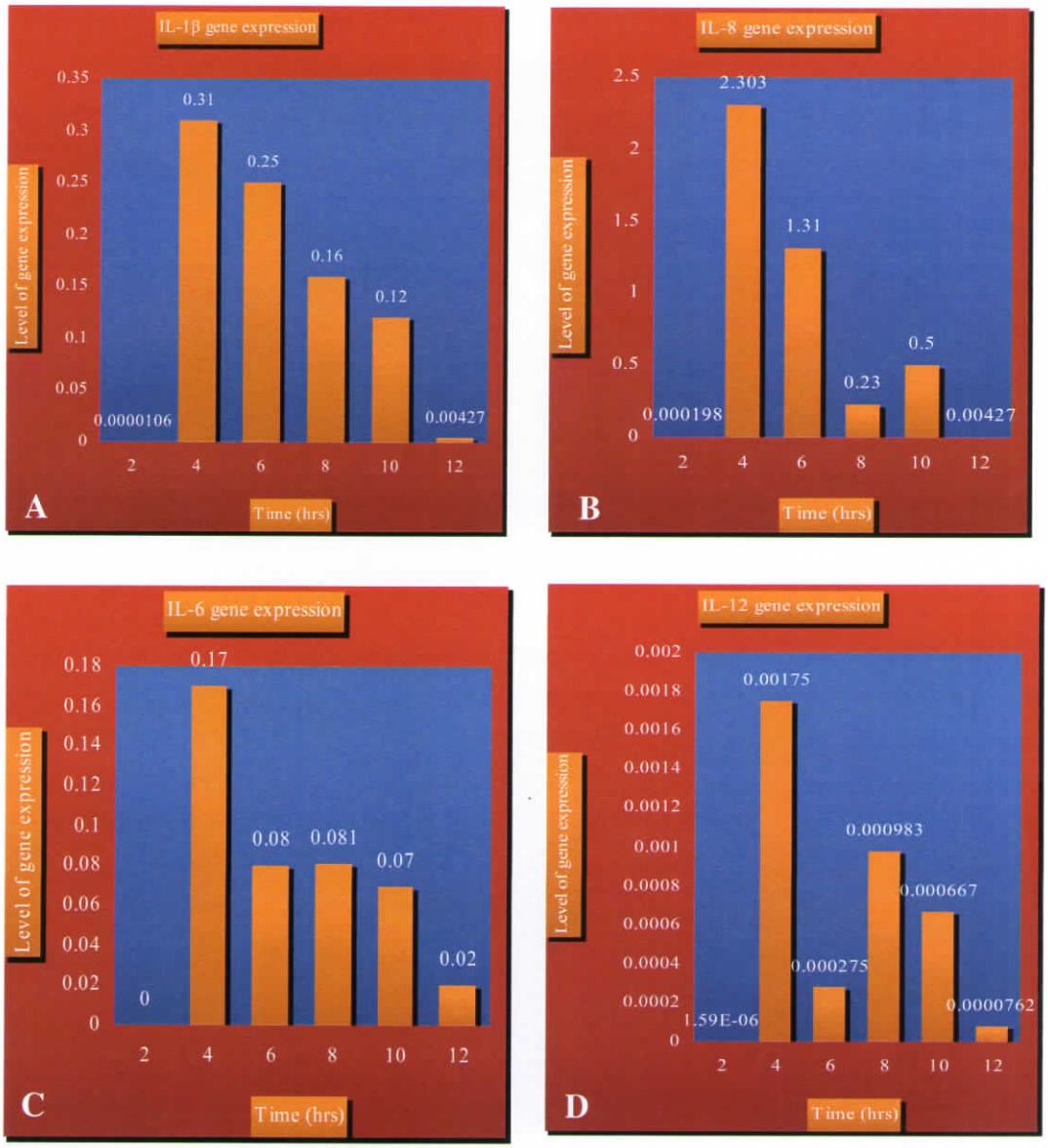


Fig:3.3 Cytokine gene expression in unstimulated PBMCs.
(A) IL-1β, (B) IL-8, (C) IL-6, (D) IL-12

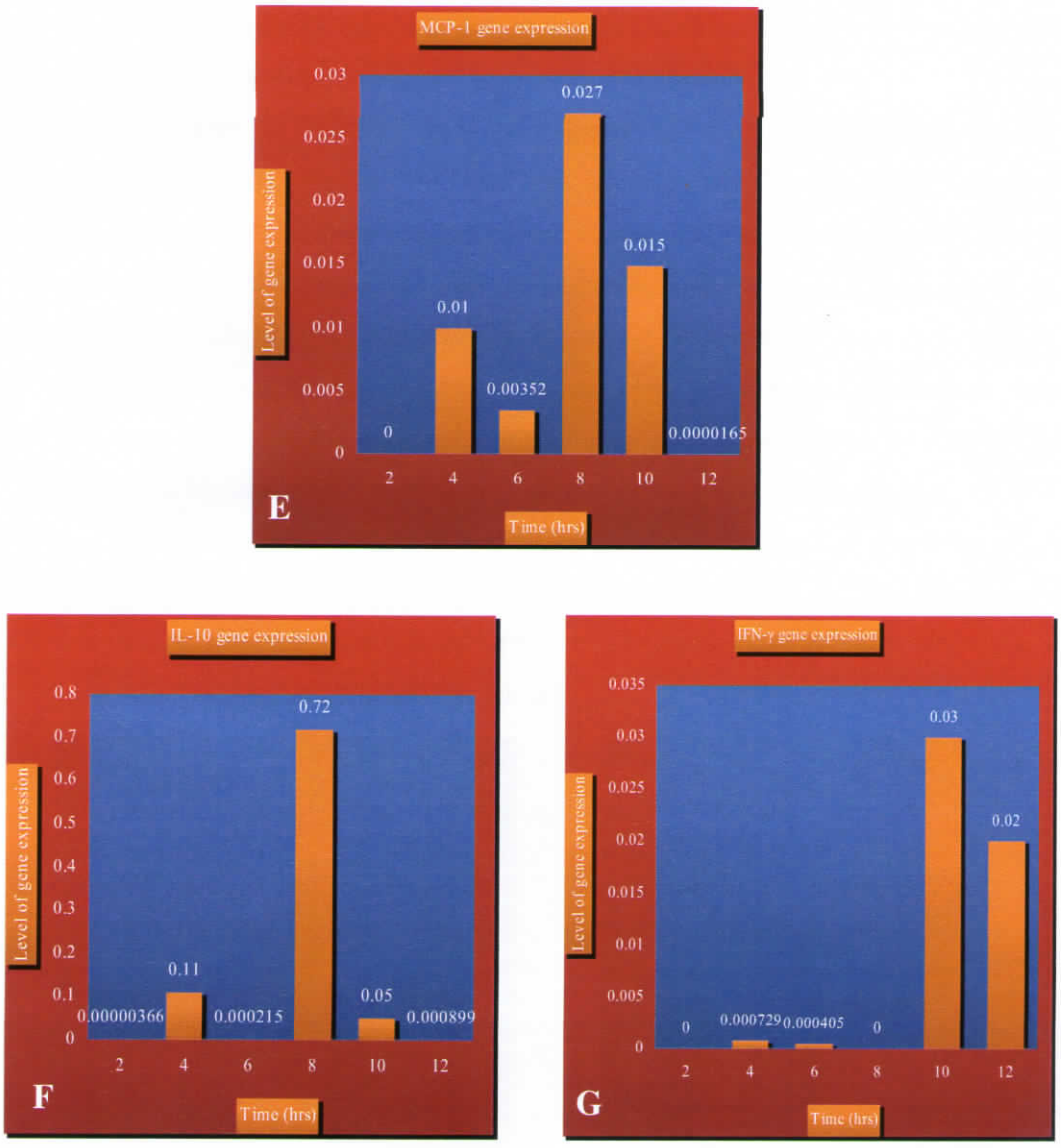


Fig 3.3: Cytokine gene expression in unstimulated PBMCs
(E) MCP-1, (F) IL-10, (G) IFN-γ

3.2 Part II

3.2.1 Morphological analysis of the interaction between PBMC cultures and Foley's latex urinary catheter

Initially on catheterization the catheter and the inflated balloon are in close proximity to the bladder mucosa and interact with it. In some cases during catheterization, the urinary catheter causes disturbances to the lining of the uroepithelial cells which leads to localized tissue inflammation and injury. As a result, immune cells come to the site of injury and produce an immune response. Our hypothesis is that since catheter is likely to be exposed to host immune cells, PBMCs may attach to the surface of urinary catheter. To understand this interaction catheter pieces were exposed to PBMC culture for 24 hours and analysed by Scanning electron microscopy (SEM).

When PBMCs come into contact with catheter surface they begin the process of adhesion and attachment which is seen in the SEM (Fig 3.4A). In SEM fig (3.4B) which is at a higher magnification, the intricate web- like net work of pseudopodia is seen which promotes adhesion onto the catheter surface.

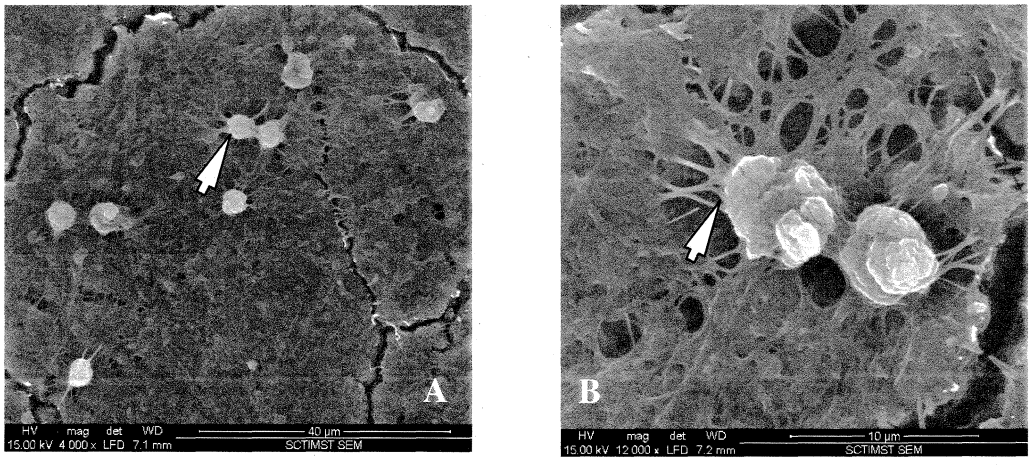


Fig:3.4 PBMC-catheter interaction showing SEM picture.(A) SEM micrograph of the process of PBMC attaching to the surface of urinary catheter.(B) Higher magnification showing web-like net work of pseudopodia.

3.2.2 Delineating cytokine gene expression in PBMCs stimulated with catheter

qrt-PCR analysis showed that TNF-α and IL-4 were not expressed at any point in the course of the study when PBMCs were stimulated by latex urinary catheters. The expression of all cytokine genes at 2nd h was quite low, although it was slightly higher than the unstimulated PBMCs (fig: 3.5). All the genes show slight increase in the expression by the 4th h with IL-8, IL-6, IL-10 showing maximum expression (fig: 3.5 A, B &C). By 6th h the gene expression profile for all the genes showed a down regulation. There was upregulation of all the genes by 8th h. IL-1β, IL-12 and MCP-1 (fig:3.5 D,E&F) have maximum expression by 8th hour. By 10th h and 12th hour the expression profile for all the genes showed a down regulation except for INF- γ. Delayed expression of IFN-γ maximized by 12th h which was similar to the observation for unstimulated PBMC (fig:3.5G). An overview of the whole expression profile gave us an idea that IL-8 was the maximally expressed cytokine in catheter stimulated PBMCs where as IL-12 was expressed minimally. This observation is similar to that of the unstimulated PBMCs but the level of expression was varied.

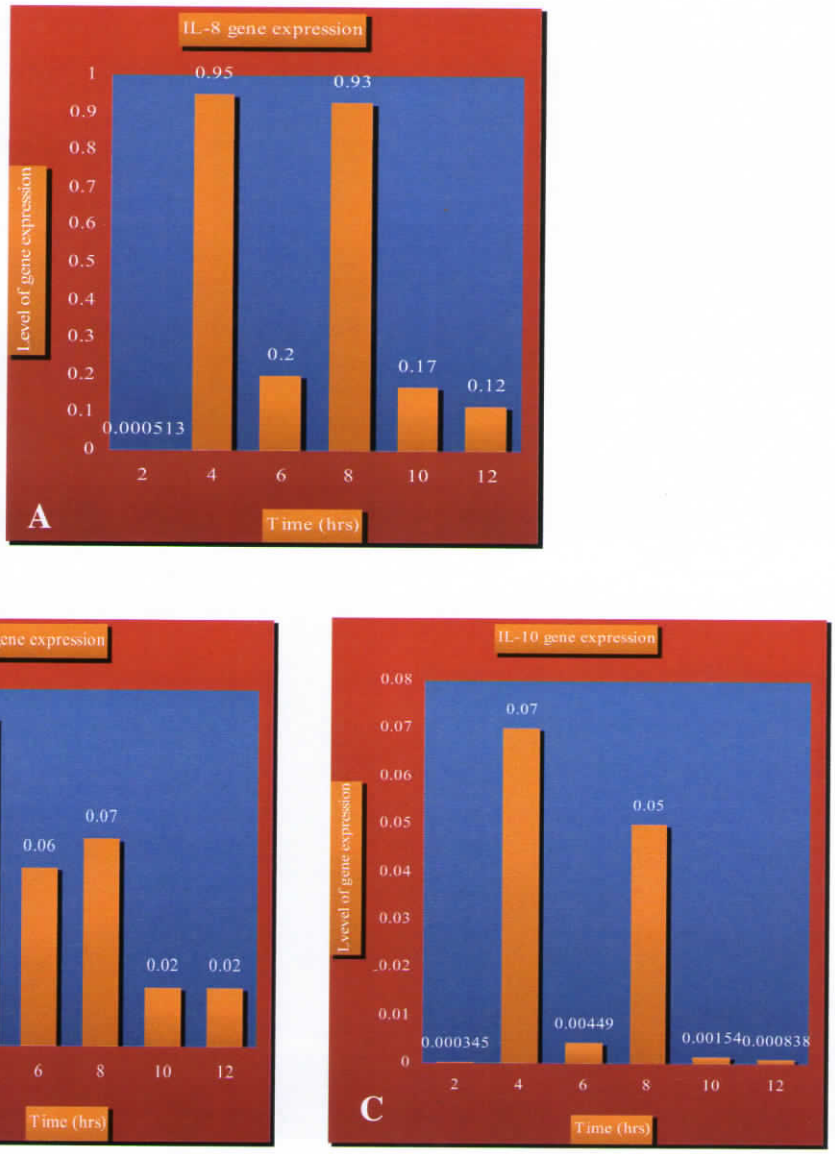


Fig: 3.5 Cytokine gene expression in catheter stimulated PBMCs.
(A) IL-8, (B) IL-6, (C) IL-10

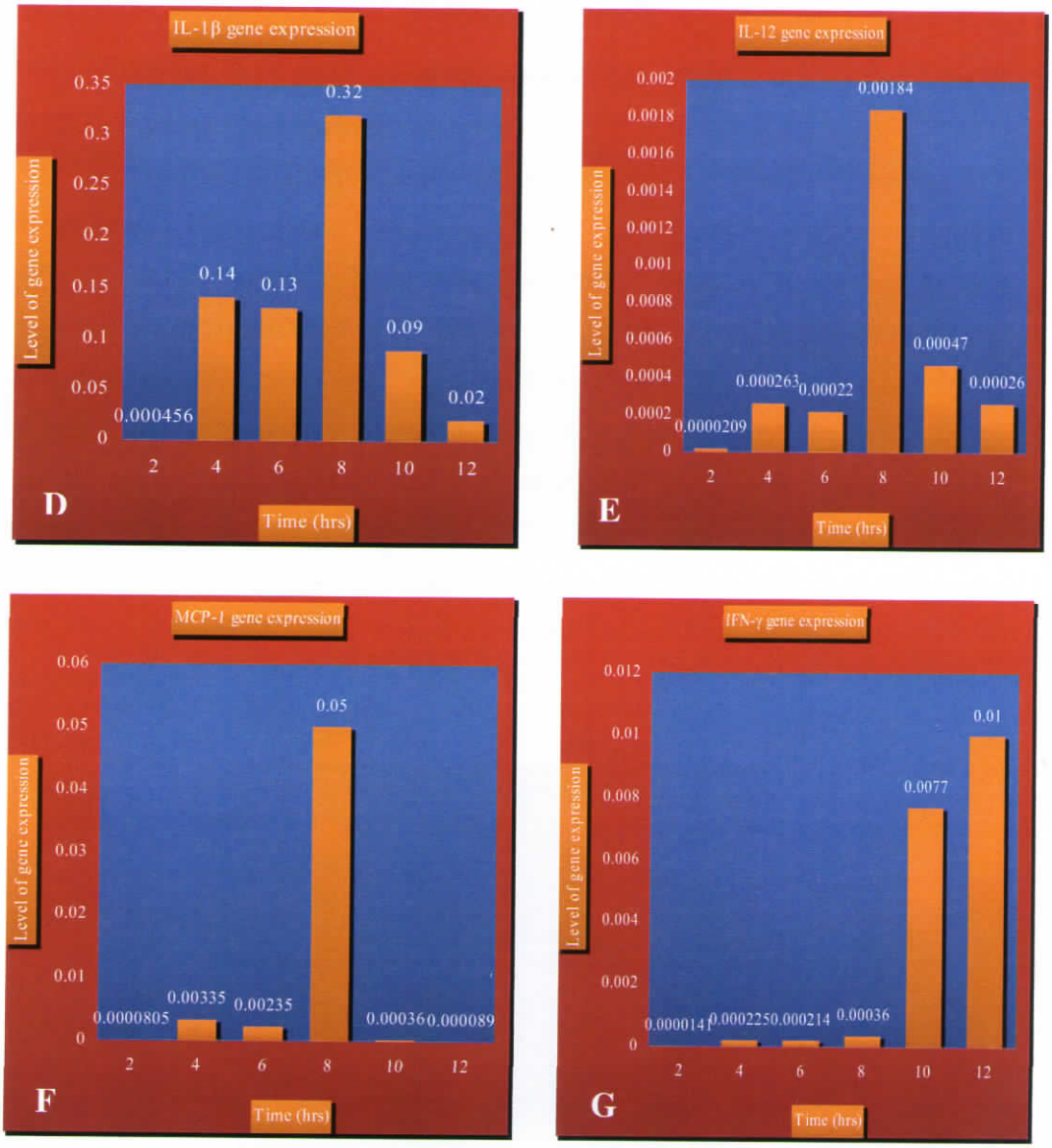


Fig: 3.5 Cytokine gene expression in catheter stimulated PBMCs.
(D) IL- 1β, (E) IL-12, (F) MCP-1, (G) IFN-γ

3.3 Part III

3.3.1 Morphological analysis of the interaction between PBMC cultures and *E.coli* 171 biofilm on Foley's latex urinary catheter

The interaction of PBMCs on the surface topography of *E.coli* 171 biofilm was investigated using SEM. During catheterization, the *E.coli* biofilm would form on the surface of the urinary catheter. Since these biofilms are likely to be exposed to host immune cells, we hypothesized that the immune cells (PBMCs) will influence the *E.coli* biofilms. Our hypothesis was tested using SEM to visualize the surface topography of both *E.coli* 171 biofilm on exposure to PBMCs, for 24 hours. Fig 3.6 shows that *E.coli* 171 biofilm architecture is maintained in the presence of PBMCs. Moreover, a cluster of bacterial cells were found surrounding the PBMCs. PBMCs appeared rounded and there was no morphological changes signifying their attachment to the biofilm in contrast to Fig (3.4). Very few PBMCs were visible on the surface of *E.coli* biofilm and none of them showed phagocytosis of *E.coli* cells (fig: 3.6).

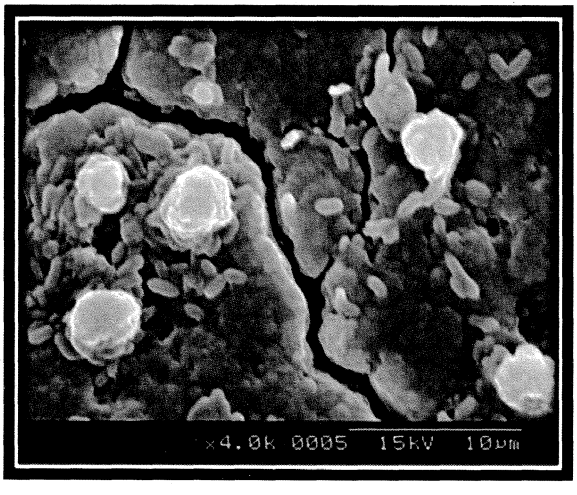


Fig:3.6. SEM micorgraph of PBMC interaction with *E.coli* biofilm. The interaction shows *E.coli* bacteria moving from biofilm congregating around the PBMCs and the typical PBMC attachment process is absent with absence of pseudopodia.

3.3.2 Modulation of cytokine gene expression in PBMCs stimulated with *E.coli* 171 biofilm on Foley's latex urinary catheters.

Here also TNF-α and IL-4 were not expressed in any time points. The expression of all cytokine genes was very low at the initial time point of 2h with IFN-γ not showing any amplification (fig: 3.7 G) and MCP-1 showing maximum expression (fig:3.7 F). All the genes showed an increase in the expression by the 4^{th} h with IL-1β, IL-8, IL-6, IL-12 and IL-10 showing maximum expression (fig:3.7A,B,C,D,&E). At 6^{th} h all the genes showed a down regulation but as seen in fig: (3.7-D), IL-12 was not amplified, which may be an experimental error and need further clarification. By 8^{th} h there was a slight decrease in the expression for some genes but IL-10 showed a slight increase (fig: 3.7 – E). MCP-1 was not amplified at 8^{th} h (fig: 3. 7F), which indicates an experimental error requiring further analysis. At 10^{th} h all genes are getting down regulated except for IFN-γ, which instead shows its maximum expression (delayed expression) (fig: 3.7G). By 12^{th} h time point all the cytokine genes were highly down regulated. An overall analysis showed that in PBMCs stimulated with biofilm, IL-8 was the maximally expressed cytokine and MCP-1 was the least expressed cytokine. These observations are similar to that of unstimulated PBMCs and catheter stimulated PBMCs but the level of expression was varied.

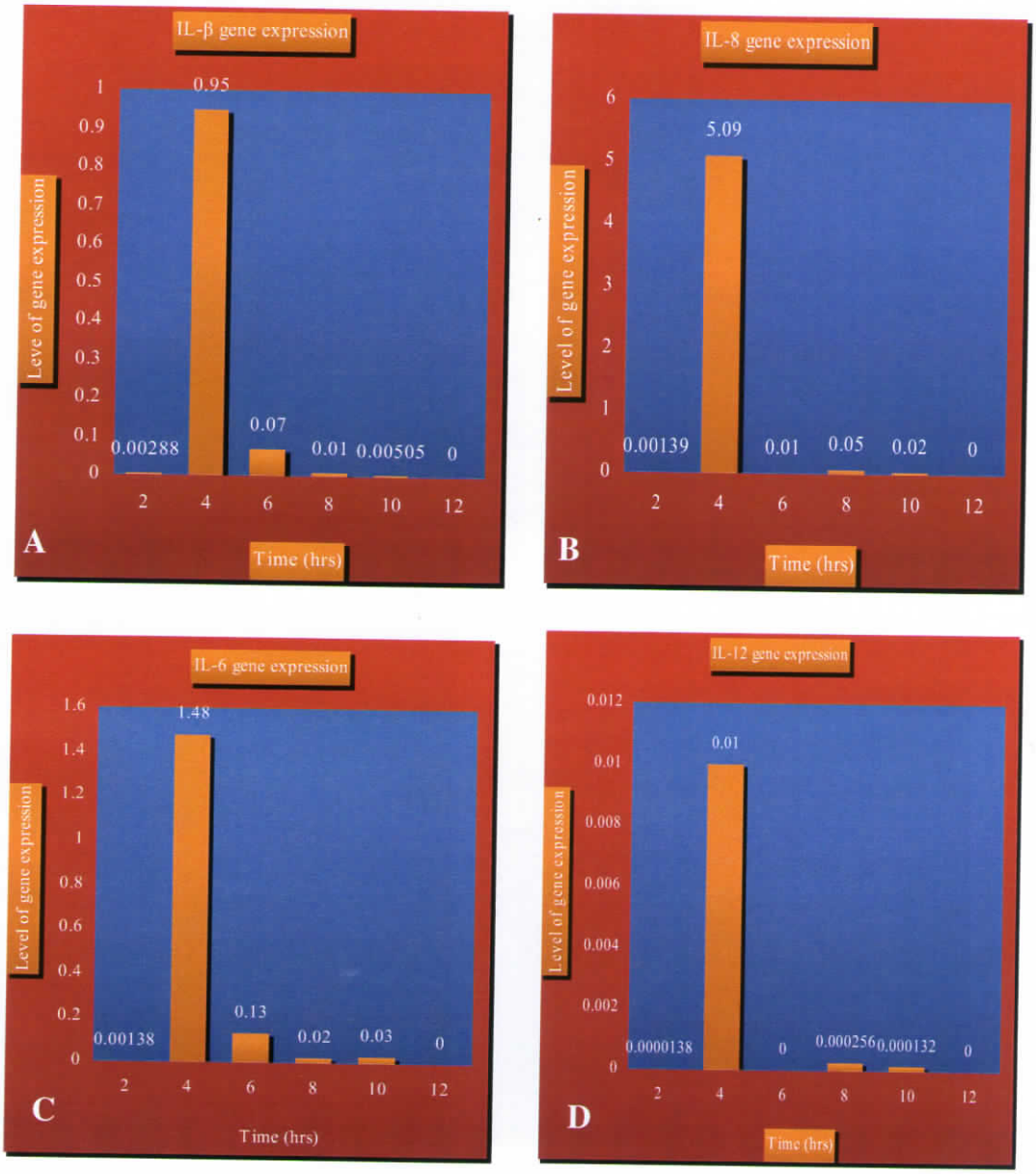


Fig: 3.7 Cytokine gene expression in PBMCs stimulated with *E.coli* 171 biofilm.
(A) IL-1β, (B) IL-8, (C) IL-6, (D) IL-12

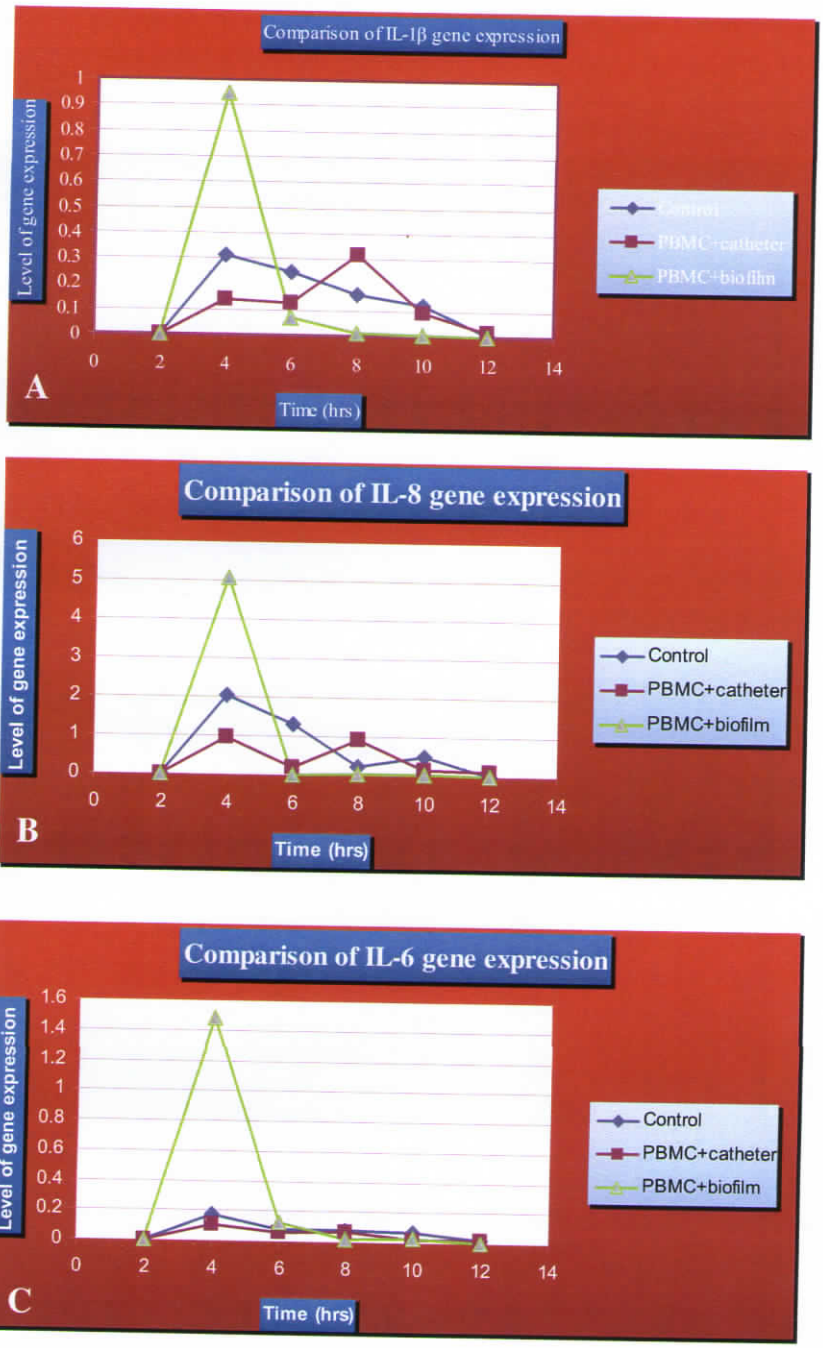


Fig: 3.8 Comparison of cytokine gene expression in unstimulated PBMCs, PBMC stimulated with catheter and PBMC stimulated with biofilm.

(A) IL-1β, (B) IL-8, (C) IL-6

3.4 Comparison of cytokine gene expression in unstimulated PBMCs, PBMC stimulated with catheter and PBMC stimulated with biofilm

In the above results we have discussed about three different conditions of experiment, i.e. unstimulated PBMCs which was the control, PBMCs stimulated with catheter and PBMCs stimulated with biofilm. Each and every gene that we analyzed showed variation in expression at different time points in these three conditions. A comparative analysis of each gene in all three different conditions was done to understand the role of immunomodulation in PBMC and on exposure to bacterial biofilm. Fig (3.8A) shows a scatter plot showing the comparative analysis of IL-1β expression in 3 different conditions. Biofilm stimulated PBMCs showed peak expression which was three times that in control and catheter stimulated PBMC by 4^{th} h, while control and catheter alone showed only minimal expression.

IL-8 gene expression (fig: 3.8B) also showed maximal expression by 4^{th} h in biofilm stimulated PBMC while catheter alone stimulated expression of IL-8 only marginally in comparison to unstimulated PBMC.

Upon comparison of IL-12 gene expression at the three different conditions (fig: 3.8D), in biofilm stimulated PBMCs the expression level was maximum than both control and catheter stimulated PBMCs by 4^{th} h. IL-6 also was expressed maximally on stimulation with biofilm by 4^{th} h (fig: 3.8 C).

MCP-1 was marginally expressed on biofilm stimulation while maximal expression was seen on catheter stimulated PBMCs (fig: 3.8F). IL-10 having an anti-inflammatory role peaked only in control by 8^{th} h (fig 3.8E). IFN-γ is over expressed both under unstimulated and biofilm stimulated condition (fig: 3.8G). The over expression at unstimulated condition may be an experimental error.

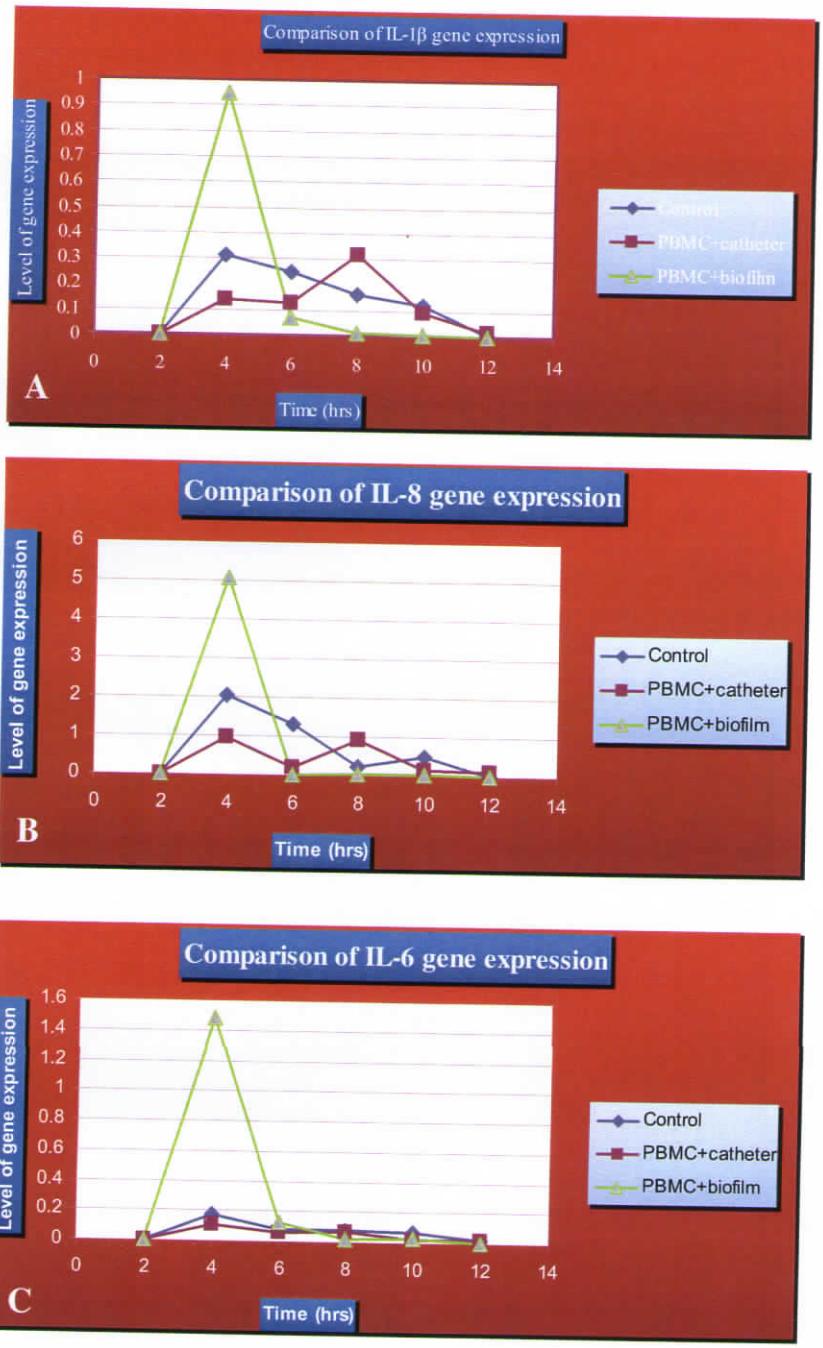


Fig: 3.8 Comparison of cytokine gene expression in unstimulated PBMCs, PBMC stimulated with catheter and PBMC stimulated with biofilm.

(A) IL-1β, (B) IL-8, (C) IL-6

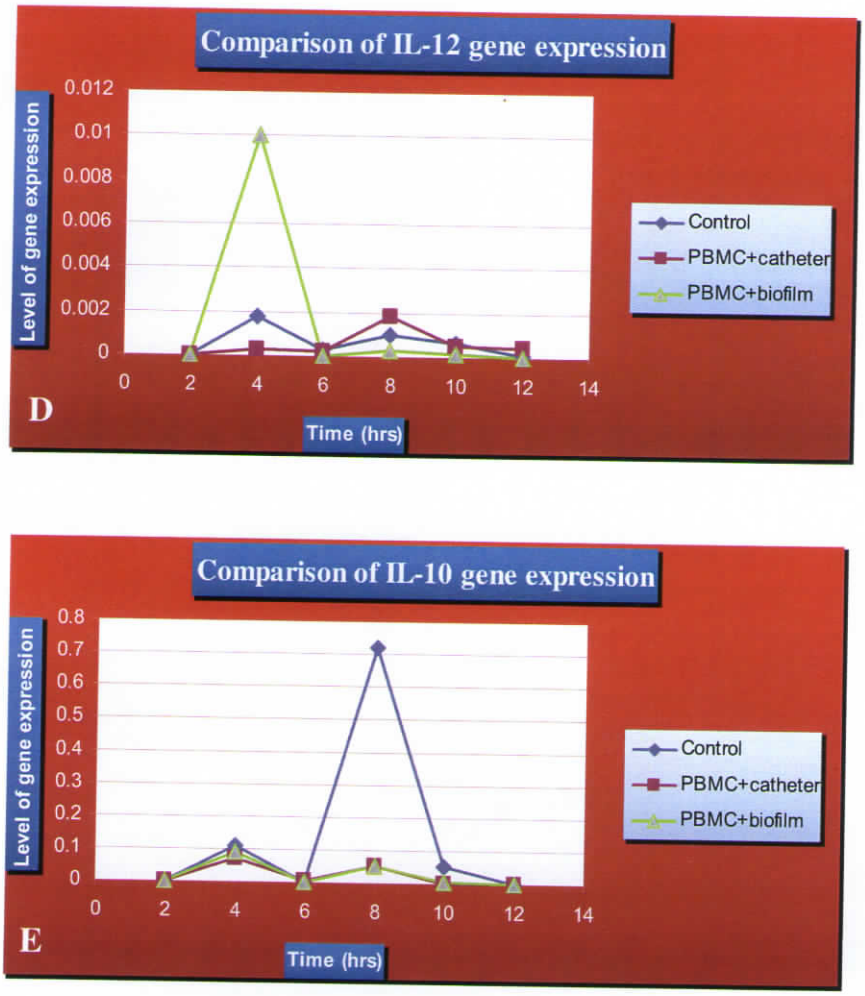
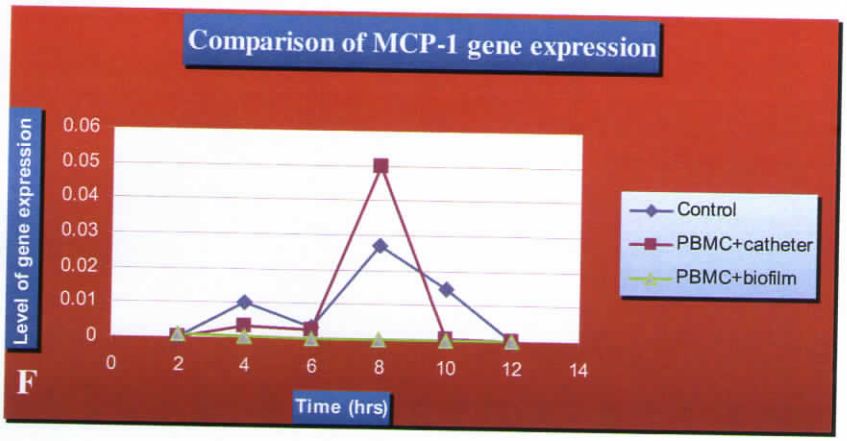


Fig: 3.8 Comparison of cytokine gene expression in unstimulated PBMCs, PBMC stimulated with catheter and PBMC stimulated with biofilm

(D) IL-12, (E) IL-10



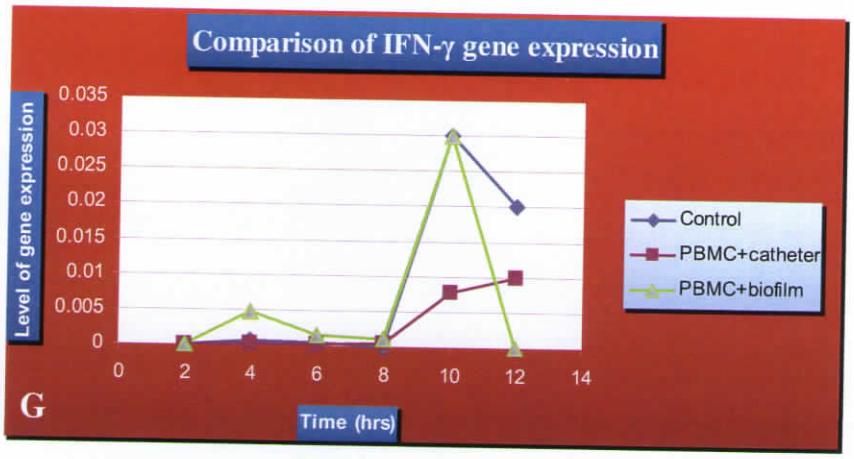
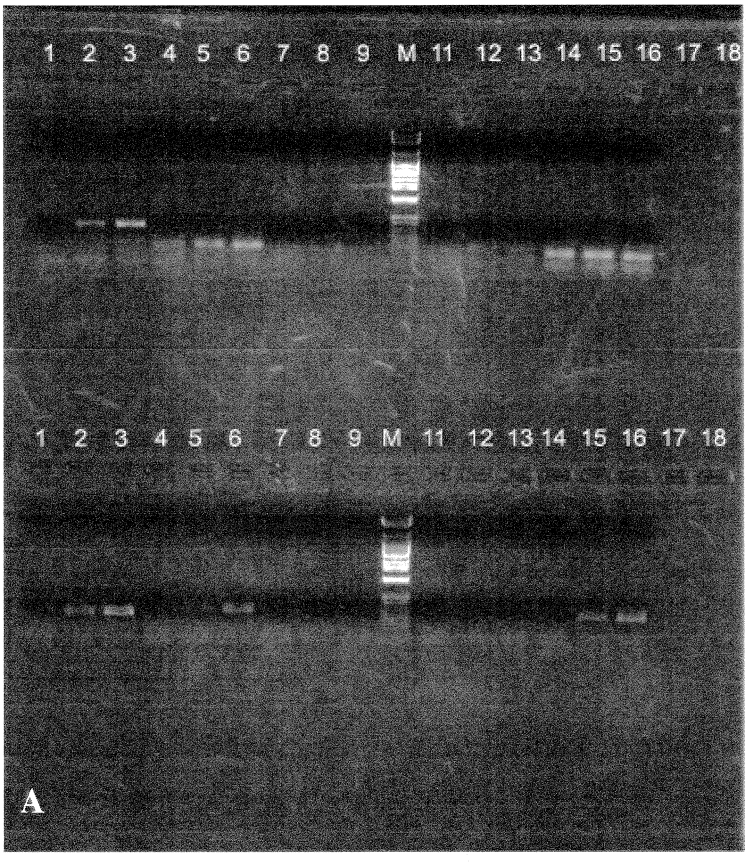


Fig: 3.8 Comparison of cytokine gene expression in unstimulated PBMCs, PBMC stimulated with catheter and PBMC stimulated with biofilm

(F) MCP-1, (G) IFN-γ

3.5 Agarose gel electrophoresis

Confirmation of PCR products were done by agarose gel electrophoresis. The PCR products of each time points were run in 1% agarose gel and it was shown in fig:3.9.



C = Control
C+C= Control+catheter
C+B= Control+biofilm
M = Marker

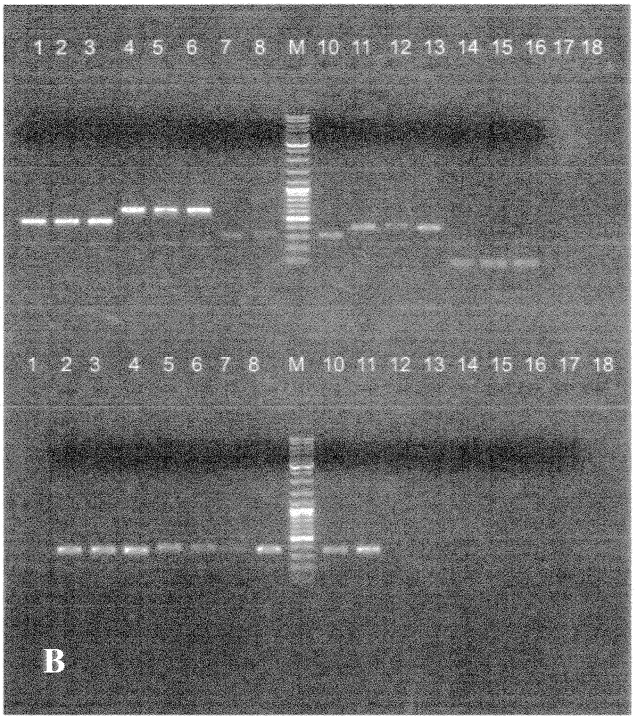
Fig: 3. 9 Agarose gel electrophoresis of PCR amplified product (A) 2hour

First row

1, 2, 3—C, C+C, C+B resp. for IL-1β
4, 5, 6—C, C+C, C+B resp.for IL-8
7,8, 9 – C, C+C, C+B resp. for IFN-γ
11, 12, 13-- C, C+C, C+B resp. for IL-12
14, 15, 16-- C, C+C, C+B resp. for β-actin

Second row

1, 2, 3-- C, C+C, C+B resp. for IL-6
4, 5, 6-- C, C+C, C+B resp. for MCP-1
7, 8, 9-- C, C+C, C+B resp. for TNF-α
11, 12, 13-- C, C+C, C+B resp. for IL-4
14, 15, 16-- C, C+C, C+B resp. for IL-10



C = Control
C+C= Control+catheter
C+B= Control+biofilm
M = Marker

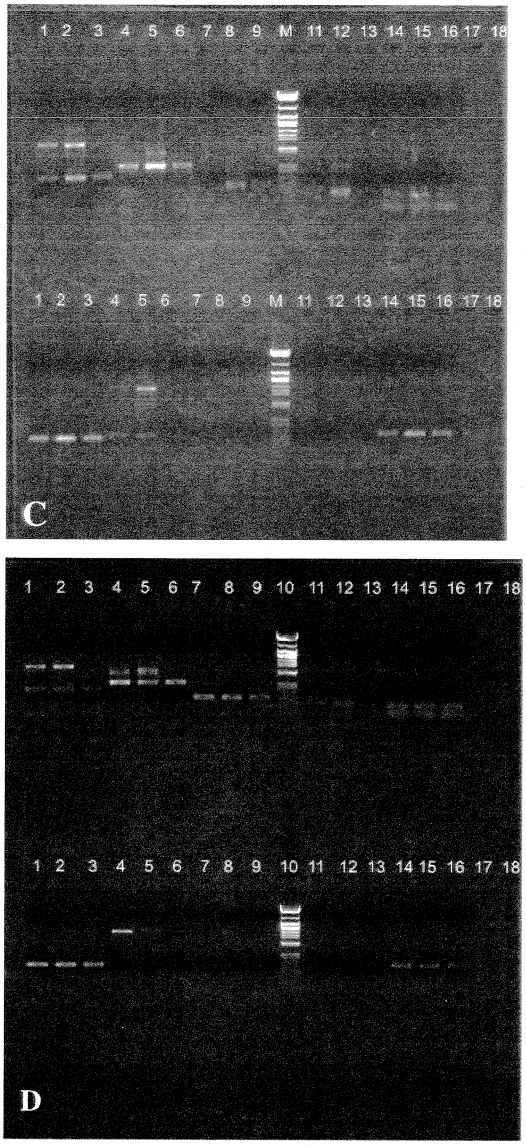
Fig: 3.9 Agarose gel electrophoresis of PCR amplified products (B) 4 hour

First row

1, 2, 3—C, C+C, C+B resp. for IL-1β
4, 5, 6—C, C+C, C+Bresp. for IL-8
7,8, 10 – C, C+C, C+B resp. for IFN-γ
11, 12, 13-- C, C+C, C+B resp. forIL-12
14, 15, 16-- C, C+C, C+B resp. for β-actin

Second row

1, 2, 3-- C, C+C, C+B resp. for IL-6
4, 5, 6-- C, C+C, C+B resp. for MCP-1
7, 8, 10-- C, C+C, C+B resp. for TNF-α
11, 12, 13-- C, C+C, C+B resp. for IL4
14, 15, 16-- C, C+C, C+B resp. for IL-10



C = Control
C+C= Control+catheter
C+B= Control+biofilm
M = Marker

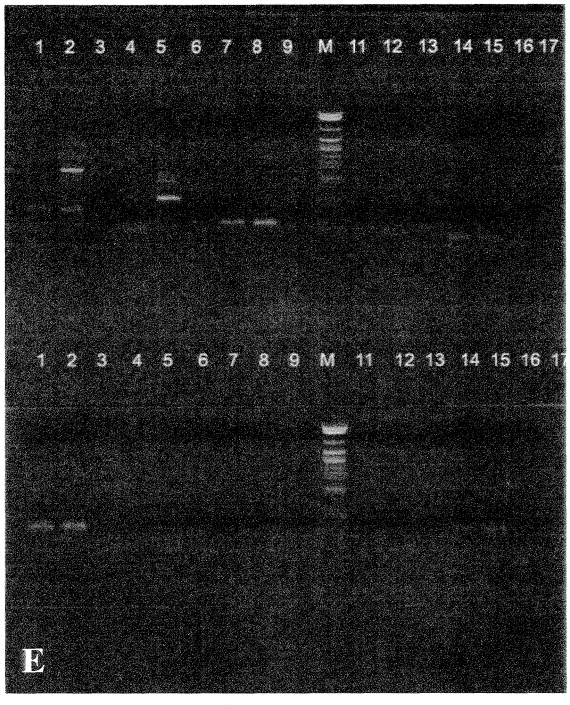
Fig: 3. 9 Agarose gel electrophoresis of PCR amplified products (C) 8 hour, (D) 10 hour

First row

1, 2, 3—C, C+C, C+B resp. for IL-1β 4, 5, 6—C, C+C, C+Bresp. for IL-8
7,8, 9 – C, C+C, C+B resp. for IFN-γ 11, 12, 13-- C, C+C, C+B resp. for IL-12
14, 15, 16-- C, C+C, C+B resp. for β-actin

Second row

1, 2, 3-- C, C+C, C+B resp. for IL-6 4, 5, 6-- C, C+C, C+B resp. for MCP-1
7, 8, 9-- C, C+C, C+B resp. for TNF-α 11, 12, 13-- C, C+C, C+B resp. for IL-4
14, 15, 16-- C, C+C, C+B resp. for IL-10



C = Control
C+C= Control+catheter
C+B= Control+biofilm
M = Marker

Fig: 3. 9 Agarose gel electrophoresis of PCR amplified products (E) 12 hour

First row
1, 2, 3—C, C+C, C+B resp. for IL-1β 4, 5, 6—C, C+C, C+Bresp. for IL-8
7,8, 9 – C, C+C, C+B resp. for IFN-γ 11, 12, 13-- C, C+C, C+B resp. for IL-12
14, 15, 16-- C, C+C, C+B resp. for β-actin

Second row
1, 2, 3-- C, C+C, C+B resp. for IL-6 4, 5, 6-- C, C+C, C+B resp. for MCP-1
7, 8, 9-- C, C+C, C+B resp. for TNF-α 11, 12, 13-- C, C+C, C+B resp. for IL-4
14, 15, 16-- C, C+C, C+B resp. for IL-10

3.6. [^{3}H] Thymidine incorporation assay in PBMCs on unstimulated and stimulated conditions.

The proliferation of immune cells is an important aspect of the cellular immune response. Cytokines play an important role in immune cell proliferation. To understand this effect a parallel experiment was carried out using unstimulated PBMCs and PBMCs stimulated with biofilm. The rate of [^{3}H] thymidine incorporation in unstimulated PBMC was $4.38x10^2$ cpm by 24 hours and for biofilm stimulated PBMCs was $1.297x10^3$ cpm by 24 hours(fig:3.10). Compared to control, [^{3}H] thymidine incorporation was higher in PBMCs on interaction with biofilm indicating that, biofilm stimulated PBMCs to proliferate probably through the various cytokines synthesized during the stimuli.

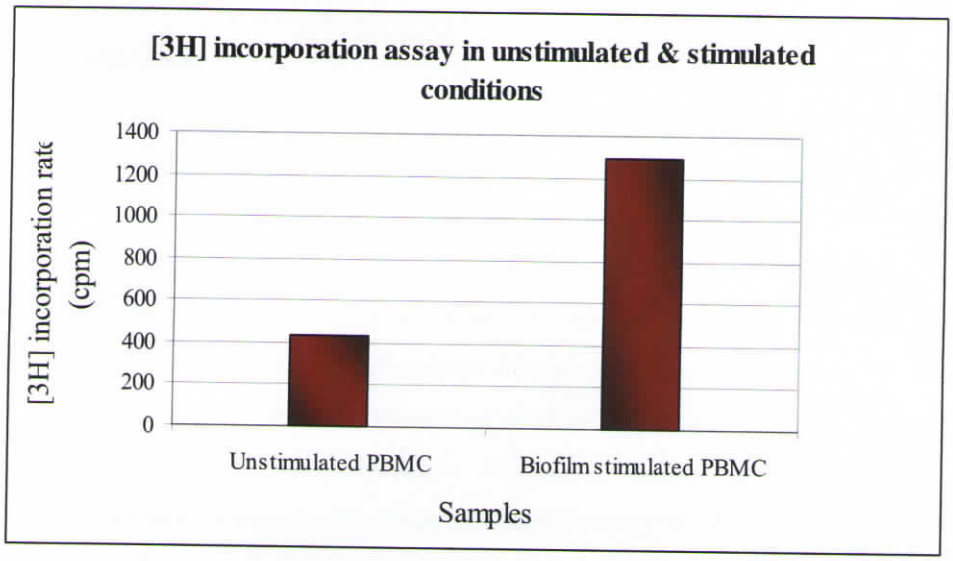


Fig: 3.10 Rate of [^{3}H] incorporation in PBMC in unstimulated and biofilms stimulated conditions

The coordinated production of cytokines following lymphocyte activation controls proliferation, differentiation and functions of cells and is crucial for regulation of the immune response [Fraser *et al.*, 1993]. Many investigators have reported by their northern blot analysis that unstimulated cells do not express cytokine mRNA [Cherwinski *et al.*, 1987; Hayashi *et al.*, 1993; Kro¨nke *et al.*, 1985]. In our present study, with the

help of highly sensitive qrt-PCR method, we observed that unstimulated PBMCs from normal healthy individuals express low levels of all seven of the cytokine mRNAs we had chosen except for TNF-α and IL-4. This decreased production may be due to low constitutive expression of these genes under culture conditions [Fan *et al.*, 1998]

We have examined the kinetics and sequence of seven cytokine genes and two chemokine genes during interaction of PBMC cultures with urinary catheter and uropathogenic *E.coli* 171 biofilm on catheter. mRNA of different cytokines/ chemokines are modulated differently when different stimuli are used (fig: 3.8). The expression level of IL-10, which is an anti-inflammatory cytokine, was maximum in control (unstimulated cells) (fig: 3.8G). This is expected as this cytokine can down regulate the expression of other proinflammatory cytokines under normal conditions. The low level expression of IL- 10 in biofilm stimulated condition may be the reason for maximal expression of other proinflammatory cytokines. MCP-1, which mediates the recruitment of monocytes [Strieter *et al.*, 1996] was maximally expressed in catheter stimulated PBMCs (fig: 3. 8F) while biofilms did not elicit any response which may point to one of the mechanisms of immune evasion and may result in persistence of biofilms leading to chronic infections. Compared to control and catheter stimulated PBMCs, the expression level of IL-1β, IL-6, IL-8, IL-12 and IFN-γ were maximum in biofilm stimulated PBMCs. All of these cytokines are pro-inflammatory cytokines. They have various roles like, recruitment of neutrophils to site of invasion and antigen phagocytosis by IL -8, IL-1 β is involved in T lymphocyte activation, IL-6 in B cell differentiation, IL-12 induces T-cell proliferation and IFN-γ production. IFN-γ is responsible for pathogen elimination [Kuby., 1997].

The production of pro-inflammatory cytokines from biofilm stimulated PBMCs causes upregulation of inflammatory responses. In CAUTI, these cytokines may be up regulated at early time periods and may cause massive inflammatory responses. Among them, the maximally expressing cytokine IL-8 is responsible for recruiting neutrophils to phagocytose the antigen. Neutrophils, are the most abundant type of white blood cells

and one of the first-responders of inflammatory cells to migrate toward the site of inflammation particularly during bacterial infections [Kuby., 1997]. The predominant cells in pus (in urine) are the neutrophils, which is a main symptom in CAUTI.

IFN-γ gene expression occurred maximally in both biofilm stimulated PBMC and control in a delayed manner by the tenth hour. IL-10 is a Th2 cytokine which down regulate the secretion of IFN-γ that may be a reason for very low and delayed expression of IFN-γ. As IFN-γ is important in pathogen elimination its low production may be one of the reasons for the persistence of biofilm as observed in morphological analysis by SEM.

The gene expression of IL-4 and TNF-α were absent in all the time points in both unstimulated PBMCs and stimulated PBMCs. IL-4 is classically associated with allergic disease, as it is the immunoglobulin E switch factor. Absence of IL-4 expression may be correlated with the non allergic conditions of study participants. TNF-α is an essential molecule for the successful control of infection and development of Th1 dependent response. Activation of macrophages by IFN-γ promotes increased transcription of the TNF-α gene and increases the stability of TNF-α mRNA. Both effects result in increased TNF-α production [Kuby, 1997]. We earlier observed that IFN-γ expression was very low and also delayed which may be a reason for absence of TNF-α gene expression.

Altogether our gene expression studies using qrt-PCR reveals that IL-8 is the major chemokine up regulated in biofilm stimulated PBMC. The main function of IL-8 is the recruitment of neutrophils to the site of inflammation. Biofilms formed on the catheter surface by microorganisms are capable of eliciting the defense mechanism of the body by activating the immune cells like monocytes and macrophages. These cells recognise the TLRs associated with the microbes and releases cytokines like IL-8 which recruits neutrophils to the inflammatory site. These results may be correlated with the inflammatory response like pus in urine which is one of the major symptoms of CAUTI.

Our study revealed that PBMC stimulated with biofilms elicited immune response at early time periods. But at twelve hour time point in biofilm stimulated PBMCs all the cytokine genes were highly down regulated.This effect may be mediated by some of the components present in the EPS of bacteria that may interfere with host immune response. A new view point in the antagonistic activity of bacterial biofilm to immune response is the cell-cell communication or quorum sensing.

CHAPTER 4

SUMMARY AND CONCLUSIONS

Urinary tract is the usual site of nosocomial infections and this is the second most common type of infection in the body. Nosocomial UTI is usually associated with catheterization and is known as CAUTI. CAUTI is one of the leading causes of morbidity and mortality in hospitalized patients. Recurrent CAUTI follows the colonization of bacteria and biofilm formation on the surface of the urinary catheter. These biofilms elicit an immediate immune response which results in many white blood cells (WBCs) or leukocytes and neutrophils being recruited to the site of infection. *Escherichia coli* is the major causative agent.

Our aim is to understand the immune modulations in PBMC by uropathogenic *E.coli* biofilms to know about the immune evasion and persistence leading to chronic CAUTI. Here we use an *in vitro* model comprising of catheter, biofilm and PBMCs. The PBMCs consist mainly of monocytes, T-cells, B-cells, small amounts of NK cells and dendritic cells (both myeloid and plasmacytoid origin). Mononuclear cells (lymphocytes and monocytes) and neutrophils are responsible for killing of the biofilm bacteria. As said earlier when the PBMCs in our system came in contact with the bacterial biofilm, an immune response was initiated. This response was seen mainly mediated by the cytokine activity rather than phagocytosis which is apparent from the SEM figures.

In this study we sought to elucidate the modulation of cytokine gene expression by normal human PBMCs, catheter stimulated PBMCs and PBMCs stimulated with *E.coli* biofilm over a twelve hour time course. We observed that unstimulated PBMCs from normal healthy individuals express low levels of all seven cytokine mRNA except TNF-α and IL-4, which were not expressed through out the study. When PBMCs were stimulated with catheter, an interaction was seen but there was only a slight modulation in the gene expression profile. But when the PBMCs were stimulated with *E.coli* 171 biofilm, a significant change was seen in the gene expression profile. Genes encoding proinflammatory cytokines like IL-1β, IL-6, IL-12 and IFN-γ and chemokines like IL-8

showed maximal expression, which would lead to cellular recruitment and activation. But as time course was increased the immune response seems to be diluted off.

This observation may explain the clinical scenario where it is noted that longer the catheterization of the bladder, higher the chances of the patient getting CAUTI. So with progress of catheterization either a development of resistance by the bacterial biofilm against host immune response or some factors may release from bacteria which help in their survival leading to a chronic infection.

Another aspect that we looked into was the proliferation of PBMCs. To find the influence of *E.coli* biofilm on PBMC proliferation, we conducted a proliferation assay in parallel using unstimulated PBMCs and stimulated with *E.coli* biofilm. Proliferation rate of biofilm stimulated PBMCs was found to be much higher than the unstimulated PBMCs. The mRNA expression of IL-1β, IL-6, IL-8 and IL-12 in biofilm stimulated PBMC may have a co-relation with PBMC proliferation.

In conclusion the morphological analysis of PBMC interaction with catheter and biofilm using SEM revealed the difference in adherence property of these cells. Normal human PBMC mRNA expression analysis using qrt-PCR showed the minimal expression of all cytokine genes except IL-10, which is an anti-inflammatory cytokine. Interaction of catheter with PBMC produced a slight modulation in cytokine gene expression profile except MCP-1, which was expressed maximally. Biofilm stimulated PBMC induced the maximal expression of the pro-inflammatory cytokines and the chemokine IL-8.

E.coli being the main causative agent of CAUTI is a potent inducer of genes encoding pro-inflammatory cytokines and chemokines, involved in the initial host response to infection. The defense mechanisms against *E.coli* involve coordinated response of cytokines. Identification of the unique kinetic profile of cytokine gene

expression in PBMCs in response to *E.coli* biofilm offers a greater understanding of the immune modulation leading immune evasion in CAUTI.

Future prospects

- Understanding bacterial virulence in biofilms could lead to anti-pathogenic drugs as against antibacterial drugs in use today.
- Enhancing innate immunity by making phagocytosis efficient i.e. by developing opsonins which might trigger biofilm phagocytosis.
- Identification of suitable candidate proteins for vaccination therapy or diagnostic to identify biofilm infections.
- Recognition of the signaling molecule involved in quorum sensing and developing antagonists to disrupt biofilms.

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Appendix

1. Congo red agar

Brain heart infusion broth (Himedia)	37g/l
Sucrose (Himedia)	50g/l
Agar (Himedia)	10g/l
Congo red (Himedia)	0.8g/l

Congo red stain was prepared as a concentrated aqueous solution and autoclaved (120°C for 15 min) separately from the other medium constituents and was added when the agar had cooled to 55°C.

2. Curli expression agar

Casaminoacids (Difco)	10g/l
Yeast extract (Himedia)	1.5g/l
$MgSO_4$ (Merck)	0.05g/l
$MnSO_4$ (Merck)	0.005g/l
$MgCl_2$ (Merck)	0.005g/l
Agar (Himedia)	20g/l
Congo red (Himedia)	20mg/l
Commassie Brilliant Blue (BDH)	10mg/l

Congo red stain and Commassie Brilliant Blue was prepared as concentrated aqueous solutions and autoclaved (120°C for 15 min) separately from the other medium constituents and was added when the agar had cooled to 55°C.

3. Acetate buffer

0.2M Acetic acid (Merck)	11.5ml/l
0.2M Sodium acetate	16.4g/l of $C_2H_3O_2Na$

Mix 255mL of 0.2M acetic acid and 245mL of 0.2M sodium acetate. pH has to be adjusted to 4.6 and make upto 1L.

4. Propidium Iodide stain	0.05mg/mL for 30 minutes
5. Luria Bertani Broth(Himedia)	25g/l
6. Tryptone Soya Agar	40g/l
7. Agarose gel	1%
8. Tris Acetate EDTA Buffer	50X
9. Ethidium bromide	0.5µg/ml