EVALUATION OF INTERLEUKIN 1β LEVELS FROM HUMAN LYMPHOCYTES IN RESPONSE TO PYROGENS

A DISSERTATION SUBMITTED

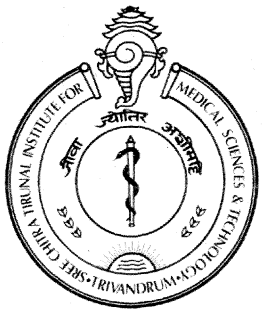
BY

LEKSHMI. N

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF

MASTER OF PHILOSOPHY



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DECLARATION

I, Lekshmi.N, hereby declare that I had personally carried out the work depicted in the dissertation entitled "**Evaluation of Interleukin 1β levels from human lymphocytes in response to pyrogens**" under the direct supervision of **Dr. PV. Mohanan, Scientist In Charge, Division of Toxicology,** Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala, India.

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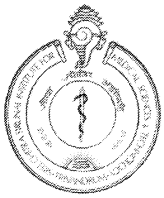

CERTIFICATE

This is to certify that the dissertation entitled "**Evaluation of Interleukin 1β levels from human lymphocytes in response to pyrogens**" submitted by **Lekshmi.N** in partial fulfillment 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 Toxicology, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram-695012.

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Date 31.7.11

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The Dissertation

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Submitted

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LEKSHMI.N

List of abbreviations

BET- Bacterial Endotoxin Test

BMT- Biomedical Technology

BSA - Bovine Serum Albumin

COX-2 - Cyclooxygenase- 2

ELISA - Enzyme Linked Immunosorbent Assay

EU - Endotoxin Unit

G-CSF- Granulocyte Colony Stimulating Factor

GM-CSF - Granulocyte - Monocyte Colony Stimulating Factor

IFN- Interferon

IL-1- Interleukin-1

IL-1β - Interleukin- 1β

IL-6 - Interleukin- 6

IL-8 - Interleukin-8

IL-12 - Interleukin-12

IPT - *In vitro* pyrogen test

IV- Intra venous

LAL- Limulus Amoebocyte Lysate

LPS - Lipopolysaccharide

LTA - Lipoteichoic acid

MIP- 1β - Macrophage Inflammatory Protein- 1β

NK - Natural killer

OVLT- Organum vasculosm of the lamina terminalis

PBS - Phosphate Buffer Saline

$(PG)E_2$ – Prostaglandin E_2

RBC - Red Blood Cell

SCTIMST- Sree Chitra Tirunal Institute for Medical Science and Technology

TLR 4 - Toll like receptor 4

TLR 6 - Toll like receptor 6

TMB - 3,3',5,5'- Tetra methyl benzidine

TNF-α- Tumor necrosis factor- α

WBC - White Blood Cell

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SYNOPSIS

Pyrogenic contaminants in pharmaceuticals or medical devices or parenterally applied drugs pose major threat to human life. Lipopolysaccharide (LPS) - gram negative pyrogens and Lipotechoic acid (LTA) - gram positive pyrogens provokes multiple biological effects by releasing cytokines, the central mediators of febrile response. The major pyrogenic cytokines include Interleukin - 1 (IL-1α & IL-1β), Interleukin - 6 (IL-6), Tumor Necrosis Factor - α (TNF- α) and Interferon-γ (IFN- γ). Cytokines have their major effect on the rich vascular network called the circumventricular organs, close to the cluster of neurons in the anterior hypothalamus. This region is called organum vasculosum laminae terminalis (OVLT). These cytokines mediate the harmful effects of the endotoxins *in vivo*, leading to endotoxemia which can result in septic shock and multiple system organ failure.

IL-1β is a potent pro-inflammatory cytokine secreted by blood lymphocytes, monocytes, tissue macrophages, keratinocytes and other epithelial cells when it comes in contact with exogenous pyrogens like LPS and LTA. This cytokine is a main mediator of the inflammatory process, which is immediately released upon exposure to minute concentration of gram negative and gram positive pyrogens. IL-1β binds to the receptors of OVLT, one of the circumventricular organs of the brain and initiates the expression of the enzyme cyclooxygenase - 2 (COX-2). This converts arachidonic acid to prostaglandin E2, triggering febrile response.

Presently, t*he in vivo* rabbit pyrogen and Limulus Amoebocyte Lysate assays are the two methods used to detect the presence of pyrogenic contaminants, which have distinct advantages and disadvantages. In the traditional rabbit pyrogen assay, the animals are injected with the compounds of interest (Pharmaceuticals, extracts of medical device etc) and monitored the rise in temperature (fever). The assay requires large number of animals and is

qualitative. The LAL assay measures the coagulation of the amoebocytes of the horseshoe crab, Limulus polyphemus, when brought into contact with the cell wall components (LPS) of gram negative bacteria. The assay cannot detect the LPS equivalents of gram positive bacteria or fungi. To cope with this situation, Hartung *et al.*1996 developed an *in vitro* human whole blood assay for detecting pyrogenicity. The assay measures the cytokine production, specifically IL-1β by human monocytes following a challenge with pyrogens. Subsequently, SCTIMST developed an indigenous ELISA method, to evaluate the pyrogenic response to gram-negative & gram-positive bacteria, chemical and biological pyrogens by measuring the IL-1β from human whole blood. In this method, the release of IL-1β, on direct contact of immune cells with the compound of interest can be detected by ELISA.

In the present study, an attempt was made to evaluate the IL-1β as a marker of pyrogenicity, in presence of freshly isolated human lymphocytes using LPS and LTA (by SCTIMST developed ELISA method). The methodology includes the collection of blood from various donors (O +ve, A +ve, B +ve and AB +ve) and isolating the lymphocyte by gradient centrifugation using Hisep 1077 solution. The viability of isolated lymphocytes was assessed using Trypan blue staining (1:1 dilution) and the cell concentration of stock lymphocyte mixture was determined using hemocytometer. Three different lymphocyte concentrations such as 4, 5 and 7 lakhs (individual blood) were used for the *in vitro* pyrogen assay and the cytokine (IL-1β) release was detected by ELISA after challenge with LPS and LTA. In pooled blood, 5 and 7 lakhs of lymphocyte concentration was used to detect the pyrogenicity.

It was observed that the release of IL-1β was at peak on 6^{th} hour, when the concentration of lymphocyte (O +ve donors) was 4 and 5 lakh cells. The extent of peak was directly proportional to the concentration of lymphocyte cells on challenge with 5EU of LPS. Similarly the release of IL-1β on challenge with 1µg/µl of LTA was at the highest level on 6^{th} hour, when the concentration of lymphocytes was 7 lakhs. A +ve donors showed the release

of IL-1β (5EU of LPS) at 5th hour of the reaction when used in lymphocytes at two different concentrations (7 lakh and 5 lakh cells). No such significant response was observed in 4 lakhs of lymphocytes stimulated with 5EU of LPS. Further, the same cells stimulated with 1µg/µl of LTA elicited an IL-1β response at 5th and 4th hour in 5 lakhs and 4 lakhs of cell concentration. The maximum release of IL-1β was obtained with 7 lakhs of lymphocytes at 4th hour of the reaction.

In pooled lymphocytes, (A +ve donors) IL-1β release elicited by 5EU of LPS was found to be maximum at 6th hour of the reaction in 7 lakhs of lymphocyte cells. Similarly, treatment with 1µg/µl of LTA in pooled sample (7lakhs) induced a maximum release of IL-1β (2 ng/µl) at 6th hour of reaction. Treatment with 5EU of LPS induced a release of IL-1β at 6th hour of reaction using lymphocytes from pooled blood of B +ve donors. Also, cells stimulated with1µg/µl of LTA induced a peak at 5th hour of reaction with 7 lakhs of lymphocyte cell concentration.

The pooled lymphocytes from AB +ve donors also showed a maximum release of IL-1β at 6th hour of reaction with 7 lakhs of lymphocytes. It was found that the release of IL-1β was at a high peak at 4 to 6th hour and then stabilizes for both the pyrogens. The results indicated that IL-1β release by 5EU of LPS and 1µg/µl of LTA release is dependent on lymphocytes concentration. It was also observed that the difference in blood group did not interfere with the IL-1β release. Based on the results obtained, it can be concluded that the isolated lymphocytes system can be used as an alternative test system to the *in vivo* rabbit pyrogen assay.

CHAPTER 1

INTRODUCTION

1.1. Background

The measurement of pyrogens is an indispensable safety control for all parenterally administered drugs, as these contaminants are life threatening. Pyrogens are chemically heterogenous group of fever inducing substances derived from gram negative and gram positive bacteria but also from viruses and fungi. They can be endotoxins or lipopolysaccharides (LPS), cell wall components of Gram negative bacteria such as E.coli, Salmonella and Shigella. Similarly lipoteichoic acids (LTA) originated from the cell walls of gram positive bacteria such as *Bacillus subtilis*, *Streptococcus pneumoniae* etc. Even in minute quantity, these substances induce elevated body temperature when injected into humans and animals. They provoke immune response by producing endogenous pyrogens such as prostaglandins and other pro-inflammatory cytokines like interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor-α. Hence, measurement of pyrogenicity is an important quality control criterion for releasing batch of medical products like parenteral drugs, medical devices and implants.

Fever is one of the cardinal symptoms of infectious diseases caused by bacteria, viruses or parasites; however induction of the fever is not constrained by live microorganisms. The discovery of a large number of mammalian receptors termed Toll-like receptors which are expressed on various cells, explains how microbial products of different pathogens can evoke the same biological response - fever. It is a common medical indication characterized by an elevation of temperature above the normal range of 36.5 to 37.5 °C. The trigger of fever results in the release of Prostaglandin E_2,

which in turn acts on the hypothalamus, generating a systemic response back to the rest of the body, causing heat-creating effects to match a new temperature level.

Cytokines are small cell signaling protein molecules secreted by lymphocytes and other cells of the immune system. Cytokines promoting inflammation are termed pro-inflammatory cytokines, whereas cytokines which suppress the activity of pro-inflammatory cytokines are anti-inflammatory cytokines. Interleukin-1β (IL-1β), Interferon-λ, Tumor Necrosis Factor-α (TNF- α) etc are pro-inflammatory, whereas IL-4, IL-6, IL-10 etc are anti-inflammatory cytokines.

Pyrogenic cytokines have their major effect on the rich vascular network called the circumventricular organs, close to the cluster of neurons in the anterior hypothalamus. This region is called organum vasculosum laminae terminalis (OVLT), one of the circumventricular organs of the brain. Pyrogens like Interleukin 1β, binds to the receptors of OVLT and initiates the expression of the enzyme cyclooxygenase-2. This results in the release of arachidonic acid, which is rapidly converted to Prostaglandin E_2 causing fever.

Multiple biological properties are the hallmark of cytokines. Cytokines like IL-1β, IL-6, TNF-α etc are of endogenous type which induces fever. Another category of cytokines are endogenous antipyretics which include α-melanocyte stimulating hormone, IL-10 and glucocorticoids. The antipyretic effects are achieved by an inhibitory influence on the action of endogenous pyrogens, or by the effects on neuronal thermoregulatory circuits that are activated during fever.

Interleukin 1β is one of the main pro-inflammatory cytokine produced by activated macrophages as a pro-protein, which is proteolically processed into its active form by caspase 1. This cytokine is a main mediator of the inflammatory process, which is immediately released upon exposure to minute concentration of pyrogens and can be readily measured by ELISA.

Traditionally, pyrogens have been measured with the *in vivo* rabbit pyrogen assay, in which the animals are administered intravenously with the compound of interest (parenteral drug, device extract etc.) and monitored for the evidence of fever reaction. However, the rabbit pyrogen assay has several limitations, which gives only a pass/fail result, which is not suitable for the detection of endotoxin limit and is not a quantitative assay.

Another assay for detecting bacterial pyrogens is the Limulus Amoebocyte Lysate assay (LAL), which measures the coagulation of the amoebocytes of the horseshoe crab, Limulus polyphemus, when contact with the cell wall components (LPS) of gram negative bacteria with a molecular weight of >8000 Daltons. The assay cannot detect the LPS equivalents of gram positive bacteria or fungi.

An innovative method for the measurement of pyrogenicity is the human whole blood assay, which bridge the gap between rabbit pyrogen and LAL assays. This has been introduced in 1995 by Hartung and Wendel, which measures the pro-inflammatory cytokines like Interleukins or Tumor necrosis factor-α and chemokines. Isolated Human lymphocytes are also used for evaluating the inflammatory response induced by pyrogenic contaminants. The detection of pro-inflammatory cytokines IL-1β using human whole blood and isolated human lymphocytes using ELISA will be more sensitive, accurate and less expensive than the traditionally used rabbit pyrogen assay.

1.2. Review of literature

1.2.1. Pyrogens History

The introduction of intravenous therapy by Sir Christopher Wren, in 1656 created an awareness of fever producing agents [Cooper *et al*, 1971]. The first rabbit pyrogen assay was developed by Hort and Penfeld in 1912 who demonstrated initially that Gram negative bacteria were pyrogenic whereas Gram positive bacteria were not. They deduced that the pyrogenic principle

was probably a heat-stable bacterial substance. The purified endotoxin principle was first introduced with a trichloroacetic acid extraction procedure. The discovery of endotoxin principle thus led to more highly purified preparations from 1952 [Westphal *et al.* 1954]. The significance of fever-inducing substances was recognized during the Second World War in large volume parenterals such as infusion therapeutics. Since then, extensive research have been developed in the area of pyrogenicity.

1.2.2. Pyrogens

Pyrogen is a substance that induces fever and has been classified as exogenous and endogenous in origin. The exogenous pyrogens act by interacting with cytokines, which are known as endogenous pyrogens [Roth & De Souza, 2001].

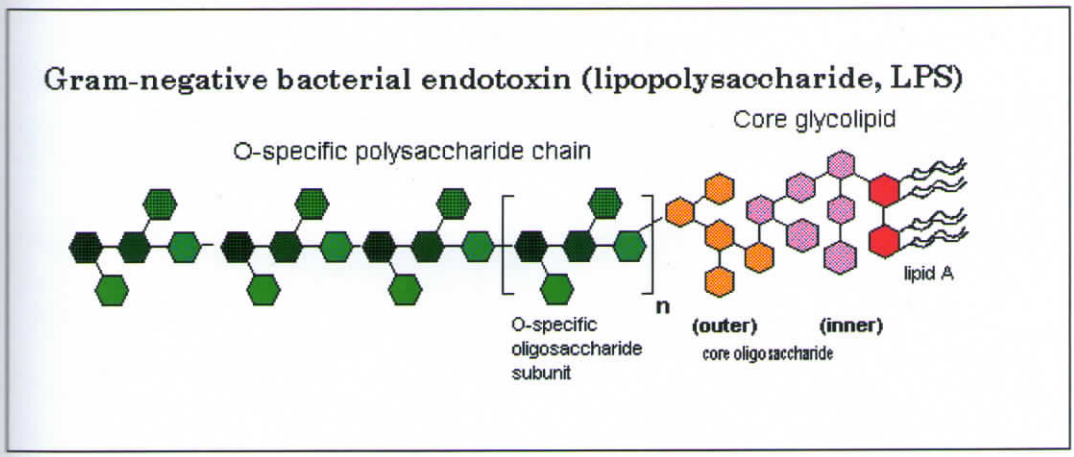
The cells that produce the endogenous pyrogens like Interleukin (IL-1β), Interleukin-6 (IL-6) and Tumor Necrosis Factor-α (TNF-α) are mainly from the monocytes. The endotoxin or lipopolysaccharide entering the blood circulation encounters a receptor termed Toll like receptor- 4 on the OVLT, triggering PGE_2 synthesis. This is the result of the ability of LPS to induce cyclooxygenase-2 [Dinarello, 2004]. PGE_2 in turn stimulates the release of cAMP in the hypothalamus and act as a neurotransmitter by raising the set point of the thermoregulatory neurons.

Exogenous pyrogens include cell wall components of microorganisms like lipopolysaccharide (LPS) from Gram negative bacteria is a major concern to the pharmaceutical industry [Ilaria Bononi, 2008]. Lipoteichoic acid (LTA) is another cell wall component of Gram positive bacteria which has potent pyrogenic effects [Ginsburg, 2002]. LTA and peptidoglycan from Gram-positive bacteria are being increasingly recognized as potent immune stimuli [Morath *et al.* 2001].

1.2.3. Lipopolysaccharides (LPS)

LPS is a major cell wall component of the outer membrane of Gram negative bacteria. The LPS molecule is toxic only when released from the bacterial cell wall. The LPS molecule consists of four different regions.

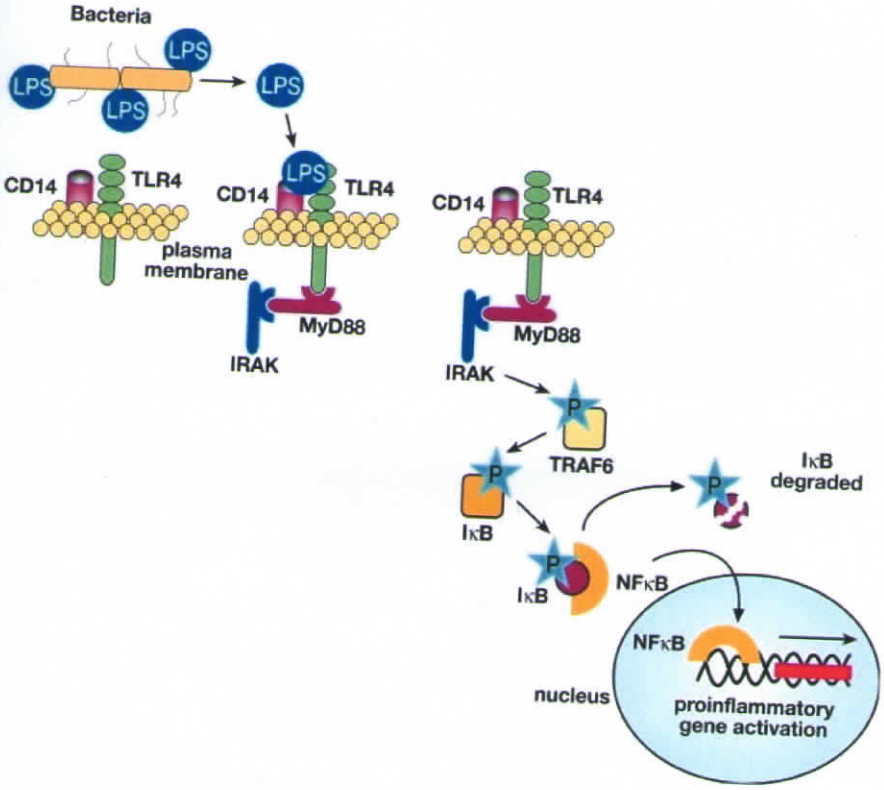
The first and the most important part is lipid A. The toxic moiety - lipid A was activated, when exposed to the immune cells and provokes inflammatory response [Van Amersfoort *et al.* 2003]. Experiments have shown that lipid A of the LPS molecule represents the pyrogenic activity [Tanamoto *et al.* 2001]. The lipid A portion is covalently attached to the core region which is divided into an outer (second region) and an inner core (third region). The inner core contains a high proportion of sugars like 2-keto-3-deoxy-D-octulosonic acid (KDO) and the outer core consists of common sugars which is more variable than the inner core. The fourth region is O-antigen, which is highly immunogenic.



Picture 1.1: Gram negative bacterial endotoxin (LPS) [www.biomin.net]

LPS is a pathogen associated molecular pattern (PAMP) that is recognized by the innate immune system. It stimulates inflammatory responses to clear bacteria that have breached the barrier defenses of the skin or mucosal epithelium. Soluble LPS released by invading bacteria, and particularly its lipid A component, interacts with the opsonic receptor CD14 and the membrane protein TLR 4 to initiate the immune signaling process. TLR 4 belongs to an

evolutionarily conserved family of receptors (TLRs) that can distinguish closely related microbially derived ligands. Although Gram-positive bacteria lack LPS, the TLR 2 receptor can recognize peptidoglycan or lipotechoic acid derived from their cell walls. TLR intracellular signaling is regulated by a group of IL-1 receptor-associated kinases (IRAKs), which bind to the TLR intracellular TIR (Toll/interleukin-1 receptor) domain, a process that requires the presence of adapter proteins (e.g., MyD88). A signaling cascade ultimately leads to the activation of the transcription factor NF-κB and its translocation to the nucleus, where it positively regulates the promoters for genes encoding several pro-inflammatory cytokines.

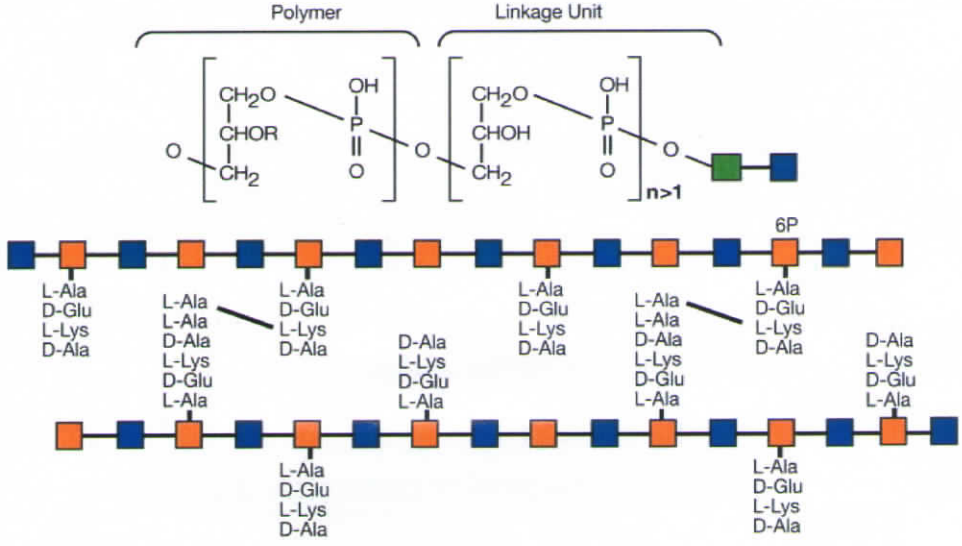


Picture1.2: Activation of immune signaling by lipopolysaccharide [www.ncbi.nlm.nih.gov]

Van Amersfoort et al reported that the immunological response to Gram negative bacteria mainly involves leukocytes and the production of cytokines like IL- β, IL-6 and TNF-α [Van Amersfoort *et al.* 2003].

1.2.4. Lipotechoic acids (LTA)

LTA can be considered as the gram negative counterpart of LPS. It contains a diacylglycerol lipid moiety instead of a phospholipid like structure as well as highly charged glycerophosphate repeating units, in contrast to the oligosaccharide repeating units in LPS. LTA molecules are inserted into the single cell membrane of gram positive cell wall. The structure of LTA from *Staphylococcus aureus* consists of a phosphoglycerol backbone esterified with D-alanine and D-N-acetylglucosamine. The hydrolysis of the D-alanine substituents revealed a crucial role of those for the biological activity of LTA from *S.aureus* [Morath *et al.* 2001].



Picture 1.3: Structure of *Streptococcus pyogenes* peptidoglycan with teichoic acid. [www.ncbi.nlm.nih.gov]

The pyrogen LTA binds to TLR 2 and causes similar cascade reactions (fever) as that in the case of LPS [Van Amersfoort *et al.* 2003].

1.2.5. Viral pyrogens

Viruses like Influenza virus, myxovirus etc play a major role in inducing febrile response. Haemagglutinin and neuraminidase, the surface glycoproteins of

influenza virus are the factors responsible for inducing pyrogenicity in humans [Kenneth *et al.* 1991]

1.2.6. Fungal pyrogens

The mechanism of fever in fungus infections was studied systematically by examining the pathogenic fungi for their capacity to induce fever in rabbits [Braude *et al.* 1960].

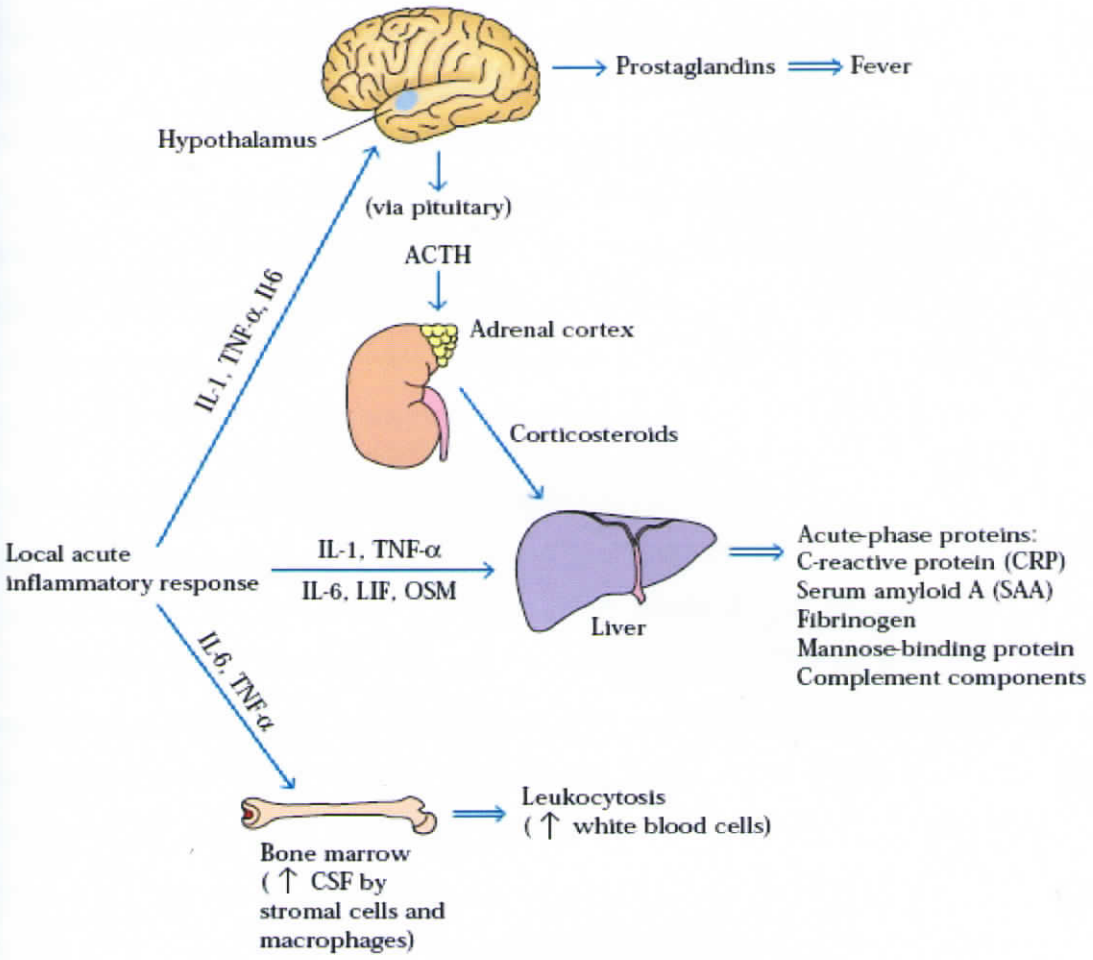
1.2.7. Chemical pyrogens

The oil burn residues remaining after combustion pose an important environmental hazard because of increased levels of pyrogenic polycyclic aromatic hydrocarbons [Garrett *et al.* 2000].

1.2.8. Fever

Fever or pyrexia is defined as an elevation of core body temperature above the normal range of 36.5 to 37.5 °C. Fever is hardly an isolated event (Dinarello, 2004). The increase in body temperature has several advantages including the bactericidal activities of neutrophils and the inhibition of bacterial growth at an elevated temperature. When foreign substances enter in to the circulation, leukocytes are stimulated to release endogenous mediators by which the signals are transmitted to increase the thermostatic set point at the level of preoptic area of the hypothalamus.

The basic event in raising core temperature is to increase the thermostat in the hypothalamus to a higher level. This is done by stimulating specific neural networks through the different neurotransmitters which are released in response to pyrogens. Endogenous pyrogens like IL-1β, TNF-α etc., are produced upon stimulation with the bacterial cell wall components. Gaining access to the circulation, the microbial products reach the OVLT of the hypothalamus. The endothelium of the OVLT expresses TLR receptors and microbial products such as endotoxins binds to their respective TLR on the OVLT and activates the endothelium.



Picture 1.4: Acute phase response [wenliang.myweb.uga.edu]

Pyrogenic cytokines IL-1, TNF-α, IL-6 and other cytokines are synthesized, processed, released and gain access to the circulation. This results in the activation of TLR and cytokine receptors which induce COX-2, resulting in the synthesis of PGE_2 on the brain side of the OVLT [Katsuura *etal.* 1990]. Increase in PGE_2 stimulates the release of cAMP and other neurotransmitters triggering the thermo sensitive neurons to raise the hypothalamic thermostatic set point. Hypothalamic signals activate peripheral efferent nerves to blood vessels resulting in the vasoconstriction of blood vessels supplying peripheral circulation. This results in the increase of body temperature.

1.2.9. Cytokines

Cytokines are pleiotropic immunologic messengers which are having multiple biological properties [Damsgaard *et al.* 2009].The term cytokine encompasses interferons, interleukins, chemokine family, mesenchymal growth factors, tumor necrosis factor and adipokines [Dinarello, 1978]. They play an important role in the progression of many diseases including cardiovascular disease and rheumatoid arthritis. The discovery of the TLRs for recognizing microbial products is intrinsic to cytokine biology because the intracellular signaling domains of TLR are nearly identical to those of the IL-1 receptor.

Cytokines have a main role in inflammation. Inflammatory cytokines are classified into two main groups: those involved in acute inflammation and those responsible for chronic inflammation [Roth & De Souza, 2001]. The cytokines involved in acute inflammation includes IL-1, TNF-α, IL-6, IL-11, IL-8 and other chemokines, granulocyte colony stimulating factor (G-CSF) and granulocyte-monocyte colony stimulating factor (GM-CSF). The chronic inflammatory cytokines include IL-4, IL-5, IL-6, transforming growth factor-β etc.

1.2.10. Interleukins

IL-1β is a major cytokine protein present in nearly all tissues containing mononuclear phagocytes, including blood (monocytes), lung (alveolar macrophages), liver (Kupffer cells), bone marrow, spleen and placenta. The IL-1β is produced by activated macrophages as a preformed molecule, pro IL-1β, which is proteolytically processed into its active form by caspase 1 or IL-1 Converting Enzyme (ICE).

IL-1β triggers fever by enhancing PGE_2 synthesis by the vascular endothelium of the hypothalamus [Dinarello, 1978]. Upon stimulation with bacterial cell wall components, macrophages and blood monocytes expressing CD14 gets activated leading to a series of signaling reactions which release a variety of inflammatory mediators, like IL-1β, TNF-α and IL-6 [Dinarello, 2004]. The IL-

1β release is one of the first responses to an immune stimulating compound [Dinarello, 2002] which leads to an increase in the set point of body temperature. Blood monocytes secrete the pro inflammatory cytokine, IL-1β in a concentration-dependent manner following exposure to pyrogenic stimulations [Daneshian *et al.* 2009]. The IL-1β released by the blood cells can be measured by ELISA upon incubation with samples contaminated with endotoxins.

1.2.11. Pyrogen assay

An important principle in the safety assessment of any parenteral drugs, medical devices and implants is the conformity for non-pyrogenicity [Mohanan *et al.* 2011]. Pyrogens have been detected so far with the rabbit pyrogen and Limulus Amebocyte Lysate (LAL) assays. The rabbit species was chosen by Siebert, who discovered the pyrogenic principle. The in vivo rabbit pyrogen assay measures the body temperature of rabbits after the administration of not more than 10ml/kg bodyweight of the substance to be tested. The rabbit has a labile thermoregulation and tends to give false positive results. This is one of the drawbacks of this assay. Pyrogen testing currently requires about 200,000 rabbits each year in Europe [Schindler *et al.* 2002]. Furthermore, the assay is qualitative and is not appropriate for biotechnological products or sera etc [Morath *et al.* 2001].

LAL test measures the coagulation of the horseshoe crab, which is initiated by the cell wall components of gram negative bacteria [Mascoli *et al.* 1979]. The lysate is prepared by placing the crabs in restraining racks and inserting a needle through the muscular hinge between the cephalothorax and the abdominal region. Hemolymph is then drawn from the cardiac chamber into a container with anticoagulant. After collection, the amoebocytes are centrifuged and the supernatant is discarded. After 2-3 washing steps, the cells can be subjected to osmotic shock by adding distilled water and the intracellular lysate is released. The product is lyophilized and stored. The

major drawback of LAL test is that, it detects only endotoxins. Contamination of products with gram positive bacteria cannot be detected by LAL assay.

The first pyrogen assay based on human whole blood was developed by Hartung and Wendel [Hartung and Wendel, 1996]. The assay is based on the principle that the sample to be tested is diluted with human blood and physiological saline. The monocytes are challenged to produce cytokines by *in vitro*, and the proinflammatory cytokines (IL-1β) produced are measured by specific ELISA's. Unlike the LAL, this assay can detect not only endotoxins, but also lipotechoic acids, fungi and super antigens such as SEB (Enterotoxin of *Staphylococcus aureus*).

The whole blood assay detects a wide variety of pyrogens and reflects their relative potency [Hartung and Wendel, 1996] Any pyrogenic activity independent of its chemical nature induces the formation of IL-1β, which can be detected and quantified using ELISA technique [Mohanan *et al.* 2011]. A rapid, accurate and cost effective ELISA method measures the pyrogenic response of gram negative, positive bacteria, chemical and biological pyrogens on human whole blood [Mohanan *et al.* 2010].

Human peripheral blood mononuclear cells (PBMC's) can be used as a measure of cytokine production [Jansky *et al.* 2003]. Isolation of peripheral blood mononuclear cells (PBMC) is simpler and less expensive. PBMC cultures are most often used to measure *ex vivo* cytokine production [Camilla *et al.* 2009]. The PBMC cultures contain a mixture of monocytes, T-cells, B-cells, NK-cells and have been shown to have a skewed monocyte: lymphocyte ratio in the isolated sample compared to whole blood [De Groote *et al.* 1992]. The advantage of using isolated human lymphocytes is that they are the critical components in the human immune system to fight towards infections and adapt to intruders.

The review of literature well suggests that the rabbit pyrogen and LAL assays have enough drawbacks in detecting pyrogenicity. To bridge the gap between

the two assays, an attempt was made in the present study to develop a novel technique by using freshly isolated human lymphocytes for the detection of the pyrogenicity using the ELISA method developed by SCTIMST.

1.3. Hypodissertation

The conventional rabbit pyrogen and LAL assays are having several disadvantages. The rabbit pyrogen assay has limitation in its utility due to insufficient accuracy and requirement of large number of animals. Both of the tests show species specificity. The LAL assay is qualitative, detects only bacterial endotoxins and is costly. To cope with such a difficult situation, it would be advantageous to have available an *in vitro* pyrogen assay utilizing human isolated lymphocyte cells as an alternative to the existing pyrogen assays. An attempt was made in the present study to evaluate the IL-1β inflammatory response of isolated human lymphocytes to Gram-negative LPS and Gram-positive LTA using the SCTIMST developed *in vitro* ELISA method.

1.4. Objectives

The main objective of the present study is to:-

Detect the Interleukin-1β levels from single and pooled human lymphocytes in response to gram negative and gram positive pyrogens.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

- Heparin extra pure(Himedia, India)
- RPMI-1640 (Himedia, India)
- Interleukin 1-β human recombinant expressed in E.coli (Sigma, USA)
- Albumin fraction V from bovine serum (BSA) for biochemistry (Merck, Germany)
- TMB plus liquid 1 component substrate (Amersco, India)
- Hisep 1077 (Sigma, USA)
- Lipoteichoic acid (Sigma, USA)
- Lipopolysaccharide (Sigma, USA)
- Anti IL -1β antibody (collected from the rabbits immunized with IL-Iβ (Sigma, USA) at Toxicology division, SCTIMST)

2.2. Equipments

- Asys Expert plus ELISA plate reader (Grenier 96 well ELISA plate) with Digiread software (Austria)
- Eppendorf centrifuge 5810R (Germany)
- Fisher Scientific AB15+, pH meter (UK)
- ESCO Air stream vertical laminar flow cabinet (Singapore)
- Olympus CH-2 microscope (Japan)
- Carl Zeiss – Axiostar microscope (USA)
- REMI Centrifuge (India)
- Heraeus Kelvitron T hot air oven (Germany)
- Deep freezer -20^{0}C (Sanyo, Japan)
- Spectrophotometer (Schimadzu, Japan)

- AKTA prime plus system (GE Healthcare, South Asia)

2.3. Methods

2.3.1 Selection of blood groups

Following blood groups were selected for the study from healthy non allergic volunteers

- O +ve
- A +ve
- B +ve
- AB +ve

2.3.2 Selection of volunteers

Healthy, non allergic individuals (personnel information) were selected for the study. Volunteers who have not taken any medication for at least two weeks prior to the blood collection were selected for the study. Informed consent was obtained from the volunteers.

2.3.3 Collection of blood

8 ml of fresh, venous blood was collected from healthy volunteers and immediately transferred into sterile, pyrogen free tubes containing heparin (2 µl/ml). Individual and pooled blood was used for the isolation of lymphocytes.

2.3.4 Isolation of lymphocyte cells

1. Collected blood (8ml) was immediately processed by diluting with equal amount of PBS (pH 7.2 to 74).
2. 2.5 ml of HiSep LSM 1077 was aseptically transferred to a clean centrifuge tube and overlayed with 7.5ml diluted blood.
3. Centrifuged at 400xg for 30 minutes with the brake off, at room temperature. Centrifugation should sediment RBC's and polynuclear

leukocytes and band mononuclear lymphocytes above HiSep LSM 1077.

4. Aspirated plasma and platelet containing supernatant above the interphase band (granulocytes and erythrocytes will be in the red pellet).
5. Using a clean glass pasteur pipette carefully aspirated the lymphocyte layer along with half of the HiSep LSM 1077 without disturbing the red pellet.
6. Two washes with isotonic phosphate buffered saline separates the cell from the suspension medium.

2.3.5 Viability of lymphocytes

Viability of lymphocytes was assessed with the vital stain, Trypan blue (0.4%). A dilution of 1:1 was used for counting the cells. In this method, the viable lymphocytes will exclude the dye, while non viable cells absorb the dye and appear blue. The viable lymphocyte cells were counted using hemocytometer.

2.3.6 Cell counting (for in vitro pyrogen test)

Hemocytometer is a device used for counting mainly blood cells. To prepare the hemocytometer for counting, the chamber and cover slip was cleaned with ethanol. The cover slip was placed over the counting surface prior to adding the cell suspension (isolated lymphocytes). Mixed well and added 10 µl of 1:1 diluted cell suspension carefully into one of the wells using a pipette. Enough liquid should be introduced in such a way that one side of the chamber should be filled. The hemocytometer was then placed on the microscope stage and the counting grid was brought into focus at low power.

One entire grid on standard hemocytometers with Neubauer rulings can be seen at 40x. The main divisions separate the grid into 9 large squares. Each square has a surface area of $1mm^2$ and the depth of the chamber is 0.1mm.

Each square represents a volume of $0.1mm^3$ or 10^{-4} cm^3. Since 1 cm^3 = 1ml, the subsequent cell concentration can be determined by the following formula.

$$\text{Concentration of cells in original mixture} = \frac{\text{Number of cells counted}}{\text{Number of squares counted} \times \text{Dilution} \times \text{Depth of chamber}}$$

The number of cells in the isolated lymphocyte sample was calculated using the above formula. The cells were subsequently diluted with appropriate volume of 1X PBS/ RPMI 1640 to obtain three different concentrations (4, 5 and 7lakhs), which was used for the *in vitro* pyrogen assay.

2.3.7 IL-1β Induction by LPS in freshly isolated human lymphocytes (Individual blood groups: O +ve and A +ve volunteers)

Freshly isolated human lymphocytes (100 µl) from O +ve and A +ve donors were incubated separately in 400 µl of RPMI-1640. LPS from *Escherichia coli* (Sigma) was added at a final concentration of 5 EU in both the samples. Freshly isolated lymphocytes were added in three different concentrations (4, 5 and 7 lakhs) as mentioned in the Table 2.1. This was used for the in vitro pyrogen assay.

Table 2.1: IL-1β Induction by LPS in freshly isolated human lymphocytes (Individual blood groups: O +ve and A+ve volunteers)

Components	Control			Test		
	1	2	3	1	2	3
RPMI (µl)	400	400	400	395	395	395
Volume of lymphocyte cells(µl)	100	100	100	100	100	100
Total Number of lymphocytes (100µl)	4,00,000	5,00,000	7,00,000	4,00,000	5,00,000	7,00,000
LPS (µl)	0	0	0	5	5	5
Total Volume (µl)	500	500	500	500	500	500

The total reaction volume was 500 µl and was prepared in sterile, pyrogen free eppendorf tubes under aseptic conditions. There were 96 tubes each for control and 5EU of LPS (for both O +ve & A+ ve donors). All the reaction tubes (96 tubes) with reaction mixtures were incubated at 37^0C up to 8 hours. At the end of each hour, all the six reaction tubes were collected and centrifuged at 500 g for 2 min at 4^0 C. The supernatant was collected in an eppendorf tube and immediately stored at - 20^0 C until analysis.

2.3.8 IL-1β Induction by LTA in freshly isolated human lymphocytes (Individual blood of O +ve and A +ve donors)

Freshly isolated human lymphocytes (100 µl) from O +ve and A +ve donors were incubated separately in 400 µl of RPMI-1640. LTA from *Bacillus subtilis* was added at a final concentration of 1 µg/µl. Live lymphocytes were added in three different concentrations such as 4, 5 and 7 lakhs and are explained in the Table 2.2.This was used for the in vitro pyrogen assay.

Table 2.2: IL-1β Induction by LTA in freshly isolated human lymphocytes (Individual blood of O +ve and A +ve donors)

Components	Control			Test		
	1	2	3	1	2	3
RPMI (µl)	400	400	400	390	390	390
Volume of Lymphocyte cells(µl)	100	100	100	100	100	100
Total Number of lymphocytes (100µl)	4,00,000	5,00,000	7,00,000	4,00,000	5,00,000	7,00,000
LTA (µl)	0	0	0	10	10	10
Total Volume (µl)	500	500	500	500	500	500

The total reaction volume was 500 µl and was prepared in sterile, pyrogen free eppendorf tubes under aseptic conditions. There were 96 tubes each for control and 1 µg/µl of LTA. All the reaction tubes (96 tubes) with reaction mixtures were incubated at 37^0C up to 8 hours. At the end of each hour, all the six reaction tubes were collected and centrifuged at 500 g for 2 min at 4^0 C. The supernatant was collected in an eppendorf tube and immediately stored at - 20^0 C until analysis.

2.3.9 IL-1β Induction by LPS in freshly isolated human lymphocytes (Pooling Individual Blood Groups: A +ve, B +ve and AB +ve donors)

Freshly isolated human lymphocytes (100 µl) from multiple donors of A +ve blood group were pooled in two different concentrations namely 4, 00, 000 and 5, 00,000 cells (Table: 2.3). Similarly, lymphocytes from B +ve and AB +ve donors were pooled and were evaluated for cytokine (IL-1β) induction using LPS.

Table 2.3: IL-1β Induction by LPS in freshly isolated human lymphocytes (Pooling Individual Blood Groups: A +ve, B +ve & AB +ve donors)

Components	Control		Test	
	1	2	1	2
RPMI (µl)	400	400	395	395
Volume of lymphocyte cells (100µl)	100	100	100	100
Total Number of lymphocytes (100µl)	5,00,000	7,00,000	5,00,000	7,00,000
LPS (µl)	0	0	5	5
Total Volume (µl)	500	500	500	500

The total reaction volume was 500 µl and was prepared in sterile, pyrogen free eppendorf tubes under aseptic conditions. There were 96 tubes each for control and 5 EU of LPS (for A +ve, B +ve & AB +ve donors). All the reaction tubes (96 tubes) with reaction mixtures were incubated at 37^0C up to 8 hours. At the end of each hour, all the six reaction tubes were collected and centrifuged at 500 g for 2 min at 4^0 C. The supernatant was collected in an eppendorf tube and immediately stored at - 20^0 C until analysis.

2.3.10 IL-1β Induction by LTA in freshly isolated human lymphocytes (Pooling Individual Blood Groups: A +ve, B +ve and AB +ve donors)

Freshly isolated human lymphocytes (100 µl) from two healthy donors of A +ve blood group were pooled in two different concentrations namely 4, 00, 000 and 5, 00,000 cells. Similarly, lymphocytes from B +ve and AB +ve donors were pooled (Table 2.4). This was then used for the detection of cytokine induction using LTA.

Table 2.4: IL-1β Induction by LTA in freshly isolated human lymphocytes (Pooling Individual Blood Groups: A +ve, B +ve & AB +ve donors)

Components	Control		Test	
	1	2	1	2
RPMI (µl)	400	400	390	390
Volume of lymphocyte cells (µl)	100	100	100	100
Total Number of lymphocytes (100µl)	5,00,000	7,00,000	5,00,000	7,00,000
LTA (µl)	0	0	10	10
Total Volume (µl)	500	500	500	500

The total reaction volume was 500 µl and was prepared in sterile, pyrogen free eppendorf tubes under aseptic conditions. There were 96 tubes each for control and 1 µg/µl of LTA (for A +ve, B +ve & AB +ve donors). All the reaction tubes (96 tubes) with reaction mixtures were incubated at 37^0C up to 8 hours. At the end of each hour, all the six reaction tubes were collected and centrifuged at 500 g for 2 min at 4^0 C. The supernatant was collected in an eppendorf tube and immediately stored at - 20^0 C until analysis.

2.3.11 Antibody (Anti IL-1 β) Purification by Affinity Chromatography

The serum isolated from the rabbit immunized with IL-1 β (Sigma) was purified using Protein A Hitrap column by Affinity Chromatography. The purification procedure involves protein ligand interactions which uses 20mM sodium phosphate buffer at physiologic pH (pH7). The second buffer, 100mM citric acid of pH 3 was added finally as elution buffer to dissociate the binding interactions and release the target molecule (antibody).

Before the start of the experiment, enable the Prime view module. Flushed the system with deionized water by placing both (A&B) inlets into it. After

washing, the two inlets were placed into binding buffer (Inlet A) and elution buffer (Inlet B). The 5ml sample loop was then connected between the ports of the injection valve and flushed with five volumes of binding buffer.

One ml of blood serum was diluted five times in binding buffer and loaded into a syringe at the fill port after injection. The HiTrap column was finally connected between the port1 of the injection valve and the upper port of the UV flow cell with suitable unions and connectors supplies along with the system.

The glass tubes used for collecting the fractions were pre-filled with 100µl of 1M Tris-Cl (pH 9). The fractions corresponding to the peak in the UV curve during elution represent the eluted purified IgG fraction bound to the Protein A column. The fractions were subjected to overnight dialysis against 5 liters of ice cold 1X PBS (pH 7.2)

2.3.12 Estimation of antibody concentration

The antibody purified using AKTA prime plus system (Affinity chromatography) was estimated using Micro Bradford method. The assay is based on the direct binding of Coomassie brilliant blue G-250 dye (CBBG) to proteins at arginine, tryptophan, tyrosine, histidine and phenyl alanine residues, measured at 595 nm.

2.3.13 Antibody-enzyme conjugation

The anti human IL-1β antibody was conjugated with horse radish peroxidase (HRP) using the EZ-Link Plus Activated Peroxidase.

2.3.14 Coating of antibody on ELISA plate

Anti-human IL-1β antibody coated using 50mM carbonate bicarbonate buffer of pH 9.6. 50µl of diluted antibody added per well of ELISA plate. The plate was then tightly sealed and incubated overnight at 4^0C. This plate was then used for detecting IL-1β.

2.3.15 Measurement of IL-1β production using Sandwich ELISA

The antibody pre-coated plates were bought to room temperature and washed twice with deionized water. The supernatant solutions collected from the reaction mixture with lymphocytes were thawed at room temperature. This reaction mixture is used as the antigen for ELISA.

The washed plates were blocked by 1% BSA solution for 1 hour. The plates were washed three times using 1X PBS with gentle shaking for 5 seconds. The antigen (supernatant solution) was diluted with 1% BSA. 50 µl of this solution was added per well and incubated for 2 hours at room temperature. The plates were then washed thrice using 1X PBS with gentle shaking.

Diluted labeled antibodies (1:200 dilution) prepared by in-house method were added 50 µl/well and incubated again for 2 hours in dark. The plates were washed thrice after incubation using 1X PBS with gentle shaking. TMB substrate was added and incubated for 30 minutes in dark. After incubation, the reactions were terminated by adding 1M H_2SO_4 and incubated for 10 minutes in dark. The plates were read at 450 nm with the corrective filter at 620 nm using ELISA reader. The mean OD values were calculated for each IL-1β concentration points on the graph.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Results

3.1.1 Detection of cell viability

The isolated lymphocyte cells were found to be viable (95%) when stained with 0.4% Trypan blue.

Figure 3.1 Separation of Human Lymphocyte by Hisep 1077 (Sigma, USA)

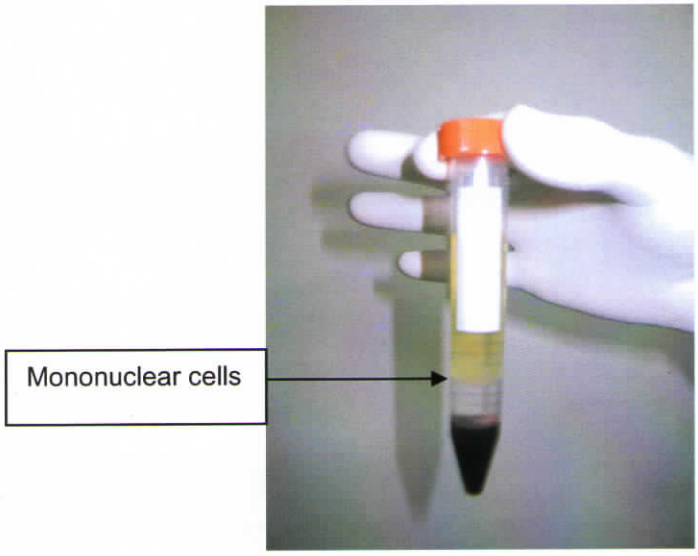
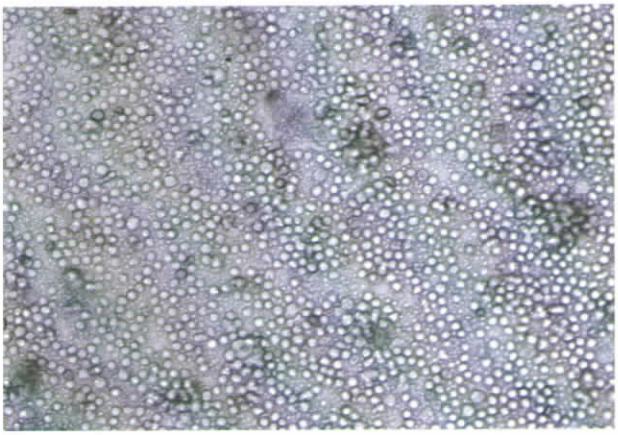


Figure 3.2 Lymphocyte cells under a bright field microscope (20X)



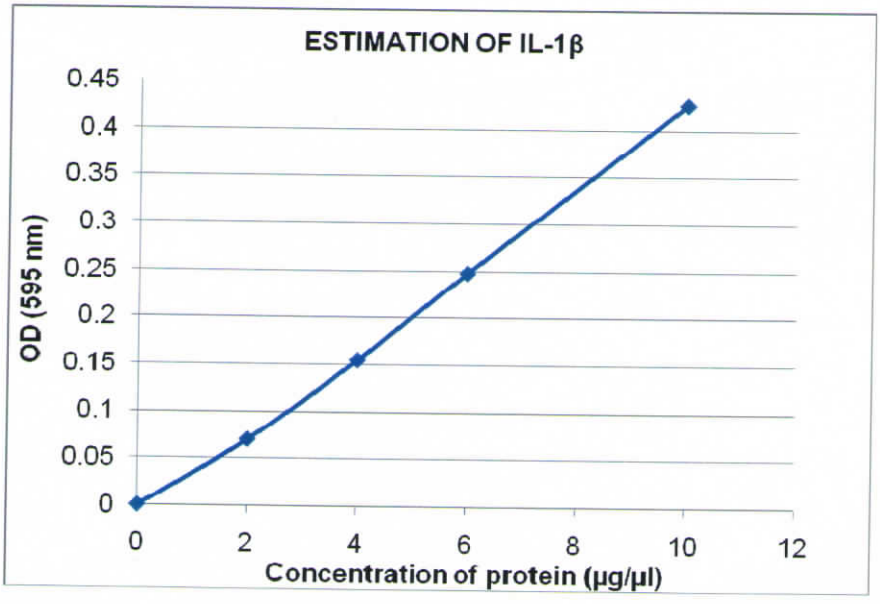
3.1.2. Protein Estimation

The serum was separated from the blood of immunized rabbit and was subjected to affinity chromatography for purification of antibody. The concentration of purified Anti human IL-1β antibody was estimated using Micro Bradford method using 1% BSA as the standard solution. A standard curve was plotted with concentration of samples (µg) in X- axis and OD in Y axis. The concentration of antibody was thus estimated from the standard curve.

Table 3.1: Estimation of protein (Anti human IL-1β antibody)

Concentration (µg/µl)	OD (595nm)
0	0
2	0.070
4	0.155
6	0.248
10	0.427
Test	0.226

Figure 3.3: Estimation of Anti human IL-1 β antibody



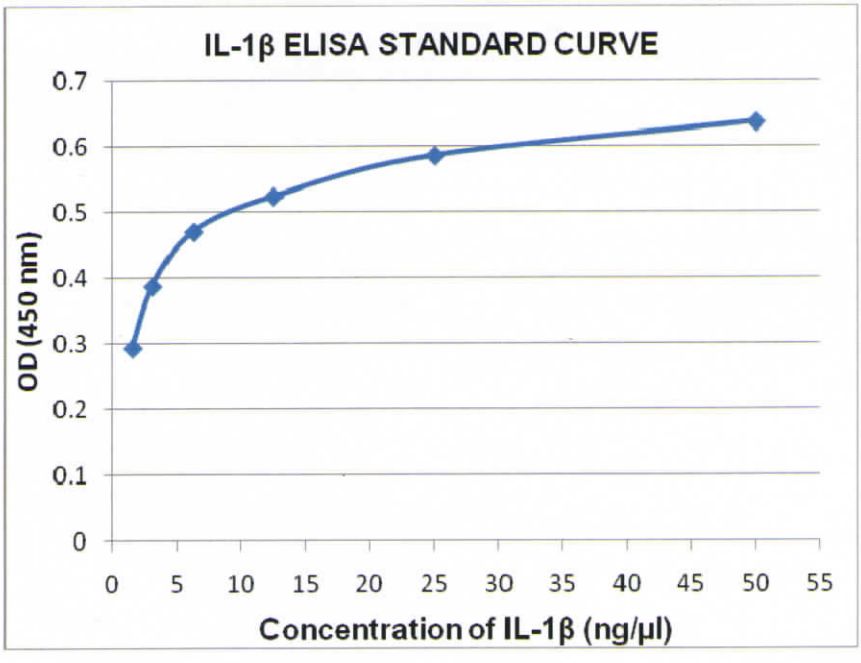
3.1.3. Estimation of IL-1β concentration

The IL-1β standards were assayed on the Sandwich ELISA method. The concentration of IL-1β released on stimulation with lymphocytes was calculated from the standard graph by plotting IL-1β concentration (ng/µl) in X-axis and OD (450nm) in Y-axis.

Table 3.2: Estimation of IL-1β concentration

Concentration (ng/µl)	OD (450 nm)
1.56	0.293
3.125	0.387
6.25	0.470
12.5	0.524
25	0.586
50	0.637

Figure 3.4: IL-1β ELISA standard curve



3.1.4. Measurement of IL-1β release, in response to Gram negative bacterial pyrogen (O +ve Donors)

The results of the study are mentioned in the Tables 3.3, 3.4 and 3.5. The time course production of IL-1β release in response to 5 EU of LPS was evaluated for 8 hours. The maximum IL-1β level in the reaction was found at 5th hour of the reaction when the concentration of lymphocyte was 7 (fig: 3.5) lakh cells. IL-1β release was dependent on the concentration of lymphocytes and a peak was obtained at 6th hour when the cell concentration was 5 (fig: 3.6) and 4(fig: 3.7) lakhs. The maximum release at a peak on 5th hour resulted in the production of 3 ng of IL-1β (Table: 3.3).

Table 3.3: Measurement of IL-1β release, in response to Gram negative bacterial pyrogen (O +ve Donors - 7 lakhs)

Time	Control	LPS (5µl)	Concentration of IL-1β (ng/µl)
1hr	0.243	0.257	1.0
2hr	0.263	0.273	1.0
3hr	0.290	0.291	1.5
4hr	0.290	0.341	2.5
5hr	0.297	0.377	3.0
6hr	0.292	0.361	2.5
7hr	0.299	0.356	2.5
8 hr	0.290	0.337	2.0

Figure 3.5: IL-1β detection with 7 lakhs of lymphocytes

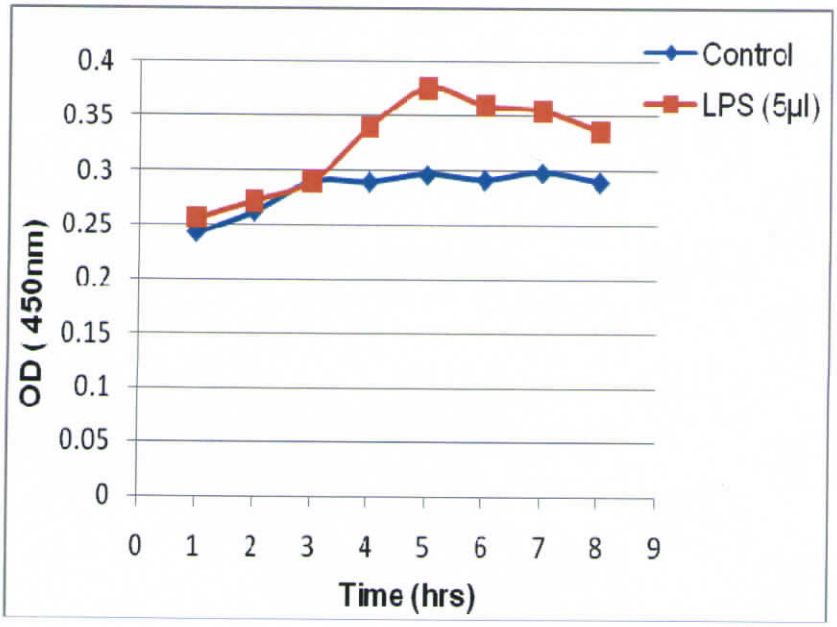


Table 3.4: Measurement of IL-1β release, in response to Gram negative bacterial pyrogen (O +ve Donors – 5 lakhs)

Time	Control	LPS (5µl)	Concentration of IL-1β (ng/µl)
1hr	0.228	0.293	1.6
2hr	0.212	0.283	1.5
3hr	0.214	0.289	1.5
4hr	0.218	0.302	1.6
5hr	0.209	0.315	1.6
6hr	0.219	0.356	2.5
7hr	0.212	0.340	2.4
8 hr	0.212	0.340	2.4

Figure 3.6: IL-1β detection with 5 lakhs of lymphocytes

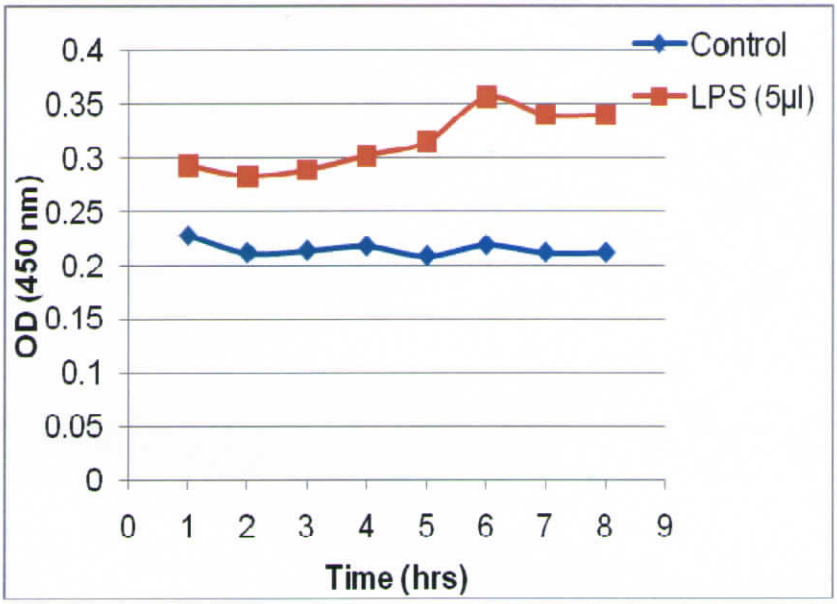
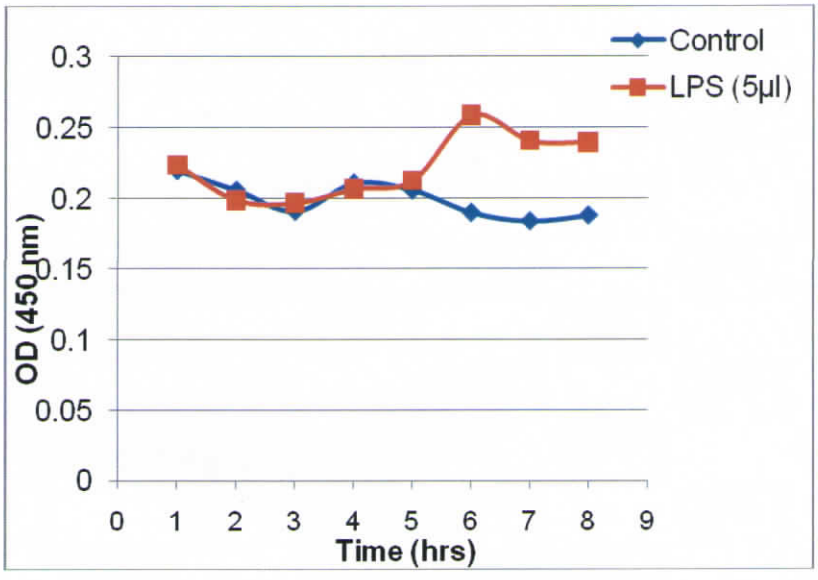


Table 3.5: Measurement of IL-1β release, in response to Gram negative bacterial pyrogen (O +ve Donors – 4 lakhs)

Time	Control	LPS (5µl)	Concentration of IL-1β (ng/µl)
1hr	0.220	0.224	1
2hr	0.206	0.199	1
3hr	0.191	0.197	1
4hr	0.221	0.207	1
5hr	0.206	0.213	1
6hr	0.190	0.259	1.5
7hr	0.184	0.241	1.4
8hr	0.188	0.240	1.4

Figure 3.7: IL-1β detection with 4 lakhs of lymphocytes



3.1.5. Measuremnet of IL-1β release, in response to Gram positive bacterial pyrogen (O +ve Donors)

The time course production of IL-1β release in response to pyrogenic stimulation with 1 µg/µl of LTA was evaluated for 8 hours. The results of the study are mentioned in the figures 3.8, 3.9 and 3.10. It was found that the maximum IL-1β level in the *in vitro* reaction was found to take place at 6th hour of reaction when the concentration of lymphocyte was 7 (fig: 3.8) and 4 lakh (fig: 3.10) cells. The cells elicited a maximum IL-1β release at 6th hour of the reaction and yielded an IL-1β concentration of 1.7 ng, when challenged with 5 lakh cell concentration (fig: 3.9).

Table 3.6: Measurement of IL-1β release, in response to Gram positive bacterial pyrogen (O +ve Donors - 7 lakhs)

Time	Control	LTA (10µl)	Concentration of IL-1β (ng/µl)
1hr	0.22	0.226	1
2hr	0.206	0.212	1
3hr	0.191	0.225	1
4hr	0.221	0.252	1.5
5hr	0.206	0.266	1.5
6hr	0.190	0.314	1.7
7hr	0.184	0.264	1.5
8hr	0.188	0.26	1.5

Figure 3.8: IL-1β detection with 7 lakhs of lymphocytes

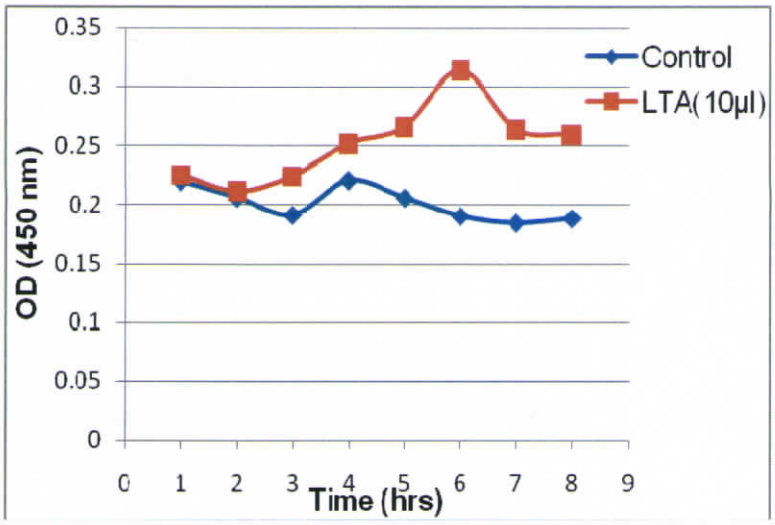


Table 3.7: Measurement of IL-1β release, in response to Gram positive bacterial pyrogen (O +ve Donors – 5 lakhs)

Time	Control	LTA (10µl)	Concentration of IL-1β (ng/µl)
1hr	0.178	0.205	1
2hr	0.170	0.202	1
3hr	0.160	0.211	1
4hr	0.161	0.209	1
5hr	0.151	0.240	1.5
6hr	0.159	0.227	1
7hr	0.180	0.211	1
8hr	0.176	0.201	1

Figure 3.9: IL-1β detection with 5 lakhs of lymphocytes

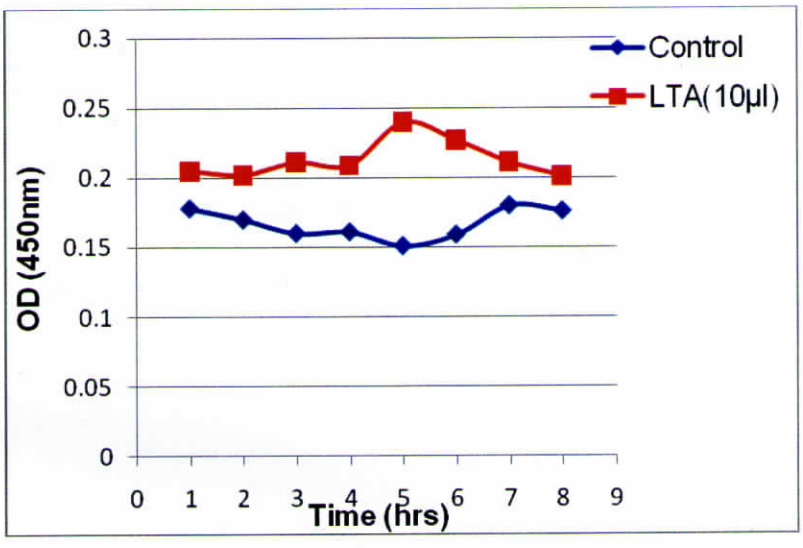
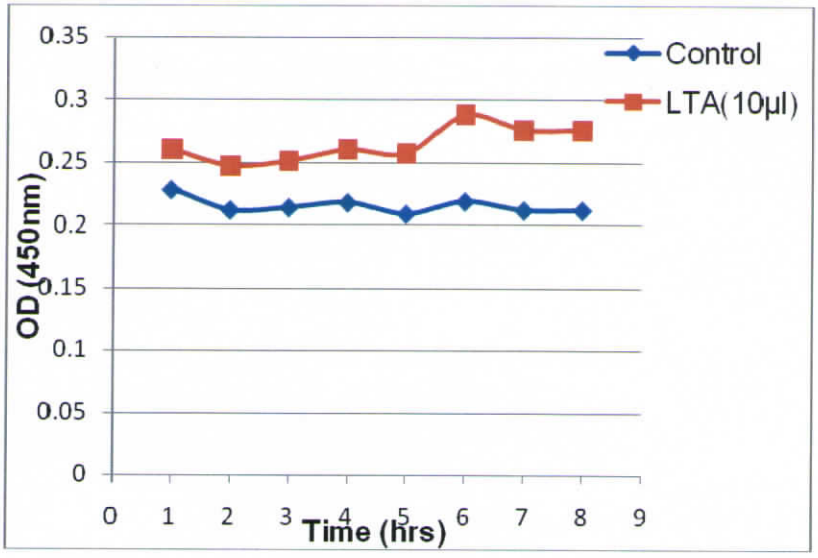


Table 3.8: Measurement of IL-1β release, in response to Gram positive bacterial pyrogen (O +ve Donors – 4 lakhs)

Time	Control	LTA (10µl)	Concentration of IL-1β (ng/µl)
1hr	0.228	0.260	1
2hr	0.212	0.247	1
3hr	0.214	0.251	1
4hr	0.218	0.260	1
5hr	0.209	0.257	1.5
6hr	0.219	0.288	1.56
7hr	0.212	0.276	1
8hr	0.212	0.276	1

Figure 3.10: IL-1β detection with 4 lakhs of lymphocytes



3.1.6. Measurement of IL-1β release in response to Gram negative bacterial pyrogen (A +ve Donors)

The lymphocytes isolated from A+ ve donors showed the release of IL-1β (5EU of LPS) at 5th hour and 4th hour when used at a cell concentration of 7 (fig 3.11) and 5 (3.12) lakh cells. No significant response of IL-1β levels was noticed when stimulated with 4 lakh cell concentration.

Table 3.9: Measurement of IL-1β release in response to Gram negative bacterial pyrogen (A +ve Donors – 7 lakhs)

Time	Control	LPS (5µl)	Concentration of IL-1β (ng/µl)
1hr	0.259	0.280	1.5
2hr	0.280	0.286	1.5
3hr	0.261	0.310	1.5
4hr	0.258	0.313	1.5
5hr	0.271	0.321	1.6
6hr	0.277	0.307	1.5
7hr	0.274	0.304	1.5
8hr	0.279	0.316	1.5

Figure 3.11: IL-1β detection with 7 lakhs of lymphocytes

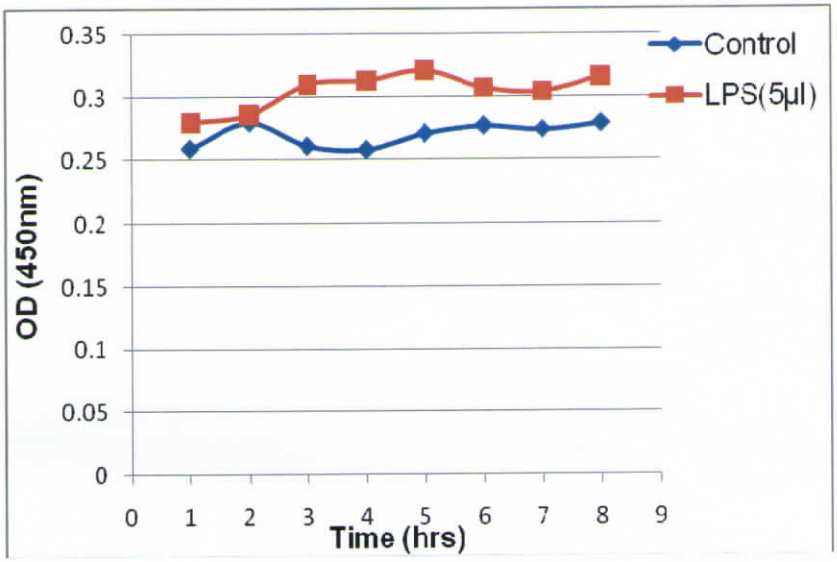


Table 3.10: Measurement of IL-1β release in response to Gram negative bacterial pyrogen (A +ve Donors – 5 lakhs)

Time	Control	LPS (5µl)	Concentration of IL-1β (ng/µl)
1hr	0.206	0.207	1
2hr	0.207	0.214	1
3hr	0.196	0.232	1
4hr	0.201	0.256	1.5
5hr	0.196	0.255	1.5
6hr	0.185	0.246	1
7hr	0.184	0.246	1
8hr	0.190	0.242	1

Figure 3.12: IL-1β detection with 5 lakhs of lymphocytes

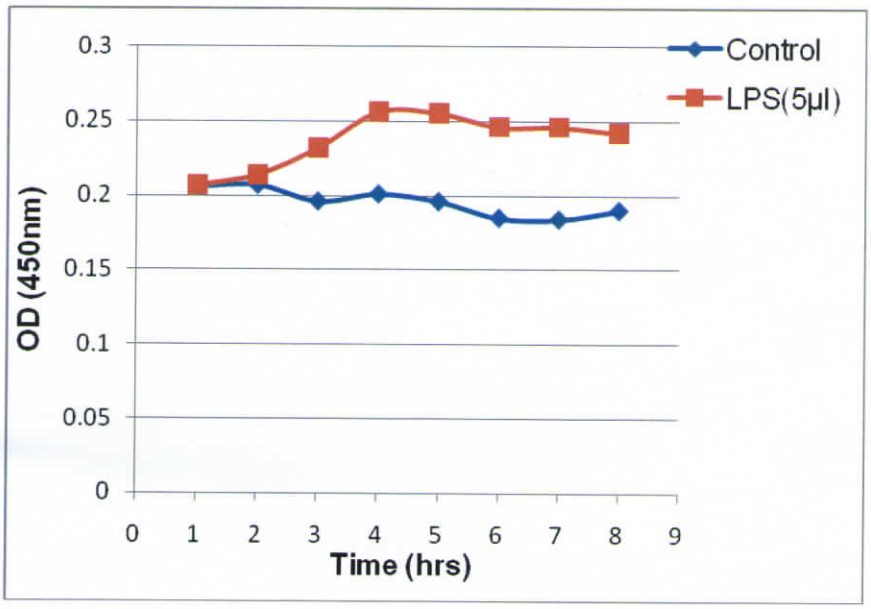
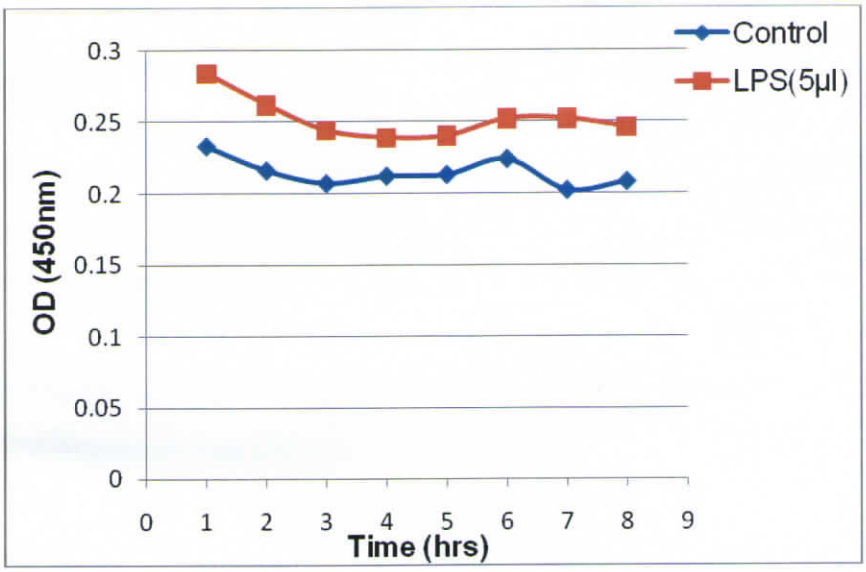


Table 3.11: Measurement of IL-1β release in response to Gram negative bacterial pyrogen (A +ve Donors – 4 lakhs)

Time	Control	LPS (5µl)	Concentration of IL-1β (ng/µl)
1hr	0.233	0.284	1
2hr	0.216	0.262	1
3hr	0.207	0.244	1
4hr	0.212	0.239	1
5hr	0.213	0.240	1
6hr	0.224	0.252	1
7hr	0.202	0.252	1
8hr	0.208	0.246	1

Figure 3.13: IL-1β detection with 4 lakhs of lymphocytes



3.1.7. Measurement of IL-1β release, in response to Gram positive bacterial pyrogen (A +ve Donors)

The LTA (1 µg/µl) stimulated human lymphocytes elicit IL-1β response at 2nd hour and gradually at a maximum on 4th hour (fig: 3.14) when the cell concentration was 7 lakhs. The cells yielded 5ng of IL-1β concentration as mentioned in table 3.12. It was found that the IL-1β was at a peak on 5th hour in 5 lakh (fig 3.15) and 4th hour in 4 lakh (fig 3.16) cell concentration.

Table 3.12: Measurement of IL-1β release in response to Gram positive bacterial pyrogen (A +ve Donors – 7 lakhs)

Time	Control	LTA(10µl)	Concentration of IL-1β (ng/µl)
1hr	0.338	0.309	1.5
2hr	0.312	0.333	1.6
3hr	0.338	0.426	4
4hr	0.333	0.457	5
5hr	0.319	0.419	4
6hr	0.344	0.406	4
7hr	0.305	0.413	4
8hr	0.315	0.418	4

Figure 3.14: IL-1β detection with 7 lakhs of lymphocytes

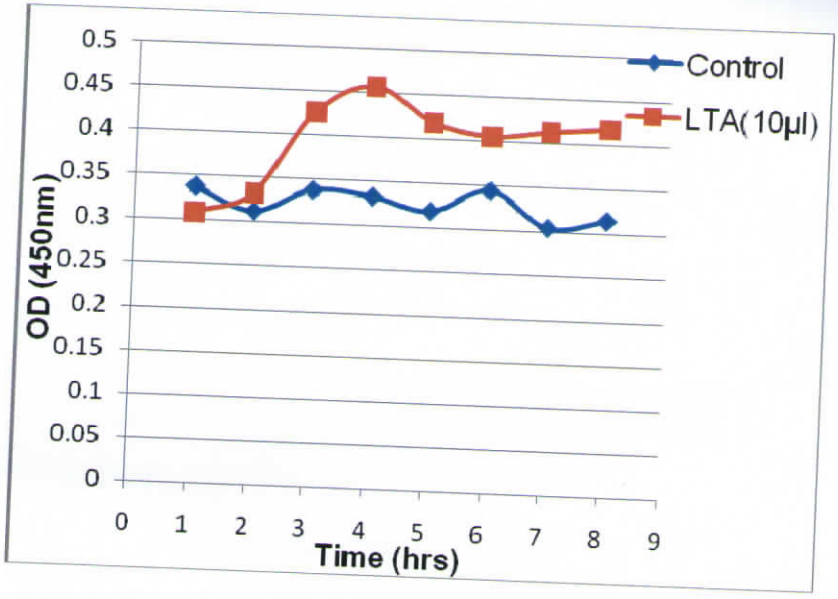


Table 3.13: Measurement of IL-1β release in response to Gram positive bacterial pyrogen (A +ve Donors – 5 lakhs)

Time	Control	LTA (10µl)	Concentration of IL-1β (ng/µl)
1hr	0.202	0.234	1
2hr	0.206	0.230	1
3hr	0.208	0.293	1.6
4hr	0.190	0.325	1.6
5hr	0.182	0.344	1.7
6hr	0.190	0.333	1.6
7hr	0.182	0.296	1.6
8hr	0.191	0.302	1.6

Figure 3.14: IL-1β detection with 7 lakhs of lymphocytes

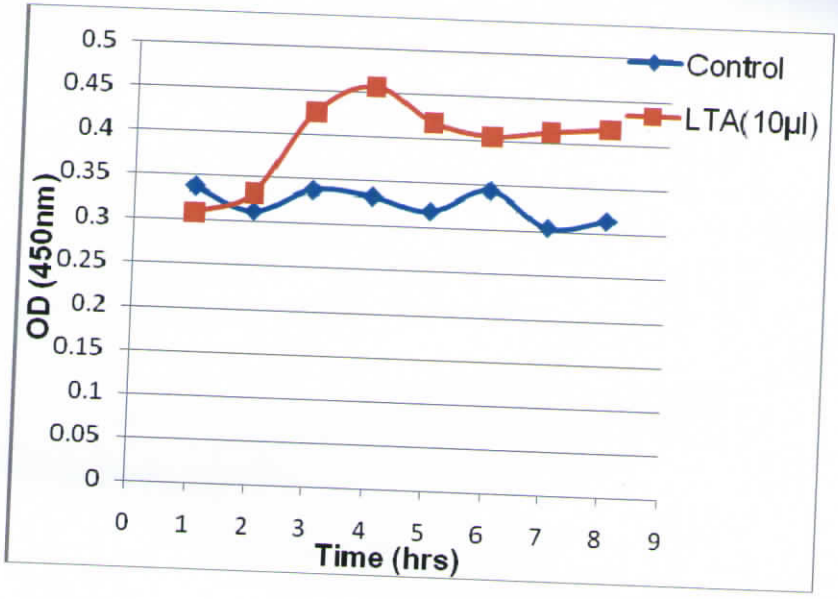


Table 3.13: Measurement of IL-1β release in response to Gram positive bacterial pyrogen (A +ve Donors – 5 lakhs)

Time	Control	LTA (10µl)	Concentration of IL-1β (ng/µl)
1hr	0.202	0.234	1
2hr	0.206	0.230	1
3hr	0.208	0.293	1.6
4hr	0.190	0.325	1.6
5hr	0.182	0.344	1.7
6hr	0.190	0.333	1.6
7hr	0.182	0.296	1.6
8hr	0.191	0.302	1.6

Figure 3.15: IL-1β detection with 5 lakhs of lymphocytes

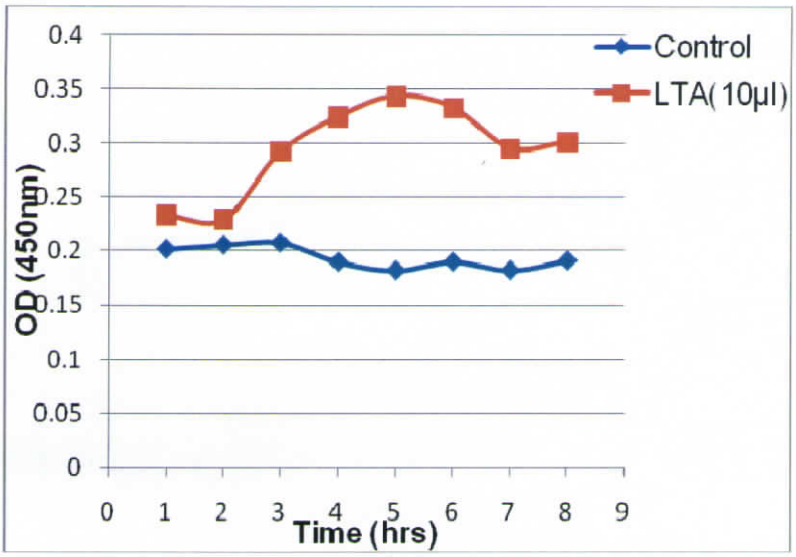
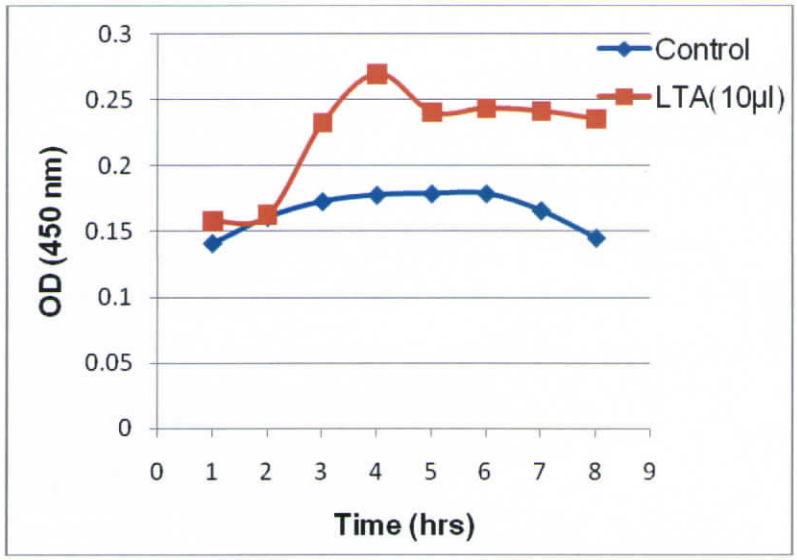


Table 3.14: Measurement of IL-1β release in response to Gram positive bacterial pyrogen (A +ve Donors – 4 lakhs)

Time	Control	LTA (10µl)	Concentration of IL-1β (ng/µl)
1hr	0.141	0.158	1
2hr	0.161	0.163	1
3hr	0.173	0.233	1
4hr	0.178	0.270	1.5
5hr	0.179	0.241	1
6hr	0.179	0.244	1
7hr	0.166	0.242	1
8hr	0.145	0.236	1

Figure 3.16: IL-1β detection with 4 lakhs of lymphocytes



3.1.8. Measurement of IL-1β release, in response to Gram negative bacterial pyrogen (Pooled sample - A +ve Donors)

In pooled lymphocytes (A +ve Donors), IL-1β release elicited by 5EU of LPS was found maximum at a peak on 6th hour of the reaction with 7 lakh lymphocyte concentration as indicated in fig: 3.17. The concentration of IL-1β was found as 3 ng during the initial period of pyrogenic stimulation and gradually increased to 20ng (table: 3.15) and gets stabilized. When 5 lakhs of cells were used (fig: 3.18), peak was obtained at 6th hour of the reaction which resulted in the production of 1.5 ng of IL-1β.

Table 3.15: Measurement of IL-1β in response to Gram negative bacterial pyrogen (Pooled sample - A +ve Donors – 7 lakhs)

Time	Control	LPS (5µl)	Concentration of IL-1β (ng/µl)
1hr	0.332	0.389	3
2hr	0.335	0.387	3
3hr	0.372	0.458	6
4hr	0.359	0.512	12
5hr	0.356	0.529	12
6hr	0.357	0.564	20
7hr	0.373	0.526	12
8hr	0.376	0.526	12

Figure 3.17: IL-1β detection with 7 lakhs of lymphocytes

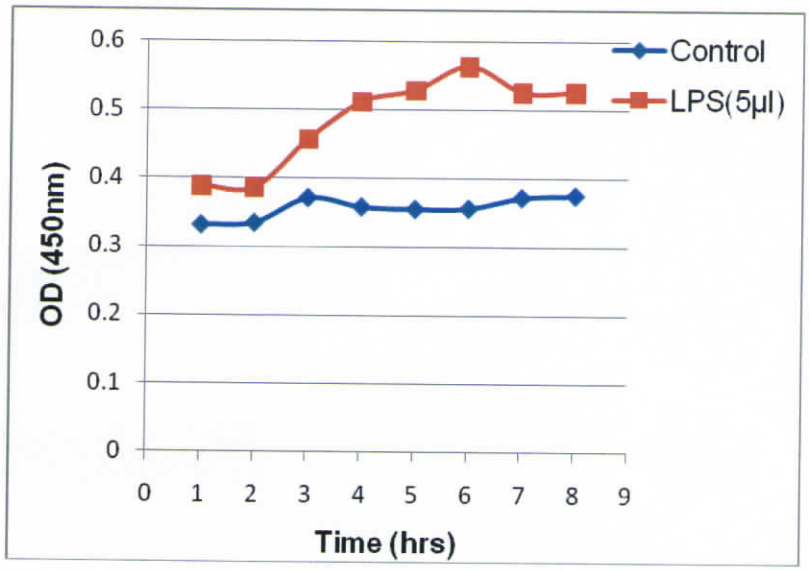
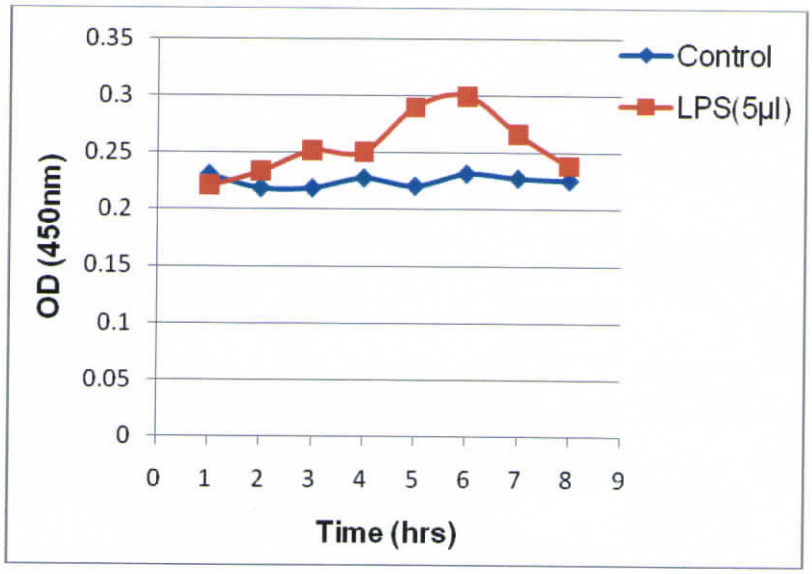


Table 3.16: Measurement of IL-1β in response to Gram negative bacterial pyrogen (Pooled sample - A +ve Donors – 5 lakhs)

Time	Control	LPS (5µl)	Concentration of IL-1β (ng/µl)
1hr	0.231	0.221	1
2hr	0.219	0.234	1
3hr	0.219	0.252	1
4hr	0.228	0.251	1
5hr	0.221	0.290	1.5
6hr	0.232	0.300	1.5
7hr	0.228	0.267	1.5
8hr	0.226	0.239	1

Figure 3.18: IL-1β detection with 5 lakhs of lymphocytes



3.1.9. Measurement of IL-1β release, in response to Gram positive bacterial pyrogen (Pooled sample - A +ve Donors)

The IL-1β response elicited by 1 µg/µl of LTA was measured by the ELISA method. The maximum release of IL-1β was at a peak on 6th hour when used in 7 lakhs (fig: 3.19) and 5 (fig: 3.20) lakhs of lymphocyte concentration. The concentration of IL-1β was 2 ng (table 3.17) in 7 lakhs of lymphocytes and 1.5 ng in 5 lakhs of lymphocytes.

Table 3.17: Measurement of IL-1β in response to Gram positive bacterial pyrogen (Pooled sample - A +ve Donors – 7 lakhs)

Time	Control	LTA (10µl)	Concentration of IL-1β (ng/µl)
1hr	0.275	0.287	1.5
2hr	0.238	0.28	1.5
3hr	0.245	0.29	1.5
4hr	0.256	0.295	1.5
5hr	0.234	0.279	1.5
6hr	0.249	0.337	2
7hr	0.247	0.27	1.5
8hr	0.256	0.27	1.5

Figure 3.19: IL-1β detection with 7 lakhs of lymphocytes

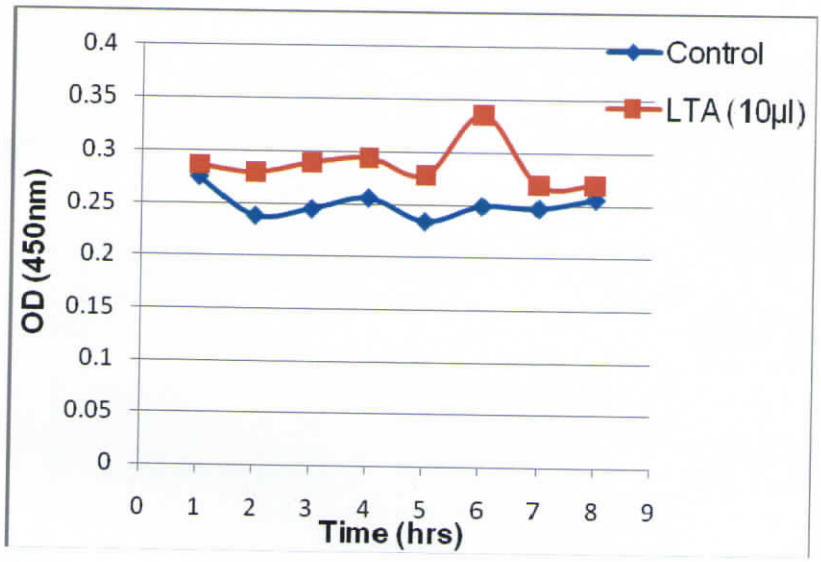
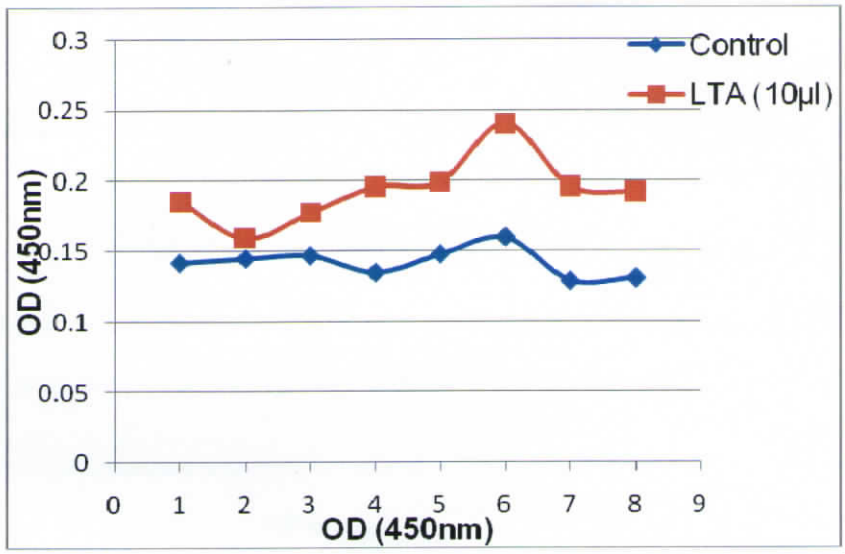


Table 3.18: Measurement of IL-1β in response to Gram positive bacterial pyrogen (Pooled sample – A +ve Donors – 5 lakhs)

Time	Control	LTA (10µl)	Concentration of IL-1β (ng/µl)
1hr	0.142	0.186	1
2hr	0.145	0.16	1
3hr	0.147	0.178	1
4hr	0.135	0.196	1
5hr	0.148	0.199	1
6hr	0.16	0.24	1.5
7hr	0.129	0.196	1
8hr	0.131	0.192	1

Figure 3.20: IL-1β detection with 5 lakhs of lymphocytes



3.1.10. Measurement of IL-1β release, in response to Gram negative bacterial pyrogen (Pooled sample - B +ve Donors)

The Figures (fig: 3.21) indicated that the LPS (5EU) stimulated lymphocytes of both the concentration displayed maximum IL-1β release within 6 hour of pyrogenic stimulation. As indicated in table 3.19, the IL-1β concentration increases during the initial period and then gets stabilized.

Table 3.19: Measurement of IL-1β release, in response to Gram negative bacterial pyrogen (Pooled sample - B +ve Donors - 7 lakhs)

Time	Control	LPS (5µl)	Concentration of IL-1β (ng/µl)
1hr	0.335	0.339	1.5
2hr	0.330	0.328	1.5
3hr	0.333	0.352	2
4hr	0.328	0.375	3
5hr	0.327	0.383	3
6hr	0.334	0.412	3.5
7hr	0.345	0.398	3
8hr	0.321	0.389	3

Figure 3.21: IL-1β Detection with 7 lakhs of lymphocytes

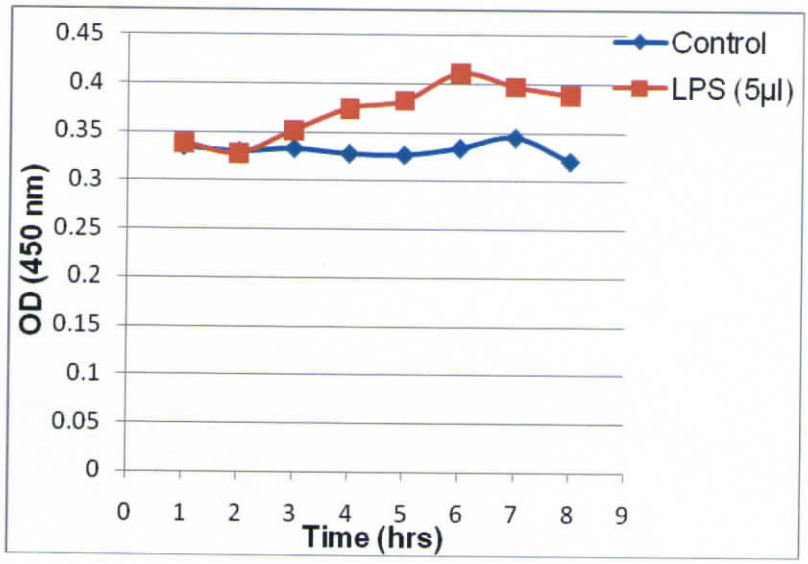
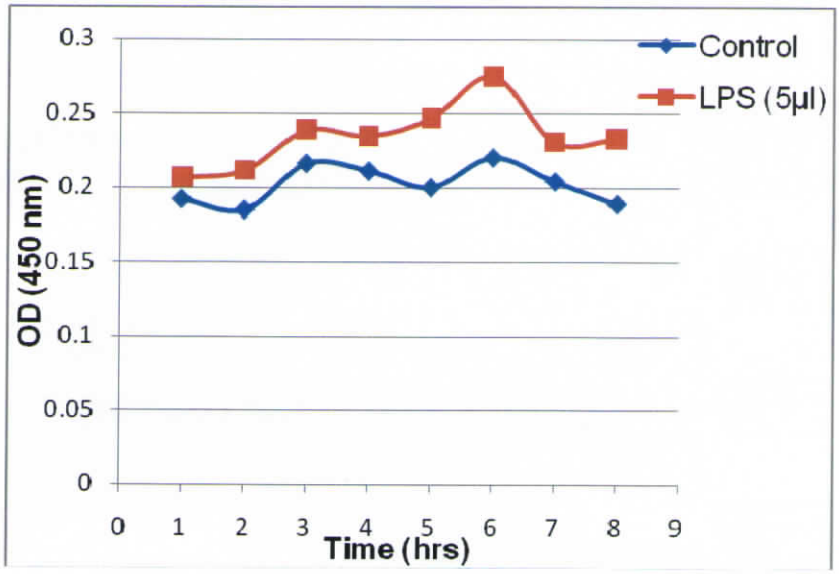


Table 3.20: Measurement of IL-1β release, in response to Gram negative bacterial pyrogen (Pooled sample- B +ve Donors – 5 lakhs)

Time	Control	LPS (5µl)	Concentration of IL-1β (ng/µl)
1hr	0.192	0.207	1
2hr	0.185	0.212	1
3hr	0.216	0.239	1
4hr	0.211	0.235	1
5hr	0.2	0.247	1
6hr	0.22	0.275	1.5
7hr	0.204	0.231	1
8hr	0.189	0.233	1

Figure 3.22: IL-1β Detection with 5 lakhs of lymphocytes



3.1.11 Measurement of IL-1β release, in response to Gram positive bacterial pyrogen (Pooled sample - B +ve Donors)

The results of the study are indicated in the figures 3.23 and 3.24. The maximum release of IL-1β (5 ng) was found at 5th hour when stimulated with 7lakhs (table 3.21) of lymphocytes, whereas peak was formed was at 6th hour of the *invitro* reaction in 5 lakhs (table 3.22) of lymphocytes.

Table 3.21: Measurement of IL-1β release, in response to Gram positive bacterial pyrogen (Pooled sample - B +ve Donors – 7 lakhs)

Time	Control	LTA(10µl)	Concentration of IL-1β (ng/µl)
1hr	0.335	0.335	3
2hr	0.330	0.357	3
3hr	0.353	0.377	3
4hr	0.328	0.403	3
5hr	0.339	0.455	5
6hr	0.334	0.380	3
7hr	0.345	0.390	3
8hr	0.321	0.385	3

Figure 3.23: IL-1β Detection with 7 lakhs of lymphocytes

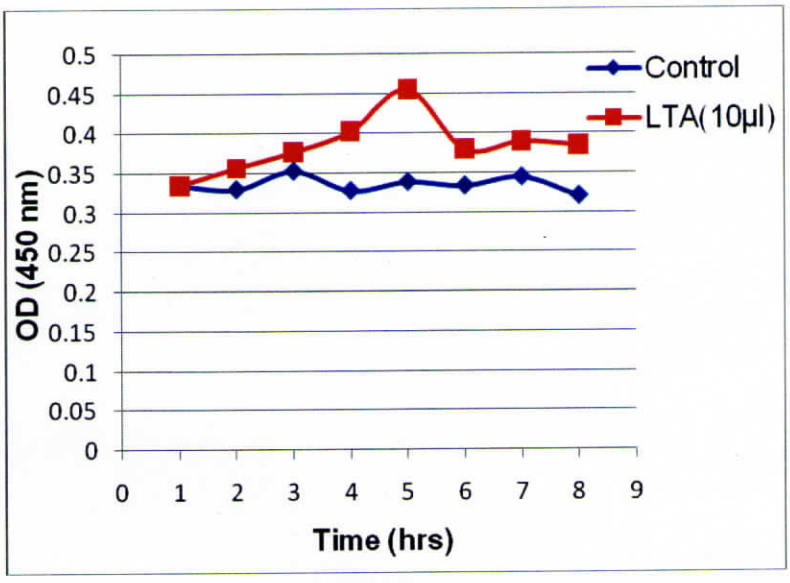
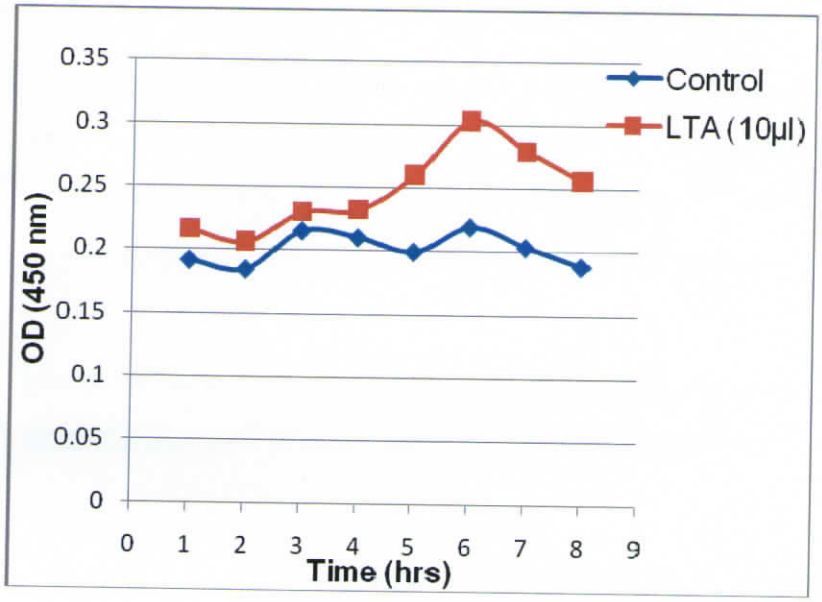


Table 3.22: Measurement of IL-1β release, in response to Gram positive bacterial pyrogen (Pooled sample - B +ve Donors - 5lakhs)

Time	Control	LTA (10µl)	Concentration of IL-1β (ng/µl)
1hr	0.192	0.217	1
2hr	0.185	0.207	1
3hr	0.216	0.231	1
4hr	0.211	0.233	1
5hr	0.2	0.261	1
6hr	0.22	0.304	1.6
7hr	0.204	0.28	1.5
8hr	0.189	0.257	1.5

Figure 3.24: IL-1β Detection with 5 lakhs of lymphocytes



3.1.12 Measurement of IL-1β release in response to Gram negative bacterial pyrogen (Pooled sample - AB +ve Donors)

The LPS (5EU) stimulated human lymphocytes induced IL-1β response at 6th hour, when 5 (fig: 3.26) and 7 (fig: 3.25) lakhs of lymphocytes concentrations were used. The concentrations of IL-1β are mentioned in the table 3.23 and 3.24.

Table 3.23: Measurement of IL-1β release in response to Gram negative bacterial pyrogen (Pooled sample - AB +ve Donors – 7 lakhs)

Time	Control	LPS (5µl)	Concentration of IL-1β (ng/µl)
1hr	0.198	0.221	1
2hr	0.188	0.209	1
3hr	0.204	0.242	1
4hr	0.220	0.266	1
5hr	0.195	0.264	1
6hr	0.218	0.318	1.5
7hr	0.211	0.281	1
8hr	0.217	0.279	1

Figure 3.25: IL-1β Detection with 7 lakhs of lymphocytes

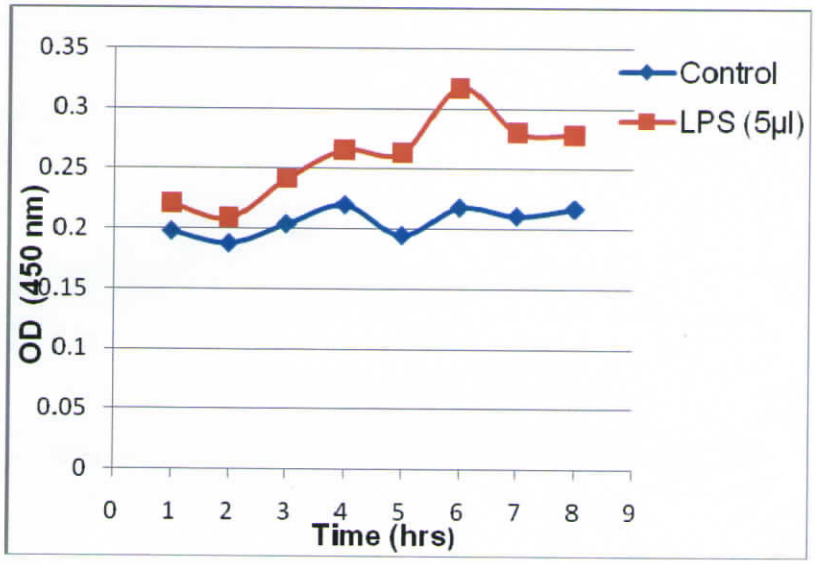


Table 3.24: Measurement of IL-1β release in response to Gram negative bacterial pyrogen (Pooled sample - AB +ve Donors – 5 lakhs)

Time	Control	LPS (5µl)	Concentration of IL-1β (ng/µl)
1hr	0.228	0.277	1
2hr	0.227	0.27	1
3hr	0.247	0.257	1
4hr	0.224	0.269	1
5hr	0.252	0.291	1
6hr	0.251	0.349	2
7hr	0.261	0.311	1
8hr	0.265	0.319	1

Figure 3.26: IL-1β detection with 5 lakhs of lymphocytes

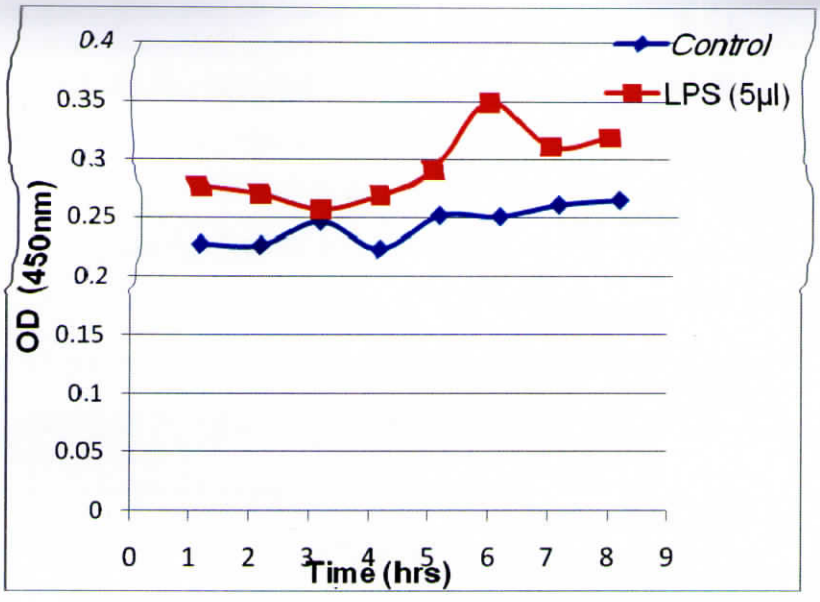


Figure 3.27: IL-1β Detection with 7 lakhs of lymphocytes

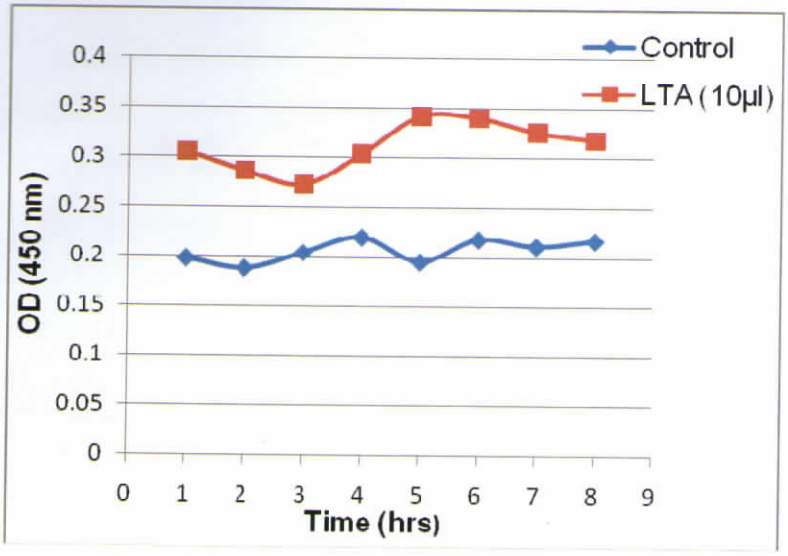


Table 3.26: Measurement of IL-1β release in response to Gram positive bacterial pyrogen (Pooled sample - AB +ve Donors – 5 lakhs)

Time	Control	LTA (10µl)	Concentration of IL-1β (ng/µl)
1hr	0.228	0.22	1
2hr	0.227	0.231	1
3hr	0.247	0.268	1
4hr	0.224	0.257	1
5hr	0.252	0.28	1
6hr	0.251	0.304	2
7hr	0.261	0.291	1
8hr	0.265	0.265	1

3.1.13 Measurement of IL-1β release in response to Gram positive bacterial pyrogen (Pooled sample - AB +ve Donors)

The results of the study are indicated in the figures 3.27 and 3.28. The maximum release of IL-1β was found at 5th hour in 7 lakhs of lymphocytes, which yielded 2 ng (Table 3.25) of IL-1 β. The stimulation of cells with 5lakh cell concentration induced a peak at 6th hour of the reaction.

Table 3.25: Measurement of IL-1β release in response to Gram positive bacterial pyrogen (Pooled sample - AB +ve Donors – 7 lakhs)

Time	Control	LTA (10µl)	Concentration of IL-1β (ng/µl)
1hr	0.198	0.306	1
2hr	0.188	0.287	1
3hr	0.204	0.274	1
4hr	0.220	0.305	1
5hr	0.195	0.343	2
6hr	0.218	0.341	2
7hr	0.211	0.327	1
8hr	0.217	0.319	1

Figure 3.27: IL-1β Detection with 7 lakhs of lymphocytes

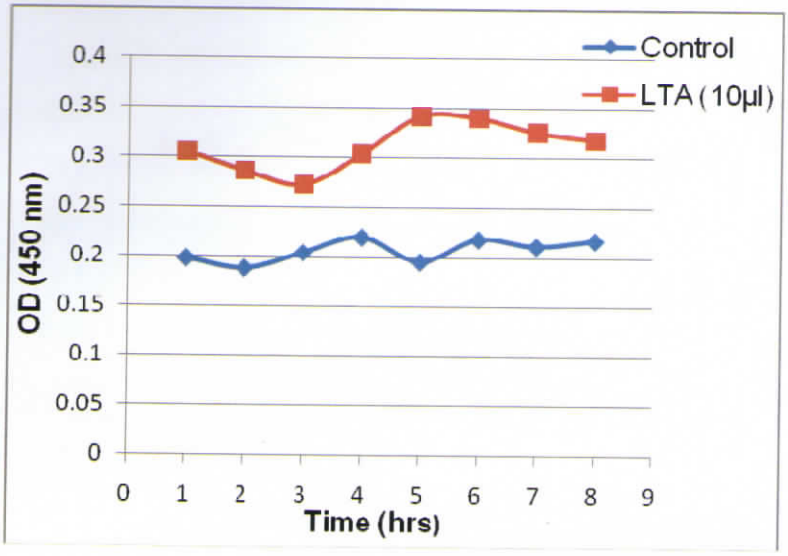
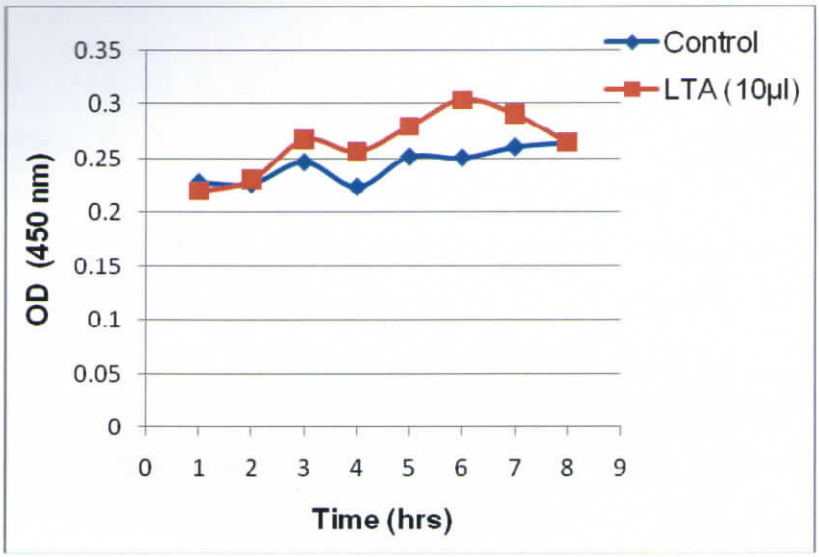


Table 3.26: Measurement of IL-1β release in response to Gram positive bacterial pyrogen (Pooled sample - AB +ve Donors – 5 lakhs)

Time	Control	LTA (10µl)	Concentration of IL-1β (ng/µl)
1hr	0.228	0.22	1
2hr	0.227	0.231	1
3hr	0.247	0.268	1
4hr	0.224	0.257	1
5hr	0.252	0.28	1
6hr	0.251	0.304	2
7hr	0.261	0.291	1
8hr	0.265	0.265	1

Figure 3.28: IL-1β Detection with 5 lakhs of lymphocytes



DISCUSSION

Cytokines are involved in several aspects of inflammatory reactions. The increasing awareness of the key role of pro-inflammatory cytokines like IL-1β in inducing pyrogenic response necessitates the development of assays for cytokine quantification which have become a rapidly expanding part of the laboratory repertoire. The selection of a suitable cytokine assay depends to a large extent on the research objective to be achieved. In the present study an attempt was made to isolate the lymphocyte cells from the human whole blood, to evaluate the release of IL-1β as a marker for pyrogenicity. The release of pro-inflammatory cytokine takes place when stimulated with 5EU of LPS and 1 µg/µl of LTA.

IL-1β is a potent pro-inflammatory cytokine secreted by blood monocytes and tissue macrophages, when it comes in contact with exogenous pyrogens (Dinarello *et al.* 1986). It has an advantage as a read out parameter for gram negative and gram positive pyrogens because of the shorter incubation time required for its release (Poole *et al.* 2003) and without pyrogenic (LPS and LTA) stimulation, no significant rise in IL-1β was observed. Furthermore, the release of IL-1β increases immediately after the initiation of incubation on challenge with exogenous pyrogens and reaches a maximum at 4 to 6^{th} hour and then stabilizes for both LPS and LTA. The data is supported by Jansky *et al.* (2003) findings, where net release of IL-1β in peripheral blood mononuclear cells increases immediately within the first few hours after the initiation of incubation with LPS and then remains stable during the 24-hour test.

The result of the ELISA method in lymphocytes collected from O +ve donors indicates that the release of IL-1β was at a peak on 6^{th} hour when stimulated with 5 EU of LPS at a cell concentration of 5 (fig:3.6) and 4 (fig: 3.7) lakhs of lymphocytes. The maximum release was at a peak on 5^{th} hour in 7 lakhs of

cells which resulted in the production of 3 ng of IL-1β. The amount of endotoxin required to generate a pyrogenic reaction is 0.5 EU/ml which is considered as the threshold of fever induction [Banerjee *et al.* 2011]. Further, it should be mentioned that the time course of IL-1β production after stimulation with LPS corresponds to the time course of fever development. Similarly, the IL-1β production in response to 1 µg/µl of LTA indicates a maximum IL-1β level at 6th hour of reaction when the concentration of lymphocyte was 7 (fig: 3.8) and 4 lakhs (fig: 3.10) cells. The cells yielded an IL-1β concentration of 1.7 ng, when challenged with 7 lakh cell concentration.

The IL-1β level was found to be fluctuating in certain control samples which created minor technical variations in the Sandwich ELISA method. The reason for the inconsistency may be due to the inhibitory factors affecting the IL-1β release. This was supported by the finding of Dinarello *et al.* (1996) that steady state levels of IL-1β increases with transient transcription and decreases due to the synthesis of a transcriptional repressor.

The results of the present study revealed a concentration dependent response of IL-1β towards lymphocytes with the pyrogenic stimulation of LPS and LTA. In O +ve donors, the concentration of IL-1β was increased from 1.5 ng to 3 ng (Tables 3.3, 3.4 and 3.5) when treated with LPS. Similarly, when treated with LTA, the IL-1 β concentration was seen increased from 1.5 ng to 1.7 ng compared to the LPS treated group. In another study conducted with A +ve donors, it was noticed that IL-1β formed a peak at 5th hour of the reaction when treated with 5 EU of LPS at a cell concentration of 7 lakhs (fig 3.11) and 5 lakhs (fig: 3.12). The reaction indicates a production of 1.6 ng of IL-1β when stimulated with 7 lakhs of lymphocytes. A similar dose response relationship study was carried out with1 µg/µl of LTA indicating a higher concentration of IL-1β (5 ng) as indicated in table 3.12. This was found to be a much higher concentration (5 ng) than the LPS induced reaction.

Furthermore, the extent of peak elicited by 5EU of LPS in inducing IL-1β release was found at 6th hour of the reaction (fig: 3.17) with 7 lakh lymphocyte concentration in pooled lymphocytes (A +ve Donors). The assay detected an IL-1β concentration of 1.5 ng during the initial period of pyrogenic stimulation which gradually increased to 20 ng. (Table: 3.15, 3.16). LTA also induced the highest IL-1β response at 6th hour in pooled sample (A +ve Donors) on stimulation with 7 lakh (fig 3.19) cell concentration.

In pooled lymphocytes (B +ve Donors), the peak IL-1β release elicited by 5 EU of LPS and 1 µg/µl of LTA took place at 6th (fig 3.21) and 5th hour (fig 3.23) of the *in vitro* reaction in 7 lakhs of lymphocytes. Similarly in pooled lymphocytes of AB +ve donors, LPS (5EU) stimulated human lymphocytes exhibited IL-1β response at 6th hour (fig 3.25, 3.26), when 5 and 7 lakhs of cell concentrations were used. Further, the IL-1β concentration was found as 2ng in 5 lakh cell concentrations, whereas 1.5 ng was present in 7 lakh cell concentration. A similar dose response relationship study was conducted with1 µg/µl of LTA which yielded 2 ng of IL-1 β in both 7 lakh (Table 3.25) and 5 lakh (Table 3.26) lymphocyte concentration.

The assay developed has a reduced incubation time and uses a different ELISA (Sandwich ELISA) detection strategy. After validation, the lymphocyte test system can be used to detect gram negative and gram positive pyrogens since it measures the IL-1β release even with small stimulations. The concentration of IL-1β and the threshold of fever induction can also be detected from the lymphocyte system. It was also observed that the difference in blood group was not interfered the IL-1β release. Based on the results obtained, it can be concluded that the isolated lymphocytes system can be a promising approach to the total replacement of animal experimentation.

SUMMARY AND CONCLUSION

Pyrogens are heterogenous group of fever and inflammation inducing substances derived from Gram positive and Gram negative bacteria, but also from fungi and viruses. They can provoke immune reaction as a part of the innate immune defense and is driven by monocytes, macrophages, neutrophilic granulocytes and also complement system. Pyrogens evoke the response by inducing pro-inflammatory cytokines like IL-1β, TNF-α etc. IL-1β binds to receptors on the blood side of circum ventricular organs of the brain and initiates the expression of enzyme cyclooxygenase-2, which converts arachidonic acid to prostaglandins (PG) E2 triggering the febrile response.

The *in vivo* rabbit pyrogen assay and Limulus Amoebocyte Lysate assays are the two methods used to detect the presence of pyrogenic contaminants. The rabbit pyrogen assay measures the change in body temperature after an intravenous injection of the sample but has limitations in its utility due to requirement of large number of animals. Meanwhile, LAL assay detects only LPS endotoxins from the cell wall components of gram negative bacteria and cannot detect the gram positive pyrogens.

The first pyrogen assay based on human whole blood stimulation by pyrogens was developed by Hartung and Wendel in 1996. Here, human blood is diluted in physiological saline and brought together with samples suspected of being contaminated. In the case of pyrogenicity, the monocytes are challenged to produce cytokines *in vitro*, and the cytokines can be measured by specific ELISAs.

The present study was carried out to evaluate the role of pyrogens in inducing inflammatory response, from human lymphocytes (O +ve, A +ve, B +ve and AB +ve donors). Lymphocytes isolated from blood of healthy individuals were

challenged with 5EU of gram negative (LPS) and 1 µg /µl of gram positive pyrogens (LTA) *in vitro* and the inflammatory cytokine, Interleukin 1β (IL-1β) release was measured by Sandwich ELISA method. Different lymphocyte concentrations (4, 5, 7 lakhs in individual blood & 5, 7 lakhs in pooled blood) were used for the *in vitro* pyrogen assay. The results suggests that the IL-1β release is concentration dependent, that is when more the cell number is, the more release of Interleukin 1β. Furthermore, the release of IL-1β increases immediately after the initiation of incubation and reaches a maximum at 4 to 6^{th} hour and then stabilizes for both the pyrogens. Based on the results obtained, it can be concluded that the isolated lymphocyte system can be used as an alternative test system to the *in vivo* rabbit pyrogen assay.

BIBLIOGRAPHY

Alluwaimi AM, Smith H, Sweet C. Role of surface glycoproteins in influenza virus pyrogenicity. Journal of General Virology.1994; 75: 2835-2840.

Banerjee S, P.V.Mohanan. Inflammatory response to pyrogens determined by a novel ELISA method using human whole blood. Journal of Immunological methods. 2011; 146-153.

Beutler B, Rietschel ET. Innate immune sensing and its roots: the story of endotoxin. Nat Rev Immunol. 2003; 3(2): 169-176.

Boivin A, Mesrobeanu L. *Rev. Immunol.* PubMed. 1936; 2: 113.

Boivin A., Mesrobeanu I, Mesrobeanu L. *C.R. Acad. Sci., Paris.* 1934; 198: 2124.

Bowie AG, Haga IR. The role of Toll-like receptors in the host response to viruses. Mol Immunol. 2005; 42(8): 859-867.

Braude AI, Mc Connel J, Douglas H. Fever from pathogenic fungi. J. Clin.Invest. 1960; 39:1266-1276.

Cooper JF, Levin J, Wagner HN. Quantitative comparison of in vitro and invivo methods for the detection of endotoxin. J.Lab.Clin.Med. 1971; 78:138-148

Camilla T Damsgaard, Lotte Lauritzen, Philip C Calder, Tanja MR Kjaer, Hanne Frokiaer. Journal of immunological methods. 2009; 340(2): 95-101

De Groote D, Zangerle PF, Gevaert Y, Fassotte MF, Beguin Y, Noizat-Pirenne F, Pirenne J, Gathy R, Lopez M, Dehart I, et al. Direct stimulation of cytokines (IL-1 beta, TNF-alpha, IL-6, IL-2, IFN-gamma and GM-CSF) in whole blood. I. Comparison with isolated PBMC stimulation. 1992; 4(3):239-48.

Daneshian M, Von Aulock S, Hartung T. Assessment of pyrogenic contaminations with validated human whole-blood assay. Nat Protoc. 2009; 4(12): 1709-1721.

Devleeschouwer MJ, Cornil MF, Dony J. Studies on the sensitivity and specificity of the Limulus amebocyte lysate test and rabbit pyrogen assays. Appl Environ Microbiol. 1985; 50(6): 1509-1511.

Dinarello CA. Infection, fever, and exogenous and endogenous pyrogens: some concepts have changed. J Endotoxin Res. 2004; 10(4): 201-222.

Dinarello CA. The IL-1 family and inflammatory diseases. Clin Exp Rheumatol. 2002 ; 20(5 Suppl 27): S1-13.

Dinarello CA. Biologic basis for interleukin-1 in disease. Blood. 1996; 87(6): 2095-2147.

Dinarello CA, Cannon JG, Wolff SM. New concepts on the pathogenesis of fever. Rev Infect Dis. 1988; 10(1): 168-189.

Dinarello CA, Wolff SM. Pathogenesis of fever in man. N Engl J Med. 1978; 298(11): 607-612.

Garrett M, Guenette C, Haith E, Prince C. Pyrogenic polycyclic aromatic hydrocarbons in oil burn residues. Environ. Sci. Technol. 2000; 34:1934-1937

Ginsburg I. Role of lipoteichoic acid in infection and inflammation. Lancet Infect Dis. 2002; 2(3): 171-179.

Hartung T, Aaberge I, Berthold S, Carlin G, Charton E, Coecke S. Novel pyrogen tests based on the human fever reaction. The report and recommendations of ECVAM Workshop 43. European Centre for the Validation of Alternative Methods. European Centre for the Validation of Alternative Methods. Altern Lab Anim. 2001; 29(2): 99-123.

Hartung T, Wendel A. Detection of Pyrogens using human whole blood. ALTEX. 1995; 12(2): 70-75.

Hartung T, Wendel A. Detection of Pyrogens using human whole blood. In vitro Toxicology. 1996; 9, 353-359.

Ilaria Bononi, Veronica Balatti, Soccorso Gaeta, Mauro Tognon. Gram-Negative Bacterial Lipopolysaccharide Retention by a Positively Charged New-Generation Filter. Appl Environ Microbiol. 2008; 74(20): 6470–6472.

Jansky L, Reymanova P, Kopecky J. Dynamics of cytokine production in human peripheral blood mononuclear cells stimulated by LPS or infected by Borrelia. Physiol Res. 2003; 52(6):593-8.

Katsuura G, Arimura A, Koves K, Gottschall PE. Involvement of organum vasculosum of lamina terminalis and preoptic area in interleukin 1 beta-induced ACTH release. Am J Physiol. 1990; 258(1 Pt 1): E163-171.

Kenneth J Jakeman, Christopher R Bird, Robin Thorpe, Harry Smith, Clive sweet. Nature of the endogenous pyrogen(EP) induce by influenza virus: lack of correlation between EP levels and content of the known pyrogenic cytokines, interleukin 1, interleukin 6 and tumor necrosis factor. Journal of general virology 1991;72, 705-709

Mohanan P, Siddharth B, Geetha C. An indigenously developed human whole blood assay for pyrogenicity: A comparative assessment. Toxicology letters. 2010; 196S: S37- S351.

Mohanan P.V, Siddharth B, Geetha C.S Journal of Pharmaceutical and Biomedical Analysis 55. 2011; 1170–1174

Mascoli CC, Weary ME. Applications and advantages of the Limulus amebocyte lysate (LAL) pyrogen test for parenteral injectable products. Prog Clin Biol Res. 1979; 29: 387-402.

Morath S, Geyer A, Hartung T. Structure-function relationship of cytokine induction by lipoteichoic acid from Staphylococcus aureus. J Exp Med. 2001; 193(3): 393-397.

Mazzotti F, Beuttler J, Zeller R, Fink U, Schindler S, Wendel A, *et al.* J Biomed Mater Res. 2007; 80 A: 276-282.

Nakagawa Y, Murai T, Hasegawa C, Hirata M, Tsuchiya T, Yagami T, *et al.* Endotoxin contamination in wound dressings made of natural biomaterials. J Biomed Mater Res B Appl Biomater. 2003; 66(1): 347-355.

Petsch D, Anspach FB. Endotoxin removal from protein solutions. J Biotechnol. 2000; 76(2-3): 97-119.

Roth J, De Souza GE. Fever induction pathways: evidence from responses to

systemic or local cytokine formation. Braz J Med Biol Res. 2001; 34(3): 301-314.

Schindler S, Bristow A, Carmell T.Comparison of the reactivity of human and rabbit blood towards pyrogenic stimuli.2002; Altex 20, 59-63.

Poole S, Yogesh Mistry, Christine Ball, Rose E.,Gaines Das, Lisa Pieti Opie, Gary Tucker, Mehul. Journal of Immunological methods. 2003; 209-220

Tanamoto Ken-ichi, Takatoshi Iida, Yuji Haishima, Satoko Azumi. Endotoxic properties of lipid A from *Comamonas testosterone.* 2001; 1087-1094

Van Amersfoort ES, Van Berkel TJ, Kuiper J. Receptors, mediators and mechanism involved in bacterial sepsis and bacterial shock. Clin Microbiol Rev. 2003;16: 379-415

Wang JE, Dahle MK, McDonald M, Foster SJ, Aasen AO, Thiemermann C. Peptidoglycan and lipoteichoic acid in gram-positive bacterial sepsis: receptors, signal transduction, biological effects, and synergism. Shock. 2003; 20(5): 402-414.

Wright SD, Ramos RA, Hermanowski-Vosatka A, Rockwell P, Detmers PA. Activation of the adhesive capacity of CR3 on neutrophils by endotoxin: dependence on lipopolysaccharide binding protein and CD14. J Exp Med. 1991; 173(5): 1281-1286.

Westphal O, Luderitz O. Chemical studies of the lipopolysaccharides of gram-negative bacteria Angew. Chem. 1954; 66:407-417.

ANNEXURE

Preparation of Reagents:

50 mM Carbonate bicarbonate buffer

Sodium carbonate - 1.59 g

Sodium bicarbonate - 2.93 g

The above constituents were dissolved in 1 litre of deionized water and the pH was adjusted to 9.6. Filtered, sterilized using 45µm filter paper and stored at 4°C.The shelf life of this buffer is one month.

10X PBS

Sodium chloride - 40g

Potassium chloride -1g

Disodium hydrogen phosphate - 5.75g

Potassium dihydrogen phosphate - 1g

The constituents were dissolved in deionized water and made up to a final volume of 500ml. The pH was adjusted to 7.2. Filter sterilized using 45µm filter paper and stored at room temperature. 10X PBS were then diluted to 1X PBS which was used as working standard.

1% BSA

1g BSA was dissolved in 100 ml of 1XPBS.

1M H_2SO_4

55.6 ml of concentrated sulfuric acid was diluted in 1 litre of deionized water.