EFFECT OF LEVAMISOLE ON HUMAN MULTIPLE MYELOMA CELL LINES *IN VITRO*- ALKALINE PHOSPHATASE ACTIVITY AS A "PUTATIVE" TARGET

Thesis submitted for the degree of

Doctor of Philosophy

By

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CERTIFICATE

This is to certify that this thesis entitled **"Effect of levamisole on human multiple myeloma cell lines *in vitro* - Alkaline phosphatase activity as a "putative" target",** submitted to The University of Hyderabad by **Ms. B.Nageshwari,** for the degree of Doctor of Philosophy, is based on studies carried out by her under my supervision. I declare to the best of my knowledge that no part of this thesis was earlier submitted for the award of any research degree of any other University / Institution.

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DECLARATION

I hereby declare that the work presented in this thesis has been carried out by me in the Department of Biochemistry, School of Life Sciences, University of Hyderabad, under the supervision of Prof. M. Ramanadham. I further declare that this work has not been submitted for the award of any research degree of any other University or Institution.

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CONTENTS

LIST OF ABBREVIATIONS

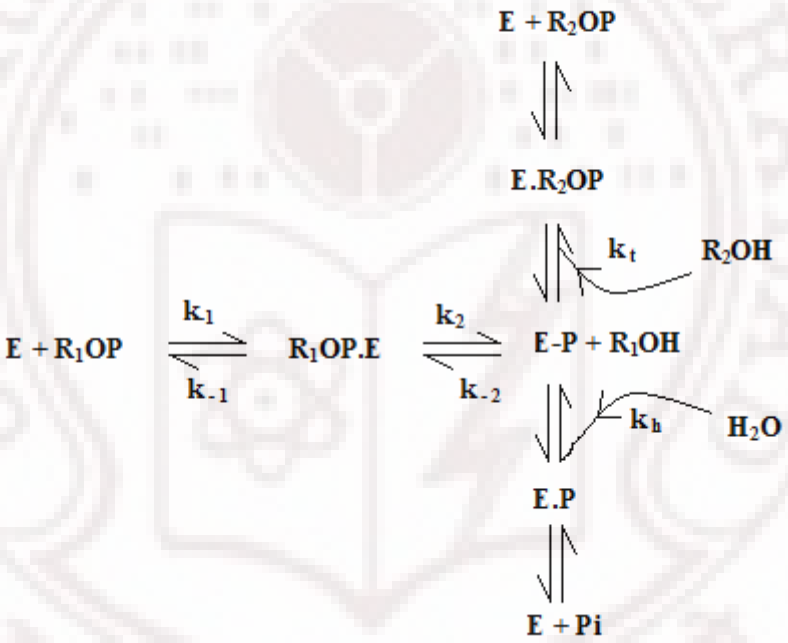
APase	Alkaline phosphatase
BCIP	5-Bromo-4-Chloro-3- Indolyl phosphate
Con A	Concanavalin A
DEA	Diethanolamine
ELISA	Enzyme linked immunosorbent assay
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
GCAPase	Germ cell alkaline phosphatase
GPI	Glycosyl phosphatidyl inositol
HPLC	High Performance Liquid Chromatography
^{3}H-Td	Tritiated thymidine
IAPase	Intestinal Alkaline Phosphatase
kDa	KiloDaltons
IL	Interleukin
LPS	Lipopolysaccharide
Lev	Levamisole
MM	Multiple Myeloma
MTT	3-[4,5-dimethylthiazole-2-yl]-2,5 diphenyl tetrazolium bromide
NBT	Nitro blue tetrazolium
PBL	Peripheral Blood Lymphocytes
PPO	2,5- Diphenyloxazole
POPOP	1,4-bis(5-phenyloxasol-2-yl)benzene
PHA	Phytohemagglutinin
PLAPase	Placental Alkaline Phosphatase
p-NPP	p- Nitro Phenyl Phosphate
PWM	Poke Weed Mitogen
RPMI	Rosewell Park Memorial Institute

SDS-PAGE	Sodium Dodecyl Sulphate- Polyacryl Amide Gel Electrophoresis
SPA	Staphylococcal protein A
TMB	3,3,5,5-Tetramethylbenzidine
TNAPase	Tissue Non Specific Alkaline Phosphatase.
NADH	Nicotinamide Adenine Dinucleotide reduced

INTRODUCTION

Alkaline phosphatase

Alkaline Phosphatase (APase, EC 3.1.3.1), an orthophosphoric-monoester phosphohydrolase, is present in most species from bacteria to man (McComb et al 1979, Millan 2006). The enzyme was first isolated from ossifying bone and cartilage (Robinson 1923). The enzyme catalyzes the hydrolysis of phosphomonoesters, including Tyr/Ser/Thr-phosphates in phospoproteins with release of inorganic phosphate and alcohol at an alkaline pH between pH 8.2 to 10.7(Schwartz & Lipmann 1961, Mc Comb et al 1979, Chan & Stinson 1986, Pezzi et al 1991, Sarroulihe et al 1992, Coleman 1992).



Reaction mechanism of Alkaline phosphatase : Scheme of the reaction path for APase (E) that takes into account both transphosphorylation (k_t) and hydrolysis (k_h). R_1OP, phosphate monoester; $R_1OP.E$, noncovalent enzyme complex; E-P, phosphoenzyme complex; R_1OH, alcohol product; R_2OH. phosphate acceptor; R_2OP, phosphorylated acceptor; Pi. Inorganic phosphate. (Taken from Coleman JE, Gettins P. Spiro TG, ed. In: Zinc enzymes, Vol. 5. pp. 153-218. New York, Wiley-Interscience, 1983).

The enzyme is inactivated under acidic conditions(Fishman & Ghosh 1967). The pH optimum is affected by the type and concentration of the substrate (Ross et al 1951, Fedde et al 1988). The catalytic activity of APases depends on a multimeric configuration of identical monomers. Each identical subunit possesses one active site, and contains two Zn^{2+} ions and one Mg^{2+} ion that stabilize the tertiary

structure. (Coleman & Calebowski 1979, Kim and Wyckoff 1991). APase is a metalloenzyme believed to be homodimeric in serum and membranes, but the tissue non-specific isoform may exist as a homodimeric and/or homotetrameric structure in membranes (Hawrylak & Stinson1987). Unlike the bacterial enzymes, mammalian APases are all anchored to the plasma membrane via a glycophosphatidylinositol moiety (Udenfriend & Kodukula 1995).

APase – Genomic localization

In humans, three out of the four APase isoenzymes are tissue specific: placental (PLAPase); germ cell (GCAPase); and intestinal (IAPase). They are 90-98% homologous at the protein level with PLAPase and GCAPase differing only by 12 aminoacid substitutions. The genes encoding these enzymes are clustered on chromosome 2q37.1. The fourth isozyme, tissue non-specific APase (TNAPase) is ubiquitous particularly abundant in bone, liver and kidney and is only about 50% identical with the other three APases (Stigbrand 1984, Harris 1989, Millan 1992).

Post-translational modification and differential glycosylation of TNAPase gives rise to tissue-specific APases of liver, bone and kidney (Komoda & Sakagishi 1978). It was hypothesized that the physiological role of the sugar moieties could be in the protection of the enzyme from rapid removal from circulation through binding to the asialoglycoprotein receptors of the liver. The different isoenzymes of APase can be differentiated based on their structure, immunological properties and sensitivity to heat and different inhibitors.

The gene encoding TNAPase is localized on short arm of chromosome 1 in humans and on chromosome 4 in mice (Terao et al 1990, Greenberg et al 1990). The gene encoding TNAPase consists of 12 exons distributed over 50kb (Weiss et al 1988a).

Intestinal and placental APase genes are present closely on chromosome 2 in both mice as well as humans (Weiss et al 1988b, Henthorn et al 1988, Knoll et al 1989).

The tissue specificity is achieved by differential transcription and subsequent co-translational and post-translational modifications controlled by two alternative exons (Terao 1990, Matsuura et al 1990, Studer et al 1991). The transcript of exon I-B is found in bone and kidney while the transcript of exon I- L is found in liver. The mechanism of tissue-specific regulation of TNAP is not yet known in detail.

All APases share the same core three-dimensional structure, as suggested by the sequence alignment of the three available structures from E.coli, human placental and shrimp. (Kim & Wyckoff 1991, Le Du et al 2001, De Backer et al 2002). However important differences exist in the peripheral domains of the molecule. The four mammalian isozymes exhibit characteristic differences in a number of functional properties, in particular in their variable heat stability (Bossi et al 1993), allosteric regulation (Holyaerts et al 1997), binding to collagen, (Wu et al 1991) and uncompetitive inhibition by various modulators (Hummer & Millan 1991, Holyaerts & Millan 1991, Holyaerts et al 1992, Kozlenkov et al 2002).

Structure of Tissue Non specific Alkaline Phosphatase (TNAPase)

Recent determination of the human PLAPase structure provides a complete view of an APase from higher organisms (Le Du et al 2001). Based on the structural evidence and functional divergence of APases conservation in mammals has been proposed (Mornet et al 2001, Le Du & Millan 2002).

The molecular and structural data suggests that APase acquired specialized functions progressively during evolution. Five functional regions have been identified in TNAPase. The active site is the centre of the catalytic activity. The N-terminal arm is essential for stability and allosteric properties of the enzyme (Hoylaerts 2006).

The crown domain is a key factor of uncompetitive inhibition heat stability and allosteric behaviour (Bossi et al 1993, Hoylaerts et al 1997, Kozlenkov et al 2004). The crown domain may also be involved in the binding of TNAP to collagen

(Holyaerts and Millan 1991, Bossi et al 1993) corroborating previous studies that suggested this property of TNAP (Vittur et al 1984, Wu et al 1991).

Mammalian APases can be inhibited uncompetitively by a wide range of compounds, which include amino acids, tetramisole, theophylline and NADH. Inhibition by amino acids is stereospecific as L- isomers only display inhibitory activity. Similarly, only the L-isomer of tetramisole (Levamisole) is a good inhibitor of certain APases (Van Belle 1976).

L-Phenylalanine and L-Tryptophan, amino acids with hydrophobic side chains, inhibit mammalian IAPases and human PLAPases with Ki values of about 1mM (Ghosh & Fishman 1966). They are not active towards TNAPase. On the other hand, amino acids with positively charged side chain, such as L-lysine, L-arginine, and especially L-homoarginine, are active as inhibitors with similar Ki values towards TNAPase but not towards other isozymes (Fishman & Sie 1970). Levamisole is also a good selective inhibitor of TNAPases, with Ki values lower than 100μM (Jalanka & Lindberg 1975).

B-Lymphocyte APase :

APase activity has been shown to be expressed specifically by mitogen-stimulated B-lymphocytes (Greaves & Janossy 1972). It has also been used as a marker of B cell activation (Garcia-Rozas et al 1982, Burg & Feldbush 1989, Marquez et al 1989). Resting B-lymphocytes and resting and activated T lymphocytes do not express APase activity (Garcia-Rozas et al 1982).

Expression of Apase activity occurs in early G1 phase (around eight hrs) after mitogenic stimulation and continues till differentiation (120 hr) (Kasyapa & Ramanadham 1992).

It has also been shown that the enhancement of APase activity correlates with proliferation and differentiation. It precedes maximal immunoglobulin secretion (Ohno et al 1986, Burg & Feldbush 1989, Marquez et al 1989, Kasyapa & Ramanadham 1992, Souvannavong et al 1994).

APase has also been proposed to be involved in the Ig transport in B-lymphocytes as it was shown to be complexed and secreted out along with IgM. A role for APase in phosphorylation/ dephosphorylation reactions in early stages of signalling in mitogen activated B lymphocytes has also been suggested (Feldbush & Lafrenz 1991, Souvannavong et al 1992).

It has also been shown that APase activity is not expressed in B-lymphocytes stimulated with incomplete mitogens like (Kasyapa 1996). Further studies have shown that APase activity is expressed in B-lymphocytes committed to proliferation. It has also been shown to be enhanced in antigen activated B-lymphocytes and the activity increases further in the antibody secreting cells (Padmaja & Ramanadham, 1998).

cAMP has been reported as a positive regulator of enhancement of APase activity in mitogen stimulated B-cells (Kasyapa & Ramanadham 1995) . Treatment of mitogen stimulated cells with antibody to APase resulted in the inhibition of proliferation and APase activity. It has been shown that the level of APase mRNA increased in mitogen stimulated cells and probably plays a crucial role in cell cycle progression.

Normal B cells do not spontaneously show APase activity but cell activation induces APase expression (Burg & Feldbush 1989, Marquez et al 1989, Souvannavong & Adam 1990). Importance of APase is indicated by the capacity of antibodies to various glycosyl Phosphatidylinositol anchored molecules to transduce activation signals(Low, 1989).

Several drugs like puromycin, actinomycin D, Colchicine enhance APase activity in culture (Moog 1964, Ikehera et al 1978, Moog & Wiemerslage 1981), Levamisole, an uncompetitive inhibitor of TNAPase has been shown to inhibit APase activity of liver, bone and kidney. 5-‘Bromdeoxyuridine, a thymidine analogue, dbtc AMP, an analogue of cAMP, Sodium butyrate and prednisolone an analogue of hydrocortisone are some of the potent inducers of APase activity

(Koyama & Ono 1971, Hamilton et al 1979, Firestone & Heath 1981, Herz & Hawler 1983).APase substrates like phenyl phosphate have also been shown to enhance APase activity.

Functions of Alkaline Phosphatase

Although APase has been studied for many years, its role has remained largely enigmatic and is still under intensive investigation. Like many other GPI anchored proteins, APase has also been proposed to be involved in transmembrane signalling function. As the GPI anchored proteins are located on the outer leaflet of the bilayer of cell membrane, they are more mobile than other cell surface proteins and may be involved in cell-cell interaction, reception or transduction of extracellular stimuli. Low & Saltiel 1988 have shown that GPI anchor acts as an apical targeting signal in the polarized epithelial cell and in this way could act as an activation antigen in the immune system.

APase has little preference for a particular substrate and will hydrolyze all the phosphomonoesters but not diesters. Catalysis includes phosphorylation of a serine residue at the active site followed by the delivery of the phosphoryl group to either water (phospo hydrolysis) or to an organic acceptor alcohol (phosphor tranferase) (Herraez et al 1980, Sarrouilhe et al 1992). However, phosphoester cleavage is faster if the transfer of phosphate is to an acceptor rather than to water.

APase has been shown to be involved in cell adhesion (Hui & Tenenbaum 1993), proliferation differentiation (Fedarko et al 1990, Owen et al 1990, Andracchi & Korte 1991), and a protein tyrosine phosphatase in some tissues (Swarup et al 1981, Lau et al 1985). In liver plasma membranes, a 18KDa phosphoprotein has been demonstrated as a substrate for dephosphorylation by APase (Chan & Stinson 1986).

It has also been shown that APase is involved in the regulation of phosphate transport in intestine, kidney and in calcium transport in the intestinal and kidney epithelial cells.

Functions of Tissue Non- specific Alkaline Phosphatase

TNAPase cleaves the extracellular substrates - inorganic pyrophosphate (PPi), Pyridoxal-5'-phosphate (PLP) and phosphoethanolamine (PEA) (Millan 2006). Function of TNAPase in bone and dental mineralisation is likely to involve hydrolysis of PPi, to maintain a proper concentration of this inhibitor to ensure normal bone mineralization and collagen and calcium binding (Whyte 1989, Hoylaerts et al 1997, Whyte 2001, Mornet et al 2001, Hessle et al 2002).

Significant physiological function of APase in mammals is provided by studies of human hypophosphatasia where a deficiency in the TNAPase is caused by deactivating mutations in its gene (Weiss et al 1986, Henthorn et al 1992, Mornet 2000) . This is associated with defective bone mineralisation in the form of rickets and osteomalacia (Whyte 2001).

Pyridoxal phosphate (PLP) is the phosphorylated form of pyridoxine. TNAPase hydrolyses PLP, and the unphosphorylated pyridoxal crosses the blood brain barrier to be regenerated as PLP in the cell (Whyte 2001). Consequently, in patients with hypophosphatasia, inability to cleave PLP has been shown to result in a localized vitamin B6 deficiency in the central nervous system causing seizures (Waymire et al 1995). TNAPase could be also involved in the intra neuronal balance between pyridoxal and PLP. In addition, studies of TNAPase activity in primate brain proposes an important role for this enzyme in neurotransmission (Fonta et al 2004, Fonta et al 2005)

Functions of Intestinal Alkaline Phosphatase (IAPase)

It is expressed in the small intestine of many species. Lymph and serum levels of IAPase increase after a fatty meal (Glickman et al 1970, Mc Comb et al 1979). IAPase is found associated with the brush border of the intestinal epithelium and

enriched in Surfactant-Like Particles (SLP) . (Goetz et al 1997, Eliakim et al 1989, Eliakim et al 1997). IAPase isozyme is likely to be involved in the intestinal absorption of lipids/nutrients across the cell membranes via its association with SLPs (Zhang et al 1996, Narisawa et al 2003).

Functions of Placental Alkaline phosphatase (PLAPase)

PLAPase has been proposed to be involved in the transfer of maternal IgG to the foetus during gestation. It acts as a Fc receptor and has been shown to be involved in the internalization of IgG in HepG2 cell line. (Makiya & Stigbrand 1992 a and 1992 b, Stefaner et al 1997).

Other studies indicate a role of PLAP in regulation of cell division (Telfer & Green 1993, She et al 2000a). PLAPase stimulates DNA synthesis and cell proliferation in synergism with insulin, zinc and calcium and that it also acts as a survival factor in combination with ATP in serum-starved mouse embryonic and human fetal fibroblast cultures (She et al 2000b). Since PLAP is synthesised in the placenta, its effects on the growth and survival of fetal cells strongly suggest that it may have a key role regulating the growth of the fetus.

Functions of Germ Cell Alkaline Phosphatase (GCAPase)

It had been postulated that germ cell APase (GCAP) may be able to interact with extracellular matrix proteins and therefore serves as the cell guidance molecule during the migration of germ cells (Millan 1990). Ligands involved in directing the cell migration via APase binding might be phosphoprotiens representing its natural substrates. The enzyme activity expression has been shown to be stage specific during embryonic development.

Alkaline Phosphatase in Diseases

An tumor marker would be one that is produced solely by the tumour and is secreted in measurable amounts in body fluids. (Jacobs & Haskell 1991). While at present no such ideal marker exists, amongst the APase isozymes, PLAPase and

GCAPase come closest to the definition given above and therefore have been evaluated extensively in various malignant conditions. TNAPase due to its ubiquitous nature fails to serve as a marker of primary malignancy and its increased level is often taken as a confirmatory finding

Human APases are abundantly expressed in tumor cells, and serum levels of APase isozymes are often used as tumor markers (Millan & Fishman 1995). Many different isozyme patterns have been reported in malignancies and renal diseases (De Broe & Van Hoof 1991). APase activity provides the clinician valuable information for diagnosis and follow up of patients during treatment.

The different mechanisms that have been suggested for the enhanced APase expression in tumor cells are:

1. Functional involvement of APase isozymes in tumorgenesis,
2. Representing one crucial factor in a multifactorial etiology.
3. A close linkage of APase gene with disease susceptibility.
4. Simultaneous deregulation with disease susceptibility gene.
5. Result of random chromosomal aberrations.

APase activity has been reported to be enhanced in cancer patients (Gordon 1993). Various tumor cell lines like teratocarcinomas and osteaosarcomas also show elevated APase activity (Hamilton et al 1979, Benham et al 1981a). Hemopoietic tumor cell lines have been shown to express high APase activity (Neumann et al 1976). Especially in B lymphoid cell lines, APase activity has been reported to be high (Culvenor et al 1981). Thus expression of APase is considered as one of the important identifiable markers of malignancy (Ruddon 1987, Millan & Fishman 1995). Deficiency of TNAPase is associated with hypophosphatasia which manifests as a rare form of rickets and osteomalacia (Henthorn et al 1992, Whyte 1995). Additionally, hypophosphatsia abnormalities in the metabolism of pyridoxal-5'-phosphate (PLP), 'putative' natural substrate of TNAPase, leads to epileptic seizures, apnea and perinatal death (Waymire et al 1995, Narisawa et al 2001). Elevated plasma TNAPase levels have been reported in osteosarcomas,

Paget's disease and osteoblastic bone metastases (Deftos et al 1991, Farley et al 1991, Demers et al 1995). Plasma TNAPase level (particularly heat labile bone derived fraction) has long been recognized as an indicator of osteoblastic activity (Leunge et al 1993). Osteosarcomas display high serum TNAPase levels and these levels have been shown to be higher in metastatic disease than in patients with localized disease (Bacci et al 1993). Leukocyte TNAPase has been shown to serve as a useful marker in cases of advanced lung cancer (Walach & Gur 1993). GCAPase is a useful immunohistochemical marker of carcinoma-in-situ of the testis and IAPase is a marker of hepatocellular carcinoma (Higashino et al 1975, Wahren et al 1979, Jeppsson et al 1984, Roelofs et al 1999). While APases are homodimeric molecules, there is re-expression in cancer cells of more than one APase isozyme in human cancer cell lines and cancer sera (Higashino et al 1972, Higashino et al 1977). The human postnatal intestine also contains heterodimers of IAP and PLAP (Behrens et al 1983). Ovarian cancer cells often express both PLAPase and GCAPase (Smans et al 1999) and cell lines derived from these tumors have been shown to express PLAPase/GCAPase heterodimers (Watanabe et al 1989, Hendrix et al 1990). Increased PLAPase activity has frequently been found in serum samples from ovarian cancer patients and testicular cancer patients (Vergote et al 1987, De Broe & Pollet 1988, Lange et al 1982). PLAPase is a marker of cancer of the ovary, testis, lung, and the gastrointestinal tract (Nathanson & Fishman 1971, Jacoby & Bagshawe 1971, Loose et al 1984).

Immune response and B lymphocyte activation

Innate and acquired immunes responses form the defense mechanisms for survival of living organisms against a wide variety of pathogens. T lymphocytes carry out cell mediated immunity while B-cell differentiation to antibody secreting cells forms the basis of the humoral adaptive immune system. The resting B lymphocyte is small, quiescent, non dividing cell with low metabolic activity and have been shown to be in G0 phase of the cell cycle. (Monroe & Cambier 1983a,b&c). They express cell surface IgM and IgD that recognise and help in specific binding of antigen leading to activation. From primary lymphoid organs mature lymphocytes circulate through blood and upon encounter with foreign antigen in the secondary

lymphoid organs, B cells are activated in either a T cell independent (TI) or T-cell dependent (TD) manner. The B cell undergoes several morphological and metabolic changes after mitogenic activation (Anderson et al 1972). TD-Ag responses often involve soluble factors that aid in activation and clonal expansion of antigen specific B cells (De Franco 1987).

In response to TD-Ag, activated B cells enter the primary follicles of secondary lymphoid organs including spleen, lymphnodes, Peyer's patches or tonsils where they undergo rapid proliferation and form germinal centers (GC). In GC, Plasma Cells and memory B cells are generated, that drive a potent immune response upon re-exposure to same antigen.

B cell antigen receptor

The B cell antigen receptor is involved in antigen recognition and signal transduction necessary for B cell activation (De Franco 1987 & 1993, Pleiman et al 1994). It endocytoses the antigen which is processed and presented to the helper T cells (Myers 1991).

Interaction of antigen with B cell antigen receptor

It is a multiplolypeptide chain composed of monomeric IgM and IgD molecules non-covalently associated with two accessory molecules Igα, Igβ and Igγ linked through disulfide linkages (Campbell & Cambier 1990, Chen et al 1990, Friedrich et al 1993, Park house 1990).

The binding of antigen to the immature B cell receptor leads to either clonal deletion or clonal anergy (Burnet 1959, Hartley et al 1991, Goodnow 1992), while binding to mature B cell leads to activation and clonal proliferation. Nature of antigen, differentiation state of the B cell and nature of additional signals by helper T cells have influence on activation pathway followed by B cell. Multivalent antigen complexed with carbohydrate induces strong activation leading to cell proliferation (Mosier & Subbarao 1982). Paucivalent and proteinaceous antigen requires additional signals from activated T cells to induce activation (Monroe & Cambier 1983c, Noelle et al 1983). Thus signal derived from ligand binding and second signal provided during the G1 phase of the cell cycle mostly by the T cell

derived soluble factors induce further proliferation and differentiation of B cell (Brestcher 1975, Anderson et al 1979 & 1980).

Signal transduction through B cell antigen receptor

B cell receptor engagement to cognate antigen results in the activation of the SRC-family protein tyrosine kinase LYN and the cytoplasmic tyrosine kinase SYK (spleen tyrosine kinase), both of which phosphorylate target sequences, such as tyrosine residues in immunoreceptor tyrosine based activation motfs (ITAMs) in the cyoplasmic tails of Igα and Igβ (Sanchez 1993, Flaswinkel & Reth 1994,). Subsequently, the SYK-mediated phosphorylation of ITAMs of BCR complexes amplifies the signal and initiates a positive feedback loop. Ultimately SYK has a central role in the activation of pathways that regulate the B-cell proliferation and differentiation.

With regard to proliferation, it has been shown that constitutively active SYK induces constitutive phosphorylation and activation of the lipid-modifying kinase phosphoinositide 3 kinase (PI3K), which regulates diverse biological processes, including cell growth, survival, proliferation, migration and metabolism.(Deane & Fruman 2004, Kanie 2004, Streubel 2006).

The signaling pathways and regulation mechanisms may vary in different species. For example, the toll-like receptor (TLR)-4 agonist lipopolysaccaride (LPS) alone efficiently activates murine naïve B cells but not human naïve B cells (Lanzavecchia 2006) because of the absence of TLR4 expression in the latter(Muzio 2000).

B cell co-receptors in signal transduction

Downstream of the BCR, SYK and SRC family protein tyrosine kinases induce phosphorylation of the co-receptor protein CD19 and /or the adaptor protein B-cell PI3K adaptor(BCAP), resulting in the recruitment and activation of PI3K (Deane & Fruman 2004, Aiba et al 2008). Subsequently, PI3K phosphorylates its

substrate phosphotidylinositol-4,5-bisphosphate (PtdIns(4,5)P2), thereby generating the second messenger, phosphatidyl-inositol-3,4,5-triphosphate (PtdIns (3,4,5)P3) (Okkenhaug & Vanhaesebroeck 2003)B, which recruits signaling molecules that have a pleckstrin-homology domain to the plasma membrane (Vanhaesebroeck & Alessi 2000). These molecules include the serine / threonine kinase protein kinase B (PKB) and 3-phosphoinositide-dependent protein kinase 1 (PDK1). PKB is dominant mediator for controlling cellular proliferation downstream of PI3K and its activating kinase (PDK1). (Manning & Cantley 2007).

Because SYK activates PI3K downstream of the BCR, tight regulation of BCR-induced signaling is important to avoid abnormal PKB activity, which can result in uncontrolled cellular expansion and malignant transformation.

Role of cAMP and cGMP during B lymphocyte activation

cAMP and cGMP, cyclic nucleotides which act as second messengers are produced and participate in signal transduction during B lymphocyte activation (Kammer 1988, Sutherland 1972). It has been shown that cAMP acts synergistically with LPS in activating the B lymphocyte proliferative response activity (Kasyapa & Ramanadham 1995).

Transcription factors that regulate B cell activation and differentiation

The ensuing differentiation of B cells during the GC reaction is regulated by a complex network of transcription factors. PC differentiation is most likely initiated by the doenregulation of PAX-5, the 'identity' gene of Bcells. B-cell lymphoma (BCL)-6, is upregulated and suppresses apoptosis and promotes proliferation. B lymphocyte induced maturation protein (BLIMP)-1, a zinc finger-containing transcriptional repressor, induced in B cells and PCs. The X-box binding protein 1 (XBP-1) is upregulated in PCs and induces secretory phenotype of the PCs. Interferon regulatory factor (IRF)-4 is involved in PC differentiation. (Shapiro-Shelef & Calame 2005, Tarlinton 2008). Importantly, B cell malignancies are often associated with aberrant expression of GC transcription factors.

Lymphokines in B lymphocyte activation

Lymphokines are a group of cytokines that regulate the activation, differentiation, maturation, migration of lymphocytes.

IL-2	secreted by activated T cells causes proliferation and activation of T and B cells. It is also involved in the cycling of B cells and enhances antibody production.
IL-4	stimulates division and differentiaton in B cells.
IL-5 and IL-6	act as B lymphocyte differentiation factors
IL-7 and IL- 11	have a role in antigen independent phase of B cell differentiation in bone marrow(Callard 1990).
IL-21	is the most potent cytokine in activation and differentiation of human B cells is secreted by helper T cells (Spolski & Leonard 2008, Ettinger et al 2008). It is also required for B - cell activation, proliferation, PC differentiation and Ab production (Bryant et al 2007, Kuchen 2007).

Phosphorylation has been recognized as a key mechanism by which the cytokine receptors induce a variety of intracellular events. The phosphorylated substrates assemble in to a complex that tranclocates to the nucleus to activate the transcription of specific genes (Keegan et al 1994).

Kinases and phosphatases involved in B lymphocyte activation

Tyrosine phosphorylation catalyzed by protein tyrosine kinases is the major event that occurs during B lymphocyte activation.

Protein tyrosine kinases, Several serine threonine kinases, cAMP –dependent kinases, mitogen activated kinases (MAP kinases), S6 kinase are some of the kinases that are activated and regulate the transcription of various genes down stream to the phosphorylation event during B lymphocyte activation (Gold et al 1990, Campbell & Cambier 1990, Lane et al 1990, Brunswick et al 1991, Burkhardt et al 1991, Yamanishi et al 1991, Campbell & Sefton 1992, Leprince et al 1992, Li et al 1992, DeFranco 1992).

Protein phosphatases are also expressed during lymphocyte activation to maintain the balance between the phosphorylation and dephosphorylation reactions.

Two types of protein phosphatases are known to operate in the cell:

1. Serine-threonine phosphatase ex: Calcineurin.
2. Tyrosine phosphatase ex: CD45.

   Among the various phosphatses that are expressed upon lymphocyte activation, alkaline phosphatase is expressed specifically in activated B lymphocytes.

Multiple Myeloma

Multiple myeloma is a B cell malignancy accounting for 1.0% of all cancer deaths globally (Parkin et al 2005) and 10% of hematologic malignancies (Kyle & Rajkumar 2004, Rajkumar & Kyle 2005). It is characterized by the monoclonal proliferation of malignant plasma cells with in the bone marrow. The disease occurs in older individuals above the age of 80 yr (Kyle et al 1994 , Kyle & Rajkumar 2004 , Jemal et al 2005, Parkin et al 2005)

Disease characteristics

Multiple myeloma is a B-cell malignancy with terminally differentiated plasma cell phenotype. The characteristic findings in MM are lytic bone disease with bone pain, renal insufficiency, anemia, fatigue, hypercalcemia, and immunodeficiency with recurrent infections (Kyle et al 2003). The myeloma cells produce an identical immunoglobulin or immunoglobulin fragment known as the monoclonal protein (M-Protein) . The myeloma proteins when produced as light chains are

excreted in the urine and are called as “Bence-Jones proteins”. The isotype of M protein is usually IgG or IgA, occasionally IgM, IgD or IgE. The M-Protein in the serum and as light chains in the serum and / or urine is the diagnostic hall mark of this malignancy.

Renal failure may develop both acutely and chronically. It is commonly due to hypercalcemia. It may also be due to tubular damage from excretion of Bence Jones proteins, which can manifest as the Fanconi syndrome (type II renal tubular acidosis). Other causes include glomerular deposition of amyloid, hyperuricemia, recurrent infections (pyelonephritis), and local infiltration of tumor cells.

The myeloma cells also generally express CD138 (syndecan-1), CD38 and other heterogenous immunophenotypic markers and secrete the cytokine IL6 as an autocrine growth factor.

Gene mutations and cytogenetic abnormalities in the aetiology of multiple myeloma

Little is known about the etiology of multiple myeloma. Both environmental and genetic factors may play a role in the development of this cancer (Riedel & Pottern 1992). Exposure to ionizing radiation has been linked with multiple myeloma. The strongest associations have been noted for Japanese atomic bomb survivors, radiologists, and radium dial workers (Matanoski et al 1975, Stebbings et al 1984, Shimizu et al 1990). Nuclear power plant workers were reported to have an increased risk for developing multiple myeloma (Darby et al 1988, Gilbert et al 1989).

Numerous epidemiologic studies have reported a link between multiple myeloma and work involving pesticides, agricultural exhausts, chemicals, dusts, or a combination of these exposures(Riedel & Pottern 1992). Other non-specific occupational exposures that have been associated with myeloma include metals, rubber, wood, leather, paint, and petroleum (Riedel & Pottern 1992). Workplace exposure to benzene, a chemical used in many manufacturing processes, may play a role in the development of multiple myeloma (Decoufle et al 1983, Rinsky et al 1987).

Prolonged stimulation of the immune system by repeated infections, allergic conditions, or autoimmune disease may also increase the risk of myeloma.

The occurrence of multiple myeloma among siblings, spouses, and family members of myeloma patients suggests that genetic factors and common environmental exposures play a role in the development of this cancer (Riedel & Pottern 1992). The discovery of specific human leukocyte antigens (HLA) , chromosome abnormalities and oncogenes among myeloma patients adds further support for a genetic predisposition for this cancer (Leech et al 1983, Gould et al 1988, Lewis & MacKenzie 1984, Ernst et al 1988, Selvanayagam et al 1988, Nishida et al 1989, Pottern et al 1992). A link between cigarette smoking and myeloma has also been suggested (Mills et al., 1990).

While alterations of proto-oncogene loci, including mutations, amplifications, chromosomal translocations, or deletions have been found associated with several types of human tumors, no such association has been firmly established for MM.
Ras gene mutations were found to occur in 47% of the patients. p53 is a tumor suppressor gene that functions as transcriptional regulator influencing cellular responses to DNA damage. Alteration or deletion of p53 gene represent an important late event in MM tumor progression. and were observed in 20% of the patients. (Antonino et al 1993, Owen et al 1997, Wei Xiong et al 2008).
The role of cytogenetic abnormalities on disease outcome has not been studied in detail. The most common and unfavourable cytogenetic abnormalities detected are-

1. del(13q), t(4;14),
2. del(17p13).
3. t (6;14)(p25;q32)
4. Loss of Chromosome 13
5. t(11;14)(q13;32)

Chromosome rearrangement of 14q32.33 occurs with variable partner sites, including 11q13.3, 8q24.1, 18q21.3, and 6p21.1 **(**Kazuhiro et al 1997). A significant number of myelomas (50% to 70%) also carry translocations targeting the switch regions of the IgH genes located at chromosome 14q32. (Bergsagel et

al 1996 and Pratt et al 1998). These aberrant rearrangements juxtapose oncogenes into the proximity of the powerful IgH enhancers, driving abnormal expression of the translocated oncogenes. These characteristics together with the known biased usage for certain V and D gene segments establish a molecular archaeology for myeloma in which positive and negative selection processes shape the Ig repertoire prior to the acquisition of the malignant phenotype. (Rettig et al 1996, Gonzalez et al 2005)

Treatment

A number of therapeutic tools have been used in the management of multiple myeloma for the last 40 years. Approximately 5% of patients were found to achieve complete remission with conventional therapy

Chemotherapy

Melphalan, Prednisone, cyclophosphamide, Vincristine, Carmustine, Adriamycin Doxorubicin, Dexamethasone.

High-Dose chemotherapy and stem cell Transplantation

High dose chemotherapy has also been used in conjunction with stem cell transplantation. High dose chemotherapy though more effective in killing myeloma cells than conventional chemotherapy also destroys normal blood forming cells in the bone marrow

Radiation therapy

This mode of treatment uses high energy radiation to induce cytosolic and cytotoxic effects. The treatment is localised and it affects only the cells in the treated area and has been found to be effective in patients with single plasmacytoma.

Supportive therapies

Supportive therapies address the symptoms and complications of the disease. Supportive therapies commonly used in multiple myeloma include bisphosphonates, growth factors, antibiotics and intravenous immunoglobulin.

Bisphosphonates work by inhibiting the activity of the bone destroying osteoclasts. Erythropoietin and Colony stimulating factors are also used to enhance the production of RBC and WBC.

Recently immunomodulatory agents like thalidomide and lenalidomide and protease inhibitor like bortezomib were introduced and investigated for their efficacy in the management of multiple myeloma. Deep vein thrombosis with thalidomide and myelosuppression and contraindication in patients with creatinine levels greater than 2.5 mg/dL in patients under the regimen of lenalidomide; and peripheral neuropathy, transient thrombocytopenia and gastrointestinal disorders in patient under treatment of bortezomib were encountered. Eventual relapse in autologous stem cell treated patients and absence of clear cut evidence in case of allogenic stem cell transplantation suggest search for new therapeutic agents.

Multiple Myeloma Cell lines Used for the study

Cell line :	**RPMI 8226**
Isotype:	lambda light chain
Biosafety	level 1
Propagation	ATCC complete growth medium: the base medium for this cell line is ATCC-formulated RPMI-1640 medium supplemented with fetal bovine serum to a final concentration of 10% .
Temperature :	37.0^{o}C
Atmosphere :	air, 95%; carbon dioxide (CO_2), 5%.
Growth properties:	suspension
Organism:	*Homo sapiens* (human)
Morphology:	lymphoblast
Source: Organ:	peripheral blood
Disease:	plasmacytoma; myeloma
Cell Type:	B lymphocyte
Cellular Products:	Immunoglobulin light chain

Cell line :	**U266B1**
Isotype:	IgE; lambda light chain
Biosafety level 1	
Propagation	The base medium for this cell line is ATCC-formulated RPMI-1640 medium supplemented with fetal bovine serum to a final concentration of 10%
Temperature :	37.0^{o}C
Atmosphere :	air, 95%; carbon dioxide (CO_2), 5%.
Growth properties:	suspension
Organism:	*Homo sapiens* (human)
Morphology:	lymphoblast
Source: Organ:	peripheral blood
Disease:	plasmacytoma; myeloma
Cell Type:	B lymphocyte
Cellular Products:	Immunoglobulin;monoclonalantibody;interleukin-6

Levamisole

Levamisole has been introduced in 1960 as an antihelmithic drug (Thienpont et al 1966, Rollo 1990.) It is the levo enantiomer of tetramisole and since then it has found many uses in the clinical medicine (Renoux & Renoux 1971). It is still being used in conjunction with 5-Fluorouracil (5-FU) as an adjuvant to treat colon cancer relapse following surgical resection (Amery & Gough 1981, Amery et al 1997, Moertel 1995, Kerr et al 2000). Levamisole and its derivatives have also been used as potent inhibitors of alkaline phosphatase, thymidylate synthase and tyrosine phosphatase (Vanbelle 1972 & 1976, Bhargava & Men 1977). It is also a good selective inhibitor of TNAPs, with Ki values lower than 100 uM (Jalanka and Lindberg 1975) in vitro. Mammalian APase activity of Liver/bone/kidney type has been demonstrated to be inhibited uncompetetively by Levamisole (Borgers 1973, Goldstein et al 1980).

Levamisole at 2mM concentration has been shown to inhibit the proliferation of cultured bone cells with concomitant inhibition in bone APase activity (Marie et al 1986). Levamisole avidly binds to the putative "substrate-APase" complex to mediate the inhibition. Levamisole has been shown to act as immunosuppressor at high doses (Artwohl et al 2000). Levamisole is currently used as an adjuvant therapy in the treatment of human cancer (Reid et al 1998). Also, levamisole was found to bring significant clinical improvement in patients with chronic infections and inflammatory diseases such as Herpes and Rheumatoid arthritis (Symoens & Rosenthal 1979). Further, it has been demonstrated that levamisole exerts anti-metastatic effects, particularly when it is used as an adjuvant to conventional anti-neoplastic therapy (Amery 1977 , Amery & Gough 1981). In vitro, levamisole has been demonstrated to potentiate anti-proliferative effect of 5-Fluorouracil in tumor cell lines (Kovach et al 1992, John et al 1992 and de Waard, et al 1998).

Chemical structure and metabolism of Levamisole

Levamisole is a synthetic drug having the structure as L-2,3,4,5-tetrahydro-6-phenyl-imidazo [2,1-b] thiazole. Levamisole has been shown to be decomposed non-enzymatically into three degradation products (Kimberly et al 1991). The

three products were purified and their structures were determined. The structures of the products are

A) 3-(2-mercapto ethyl)-5-phenyl imidazolidine-2one or dl-2-oxy-3-(2-mercaptoethyl)-5-phenyl

B) 6-phenyl-2,3-dihydroimidazo(2,1-b)thiazole

C) bis(3-(2-oxo-5-phenyl imidazolidine-1-)ethyl) disulfide.

The decomposition of levamisole in to the above products depends on temperature and pH. Upon oral administration, levamisole is well absorbed from the gastrointestinal tract and is extensively metabolised by the liver.

Levamisole as an anti-helminthic drug

Levamisole probably owes its anti-helminthic property to specific binding to nicotinic-acetylcholine receptors of muscles of nematodes. As a result of this interaction, Levamisole muscular hypercontractions which leads to paralysis and elimination of the worms (Lewis et al 1987a & 1987b). Levamisole is used in human medicine as an antihelminthic in a single dose form at 2.5 mg / Kg. Levamisole has been used in humans for the following indications: antihelminthic , rheumatoid arthritis, inflammatory disease, infectious disease and cancer therapy. The most important side effects are hematologic, including reversible leukopenia, agranulocytosis and thrombocytopenia.

Levamisole induced apoptosis

LMS affects antitumor cytotoxicity by inducing apoptosis in some tumor cells. Levamisole has been shown to induce apoptosis in adult human endothelial cells of veins and uterine capillaries (Artwohl et al 2000). Levamisole increased intergrin-dependent matrix adhesion and reduced the expression of survival factors such as clusterin, endothelin-1, bcL-2, endothelial NO-synthase and retinoblastoma protein (pRb). It also caused growth arrest and increased death signals such as p21 and bak. Levamisole induced apoptosis and regulation of pRb expression was inhibited by glutathione and N-acetyl cysteine suggesting that reactive oxygen species plays a role in this process (Cruchand et al 1979).

Direct toxic effects of levamisole alone on cancer cell lines have been found only with suprapharmacologic concentrations of the drug (Grem & Allegra 1989). Levamisole was found to be bound to plasma proteins.

AIM AND SCOPE OF THE PRESENT WORK

The enzyme, Alkaline phosphatase (APase) is involved in several physiological functions of the cell like- transmembrane signaling function, transport of nutrients across cell membranes, ossification of bone, protein tyrosine phosphatase, phosphotranferase, cell adhesion ,proliferation and differentiation. Mitogen / antigen stimulated B lymphocytes express Alkaline Phosphatase activity (in mice and rats). In resting B cells and in resting and activated T cells APase activity is negligible. Enhanced enzyme activity has been observed during the proliferative phase and differentiation of B cells. Normal human peripheral blood lymphocytes do not show APase activity even upon mitogenic stimulation while certain cancers and cancer cell lines of both murine and human origin show enhanced APase activity. This enhancement of APase activity could be due to functional involvement of APase isoenzymes in tumorgenesis and might be a crucial factor in the etiology of the disease. However the function of APase in malignancy remains largely unknown. Myeloma cells (malignant differentiated B cells) offer an experimental system to understand the role of APase in B cell differentiation and carcinogenesis. Levamisole is an inhibitor of tissue non specific alkaline phosphatase of mammalian type .Levamisole has also been shown to have anticancer property. It is presently being used as an adjuvant along with 5-FU in the treatment of colorectal carcinoma. However the exact mechanism of action in cancers especially the B cell tumor, multiple myeloma has not been completely unravelled so far. Based on the review of literature and the work carried out earlier, in the present study, effect of levamisole on human multiple myeloma cell lines, has been studied *in vitro* using Alkaline phosphatase activity as a "putative" target with the following approach :

- To screen human myeloma cell lines for APase activity.
- To examine the effect of levamisole on the growth, proliferation and viability of myeloma cells
- To determine the cytotoxic mechanism exerted by levamisole .
- To evaluate the fate of levamisole in cell culture by HPLC
- To examine the role of APase activity in immunoglobulin secretion.
- To study the binding of levamisole to myeloma cells in culture using ^{3}H-levamisole.

MATERIALS AND METHODS

Media preparation:

1. RPMI-1640 Medium

Powdered RPMI-1640 medium was dissolved in ultra pure distilled water and 2gm of sodium bicarbonate was added, pH was adjusted to 7.2 and the volume made up to 1 liter. 100U/ml Penicillin G, 100 units /ml and streptomycin, 100ug/ml were added to the medium before use. The medium was sterile filtered through 0.22µ membrane filter using Sartorius filtration unit and stored at 4^oC until use.

2. Cell lines and culture conditions

U266B1 and RPMI 8226 cell lines were obtained from National Center for Cell Sciences, Pune. They were maintained in RPMI-1640 tissue culture medium (Sigma) supplemented with L-glutamine (2mM), and 10 % heat inactivated Fetal Calf Serum (Hi Media) in $25cm^2$ tissue culture flasks (Orange Scientific) and incubated at 37^oC in a humidified atmosphere of 95% air and 5% CO_2 in a CO_2 incubator (Forma scientific): everyday the cultures were examinedfor microbial contamination using an inverted microscope (Unico, USA).

3. Cell counting

A small volume of the cell suspension was diluted appropriately in trypan blue solution (0.2%W/V in 0.9% NaCl) and the cell count was done microscopically using a Neubauer's chamber. The cell count was calculated and expressed as $X10^6$ / ml of suspension. (Barbara & Stanley, 1980).

4. Cytochemical staining of Lymphocytes for APase activity

Chemicals and reagents : Tris buffered saline, BCIP / NBT, 0.9% NaCl.

Myeloma cells were centrifuged at 200g and the pellet was washed with saline. To the pellet BCIP/NBT in Tris buffered saline was added and incubated for 10 min at 37^0C in humidified incubator. The cells were washed thrice with saline to remove the excess substrate and the cells positive for APase were observed using an inverted microscope.

5. Activity staining of Alkaline Phosphatase on gel

Chemicals and Reagents : Tris, $MgCl_2$, TX-100, BCIP/NBT.

Procedure: Cell suspension was harvested by centrifugation at 400g for 10min. The cells were washed twice with saline and then suspended in 10mM Tris buffer

containing 2mM $MgCl_2$ pH7.4. The cells were sonicated at half –maximal amplitude for 15sec for 15 cycles with 30sec interval. To the sonicate, TX-100 was added to a final concentration of 0.5% and centrifuged at 15000xg for 20 min. The supernatant was collected and loaded on a 7.5% Native polyacrylamide gel and electrophoresis was carried out at a constant voltage of 60V for 2h. The gel was then incubated in APase substrate solution BCIP/NBT. The reaction was terminated by washing the gel with water (Ding Yi et al 2003).

6. Isolation of murine splenic lymphocytes

Chemicals and Reagents: Ether, Ethanol, Histopaque.

Procedure: The mouse was killed using ether anesthesia. Its' left side of the abdomen was then wiped with 70% alcohol a cut was made through the skin and the spleen was then separated from the vessels and connecting tissue with scissors. The spleen was then placed in RPMI-1640 medium on a stainless steel mesh kept on a petridish and then minced in to small pieces. The minced tissue was teased carefully using teasers with regular addition of medium on to the tissue. The cell suspension was transfered to a sterile tube for the large clumps to settle down. The cell suspension was taken in to a fresh tube and centrifuged at 400g for 10min. was kept on ice. The pellet was then resuspended in RPMI 1640 and was carefully layered on Histopaque (d=1.077, half the volume of the suspension) and centrifuged at 800g for 10 minutes. The lymphocytes separated were at the interface of RPMI-1640 and gradient solution were collected carefully and the centrifugation step was repeated twice as described above. The final cell pellet was suspended in RPMI-1640 with 5% FCS.

7. Isolation of human peripheral blood lymphocytes (PBLs)

Chemicals and Reagents : Heparin: - A stock solution of 10000u/ml in 0.9% Nacl, Histopaque.

Procedure: PBLs were isolated from venous blood collected into heparinised tubes (10u/ml). The blood was diluted 1:1 with saline and 8ml of it was layered over 3ml histopaque and centrifuged at 500g for 20min. The cells at the interface were collected and washed thrice with complete medium and suspended in the same medium.

8. Isolation of lymphocytes from human bone marrow samples

Procedure: The bone marrow aspirate samples were collected in 10-20ml of sterile RPMI 1640 medium and were kept at 4^0 C till they were processed. The samples were first brought to room temperature and then were dispensed by gentle aspiration using a pasture pipette . The clumps were allowed to settle down for 5 min and the supernatant cell suspension was layered on histopaque (d =1.077g/mL). The gradient was centrifuged at 600g for 15 min at room temperature. The cells at the interface were collected in to RPMI-1640 with 5% FCS. The cell suspension was centrifuged at 250g for 10 min and the cells were resuspended in RPMI 1640. This step was repeated once more and the final cell pellet was suspended in RPMI-1640 and assayed for APase activity.

9. ^{3}H-Thymidine incorporation assay

Chemicals and Reagents: complete medium RPMI 1640 supplemented with 5% FCS, ^{3}H-thymidine, Glass fibre filter, Scintillation cocktail: 4g PPO and 200mg POPOP in 1 litre of scintillation grade toluene.

Cell were cultured in triplicate in 200ul of complete medium in 96 well flat bottomed microtitre plates. Cultures were kept at 37^{o}C in a humidified incubator with 5%CO_2. The cultures were pulsed with 0.5uCi of ^{3}H-thymidine for the last 24h of the culture period and were harvested on to glass fibre filter using Skatron automatic cell harvestor. The dried filters were transferred into toluene based scintillation cocktail and the radioactivity was measured using Beckman scintillation counter.

10. Alkaline phosphatase assay

Principle: p-nitrophenol (p-NP) is released from p-nitrophenul phosphate (p-NPP) by APase. The absorbance of p-NP is measured at 405nm and is directly proportional to the amount of p-NP released.

Chemicals and reagents: 0.1M bicarbonate buffer -(containing 2mM $MgCl_2$, pH 9.8), 1N NaOH, 0.9% NaCl, 2.5mM p-nitrophenyl phosphate in bicarbonate buffer, p-nitrophenol.

Procedure: Alkaline phosphatase activity was determined by p-NPP hydrolysis. The absorbance of p-nitro phenol (p-NP) produced was measured at 405nm. Cells

were dispensed in to wells of microtitre plate and centrifuged at 450g for 10min at 4°C. The cells were suspended in 0.9% saline and the centrifugation step was repeated. To the pellet 180µl of 1mg/ml p-NPP in 0.1M bicarbonate buffer with 2mM $MgCl_2$ was added. Incubation was carried out at 37^0C in a humified incubator. After 30min the reaction was stopped by the addition of 20µl of 1N NaOH and the absorbance was measured at 405nm using ELISA plate reader. The amount of p-NP released was calculated form a standard graph. The results were expressed as nmoles of p-NP released / $0.2X10^6$ cells.

11. Dot Blot

Principle: This is a technique used for detecting, analyzing, and identifying proteins, similar to the western blot technique but differing in that protein samples are not separated electrophoretically but are spotted through circular templates directly onto the membrane. Concentration of proteins in crude preparations (such as culture supernatant) can be estimated semiquantitatively by using "Dot Blot" method using specific antibody against it.

Chemicals and Reagents : Nitrocellulose membrane (Sartorius), Lactalbumin peptide solution, Tris- buffered saline pH 7.4 (TBS) with 0.02% Tween-20 (TBST), Goat Anti mouse IgM – Horse radish Peroxidase (HRPO) conjugate (Sigma Immuno chemicals) , anti- human IgE antibody-Alkaline phosphatase conjugate, 3,3',5,5'-tetramethylbenzidine hydrochloride / hydrogen peroxide (TMB / H_2O_2), 5-Bromo-4-Chloro-3-Indolyl Phosphate / Nitro Blue Tetrazolium (BCIP/NBT), RPMI-1640 with 5% FCS.

Procedure : Cell free supernatant containing immunoglobulin 5ul sample was spotted on to the nitro cellulose membrane and dried for 1hour at room temperature, The membrane was then washed thrice with TBS for 5 minutes each time. The membrane was then blocked with 5% peptide solution made in TBST for 1hour. It was then incubated for 4-6 hours with either anti-human IgM-HRPO conjugate or anti-human IgE – alkaline phosphatise conjugate antibody to immunoglobulin, diluted 1:2000 in 1% w/v of peptide solution (w/v) at room temperature. Then the blot was washed with TBST 4 times for 5 minutes each time and finally incubated with substrate. The colored spots were then scanned and quantitated using a densitometer (Sulimenko & Dráber 2004).

12. Detection of intracellular IgE by western Blot

Procedure: For determining intracellular IgE. Cell lysis buffer mentioned in Caspase assay was used. Lysates were prepared by lysing untreated and levamisole treated U266 B1 cells. lysate was separated on a 7.5 % SDS-PAGE, and after electrophoresis, proteins on the gel were transferred to nitrocellulose membrane and probed with monoclonal anti human IgE antibody ALP conjugate. The blot was developed using the substrate BCIP/NBT (Liu et al 1996).

13. MTT assay

Principle : Cytotoxicity / Cell proliferation was determined by a colorimetric assay using MTT dye which is reduced by the mitochondrial dehydrogenase enzymes of the living cells to a purple formazan. The amount of the purple colored product formed is proportional to the number of viable / proliferating cells.

Chemicals and Reagents : 3-[4,5-dimethylthiazole-2-yl]-2,5 diphenyl tetrazolium bromide (MTT), Sodium Dodecyl Sulfate, Phosphate Buffered Saline.

Procedure: At the end of the incubation period, the microplate was centrifuged at 250g for 10 min and the medium was discarded and 0.10 ml of PBS supplemented with glucose and $MgCl_2$ was added. 20µl of MTT (5mg/ml) solution was added and incubated for 4hr at 37^0 C. Later, 100µl of acidified SDS was added and incubated overnight to solubilise the purple formazan product and the absorbance was measured at 570-630nm dual wavelength mode in a ELISA reader. (Lichtenstein et al 1995).

14. Assay of Caspase-3 Activity

Chemical and reagents: Lysis buffer (20mM Tris-HCl pH 7.8, 1mM $MgCl_2$, 1mM DTT, 80mM KCl, 10µg/ml of pepstatin, 10µg/ml of leupeptin, and 1mM PMSF). Caspase-3 activity is determined by incubating cell lysate (150µg total protein) with 200uM fluorogenic susbstrate in protease assay buffer (20mM Hepes buffer pH 7.5, 10mM DTT, 1mM EDTA, 0.1% Triton X-100 and 10% Sucrose).

Procedure: Cells were harvested by centrifuging at 4000 g for 10 min. Cell extracts were prepared by lysing treated and untreated cells in lysis buffer. The protein was estimated using Lowry's method. About 150µg of protein and 10µg (200µM) of AC-DEVD-AFC, a fluorogenic Caspase 3 substrate were added to the protease assay buffer and incubated for 1hr at 37^0C. A control reaction is set up by

the addition of 10µg of AC-DEVD-CHO, Caspase 3 specific inhibitor with incubation at 37^0C for 30 min prior to the addition of 10µg of AC-DEVD-AFC. Intensity of AFC liberated due to the cleavage of AC-DEVD-AFC by Caspase 3 was measured using a spectrofluorimeter with an excitation wavelength of 480 and emission wave length of 520nm (Bhuyan et al 2001).

15. DNA fragmentation assay

Reagents :

a. Lysis buffer: 50mM Tris-HCl pH 8.0, 10mM EDTA, pH 8.0, 0.5%(w/v) Sodium lauryl sarcosine.

b. Proteinase K : 20mg/ml, added freshly to a final concentration of 0.5mg/ml of lysis buffer.

c. RNase A: 10mg/ml, added freshly to a final concentration of 0.5 mg/ml of lysis buffer**.**

Procedure : Cells, 1 x 10^6 were centrifuged at 4000 g for 5 min at 4^oC and the supernatant was discarded. The cells were resuspended on ice in 20 µl of lysis buffer containing proteinase K and pipetted repeatedly to ensure complete lysis. The lysate was incubated at 55^oC for 1h after which the cell debris is pelleted and 10 µl of lysis buffer containing RNase A was added to the supernatant and incubated for further 1h. The sample was briefly spun to pellet any further cell debris and the supernatant was collected and heated to 70^oC for a few minutes and mixed with 10 µl of loading buffer (preheated to 70^oC). The sample was loaded on a 2% agarose gel containing 0.5 µg /ml ethidium bromide and run at 40V for 2h and visualized under UV light in a trans–illuminator. The gel pattern was photographed.

16. Interleukin 6 assay

Chemicals and Reagents : Na_2CO_3 , Tween-20, TMB/H_2O_2, $NaH_2PO_4.H_2O$, $Na_2HP0_4.7H_2O$, NaCl.

Coating Buffer – 0.1M Bicarbonate buffer, pH 9.5. Freshly prepared and stored at 2-8 ^{o}C.; Assay Diluent – PBS with 10% FBS, pH 7.0 ; Wash Buffer – PBS with 0.05% Tween-20. Freshly prepared or use within 3 days of preparation, stored at 2-8 ^{o}C. ; Substrate Solution – Tetra Methyl Benzidine / H_2O_2. ; Stop solution – 2N H_2SO_4 ; Capture antibody – Purified antihuman IL6 antibody (BD biosciences);

Detection antibody- Biotin anti-human IL-6.; Enzyme concentrate Streptavidin – HRP.

Procedure : Microwells were coated with 100ul per well of capture antibody (anti human IL 6) diluted in coating buffer and the plate was sealed and incubated overnight at 4^{o}C. The solution was aspirated and washed thrice with 200ul per well of wash buffer. The wells were blocked with 200ul per well of assay diluent and incubated at room temperature for 1 hour. The wells were aspirated and washed thrice with 200ul per well of wash buffer. The samples were diluted in assay diluent and 100ul was pipetted in to wells and incubated for 2 hrs at room temperature.The wells were aspirated and washed five times with 200ul per well of wash buffer.

100ul of working detector (anti human IL 6 antibody + streptavidin-Avidin-Horse Radish Peroxidase- mixed just before addition) was added to each well and incubated for 1hr at room temperature. The wells were aspirated and washed seven times with 300ul per well of wash buffer. 100ul of substrate solution was added to each well and incubated for 30min at room temperature in dark.50ul of stop solution was added to each well and the absorbance read to 450nm with in 30 min of stopping the reaction.

17. CD138 expression on myeloma cells by flow cytometry

Cell culture :

The myeloma cell lines RPMI 8226 and U266B1 were cultured in T-25 flasks in RPMI-1640 medium supplemented with 10% FCS and treated with various concentrations of levamisole (control, 0.5mM, 1mM, 2.5mM) and the cells were harvested after 48hrs. CD138 positive cell number was determined by FACS using FITC conjugated anti- human CD138 antibody.

Chemicals and Reagents :

Wash buffer : Phosphate buffered saline (PBS) (Dulbecco's PBS without calcium, magnesium, or phenol red, pH 7.2) containing 0.1% sodium azide. Filtered through a 0.2 μ filter prior to use and stored refrigerated.

Staining buffer : PBS containing 0.1% sodium azide and 2% fetal bovine serum (FBS).

Procedure : 1 x 10^6 cells treated cells were centrifuged at 200 x g for 10 min and washed with wash buffer. The cells were then resuspended in 50 µl of staining buffer to this suspension, 20 µl of fluorescein conjugated anti-CD138 antibody was added and incubated for 45 minutes on ice. Then the suspenstion was centrifuged at 200 x g for 10 minutes at 4^0 C. The supernatant was removed and the cells were suspended in 1 ml of cold buffer and centrifuged as above. The step is repeated twice. The pellet was resuspended in 0.5ml of PBS and stored at 4^0C until analyzed.

The expression of CD138 was assessed using a FACS flow cytometer using a gating protocol set for measuring mean FITC fluorescence intensity of labeled cells. Ten thousand events were counted and the data is presented as scatter plots. Appropriate isotype controls were prepared and processed in an identical manner.

18. Assay for cytochrome C

Procedure: For determining cytochrome C, cells lysis buffer mentioned in Caspase assay was used. Extracts were prepared by lysing untreated and levamisole treated U266 B1 cells. 50 µg protein from lysate was separated on a 10 % SDS-PAGE, and after electrophoresis, proteins on the gel were transferred to nitrocellulose membrane and probed with monoclonal anti-cytochrome C antibodies ALP conjugate. The blot was developed using the substrate BCIP/NBT (Liu et al 1996).

19. Statistical analysis:

For all experiments, the data was calculated and presented as Mean ± SEM. The figures were drawn using Sigma Plot 10. Wherever applicable, the data was analysed for statistical significance using Student's "t" test.

CHAPTER - 1

Alkaline Phosphatase Activity of normal and malignant human lymphocytes

Enhancement of APase activity has been demonstrated to be an intergral feature of B-cell activation and differentiation. In an activated B cell, probably, it aids in the metabolite transport to fulfil the demands of the growth and proliferation. Although many other functions have been attributed to APase, its role in malignancy needs to be evaluated.

A change in the level of serum APase has been used as an important diagnostic marker in several disease states. APase activity provides the clinician, valuable information for diagnosis and follow up of patients during treatment. APase isozyme patterns have been reported in malignancies and renal diseases (De Broe & Van Hoof 1991). Tumorogenicity has been correlated with high levels of APase expression (Lantham & Stanbridge 1990). Human APases are abundantly expressed in tumor cells, and serum levels of APase isozymes are often valuable tumor markers (Millan & Fishman 1995).

Increased level of APase activity has been reported in neutrophlis in chronic myeloproliferative diseases (Rosenblum & Petzold 1975). Hemopoietic tumor cell lines have been shown to express high APase activity (Neumann et al 1976). Especially in B lymphoid cell lines APase activity has been reported to be high. APase activity has been demonstrated in several murine tumor cell lines of B lymphoid lineage and in few human lymphoid cell lines (Neumann et al 1976, Culvenor et al 1981, Harb et al 1991). Thus, expression of APase is considered as one of the important identifiable markers of malignancy (Ruddon 1987). Various tumors and tumor cell lines such as teratocarcinomas, choriocarcinomas and osteosarcomas express APase activity (Van Hoof & De Broe 1994, Millan & Fishman 1995).

In the present study, normal human peripheral blood lymphocytes and mononuclear cells from bone marrow of multiple myeloma patients were screened for the presence of APase activity. Also two human myeloma cell lines RPMI 8226 and U266 B1 were screened for APase activity and for preliminary characterisation of the enzyme.

Methods:

1. Preparation of mouse splenic lymphocytes

Lymphocytes from spleen of mice were prepared as described in materials and methods.

2. Mitogenic stimulation and Proliferative response

Splenic lymphocytes (in triplicate), 0.2 X 10^6 per well were cultured in 96 well microtitre plates with 5 µg LPS, and 2 µg Con A. The cultures were pulsed with 0.5µCi of ^{3}H-thymidine for the last 24h of 72h culture period and processed as described in materials and methods.

3. Assay of APase activity

APase assay was performed at the end of 48h as described in materials and methods.

4. Isolation of Human peripheral blood lymphocytes (PBL)

The PBL were isolated as described in materials and methods.

5. Mitogen induced proliferative response and APase activity of human peripheral blood lymphocytes

Human PBL were cultured with the indicated concentrations of Pokeweed mitogen (PWM), Phytohemaglutinin (PHA) and Staphylococcal protein A (SPA) and the proliferative response was measured using ^{3}H-Thymidine incorporation into DNA. The cultures were processed as described in Materials and Methods . APase activity was estimated at the end of the culture period as described in materials and methods.

6. Screening of lymphocytes from bone marrow samples of multiple myeloma patients

The bone marrow aspirate samples were obtained from Department of Pathology, Nizam's Institute of Medical Sciences, Hyderabad. The sample was a leftover sample of aspirate taken for diagnostic purpose. No special ethical clearance was

obtained as the samples were primarily taken for diagnosis.The sample was kept in RPMI 1640 at 4^{o} C till they were processed. The samples were first brought to room temperature and then were dispensed by gentle aspiration using a Pasteur pipette. The clumps were allowed to settle down for 5 min and the supernatant cell suspension was collected and lymphocytes were isolated and assayed for APase activity.

7.Screening of myeloma cell lines for APase activity

The cell lines were procured form National Centre for Cell Sciences, Pune, India. They were maintained as described in materials and methods and were used for assay of APase activity using p-NPP as substrate.

8.Fluorescence immuno staining of cells

RPMI 1640 medium containing 0.05% sodium azide is used during staining for washing cells. Human lymphocyte culture / myeloma cells (one million cells) were centrifuged at 400g for 10 min and the cell pellet was washed twice with 1ml of medium. To the cell pellet, 5µg of antibody to human APase was added and incubated for 1 hr at 4^{0}C. The cells were washed twice with medium to remove the unbound antibody by centrifugation. To the cell pellet, 10µl of 1:10 diluted goat anti mouse IgG FITC conjugated antibody was added and incubated for 1 hr at 4^{0}C. The cells were washed twice with medium to remove the unbound FITC conjugated antibody. The cells stained for APase were analysed by fluoroscence microscopy.

9.Enzyme cytochemical staining

Multiple myeloma cell lines were analyzed for the presence of APase activity by staining with BCIP/NBT as substrate as described in materials and methods.

10. Activity staining of enzyme on polyacrylamide gel

The cell lysate was electrophoresed using a native 7.5% polyacrylamide gel and stained with BCIP/NBT as substrate as described in material and methods.

11. Determination of Km and effect of inhibitors

All the assays were carried out in triplicates. Substrate blank were included in each experiment to measure the non enzymatic hydrolysis of substrate.

Determination of Michaelis-Menten constant (Km)

For the determination of Km, the reaction rate was measured at the following substrate (pNPP) concentrations 2.5mM, 5.0mM, 10mM, 20mM. The data was then fitted to the Michaelis-Menten equation using non linear regression to find Km values. APase activity was estimated to be higher in diethanolamine buffer as compared to bicarbonate buffer, pH 10.0. Hence, for kinetic experiments, diethanolamine buffer was used. The substrate, p-NPP was prepared in 0.10 M Diethanolamine buffer, pH 10.0 with 5mM $MgCl_2$. OD was taken at 405nm after 1hr incubation at 37^oC. A Line weaver-Burk plot, 1 / [V] Vs 1 / [S] was plotted. The value of X-inercept was used to calculate Km. The formula, X-intercept= -1/Km.

Determination of inhibition constant (Ki)

The enzyme activity was measured in the presence of varying concentrations of the inhibitors - levamisole, L-homoarginine, L-phenyl alanine and theophylline

To determine Ki, APase activity was measured as residual activity compared to the activity in the absence of inhibitor, and the inhibition constants were calculated by non linear regression analysis.

Results :

Mitogen induced proliferative response and APase activity of murine splenic lymphocytes and human peripheral blood lymphocytes

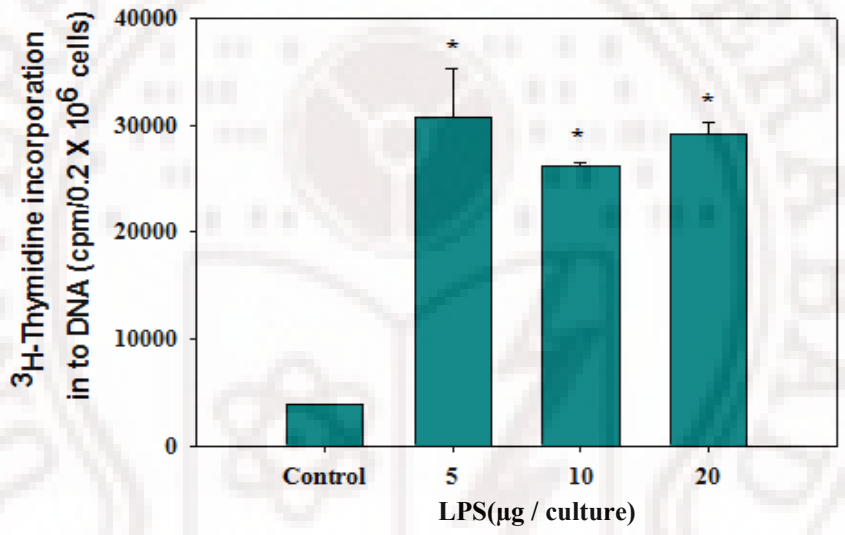
Murine splenic lymphocytes were stimulated with mitogens Con A (T cell-specific) and LPS (B cell- specific) for 72h. The proliferative response was measured by ^3H-thymdine incorporation in to DNA. Simultaneously, APase

activity of mitogen stimulated and unstimulated cultures was measured. The results are presented in Fig 1.1, A-D.

A significant proliferative response was induced with both the mitogens in a dose dependent manner Fig1.1 A & C. However, significant enhancement in APase activity was observed only in LPS stimulated cultures, but not in ConA stimulated lymohocytes Fig 1.1B & D. These results were in agreement with those obtained earlier with murine splenic lymphocytes.

Fig - 1.1A

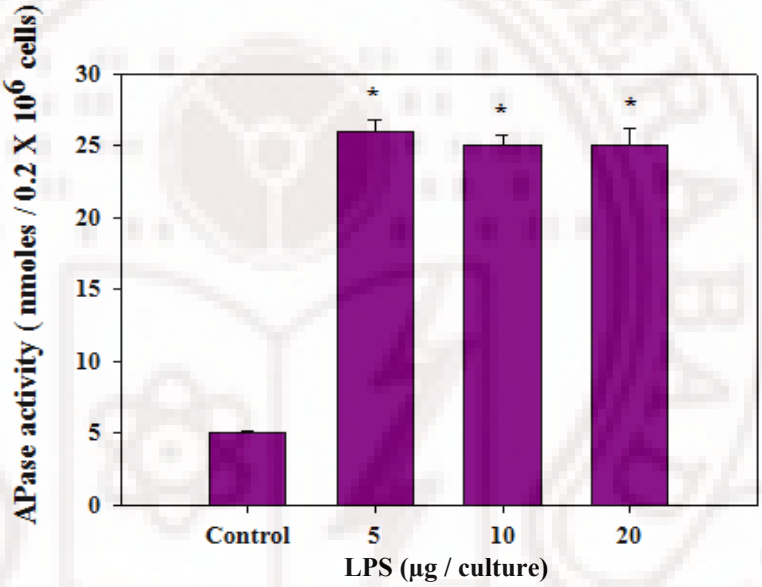
Proliferative response of mitogen stimulated murine splenic lymphocytes



Values represented are mean ± SEM of three experiments. *values significantly differ from the respective control, $p<0.05$.

Fig - 1.1 B

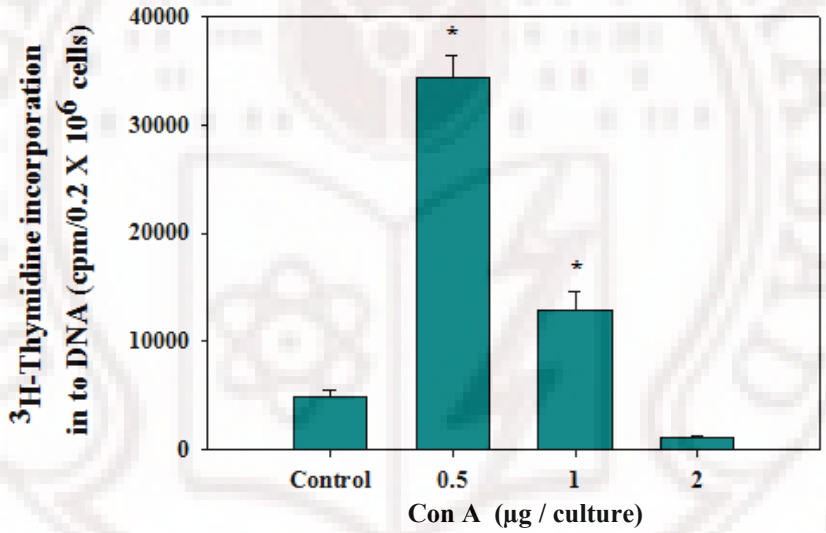
APase activity of mitogen stimulated murine splenic lymphocytes



Values represented are mean ± SEM of three experiments. *values significantly differ from the respective control, $p<0.05$.

Fig - 1.1 C

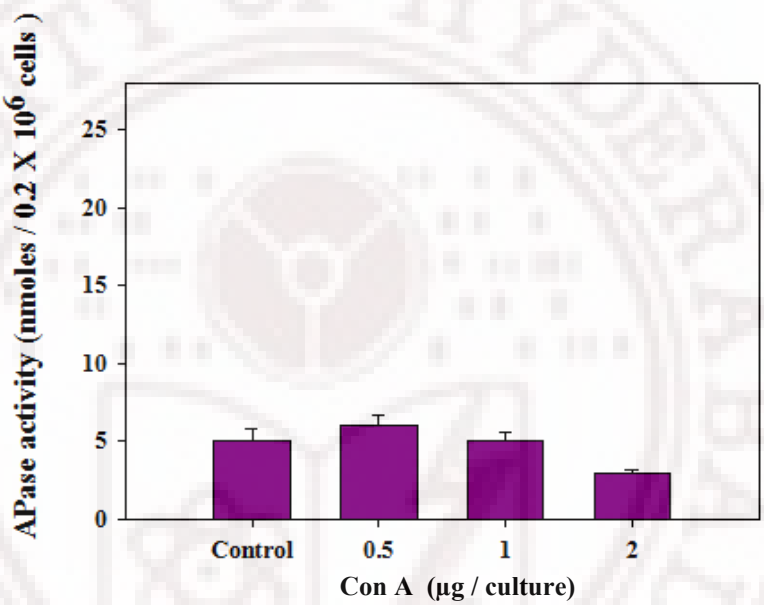
Proliferative response of mitogen stimulated murine splenic lymphocytes



Values represented are mean ± SEM of three experiments. *values significantly differ from the respective control, $p<0.05$.

Fig - 1.1 D

APase activity of mitogen stimulated murine splenic lymphocytes



Values represented are mean ± SEM of three experiments. *values significantly differ from the respective control, $p<0.05$.

Human PBLs were cultured with and without mitogens, PWM (B cell specific) , SPA and PHA (both T cell specific) for 72h. The results of Proliferative response and APase activity are presented in Fig 1.2, A-F. A significant increase in the proliferative response was observed with all the mitogens in a dose dependent manner Fig 1.2 A, C, E. However, no enhancement of APase activity was detectable with any of the mitogens Fig 1.2 B, D, F.

APase activity of lymphocytes isolated from bone marrow samples of multiple myeloma patients

Bone marrow aspirates form patients with multiple myeloma were obtained and lymphocytes were isolated using density gradient centrifugation. The APase activity of the samples ranged from 12 – 88 nmoles / $0.2X10^6$ cells as compared to the very low activity present in normal human PBL. The results are presented in Table 1.1.

Screening of cell lines for APase activity

Based on the above observations, human myeloma cell lines RPMI 8226 and U266 B1 and SupT1, a human T cell lymphoma cell line were screened for APase activity. The results are presented in Table 1.2. It is observed that both the human myeloma cell lines had significant APase activity which is constitutive.

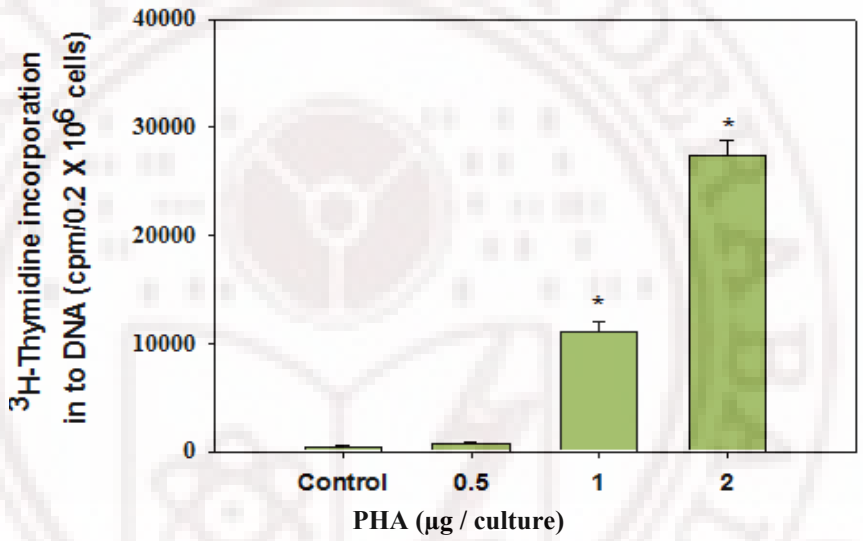
Immunofluorescence analysis of mitogen stimulated normal human peripheral blood cells and myeloma cell lines for APase

The above observations demonstrated the presence of APase in myeloma cell lines based on the measurement of catalytic activity. Indirect immunofluorescence analysis was carried out to detect the presence of the enzyme protein on the membrane using a monoclonal antibody to human bone APase.

The unstimulated human peripheral blood lymphocytes and PHA, PWM and SPA stimulated human peripheral blood lymphocytes were treated with the unconjugated monoclonal antibody (mouse) for human APase followed by the FITC conjugated anti-mouse Ig G1 secondary antibody. The results are presented in Fig 1.3 (A-H). The results show that unstimulated and mitogen stimulated human PBL were negative for the presence of membrane APase. On the other hand, U266 B1, human myeloma cells were positive for surface APase Fig 1.4.

Fig - 1.2 A

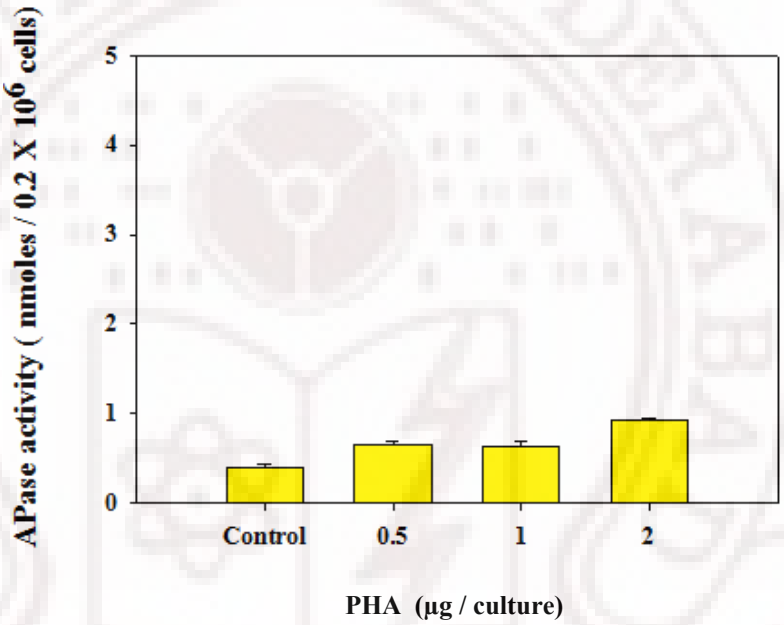
Proliferative response of mitogen stimulated normal human peripheral blood lymphocytes



Values represented are mean ± SEM of three experiments. *values significantly differ from the respective control, $p<0.05$.

Fig - 1.2 B

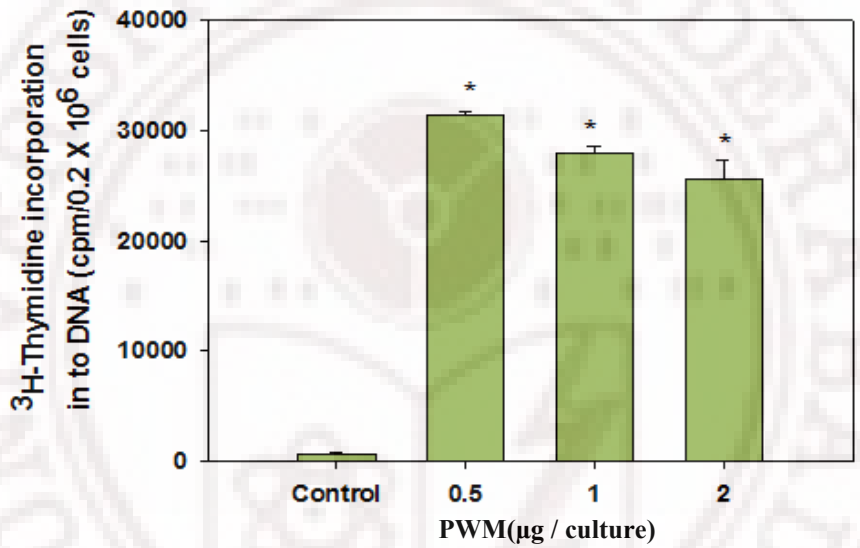
APase activity of mitogen stimulated normal human peripheral blood lymphocytes



Values represented are mean ± SEM of three experiments. *values significantly differ from the respective control, $p<0.05$.

Fig - 1.2 C

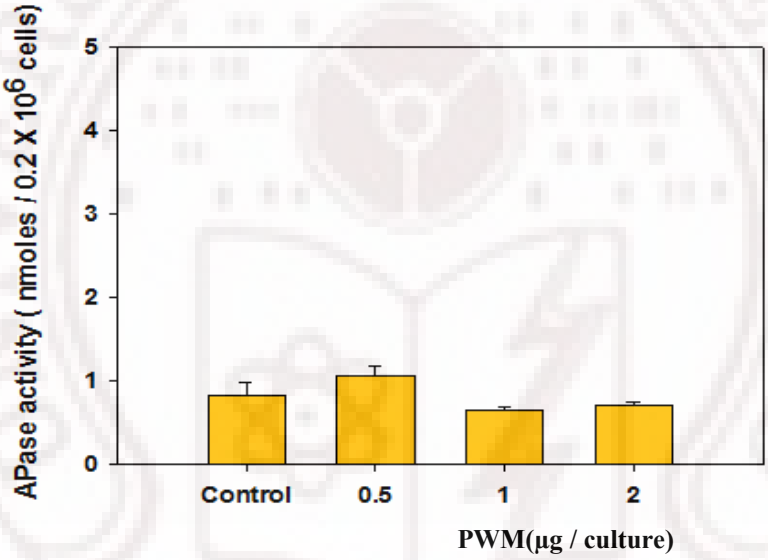
Prolierative response of mitogen stimulated normal human peripheral blood lymphocytes



Values represented are mean ± SEM of three experiments. *values significantly differ from the respective control, $p<0.05$.

Fig - 1.2 D

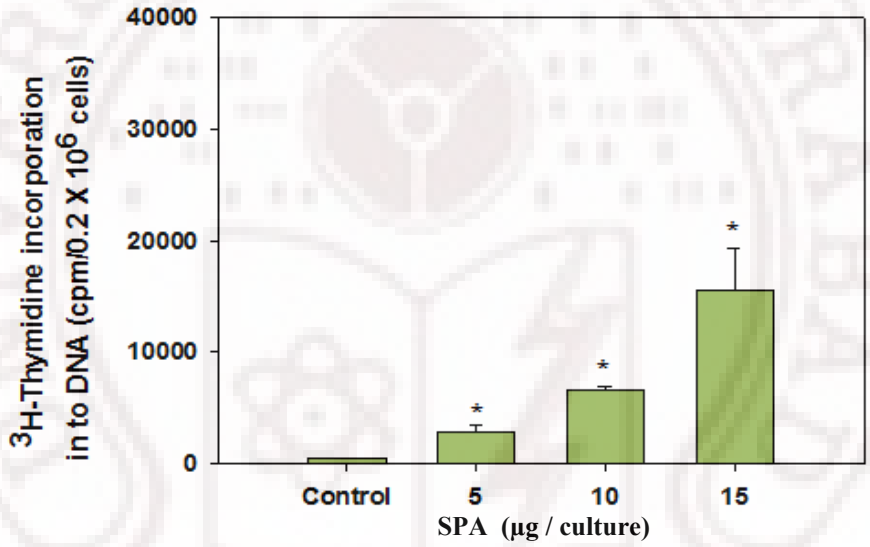
APase activity of mitogen stimulated normal human peripheral blood lymphocytes



Values represented are mean ± SEM of three experiments. *values significantly differ from the respective control, p<0.05.

Fig - 1.2 E

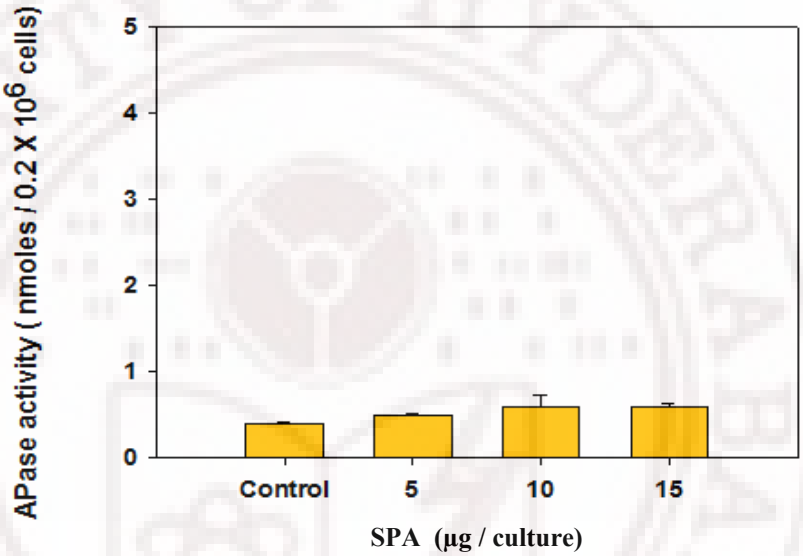
Proliferative response of mitogen stimulated normal human peripheral blood lymphocytes



Values represented are mean ± SEM of three experiments. *values significantly differ from the respective control, $p<0.05$.

Fig - 1.2 F

APase activity of mitogen stimulated normal human peripheral blood lymphocytes



Values represented are mean ± SEM of three experiments. *values significantly differ from the respective control, $p<0.05$.

Table - 1.1

APase activity of B cells isolated from human peripheral blood and from bone marrow aspirates of Multiple Myeloma patients.

Sample		APase activity nmoles / $0.2X10^6$ cells
B cells from peripheral blood		3.55 ± 0.77 #
Myeloma bone marrow cells *		
Sample	1	12.50 ± 0.07
"	2	87.90 ± 4.97
"	3	12.30 ± 2.72
"	4	20.30 ± 1.20
"	5	13.80 ± 4.40

Values presented are mean ± SEM of five samples. * Values presented are mean ± SEM of triplicates.

Table - 1.2

APase activity of human cell lines

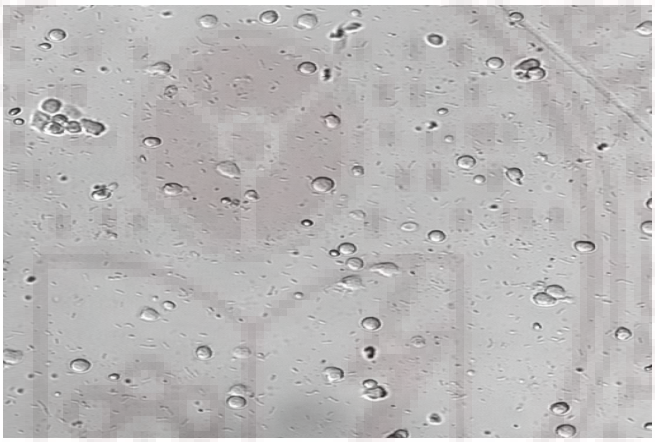
Cell line	Cell conc (X 10^5 cells)	APase activity (nmoles / 30 min)
RPMI 8226 *		
	0.5	3.25 ± 0.23
	1.0	7.30 ± 0.33
	2.0	13.5 ± 0.1
U266B1*		
	0.5	4.5 ± 0.01
	1.0	8.0 ± 0.02
	2.0	15.0 ± 0.64
SupT1#	ND	ND

* Myeloma # T cell lymphoma
Values represented are mean ± SEM of triplicates.
ND: Not detectable

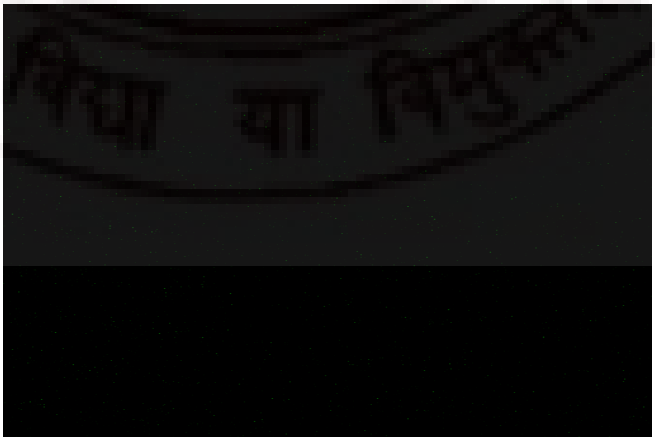
Fig - 1.3

Indirect immunofluorescence analysis of unstimulated human PBL for surface APase

A. Phase contrast (40 x)

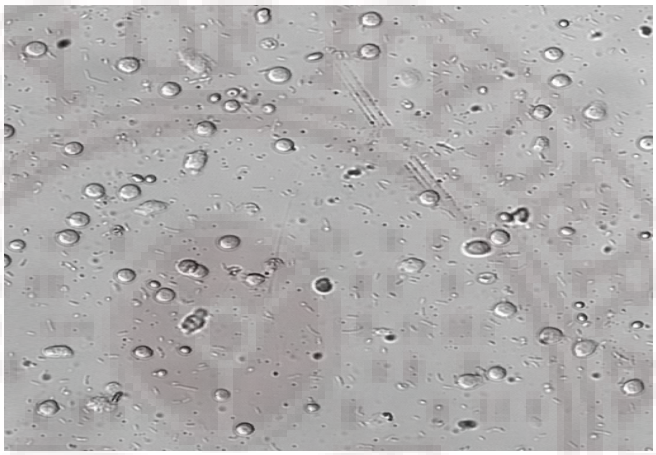


B. Fluorescence (40 x)

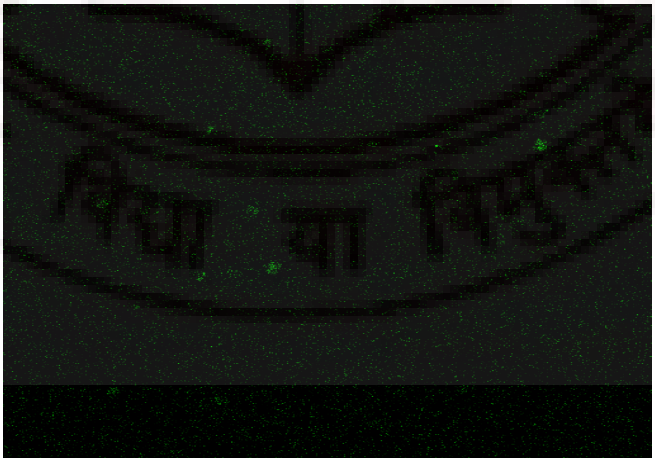


Indirect immunofluorescence analysis of PHA stimulated human PBL for surface APase

C. Phase contrast (40 x)

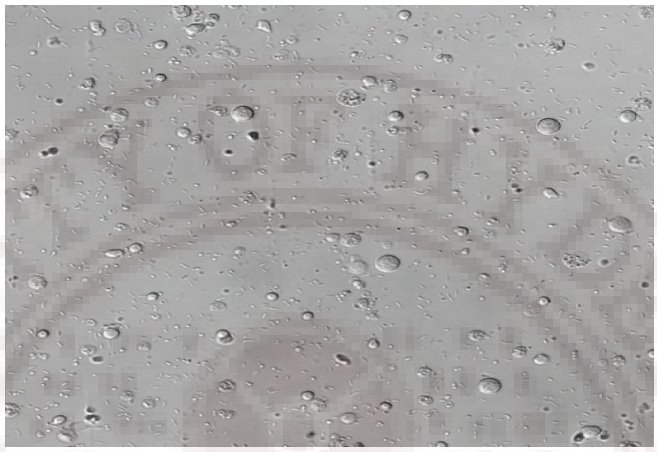


D. Fluorescence (40 x)



Indirect immunofluorescence analysis of PWM stimulated human PBL for surface APase

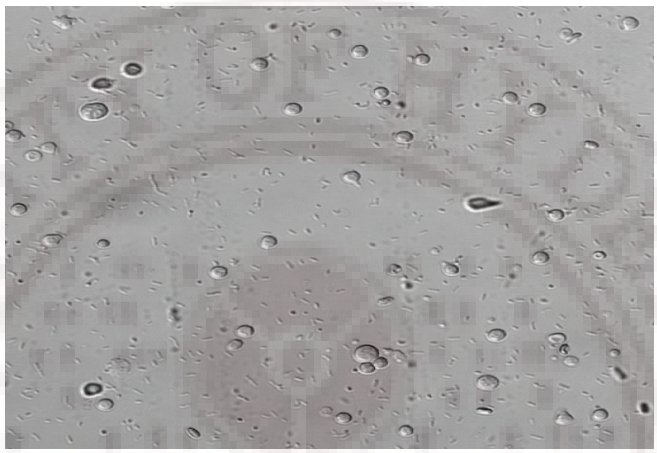
E. Phase contrast (40 x)



F. Fluorescence (40 x)

Indirect immunofluorescence analysis of SPA stimulated human PBL for surface APase

G. Phase contrast (40 x)



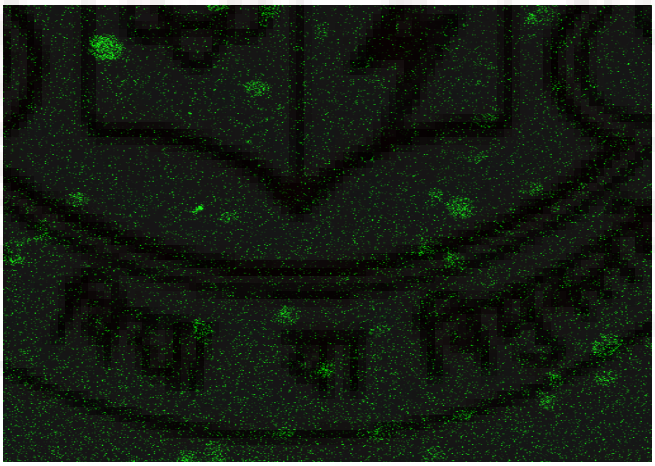
H. Fluorescence (40 x)

Fig - 1.4

Indirect immunofluorescence analysis of U266 cells for surface APase

A. Phase contrast (40 x)

B. Fluorescence (40 x)



Cytochemical staining

Myeloma cell line, U266 B1 was incubated with BCIP/NBT as substrate in Tris-buffered saline to visualize the presence of APase activity. SupT-1 cells , a T cell lymphoma which were known to have no APase activity were used as a control for comparison. The results are presented in Fig 1.5. U266 B1 cells were stained positive for APase activity and the staining was uniform in all the cells. SupT1 cells did not show any staining indicating the absence of the enzyme activity. These results suggest the presence of catalytically active enzyme on the membrane of the intact myeloma cells.

Activity staining of APase on polyacrylamide gel

Further biochemical analysis was carried out using lysates of myeloma cell line U266B1 and RPMI 8226. The lysates were electrophoresed under non-denaturing conditions on a native polyacrylamide gel and was stained with substrate BCIP/NBT. The results are presented in Fig 1.6. Both lysates showed significant enzyme activity.

Kinetic characterisation

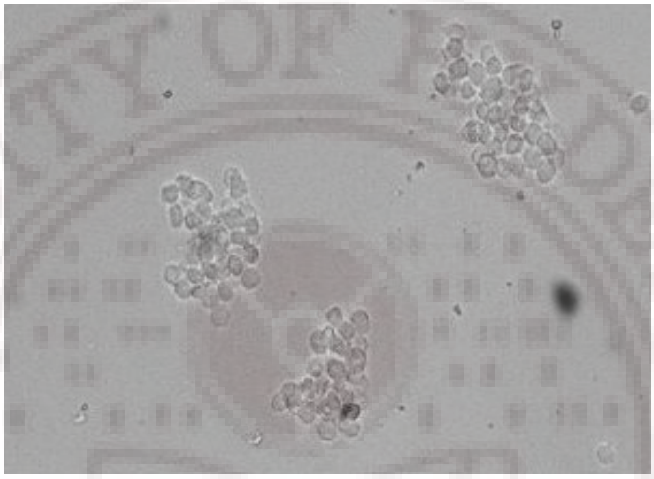
Preliminary kinetic characterization of APase activity was carried out using U266 cells. Apart from bicarbonate buffer, diethanolamine (DEA) buffer was used to determine activity. The specific activity in DEA buffer was higher (26 nmoles / 0.2 X 10^6 cells) as compared to that obtained with bicarbonate buffer (9.7 nmoles / 0.2 X 10^6 cells). In all further experiments on characterization of APase, DEA buffer was used.

Linweaver – Burk plot analysis of the data using p-nitrophenyl phosphate as substrate showed that Km value was 3.57mM Fig 1.7A. The apparent Ki was determined in the presence of varying concentrations of three inhibitors - levamisole, L-homoarginine, and theophylline. Levamisole was found to be a potent inhibitor of APase with an apparent Ki of 22µM (Fig 1.7B). The inhibition constants of other inhibitors used is presented in Table 1.3. To determine the type of inhibition by levamisole.the enzyme velocity was measured at single concentration of substrate pNPP (2.5mM) and with varying concentrations of levamisole. The kinetic plot indicate that the inhibition observed with levamisole is of uncompetitive type (Fig 1.7C).

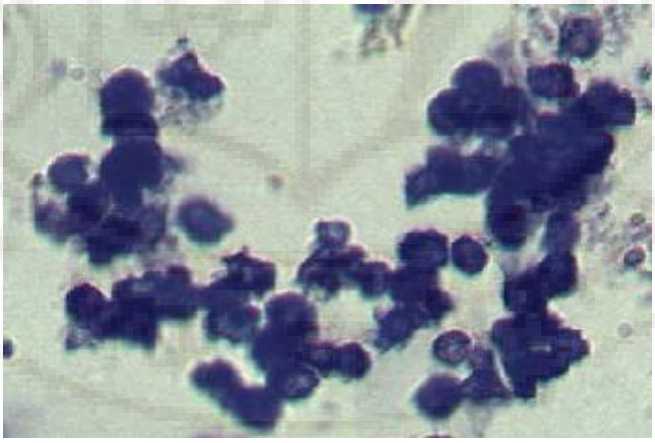
Fig - 1.5

Cytochemical staining for APase activity

A. Sup T1



B. U266 B1



Magnification- 40X : BCIP/NBT was used as substrate.

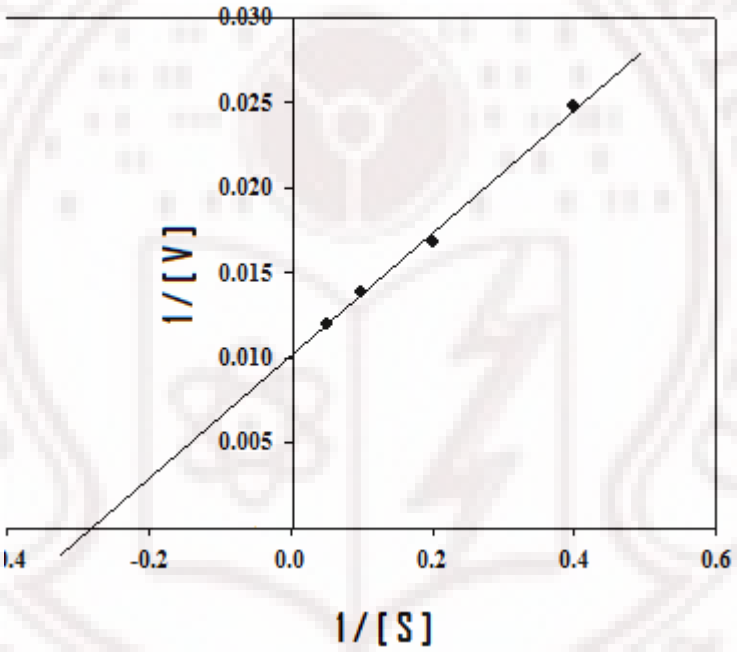
Fig 1.6

Activity staining of APase

Lane 1. Purified Intestinal APase (bovine).
Lane 2. Myeloma cell lysate. A: U266B1, B: RPMI 8226.

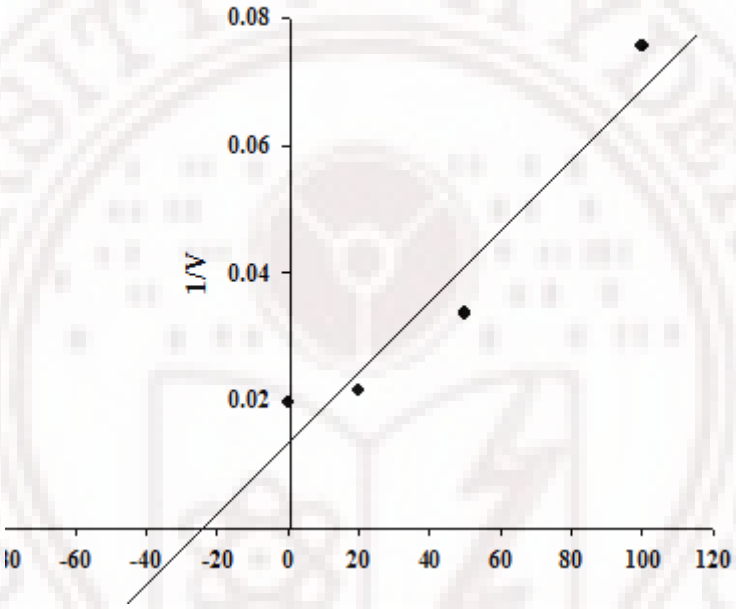
Fig - 1.7

A. Lineweaver-Burk plot of APase (U266) activity using p-nitrophenyl phosphate as substrate.



Km=3.57mM

B. Inhibition of APase activity of U266 cell lysates by Levamisole



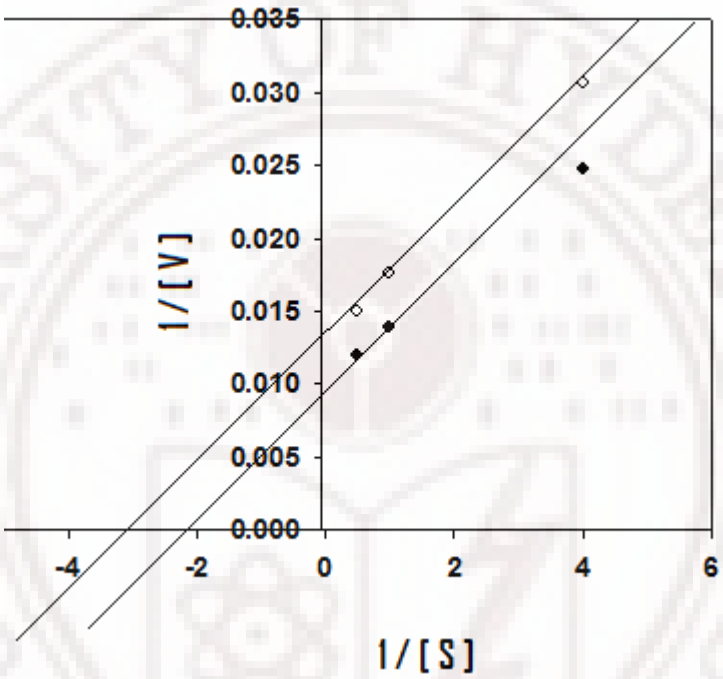
Lev (µM)
Apparent Ki= 22µM

Table - 1.3

Inhibition constant (K_i) of APase activity

Inhibitior	Ki (μM)
Levamisole	22
L-Homoarginine	4000
Theophylline	600

C. Kinetic analysis of inhibition of APase activity by levamisole



◇ p-Nitrophenylphosphate

◆ " + Levamisole (20uM)

Discussion :

Distribution of APase is ubiquitous amongst different cell types and tissues in almost all living organisms. In mammals four isoenzymes with tissue-specific distribution have been well characterized (Fishman 1974). Several physiological functions have been proposed for APase. In bone, TNAPase is thought to mediate phosphate assimilation (Narisawa et al 1997, Zurutuza et al 1999). TNAPase may also play a role in the renal transport of phosphate (Petitclerc & Plante 1981). In the intestine, IAPase has been proposed to participate in the absorption and transport of lipids and nucleotides (Young et al 1981). IAPase may also function as a Pi binding protein under phyiological conditions in the brush border membrane vesicles (Hirano et al 1985).

In humans, the role of APases *in vivo* has been demonstrated by studies on the defeciency of TNAPase resulting in rickets and osteomalacia. Reduction in TNAPase activity has been implicated in the defective mineralization of bone (Fraser 1957, Whyte 1994 & 1995). The role of TNAPase seems to be in the generation of Pi needed for hydroxyapatite crystallization (Robinson 1923, Majeska and Wuthier 1975, Fallon et al 1980). TNAPase aslo hydrolyzes the minerlisation inhibitor, pyrophosphate (PPi)(Meyer 1984). Hence, defeciency of TNAPase aslo leads to elevated levles of extracellular PPi . The role of TNAPase in other organs e.g. liver, kidney and skin, are much less understood. Some recent reports have suggested a role for TNAPase in the regulation of secretory activity of intrahepatic biliary epithelium(Alvaro 2000).

Apart from PPi, pyridoxal phosphate has been shown to be a physiological substrate for TNAPase. A role for TNAPase in phosphate resorption through the brush border of proximal convoluted kidney tubules has been proposed (McComb et al 1979). TNAPase activity on endothelial cell surfaces appears to protect tissues from ischemic damage that results from injury. Also TNAPase has been postulated to play a major role in exrtacellular nucleotide catalysis (Gijsbers et al 2001 & 2003).

APase isozymes have also been used as disease markers**.** PLAPase was one of the first enzymes to be recognized as an onco-fetal protein in a patient with a squamous cell carcinoma of the lung (Fishman et al 1968 a & b). A rare variant of

PLAPase was detected in serum of a patient with pleuritis carcinomatosa (Nakayama et al 1970). Many samples derived from sera of patients with seminoma of the testis showed the presence of PLAPase isoenzyme. Numerous associations have been reported between the expression of GCAPase and PLAPase and malignancy.

Some human tumor cell lines (HeLa) showed an enhanced expression of PLAPase (Benham et al 1981a). Also, high TNAPase activity was detected in cell lines derived from human testicular germ cell tumors (Benham et al 1981b). In some choriocarcinoma cell lines the transformation of normal to malignant trophoblast is associated with a switch in expression from PLAPase to GCAPase. The ectopic expression of PLAPase has been observed in cancer of the lung, ovary, uterus and gastrointestinal tract (Mc Comb et al 1979, Loose et al 1984). Germ cell neoplasms and somatic tumors were found to express PLAPase / GCAPase (Wick et al 1987).

Seminomas, embryonal carcinomas and yolk sac carcinoma, gonado blastomas were positive for PLAPase/GCAPase. All seminomas and malignant teratomas were positive for PLAPase (Epenetos et al 1984). GCAPase is particularly good marker to diagnose carcinoma in situ of testis (Wahren et al 1979, Lange et al 1982, Paiva et al 1983, Jeppsson et al 1984). PLAPase / GCAPase can serve as useful tumor markers. PLAPase also showed rising levels during progression of colorectal carcinoma.

IAPase was found in teratocarcinomas and trophoblastic giant cells of two seminomas (Paiva et al 1983). Intestinal-like APase variant was found in patients with hepatocellular carcinoma (Higashino et al 1972, 1974, 1975a & 1977). It was also detected in renal cell carcinoma (Higashino 1975a, Hada 1979). The loss of a tumor suppressor on chromosome 11 was closely linked to the expression IAP (Lantham & Stanbridge 1990). The significance of IAP as tumor marker is evident from its apparent strong association with a tumor suppressor locus (Lantham and Stanbridge 1992).

TNAPase has been used for many years as a biochemical marker of bone turnover, specifically bone formation, and for monitoring the treatment of patients with metabolic bone disease. Bone APase has been used to identify renal bone

disease common in chronic renal failure. Osteosarcomas display high serum TNAPase levels and these levels are significantly higher in metastatic disease than in patients with localized disease (Bacci et al 1993). Patients with cholestasis have greatly elevated tissue and serum levels of liver- derived TNAPase. TNAPase levels can also be informative in other pathologies like in patients with hyperthyroid Graves disease , patients with rheumatoid arthritis and Paget's disease.

TNAPase is also expressed in cells of the hematopoietic lineage. In the normal hematopoietic system, the post mitotic neutrophilic granulocyte is the only cell type that expressed leukocyte APase under basal conditions. Culvernor et al 1981 have demonstrated that significant APase activity is expressed in cell lines of B lymphoid lineage, including Abelson pre-B , B lymphoma and plasma cell tumor lines. In contrast, T lymphoid and non lymphoid hematopoietic lines had very low activity. Hence TNAPase seems to be a useful marker of B lymphoid lineage.

Despite these observations, the function of TNAPase in malignancy remains largely unknown.

Mitogens are a group of activators which stimulate the lymphocytes in a non-specific or polyclonal manner, unlike antigens which activate a single or a few clones of lymphocytes. Subsets of lymphocytes show differential reactivity to different mitogenic stimuli. Some mitogens activate only certain subset of lymphocytes and some stimulate lymphocytes of certain species only.

It has been demonstrated earlier that mitogenic stimulation of murine B lymphocytes leads to proliferation and concomitant increase in APase activity (Padmaja & Ramanadham 1998). The expression of APase in activated murine B cells was identified as a late marker associated with cell proliferation, progression to differentiation and preceded immunoglobulin secretion (Grayson et al 1981, Burg & Feldbush 1989, Marquez et al 1989, Feldbush & Lafrenz 1991, Souvannavong et al 1997).

Also, it has been observed that the enhancement of APase activity was B cell specific. Stimulation of murine T cells with Con A , a T –cell specific mitogen did not induce alkaline phosphatase activity (Kasyapa & Ramanadham 1992). These earlier observations were confirmed in the present work. As an extrapolation

from the murine studies, normal human peripheral blood lymphocytes were stimulated with T and B cell specific mitogens (PHA, PWM and SPA) and the proliferative response and APase activity were measured. Surprisingly, the resting and mitogen stimulated proliferating T and B-lymphocytes from normal humans did not show enhancement in the APase activity.

The lymphocytes isolated from bone marrow samples of multiple myeloma patients and multiple myeloma cell lines RPMI 8226 and U266B1 were screened for APase activity. Significant activity was found in these malignant B cells. The enhanced expression of APase activity in myeloma cells was confirmed by APase assay, biochemical staining with BCIP/NBT and immunostaining with monoclonal antibody to human TNAPase analyses.

The characterization of the APase activity of myeloma cells in terms effect of different known inhibitors indicated the enzyme belongs to the tissue non-specific isozyme. Earlier experiments on murine B lymphocytes and hybridomas have also shown that the APase present in these cells belongs to tissue non-specific category (Marquez et al 1989 & 1998). Levamisole inhibited the enzyme activity in a uncompetitive manner (Pappas & Leiby, 1989).

The salient findings of the studies described in this chapter are as follows:

1. Neither the unstimulated nor the mitogen stimulated lymphocytes from peripheral blood of normal humans show appreciable APase activity.

2. The lymphocytes from bone marrow of multiple myeloma patiens as well as myeloma cell lines- RPMI 8226 and U266 B1 express APase activity constitutively.

3. The characterization of the APase activity of myeloma cells revealed the enzyme to be tissue non-specific type.

4. Levamisole inhibited the APase activity of myeloma cell lines significantly in an un-competitive manner.

Based on the above results, further experiments were planned to understand the role of APase activity in B cell differentiation and malignancy using myeloma cell lines and levamisole.

CHAPTER - 2

Role of APase activity in the proliferative and differentiative response of myeloma cells

The B lymphocyte is the principal cell involved in mediating specific humoral immune response to infection. Prior to onset of immune response, lymphocytes are quiescent, non-dividing cells circulating in the blood and reside in the secondary lymphoid organs. Upon stimulation with an antigen B lymphocytes go through three distinct phases of cell cycle–early activation (G1 phase), proliferation (S phase) and differentiation to immunoglobulin secreting plasma cells (De Franco 1987).

APase is a membrane bound glycoprotein which has been shown to be expressed in B lymphocytes upon activation and has been used as a marker of B cell activation (Garcia-Rozas et al 1982, Ohno et al 1986, Burg & Feldbush 1989, Marquez et al 1989). It has also been shown that the enhancement of APase activity correlates with proliferation (Ohno et al 1986) and differentiation in murine B cells (Burg & Feldbush 1989, Marquez et al 1989, Souvannavong et al 1994). Further studies have shown that APase activity is expressed in B-lymphocytes committed to proliferation and the activity increases further in the antibody secreting cells (Marty & Feldbush 1993).

Although APase activity has been studied for many years in different tissues, its physiological role has remained largely enigmatic and is still under investigation. Earlier it has been hypothesized that APase may be involved in the transport of Ig molecules (Feldbush & Lafrenz 1991) and in phosphorylation/ dephosphorylation reactions (Souvannavong et al 1992)

It has been proposed that Apase may have a physiological role in the activated B cell in terms of-

A) As a phosphotyrosine phosphatase in the early stages of signaling,

B) Involvement in the proliferation and differentiation,

C) Transport of Ig molecules from the plasma cells.

In the present work it has been observed that multiple myeloma cells lines RPMI 8226 and U266 B1 express APase activity. U266 B1 secretes IgE and RPMI 8226 secretes λ light chains. We used these cell lines to study the role of B lymphocyte APase in proliferation and differentiation and to assess if the APase activity can be targeted to inhibit the proliferation of these cells. An uncompetitive

inhibitor of APase, levamisole has been chosen for the study as in addition to having the property of inhibiting APase it has also been found to have anti-cancer properties.

In the present experiments levamisole has been used to study the role of APase in proliferation, differentiation leading to immunoglobulin secretion.

The Objectives are to study :

1.The effect of levamisole on proliferative response and APase activity of LPS stimulated murine splenic lymphocytes.

2.The effect of levamisole on immuoglobuin secretion by LPS stimulated murine splenic lymphocytes

3. The effect of levamisole on immunoglobulin secretion by myeloma cell lines.

Methods :

1.Preparation of mouse splenic lymphocytes

Murine splenic lymphocytes were prepared as described in Materials and Methods.

2. Proliferaive response and APase activity assay of murine splenic lymphocytes

Splenic lymphocytes, 0.2 X 10^6 cells / well were cultured with 5µg LPS and with varying concentrations of levamisole. The cultures were pulsed with 0.5µCi of ^{3}H-thymidine for the last 24h of 72h culture period and were processed as described in Materials and Methods.

APase assay was performed at the indicated time points as described in Materials and Methods.

3. Proliferaive response and APase activity assay of myeloma cells

RPMI 8226 and U266 B1, 5 X 10^4 cells in triplicate were cultured with various concentrations of levamisole in 200 µl of complete medium. At 48 h, MTT assay was carried out as described under Materials and Methods.

RPMI 8226 and U266 B1, 5 X 10^4 cells in triplicate were cultured with various concentrations of levamisole in 200 µl of complete medium. The cultures were pulsed with 0.5 µCi of thymidine for the last 24h of 72h culture period. The cells

were then harvested and radioactivity was measured as per the protocol given in Materials and Methods.

Myeloma cells, 5 X 10^4, in triplicate were cultured with various concentrations of levamisole in 200 µl of complete medium. APase assay was carried out as described in Materials and Methods at the indicated time points.

4. Estimation of secreted Immunoglobulin in the supernatant by Dot Blot

Mouse IgM detection by Dot Blot

a. Culture supernatant of LPS stimulated murine splenic lymphocytes was collected at the end of 7 days and the IgM was detected by dot blot assay as described in Materials and Methods.

Detection of Human IgE from U266 B1 by Dot Blot

b. U266B1 cells were seeded at a concentration of 5 X 10^4 cells / well in 200 µl medium in a 96 well microtitre plate. Culture supernatant of untreated and levamisole treated U266 B1 cells were collected at the end of 48h and the IgE was detected by dot blot assay.

5. Analysis of light chains by SDS-PAGE:

U266 B1 and RPMI 8226 were cultured at 0.25 X 10^6/ml with and without 1mM levamisole for 48h and 15ml of the culture supernatant was collected and lyophilized. The lyophilized samples were reconstituted in 1ml of PBS and dialysed. Electrophoresis was carried out on 30µl of the dialysed samples. SDS-PAGE was performed according to the method of Laemmli et al 1970. The gels were silver stained according to the method of Blum et al 1987.

6. Analysis of intracellular IgE by Western Blot:

The intracellular IgE content was quantified by Western blot assay. Control and levamisole treated cells, 1 x 10^6 were harvested at 48 h of culture. The cells were centrifuged and washed twice with isotonic saline. The cells were then lysed with the lysis buffer as described in Materials and Methods.

7. Effect of Levamisole on the proliferative response of mitogen stimulated normal human peripheral blood lymphocytes

Normal peripheral blood lymphocytes were isolated and cultured along with PWM and levamisole as described Materials and Methods.

Results :

Effect of levamisole on proliferative response of mitogen stimulated murine splenic lymphocytes.

Murine splenic lymphocytes were stimulated with B cell specific mitogen LPS and the proliferative response was measured by ^{3}H-thymdine incorporation in to DNA at 72 h.

Levamisole was added at two concentrations, 0.75 and 1.0 mM. The results are presented in Fig 2.1a. The proliferative response was inhibited by levamisole in a dose-dependent manner ($p< 0.05$ at 0.75 mM conc.). Simultaneously, APase activity of mitogen stimulated and unstimulated cultures was measured. The results are presented in Fig 2.1 b. APase activity was also siginificantly inhibited by levamisole ($p<0.05$).

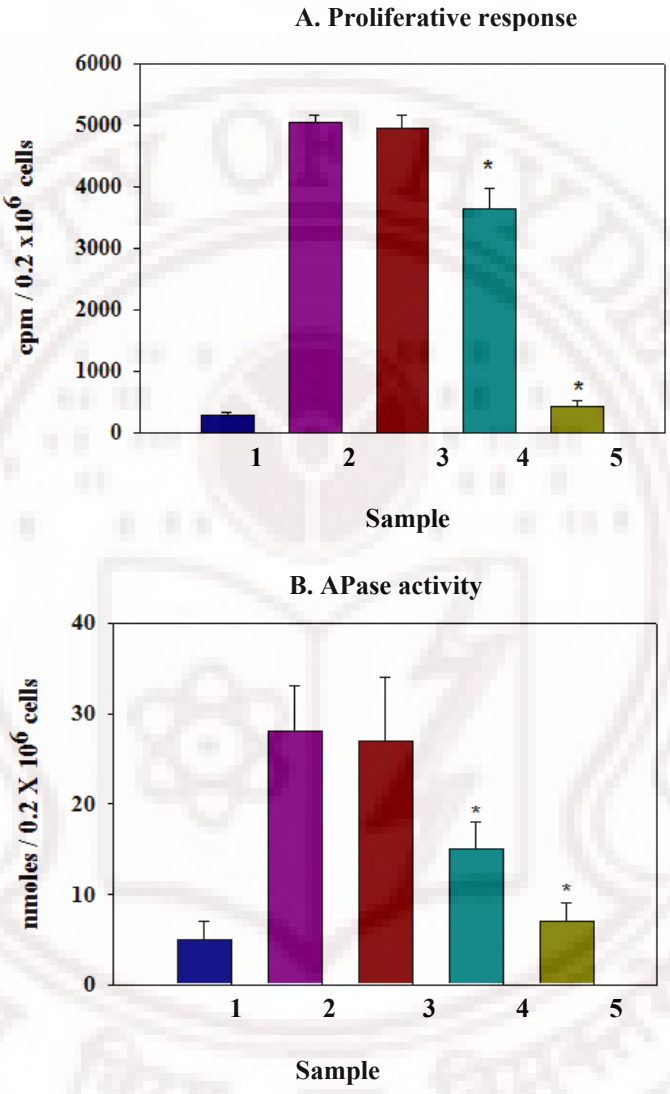
Effect of levamisole on Immunoglobulin secretion by mitogen stimulated murine splenic lymphocytes in to the supernatant .

LPS activated B lymphocytes differentiate in to antibody secreting cells and secrete IgM. The IgM secreted in to the cell culture supernatant was estimated using a Dot Blot assay using a polyclonal anti-IgM antibody (μ - chain specific) conjugated to horse radish peroxidase.

The effect of levamisole on IgM secretion by LPS stimulated murine splenic B cells was studied and the results are presented in (Fig-2.2a). The antibody used for the assay was highly specific as it did not show any cross-reactivity with bovine IgM present in FCS or any other protein. The blot was scanned by densitometry and the results are presented in Fig 2.2b. The amount of IgM secreted by LPS stimulated cells was significantly inhibited by levamisole ($p< 0.05$).

Fig - 2.1

Effect of levamisole on proliferative response , APase activity of LPS-stimulated murine splenic B lymphocytes (48h culture period)

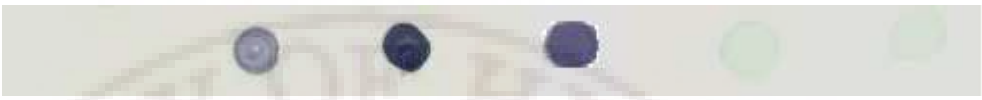


Values presented are mean ± SEM of 3 experiments. *values significantly differ from the respective control, p<0.05.

Sample
1. Control
2. LPS 10µg
3. LPS + 500 µM Lev
3. LPS + 750 µM Lev
4. LPS + 1. 0 mM Lev
*** p< 0.05, 2 Vs 4 and 5**

Fig - 2.2

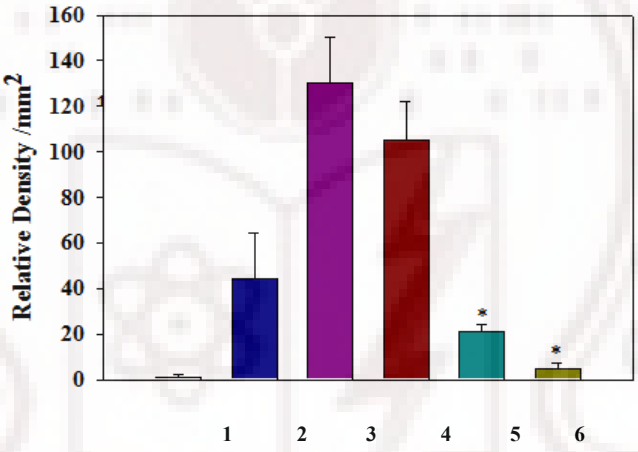
A. Dot blot of IgM secreted by LPS stimulated murine splenic lymphocytes



1 2 3 4 5 6

Sample

B. Densitometric analysis of dot blot



Samples

1. FCS
2. Culture supernatant - untreated cells
3. " - LPS treated cells
4. " - LPS treated cells +500 µM Lev
5. " - LPS treated cells +750 µM Lev
6. " - LPS treated cells +1.0 mM Lev

In all cases 5 µl of samples was used. * $p < 0.05$, 3 Vs 5 and 6. Mean ± SEM of 3 experiments

Effect of levamisole on Proliferation

The proliferative response of myeloma cell lines in presence of levamisole (1 μM – 2.5 mM) was studied using MTT assay and ^{3}H-thymidine incorporation.The results are presented in the Fig 2.3 A&B, 2.4 A&B and 2.5A&B.. No inhibition of proliferative response was observed up to a 500 μM concentration of levamisole. However, the proliferation was inhibited in a dose dependent manner from 0.5 – 2.5 mM levamisole.

Effect of levamisole on APase activity

Both the myeloma cell lines (RPMI 8226 and U266 B1) constitutively express APase activity. In the presence of levamisole, the APase activity was inhibited in a dose dependent manner. The results are presented in Fig 2.6 A&B.

Effect of levamisole on IgE and light chain (λ) secretion by multiple myeloma cells in to the supernatant .

U266 B1 myeloma cells secrete IgE and RPMI 8226 cells secrete λ light chains constitutively in to the culture supernatant. IgE secreted by U266 B1 in to the culture supernatant was also estimated by Dot Blot assay using anti human IgE antibody and the results are presented in Fig 2.7 A. The secretion of IgE was inhibited by levamisole in a dose dependent manner as assessed by densitometry Fig 2.7 B . However the western blot analysis showed that much of the immunoglobulin remained intracellular when levamsiole was added (Fig 2.8).

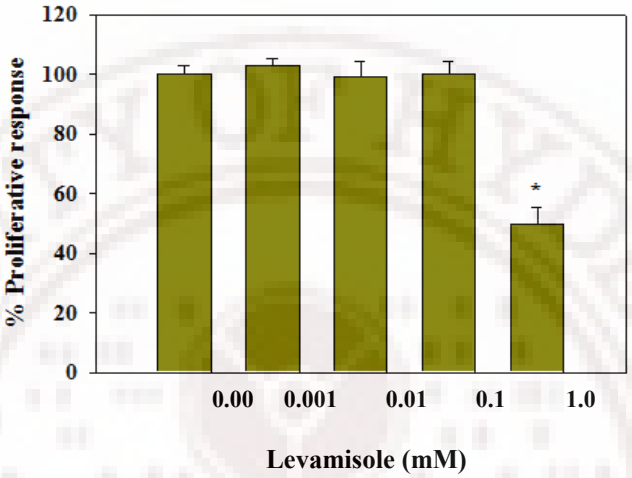
The cell line RPMI 8226 secretes immunoglobulin light chains of λ type, and U266 B1 secretes IgE. Both the cell lines were incubated in a serum-free medium and the culture supernatants were collected and concentrated. The samples were electrophoresed and the results are presented in Fig 2.9

The stained bands in lane 1 are due to molecular weight marker, lane 2 shows two bands one at 29 KDa due to light chain of IgE and other at 66KDa due to heavy chain of IgE from untreated cells. Lane 4 shows a decrease in the concentration of IgE light and heavy chains from levamisole treated U266B1 cells. Lane 5 shows a single band due to λ light chains secreted by untreated RPMI 8226 cells and lane 6 indicated that secretion of λ light chains is inhibited in levamsiole treated RPMI 8226 cells.

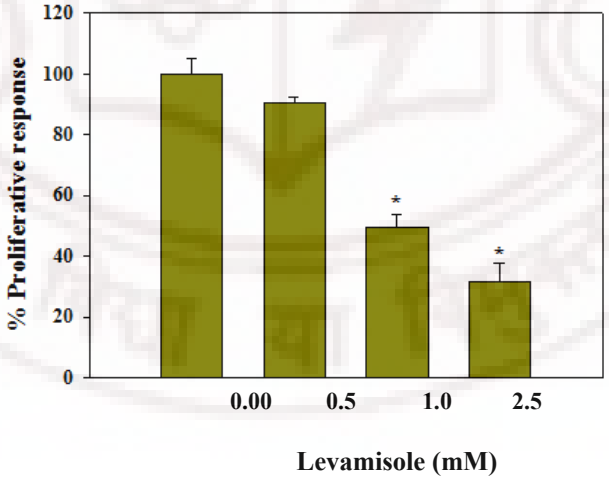
Fig – 2.3

Effect of levamisole on proliferative response of U266 cells(MTT assay)

A



B

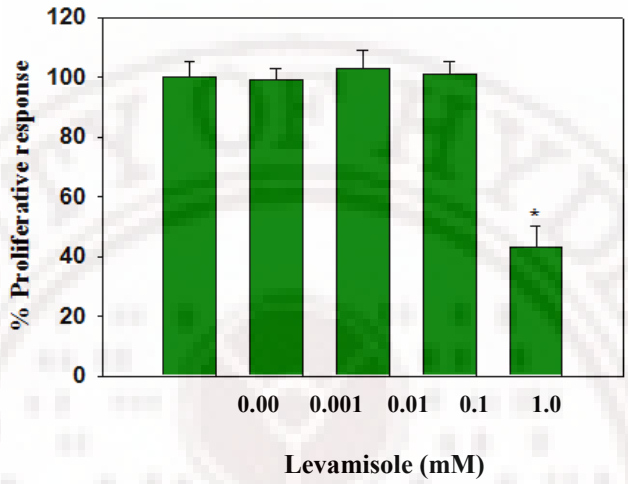


Values represented are mean ± SEM of three experiments. *values significantly differ from the respective control, $p<0.05$.

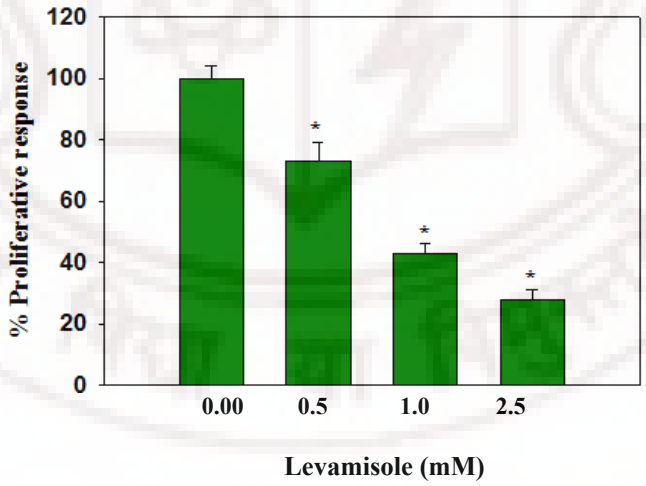
Fig- 2.4

Effect of levamisole on proliferative response of RPMI 8226 cells(MTT assay)

A



B

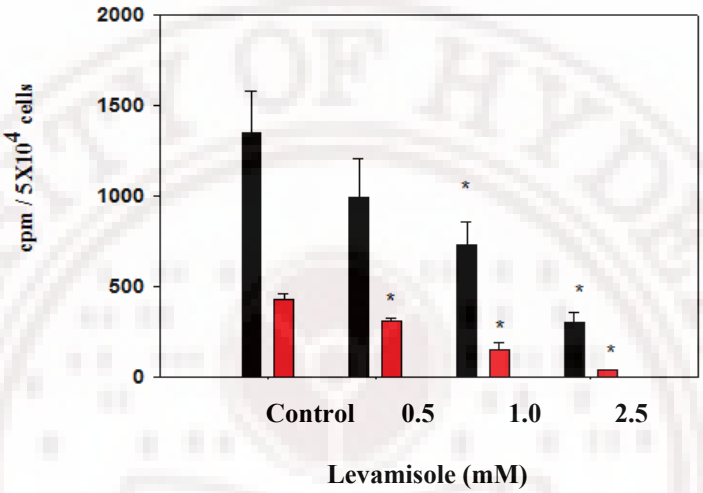


Values represented are mean ± SEM of three experiments. *values significantly differ from the respective control, $p<0.05$.

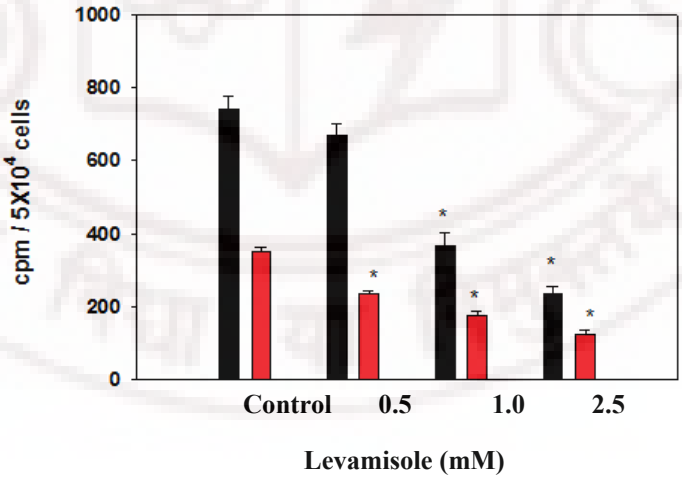
Fig – 2.5

Effect of Levamisole on proliferative response of myeloma cell lines by ^{3}H-Thymidine incorporation in to DNA (48-72hr pulse).

A. RPMI 8226



B. U266 B1



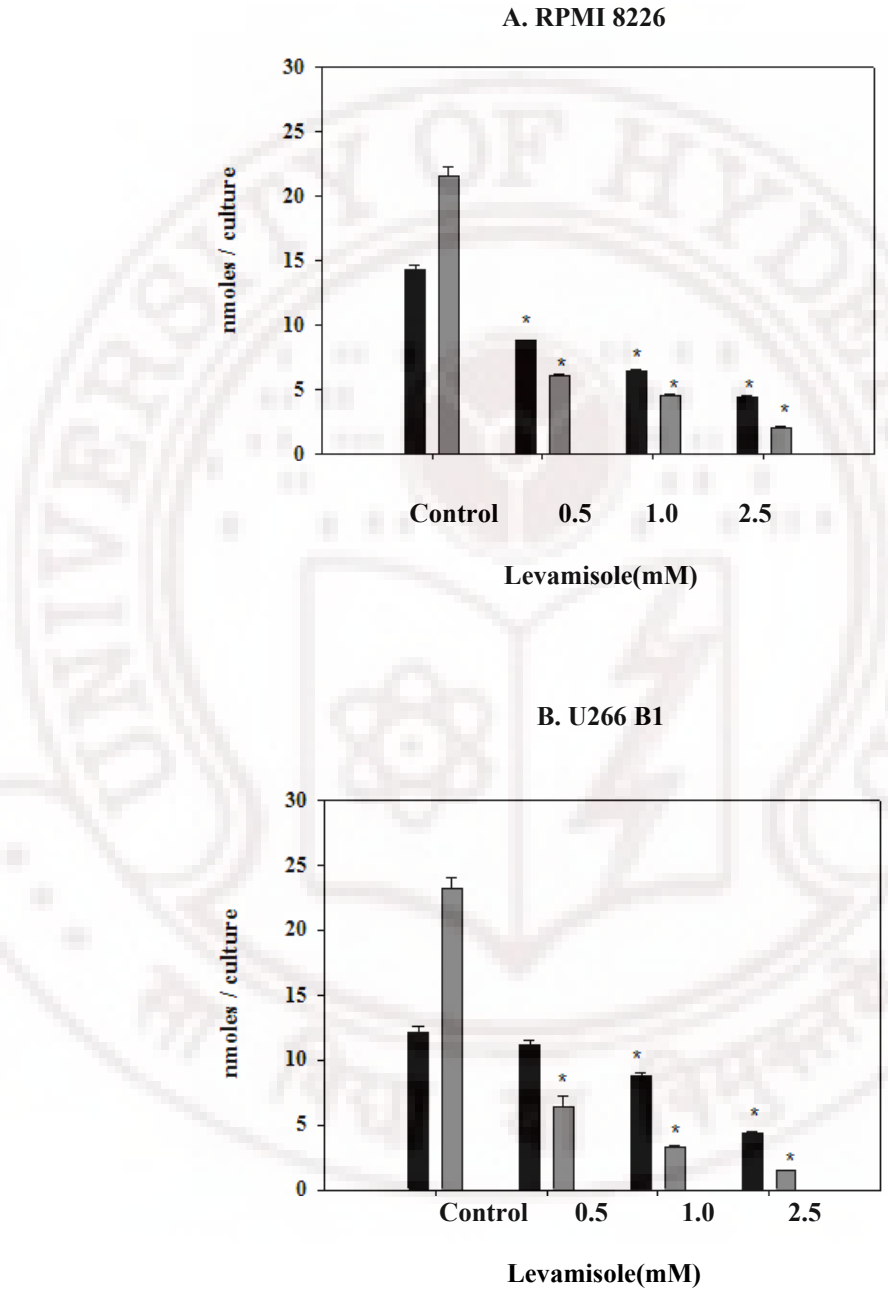
+ FCS
- FCS

Control : No levamisole addition.

Each value represents the mean ± SEM of expriments. *values significantly differ from the respective control, $p<0.05$.

Fig – 2.6

Effect of Levamisole on APase activity of multiple myeloma cell lines.

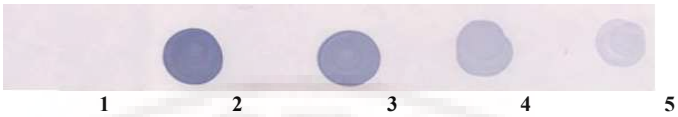


Control : No levamisole addition.

Each value represents the mean ± SEM of expriments. *values significantly differ from the respective control, p<0.05.

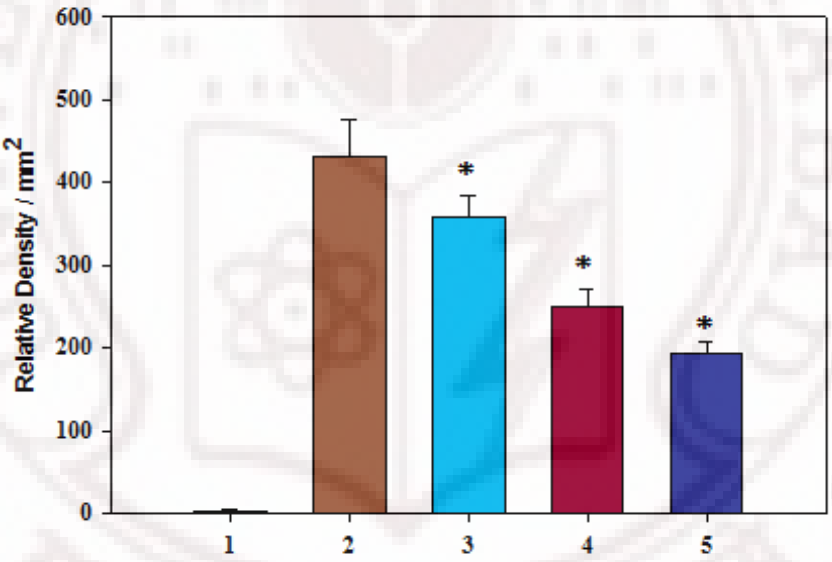
Fig - 2.7

A.Dot Blot of IgE secreted by U266 B1cells (48h)



Samples

B. Densitometric analysis of dot blot



Samples

Samples-

1. **FCS, 5 µl**
2. **Culture supernatant, 5 µl, untreated cells**
3. **" from cells treated with 0.5mM Lev**
4. **" 1.0mM Lev**
5. **" 2. 5mM Lev**

Mean ± SEM of 5 experiments *values significantly differ from the control, $p<0.05$.

Fig – 2.8

Western Blot analysis of IgE in U266 B1 cell lysate

a: Lane 1: U266 B1 control cells

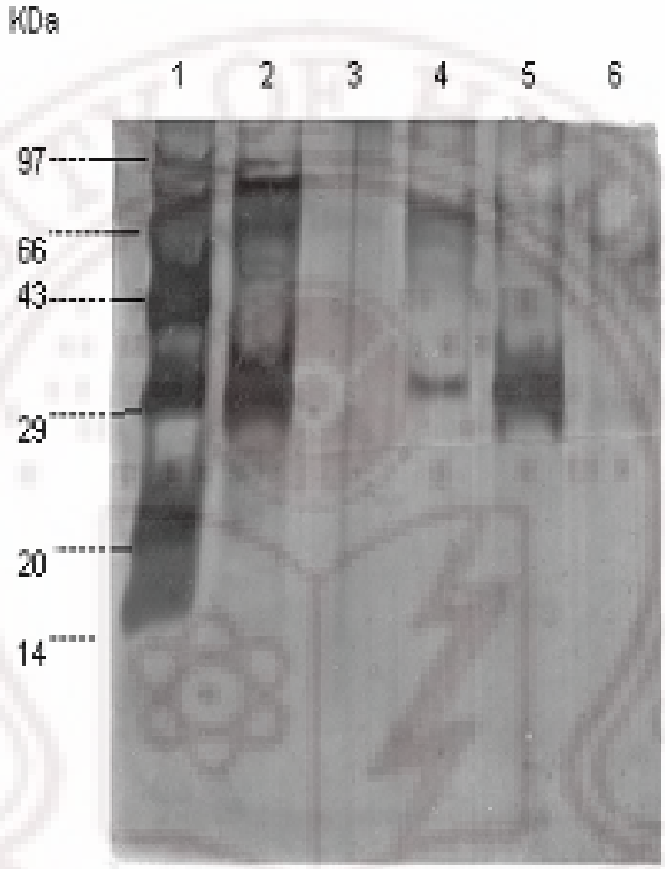
Lane2: U266 B1 treated with levamisole 0.5mM

Lane3: U266 B1 treated with levamisole 1.0mM

b: β actin from the above three treated samples

Fig - 2.9

Electrophoretic analysis of IgE and λ light chain in the culture supernatant of myeloma cell lines treated with levamisole



Sample (concentrated culture supernatant 40µl was loaded.)

Lane 1: Molecular weight markers
Lane 2: U266 B1
Lane 3 : Blank
Lane 4: U266 B1 + levamisole, 1.0mM
Lane 5: RPMI 8226
Lane 6: RPMI 8226 + levamisole 1.0mM

Effect of Levamisole on the proliferative response of mitogen stimulated normal human peripheral blood lymphocytes.

Levamisole was chosen for the studies as it is an inhibitor of APase. It was found that levamisole affects myeloma cell growth and proliferation via inhibition of APase. In order to evaluate if levamisole adversely effects normal cells which do not express APase activity, Mitogen (PWM) stimulated normal human peripheral blood lymphocytes were cultured with and without levamisole. The results are presented in Table 2.1. Levamisole did not inhibit the proliferation in PWM mitogen stimulated peripheral blood lymphocytes.

Table – 2.1

Effect of Levamisole on the proliferative response of Poke weed mitogen (PWM) stimulated normal human peripheral blood lymphocytes

Addition	^{3}H- Td incorporated in to DNA (cpm / 0.2 x 10^{6} cells)
None	500 ± 50
PWM, 2.0 ug	27000 ± 3250
PWM, 2.0 ug + Lev, 0.5 mM	26000 ± 3725
PWM, 2.0 ug + Lev, 1.0 mM	26500 ± 3315

Values represented are mean ± SEM of three experiments

Discussion:

Mitogens induce lymphocytes to undergo progressive enlargement, nucleotide synthesis , nuclear protein phosphorylation, histone acetylation and mitosis. This results in transformation of the normally dormant cells to a rapidly proliferating blast like state. During the early phase of lymphocyte activation, phosphorylation/ dephophorylation reactions and phosphate transport may play a crucial role in progression of cell through cell cycle. APase distribution is ubiquitous among cell types and tissues. In bone, APase is thought to mediate phosphate assimilation (Narisawa et al 1997 , Zurutuza et al 1999). In intestine, APase is assumed to participate in absorption and transport of lipids and nucleotides (Young et al 1981). APase may play a role in the renal transport of phosphate (Petitclerc & Plante 1981). A possible role in phosphate binding has also been proposed (Hirano et al 1985). It is observed that APase activity is enhanced when murine splenic lymphocytes are stimulated with LPS, a polyclonal B cell mitogen. LPS induces the phenotypic maturation of pre-B cells and immature B lymphocytes to the mature B-cell stage and induces mature B-cells to proliferate and differentiate in to antibody secreting plasma cells *in vitro*.

Earlier studies in murine system have shown that following B cell activation and differentiation, enhancement of APase activity was associated with immunoglobulin secretion (Grayson et al 1981, Burg & Feldbush 1989, Marquez & Toribio 1989, Feldbush & Lafrenz 1991, Souvannavong et al 1997). Increase in APase activity is an integral feature of B-cell activation and differentiation and perhaps aids in the metabolite transport to fulfill the demands of the growing cell. It could also be involved in the Ig transport in B-lymphocytes as it was shown to be complexed and secreted out along with IgM (Lafrenz & Feldbush 1991). In view of its pleiotropic role, the enhancement of APase activity appears to be a physiological phenomenon in activated B-lymphocytes.

But, its function in malignant cell was largely unknown. In this part of study an attempt has been made to explore the role of APase in B cell differentiation and possible patho-physiological significance in malignancy.

In the previous chapter it was demonstrated that APase activity is constitutively present in myeloma cells and mitogen stimulated splenic lymphocytes, the

proliferative response was associated with concomitant increase in the APase activity. In this chapter, we have extended our previous observations and studied the role of APase in immunoglobulin secretion by LPS stimulated lympocytes and myeloma cells. To begin with we studied the role of APase using mitogen stimulated murine lymphocytes, where levamisole inhibited the Immunoglobulin secretion, indicating that APase is involved in immunoglobulin secretion .

It is hypothesized that APase may be involved in the constitutive proliferation as well as in immunoglobulin secretion of myeloma cells. It was observed that murine splenic lymphocytes showed an increase in proliferative response concomitant with an increase in the APase activity and immunoglobulin secretion upon mitogenic stimulation. Levamisole signficantly inhibited the APase activity when added to the LPS stimulated cells at the initiation of the culture. In these cells, the proliferative response and immunoglobulin secretion was also inhibited in a dose dependent manner suggesting that inhibition of APase activity results in inhibition of immunoglobulin secretion.

Recently it has been demonstrated that alkaline phosphatase activity is expressed in a subset of $CD19^{+}$ BAP^{+} normal human B cells (Hossain & Jung 2008). It has also been shown that upon PWM stimulation of human PBMC, a fraction of proliferating B cells expressed alkaline phosphatase immunologically similar to bone cell APase. The expression of APase correlated with Ig secretion.

In the present work, levamisole added at the initiation of the culture had no effect on the proliferation in normal PBL even at 1mM concentration. This could probably be due to the absence of APase activity in these cells. In order to investigate the role of APase in proliferation and immunoglobulin secretion by myeloma cells, the effect of levamisole was studied. It was found that levamisole inhibited the APase activity, proliferation and immunoglobulin secretion in a dose dependent manner. These results suggest that APase may have a significant role in B cell proliferation and immunoglobulin secretion. A desirable target in any disease would be a factor uniquely present in and associated with the disease. The presence of APase activity with probable pleiotropic functions in malignant human myeloma cells and its absence in normal B cells makes APase an attractive target for the modulation of proliferation of myeloma cells. The results of the

present studies suggest that APase activity could be used as a target to control the proliferation and differentiation of myeloma cells.

CHAPTER - 3

Anti-proliferative and cytotoxic effect of levamisole on multiple myeloma cells

Multiple myeloma is characterized by the proliferation of malignant monoclonal B cells in the bone marrow, renal insufficiency, anemia, hypercalcemia and immunodeficiency. For the treatment of multiple myeloma a number of chemotherapeutic agents have been used for the last 40 years. But only about 5% of the patients were found to achieve complete remission with this therapy. A number of side affects and eventual relapse are encountered with anti-myeloma drugs. An ideal anti-cancer drug is usually designed towards a molecular target which is unique to cancer cells which and is involved in pathogenesis.

It has been reported that high levels of APase activity has been observed in different types of malignant cells and in serum samples of cancer patients (Millan 1992, Millan & Fishman 1995). The enhancement of APase activity could be due to the functional involvement of APase isoenzymes in tumorogenesis and might be a crucial factor involved in the etiology of the disease. Further, antibody to APase inhibited the proliferative response in mitogen induced B lymphocytes suggesting a role for APase in cell cycle (Marty and Feldbush 1993, Padmaja & Ramanadham 1999).

Levamisole which is used as an adjuvant along with 5-FU in the treatment of cololorectal carcinoma has also been shown to have anti-cancer and immunosuppressive actions (Moertel et al 1995). Levamisole can exert its anti-neoplastic effects when used as an adjuvant to conventional anti-neoplastic therapies-surgery and radiotherapy . However the mechanism by which levamsiole induces cytotoxicity is still not clear.

Earlier studies from this laboratory have shown that levamisole inhibits the proliferative response and APase activity of lipopolysaccharide stimulated murine B-lymphocytes in a dose-dependent manner. However, human peripheral blood B lymphocytes did not express APase activity upon mitogenic activation. Some human B cell lines and leukemic B cells have been shown to express APase activity. These results suggested that APase activity is preferentially expressed in malignant cells and thus could be used as a molecular target for therapy.

Hence, in the present study, we have investigated the effect of levamisole on myeloma cell lines RPMI 8226 and U266 B1 *in vitro* using APase activity as a molecular target. The effect of levamisole on myeloma cells in culture in terms of

their growth, viability, proliferation, and induction of apoptosis have been evaluated.

The objectives of the present work are:

1. Effect of Levamisole on the proliferative response and APase activity of cell lines.
2. Cytotoxic effect of levamisole .
3. Mechanism of cell death induced by levamisole.
4. Reversibility of the effect of levamisole on proliferation and effect of levamisole in combination with Atorvastatin on the proliferative response.

Methods :

1.Cell viability

5 X 10^4 cells (RPMI 8226 and U266 B1) in triplicate were cultured with various concentrations (0, 0.5, 1.0, 2.5 mM) of levamisole in 200µl of complete medium. Cell viability was determined every 24h for over a period of 96 hours by trypan blue assay as described in Material and Methods.

2.Cytotoxicity by MTT assay

5 X 10^4 cells in triplicate were treated with various concentrations (0,0.5,1.0,2.5mM) of levamisole in 200µl of culture medium with and without FCS. MTT assay was done at a interval of 24h over a 96h culture period. MTT assay was done as described in Material and Methods.

3.Cell number

5 X 10^4 cells in triplicate were treated with various concentrations (0,0.5,1.0,2.5mM) of levamisole in 200µl of culture medium with and without FCS. The cell number at the intervals of 24h, over a culture period of 96h was determined by counting both live and dead total cells. The cells were counted using inverted microscope, Neubauer chamber and trypan blue dye were used.

4.DNA fragmentation assay

0.25 X 10^6 cells / ml were incubated with 1.0mM levamisole for a period of 48 and 72h. Cells treated with Vincristine at concentration of 10^{-6} M was taken as positive control. DNA was extracted from the treated cells and fragmentation assay was performed as per the protocol given in Materials and Methods.

5.Cytochrome *c* assay

Cytochrome c assay was done as described in Materials and Methods.

6.Caspase 3 activity

0.25 X 10^6 cells / ml were treated with 1.0mM and 2.5mM levamisole for 24h. Cells treated with Vincristine at concentration of 10^{-6} M was taken as positive control. The treated cells were then harvested and caspase 3 activity was determined as per the protocol given in Materials and Methods.

7.Reversibility of cytotoxixity

5 X 10^4 cells intriplicate were treated with various concentrations (0.0, 0.5, 1.0, 2.5mM) of levamisole in 200µl of complete culture medium.

In the first set of culture levamisole containing supernatant was removed after 24h and to the cell pellet fresh culture medium without levamisole was added and incubated for next 48h.

In the second set of culture levamisole containing supernatant was removed after 48h and to the cell pellet fresh culture medium without levamisole was added and incubated for next 24h.

The third set of culture was incubated with levamisole for complete 72h of the culture period.

8.Anti proliferative activity of levamisole in presence of atorvastain

To the cells seeded at a concentration of 5 X 10^4 / well different concentrations of atorvastatin (0, 10, 30, 50, 70uM) was added to each of the levamisole concentration (0.0, 0.5, 1.0, 2.5mM).

Results :

Effect of levamisole on viability

As shown in Fig 3.1 A&B and Fig 3.2 A&B, the addition of levamisole significantly inhibited the viability of RPMI 8226 and U266B1 myeloma cell lines in a dose dependent manner over a period of 72 h as assessed by trypan blue dye exclusion. The cell viability was not affected at 0.5 mM concentration of levamisole. However, the viability was significantly decreased with 1.0 and 2.5 mM levamisole concentration. These results indicated that levamisole is cytotoxic to myeloma cell above 1.0 mM concentration. When the myeloma cells were cultured in the absence of fetal calf serum, the viability was significantly decreased in presence of levamisole as compared to those cultured with FCS.

Cytotoxic effect of levamisole

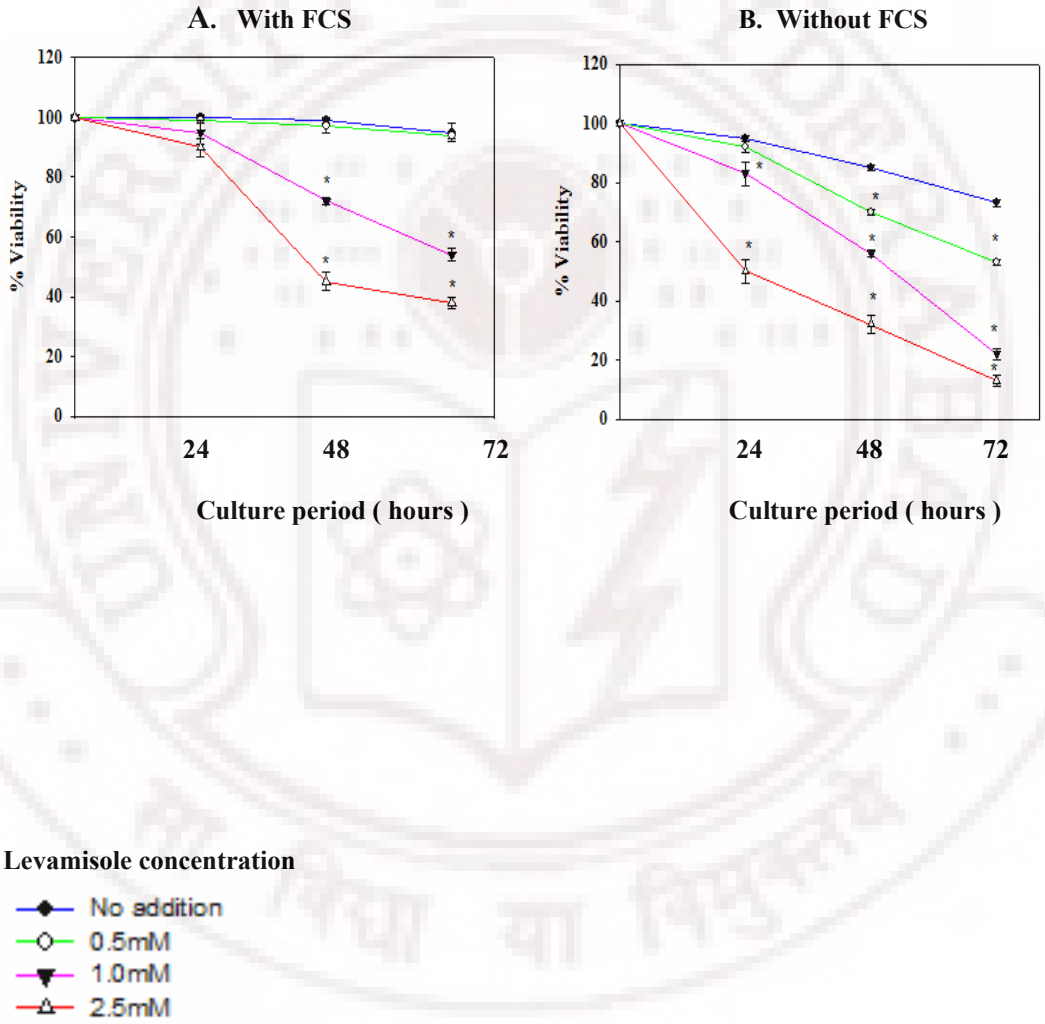
The cytotoxic effect of levamisole on multiple myeloma cells was determined by MTT assay. There is a significant dose-dependent cytotoxic effect levamisole on both the cell lines at 1.0 mM and 2.5 mM concentration. The results are presented in the Fig 3.3 A&B and 3.4 A&B. The effect observed was also time-dependent as the cytotoxic effect increased significantly with time, i.e. from 24 - 72 h of culture period. The cytotoxic effect of levamsiole was found to be significantly higher in the absence of serum than in medium with serum. The IC_{50} value of levamisole was around 2.5mM at 48h and 1mM at 72h.

Effect of levamisole on cell number

The effect of levamisole on cell number was determined by counting total number of cells at 24, 48 and 72 h of culture period. The results are presented in Fig 3.5 A&B and 3.6 A&B.

Fig - 3.1

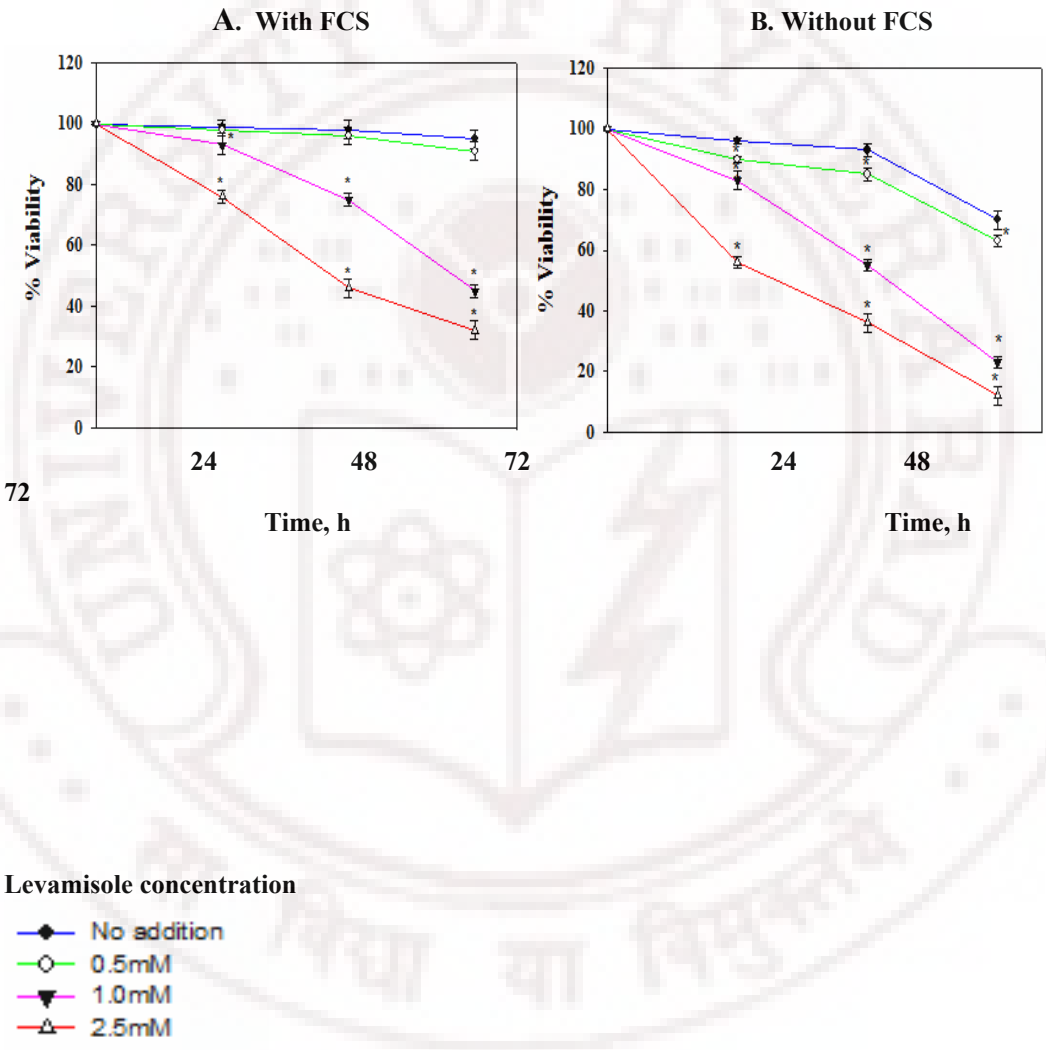
Effect of levamisole on Viability of U266B1 cells



Viability was determined by trypan blue dye exclusion assay. Each value represents the mean ± SEM of 3 experiments. * * values significantly differ from the respective control, p<0.05

Fig - 3.2

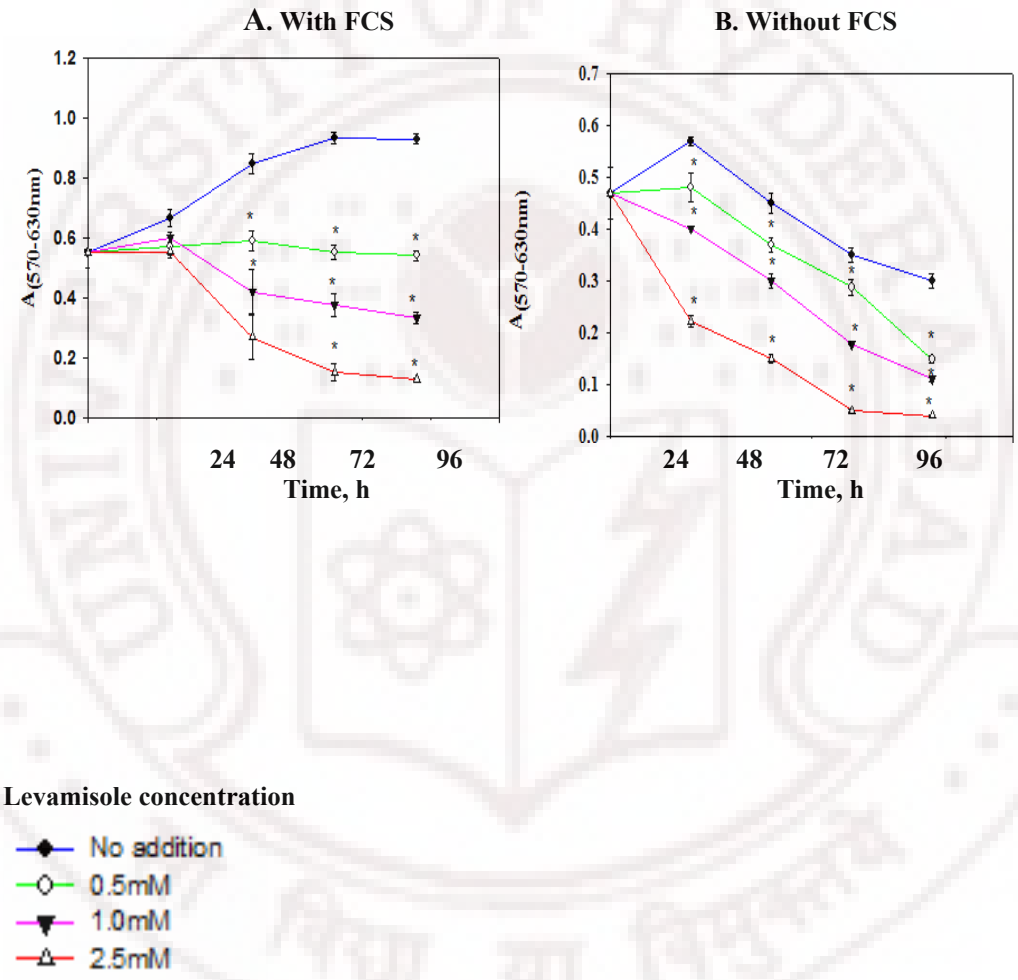
Effect of levamisole on Viability of RPMI 8226 cells



Viability was determined by trypan blue dye exclusion assay. Each value represents the mean ± SEM of 3 experiments. * values significantly differ from the respective control, $p<0.05$

Fig - 3.3

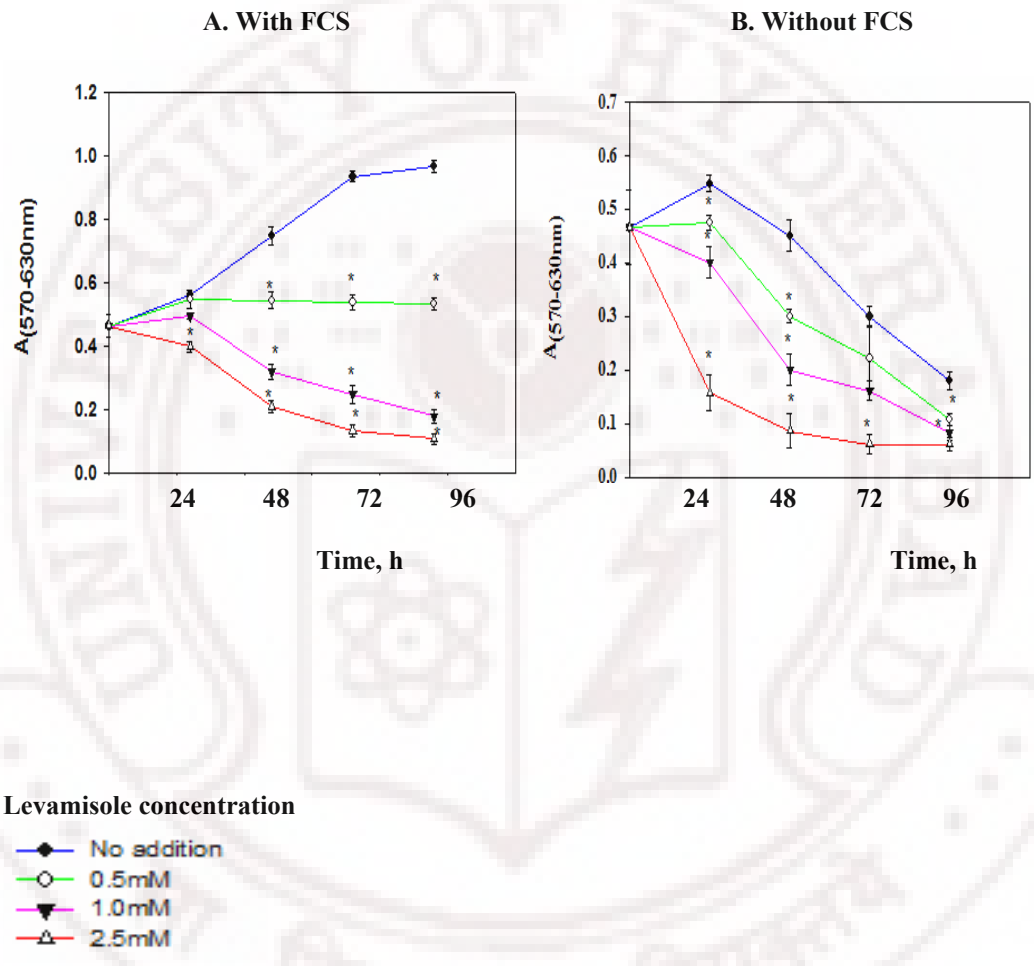
Cytotoxic effect of levamisole on U266 B1 cells - MTT assay



Each value represents the mean ± SEM of 3 experiments . * values significantly differ from the respective control, p<0.05

Fig - 3.4

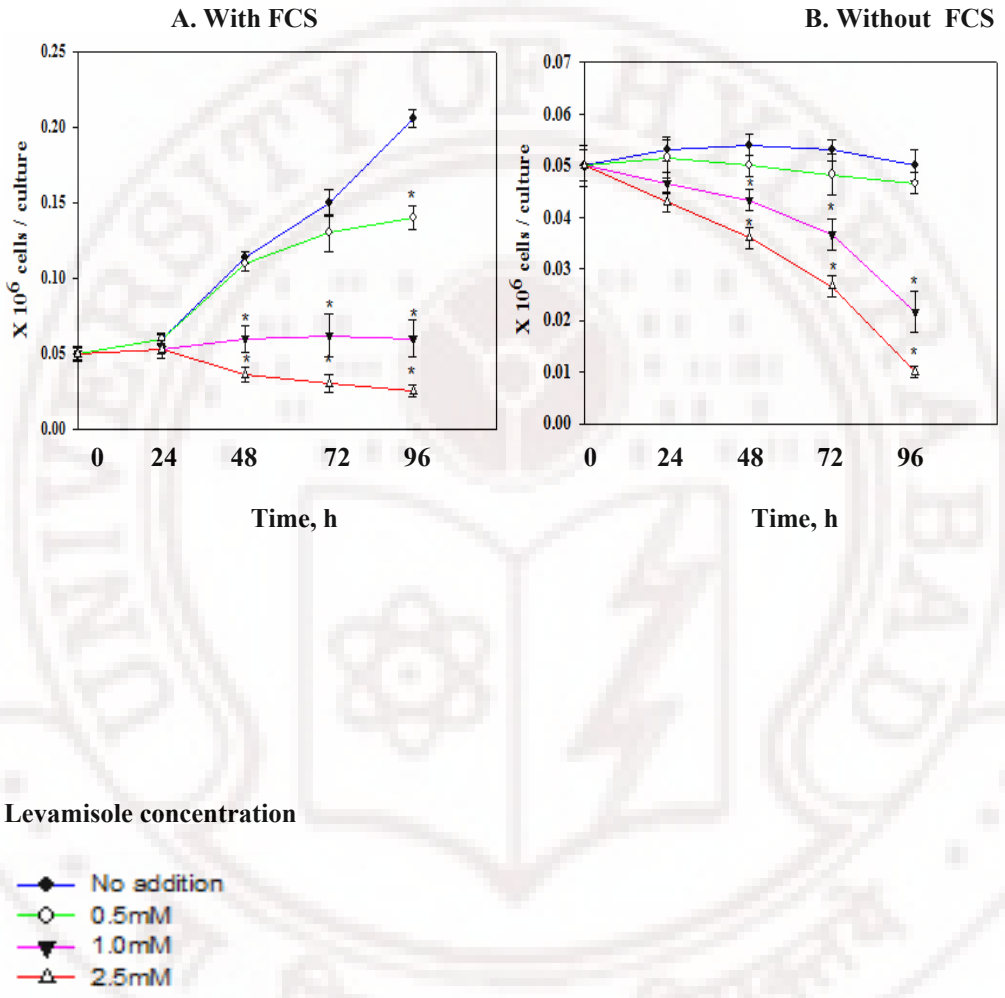
Cytotoxic effect of levamisole on RPMI 8226 cells - MTT assay



Concentration and time dependent effect of Levamisole on RPMI 8226 cell line. Each value represents the mean ± SEM of 3 expriments. * values significantly differ from the respective control, $p<0.05$

Fig - 3.5

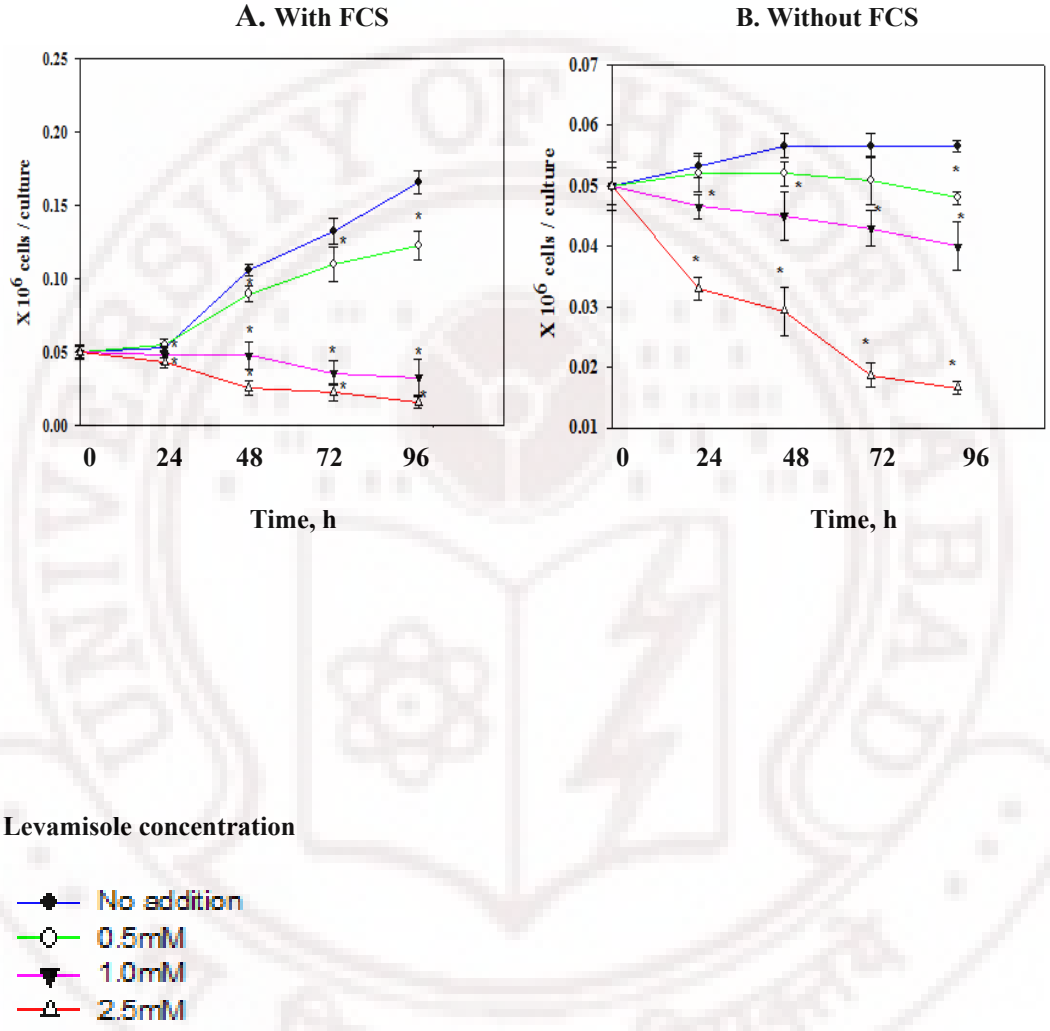
Effect of levamisole on cell number - U266 B1



Each value represents the mean ± SEM of 5 experiments. * values significantly differ from the respective control, $p<0.05$.

Fig - 3.6

Effect of levamisole on cell number - RPMI 8226



Each value represents the mean ± SEM of 5 experiments. * values significantly differ from the respective control, p<0.05

In complete medium, the cell number showed an increase with increase in time period of culture. At 0.5mM levamisole concentration there was no effect on the cell number at 48 and 72h as compared to the controls in complete medium as well as in serum-free medium. At 1.0 mM concentration of levamisole, the cell number was significantly lower as compared to the zero time value indicating loss of cells.

DNA fragmentation assay

In order to examine the mechanism of cell death induced by levamisole, DNA fragmentation assay was performed. DNA was isolated from myeloma cell lines treated with 1.0 mM levamisole and 1.0 μM vincristine for 48 and 72 h. The results are presented in Fig 3.7 A&B. DNA fragmentation indicative of apoptotic cell death was observed in levamisole treated cell lines as compared to the DNA from control cells which was intact.

Identification of cytosolic Cytochrome C

Levamisole (0.5mM and 1.0mM) treated cells were harvested at the end of 24h and were assayed for cytosolic cytochrome C. Cytochrome C was identified by Western blotting using anti- human cytochrome C antibody. The results are presented in Fig 3.8. An increase in the level of cytochrome C was seen in levamisole treated cells compared to untreated cells. β- actin was used as a control to indicate equal protein loading of the samples for analysis.

Assay of caspase activity

The caspase 3 activity of levamisole treated U266 cells was measured using a fluorogenic substrate. In order to see the specificity of the assay, DEVD-CHO (an inhibitor of caspase) was added prior to the addition of the substrate. The results are presented in the Fig 3.9. Cells exposed to UV-B radiation for 30 sec were used as positive control. The caspase activity is enhanced in levamisole treated cells indicative of apoptosis.

Effect of serum on effect of levamisole in culture

Levamisole has been shown to bind to proteins. Hence, the free concentration of levamisole available for interaction with cells could be lower than the final concentration

Fig - 3.7

Agarose gel electrophoresis of DNA from levamisole treated cells

A. RPMI 8226

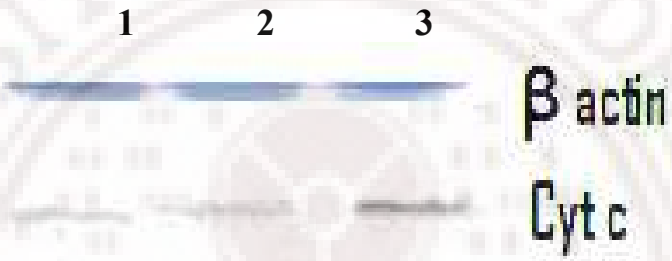
Lane 1 : DNA ladder (1-10 Kb)
Lane 2 & 3 : Untreated cells at 48 h and 72 h
Lane 4 & 5 : Cells treated with 1.0mM levamisole for 48 h and 72 h
Lane 6 & 7 : Cells treated with vincristine (10^{-6}M) for 48 h and 72 h

B. U266 B1

Lane 1 : DNA ladder (1-10 Kb)
Lane 2 & 3 : Untreated cells at 48 h and 72 h
Lane 4 & 5 : Cells treated with 1.0mM levamisole for 48 h and 72 h
Lane 6 & 7 : Cells treated with vincristine (10^{-6}M) for 48 h and 72 h

Fig - 3.8

Western blot analysis cytosolic Cytochrome-C of U266 B1 cells treated with levamisole.



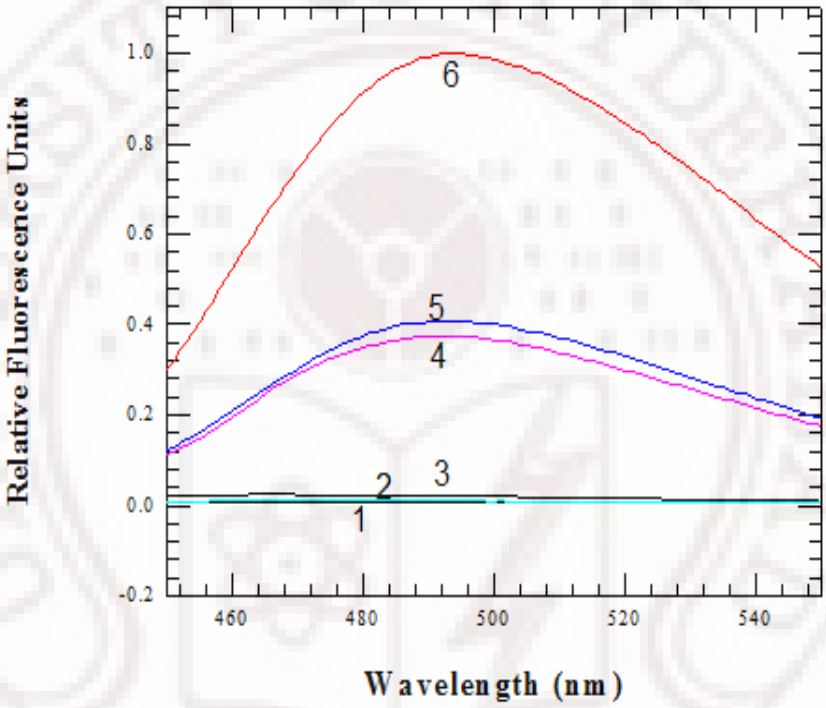
Lane 1: No addition

Lane2: 0.5mM Lev

Lane3: 1.0mM Lev

Fig - 3.9

Caspase 3 activity of U226 B1 cells treated with levamisole



Caspase-3 activity was determined using a fluorogenic substrate .

1. **Blank,**
2. **Untreated cells,**
3. **DEVD-CHO,**
4. **1.0mM levamisole,**
5. **2.5mM levamisole,**
6. **UV-B(60 sec)**

in the culture as a result of protein binding. In order to examine the effect of serum, cells were cultured without and with 5% fetal calf serum. The results of MTT assay, cell number, proliferation and viability indicate that the effect of levamisole on myeloma cells is independent of the serum concentration.

Reversibility of levamisole effect on proliferation of cell lines

To see the reversibility of the effect induced by levamisole, it was removed form the culture by replacing the supernatant with fresh complete medium at various time points 24h, 48h . At the end of 72h MTT assay was done on all the three sets. The results are presented in Table 3.1 . The effect of levamisole was observed to be irreversible.

Effect of Levamisole in combination with Atorvastatin on the proliferative response of myeloma cell lines.

The effect of levamisole in presence of atorvastatin on the proliferative response of both the cell lines was evaluated using varying concentrations of levamisole and atorvastatin. The results are presented in Table 3.2 A&B. Levamsiole had a marginal additive inhibitory affect when combined with atorvastatin in both the myeloma cells lines. However, the effect observed was not statistically significant.

Table - 3.1

Reversibility of cytotoxic effect of levamisole on RPMI 8226 cells

(mM) Time of levamisole removal (hours)	Concentration of levamisole 0.0	0.5	1.0	2.5
			A (570-630 nm)	
24h	100	95 ± 2	76 ± 3 *	60 ± 5*
48h	100	90 ± 3	68 ± 2*	26 ± 5*
72**	100	89 ± 8	39 ± 6 *	16 ± 4*

** Samples incubated with levamisole for 72hours without replacement of medium

The Values obtained without levamisole are taken as 100 % at each time point (24h : 48h : 72h:)

Each value represents the mean ± SEM of 3 experiments. * values significantly differ from the respective control, $p<0.05$

Table 3.2

A. Effect of Levamisole in combination with Atorvastatin on the proliferative response of U266 B1 cell line -by MTT (48h)

Conc of Levamisole (mM)	Conc. of Atorvastatin (uM)				
	0	10	30	50	70
	% proliferative response				
0.00	100	88	52	40	22
0.50	91	80	44	32	19
1.0	61	78	44	29	15

A570-630nm value of 0.50 for untreated cells is taken as 100%.

Table 3.2

B. Effect of Levamisole in combination with Atorvastatin on the proliferative response of RPMI 8226 cell line –by MTT (48h)

Conc. of Levamisole (mM)	Conc. of Atorvastatin (uM)				
	0	10	30	50	70
	% proliferative response				
0.0	100	100	65	33	9
0.5	65	65	55	25	8
1.0	49	54	39	20	7

A570-630nm value of 0.372 for untreated cells is taken as 100%.

Discussion:

The data presented in the previous chapters clearly indicates that the APase activity is involved in the proliferative and differentiative response of myeloma cells. Inhibitors of APase activity have found important applications in clinical practice and research.

Since APase activity is possibly involved in the malignant state of myeloma cells, leading to associated clinical complications, it becomes an attractive target for treatment of multiple myeloma. Levamisole was found to be good inhibitor of tissue non-specific APase activity (Pappas & Leiby, 1989). It was also found to have anti-cancer properties (Amery, 1977). It is also used as an adjuvant along with 5-fluorouracil in the post-surgical treatment of colorectal carcinoma (Cafiero et al 2000). In an experimental rat model of colonic inflammation, significant increase in APase activity was observed mainly in the epithelial cells and leukocyte population. Inhibition of enzyme activity using levamisole or a monoclonal antibody to APase resulted in significant protection from the inflammation (Sanchez et al 2004). These observations strongly suggested that levamisole could be used as an inhibitor of APase *in vivo*.

We have used levamisole in a similar manner *in vitro* to see its effects in myeloma cell lines which was brought about via inhibition of APase.

Levamisole, was used at various concentrations to evaluate its anti-proliferative activity. In addition to inhibiting the cell proliferation, levamisole at more than 0.50 mM concentration induced cytotoxicity. The possible mechanism for the cytotoxic effect of levamisole could be through the induction of apoptosis. A study reported earlier has shown that levamisole had an apoptotic effect on vascular endothelial cells (Artwohl et al 2000). Myeloma cells treated with levamisole were evaluated for apoptosis using caspase-3 activity, cytosolic Cytochrome C release from mitochondria and DNA fragmentation analysis . The DNA fragmentation analysis revealed a ladder pattern typical of cells undergoing apoptosis. The results on Caspase-3 activity and cytochrome *c* release also indicated that levamisole induced cell death follows the mitochondrial mediated pathway.

The observations reported here indicate that levamisole exerts a potent anti-proliferative activity as well as cytotoxic effect by apoptosis on myeloma cells. Thus inhibition of APase may have important application in management of pathological conditions in multiple myeloma. However, it is important to remember that levamisole has a number of other properties that may also be involved in its anti-proliferative activity, such as potassium channel inhibition, induction of apoptosis, inhibition of NF-kB, etc (Kim et al 1999, Artwohl et al 2000, Liu et al 2004). Nevertheless, the fact that APase is expressed only in case of malignancy in humans and that levamisole has a significant inhibitory effect on myeloma certainly suggests that APase is involved in the response and that it might constitute a relevant target for drug therapy.

Statins are cholesterol-lowering drugs that exert pleiotropic functions by preventing the synthesis of mevalonic acid that plays a key role in a number of cellular processes(Goldstein & Brown 1990, Endo 1994). They exert anti-prolferative function by reducing the isoprenylation of proteins involved in cell signal transduction. Statins have been shown to promote anti-proliferative and cytotoxic effects in human, rat and rabbit cells *in vitro* (Sonoda et al 1994, Van et al 1996, Mutoh et al 1999, Knapp et al 2000). The cytotoxic mechanism involved the suppression of the cell cycle by blocking cell proliferation in the G0/G1 phase and by an increase in protein tyrosine phosphorylation which regulates the intracellular signal transduction leading to apoptosis (Keyomarsi et al 1991).

In myeloma cells stains activate the mitochondrial pathway of apoptosis (Paola et al 2005). In the present study, when atorvastatin was used in association with levamisole no synergistic cytotoxic effect observed. Only an additive effect was observed. The cytotoxic effect of levamisole was observed to be irreversible as the removal of the drug from the culture after 24 and 48 h period did not reverse the inhibitory effect. Hence, it is possible that levamisole exerts an irreversible cytotoxic effect within 24 hours of exposure to the cells.

CHAPTER - 4

Effect of Levamisole on CD138 and Interleukin 6

Syndecan-1 (CD138), a transmembrane heparan sulphate glycoprotein is expressed on different types of adherent and non adherent cells including epithelial, keratinocytes, endothelial, vascular smooth muscle cells and also on myeloma and tumor cells. It binds extracellular matrix components including interstitial collagens type I, III, and IV, fibronectin , thrombospondin , and growth factors, particularly basic fibroblast growth factor (Sun et al 1989, Bernfield et al 1992, Kiefer et al 1990, Salmivirta et al 1992, Steinfeld et al 1996, Kishimoto et al 1997, Saunders & Bernfield 1998). Syndecans participate in the binding of growth factors and interaction between cells and the extra cellular matrix (Bernfield et al 1992, Ridley et al 1993, Ruoslahti 1988, Liebersbach & Sanderson 1994, Bankfalvi 1996).

These are present as surface molecules anchored in plasma membrane as components of extra cellular matrix. The core protein comprises of 251, 34 and 25 aminoacid residues in the extracellular, transmembrane and intracellular domains. CD138 is used as a standard marker for identification of tumour cells (Zhan et al 2002). It has been shown that CD138 may act as a multifunctional regulator of cell behaviour in the tumor milieu; mediates cell-cell adhesion, binding of myeloma cells to type I collagen and tumour cell invasion (Bernfield et al 1992)(Rapraeger 1993) (Elenius & Jalkanen 1994).

CD138 is expressed in distinct stages of B cell differentiation which include pre-B cells and immunoglobulin-producing plasma cells in normal bone marrow. It is also expressed on myeloma cells, Hodgkin's lymphoma and in HIV-associated lymphomas. By fine tuning the function of regulatory proteins and cell signalling, CD138 regulates cell behaviour in normal and pathological processes like tumor growth and metastasis by sequestering chemokines and growth factors (Bernfield et al 1999, Lozzo 2001a, Lozzo & San Antonio 2001 b).

CD138 has been shown to be a marker in prostrate, lung, myeloma and cervical carcinomas potentially by imbuing the cancer with an aggressive phenotype. Enhanced CD138 expression is associated with cell proliferation.

CD138 expressed dominantly on the surface of most primary myeloma cells has been used for the identification and purification of myeloma cells from clinical samples (Borset et al 1996).

CD138 has been shown to mediate adhesion of myeloma cells to type-I collagen Via its heparan sulfate chains (Ridley et al 1993, Numa et al 1995, Pulkkinen et al 1997, Inki & Jalkanen 1996). CD138 can also influence the cell shape, cell-cell adhesion, proliferation and differentiation. (Michelle et al 1995, David et al 1992). CD138 is also expressed by human plasma cells while its expression is lost in cells undergoing apoptosis (Madhav et al 1998).

In addition to CD138 several cytokines and growth factors have been suggested to stimulate the growth of human myeloma cells, among them interleukin-6 seems to be the major growth factor for myeloma cells and can inhibit apoptosis of these malignant cells (Nilsson et al 1990, Hirano 1991, Gado et al 2000 and Lichtenstein et al 1995).

Interleukin-6 (IL-6), is a protein of 180 a.a., produced by different types of cells like monocytes, T cells, leukocytes, lymphocytes and smooth muscle cells. It is a cytokine with pleiotropic activities such as regulation of cell growth, differentiation and maturation in a wide variety of cell types(Barton 1996). It plays an important role in diverse host defence mechanisms such as the immune response, haematopoiesis and acute reactions (Akira et al 1993)(Narazaki & Kishimoto 1994). It was originally described as B cell differentiation factor or plasmacytoma growth factor which promoted differentiation of normal B cells to antibody-producing cells (Akira et al 1993). In different clinical conditions including myeloma, role of IL- 6 has been recognised (Simpson et al 1991).

Several in vitro and in vivo studies have shown that IL-6 is one of the major growth factors involved in the pathogenesis of human multiple myeloma and mouse plasmacytoma cells (Nilsson et al 1990, Hirano 1991, Klein et al 1992 & 1995). Previous studies have demonstrated that there is both an autocrine and

paracrine IL-6 mediated growth and survival in multiple myeloma cells and derived cell lines in vitro (Kawano et al 1988, Hata et al 1993, Hardin et al 1994, Lichtenstein et al 1995, Klein 1995 et al, Chauhan 1995, Chauhan et al 1997a,b).

Exogenous addition of IL-6 has been shown to be essential for the proliferation of freshly isolated myeloma cells cultured *in vitro* (Chauhan et al 1996, Lokhorst et al 1994, Uchiyama et al 1993). Serum levels of IL-6 have been shown to be associated with disease activity in myeloma, with high levels indicating severity (Bataille et al 1989, Nachbaur et al 1991). In addition, IL-6 appears to have broad anti-apoptotic activity in several B cell tumors including multiple myeloma (Reittie et al 1996, Chauhan et al 1997a).

Therefore, drugs which affect CD138 and IL-6 function are expected to be useful as therapeutic agents in patients with this lethal disease. Anti-IL-6 monoclonal antibody therapy can transiently reverse disease manifestations in patients with myeloma (Bataille et al 1995). Several studies suggest potential therapeutic utility of inhibiting IL-6 in myeloma: Antisense oligonucleotides to IL-6 gene inhibit the growth of human myeloma cell lines in vitro (Simpson et al 1991)

In view of its effects on tumor cell growth, survival, adhesion and invasion, CD138 and Interleukin 6 may be potential beneficial regulators of myeloma pathobiology.

Levamisole was found to inhibit alkaline phosphatase activity, proliferation and induces apoptosis in myeloma cells. In addition to these anticancer properties in the present study CD138 expression and IL-6 secretion levels have been assessed in levamisole treated multiple myeloma cell lines.

Methods:

1.Syndecan-1 expression on myeloma cells by flow cytometry

Human multiple myeloma cell lines RPMI 8226 and U266B1 were cultured in RPMI 1640 medium supplemented with 10% FCS and treated with various concentration of levamisole (Control, 0.5 , 1.0, 2.5mM) and the cells were harvested after 48h. The cells were stained with FITC conjugated anti human

Syndecan-1 antibody as per the protocol given in Materials and Methods. CD138 positive cell number was determined by FACS.

2.Interleukin 6 assay

5 X 10^4 cells in triplicate were treated with various concentrations (0.0, 0.5, 1.0, 2.5mM) of levamisole in 200µl of complete culture medium. IL- 6 in the culture supernatants was determined by ELISA as described in Material and Methods.

Results :

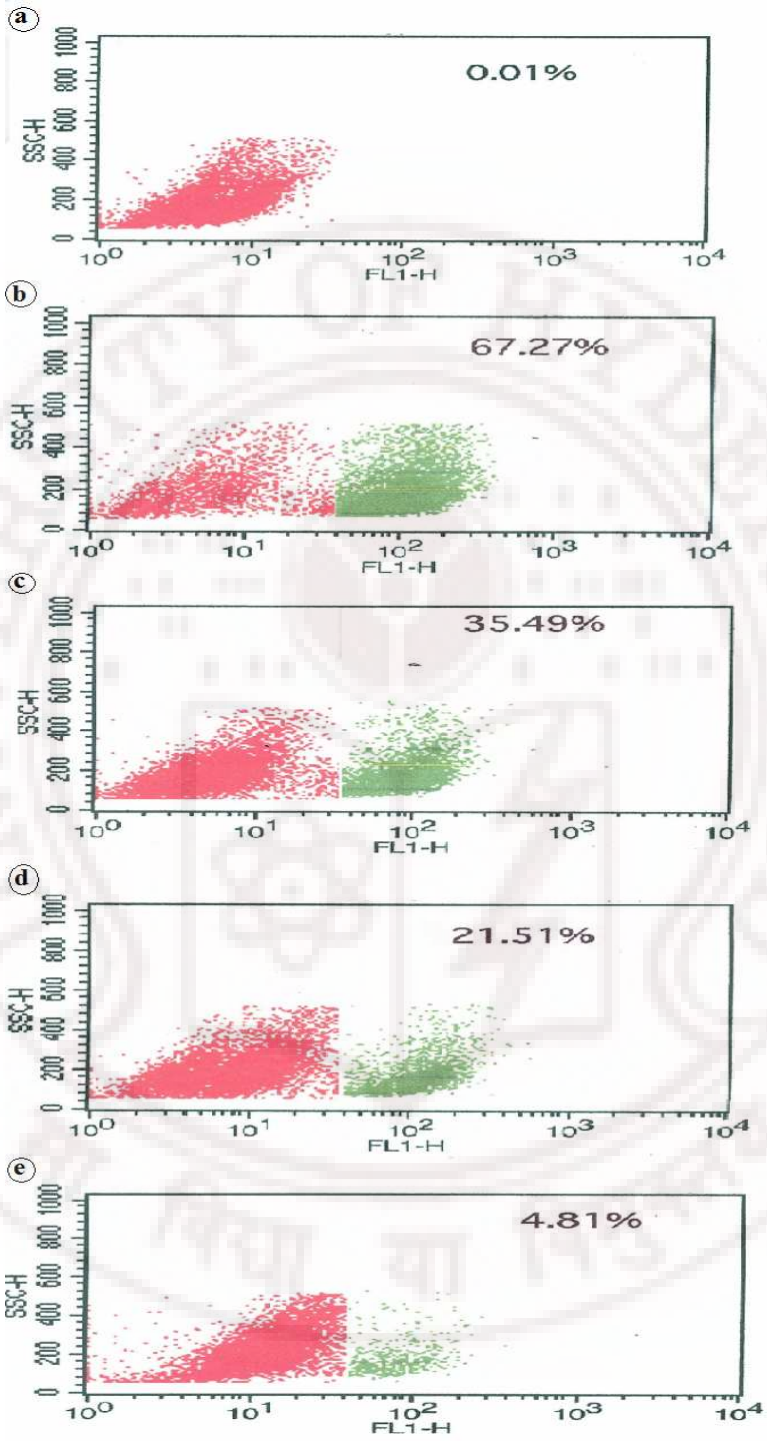
Effect of levamisole on CD 138 expression in myeloma cells

CD 138 expression on untreated and levamisole treated myeloma cells at different concentrations for 48h of culture period was determined using FITC- conjugated anti- CD138 monoclonal antibody . Flow cytometric analysis showed that in case of untreated cells, 63 % of U266 and 51 % of RPMI 8226 cells were CD 138 positive. The percentage of CD138 positive cells decreased significantly with increasing concentrations of levamisole (Figs 4.1 A&B & 4.2 A&B, $p< 0.05$).

Quantitation of Interleukin - 6 in U266 B1 cell culture supernatant

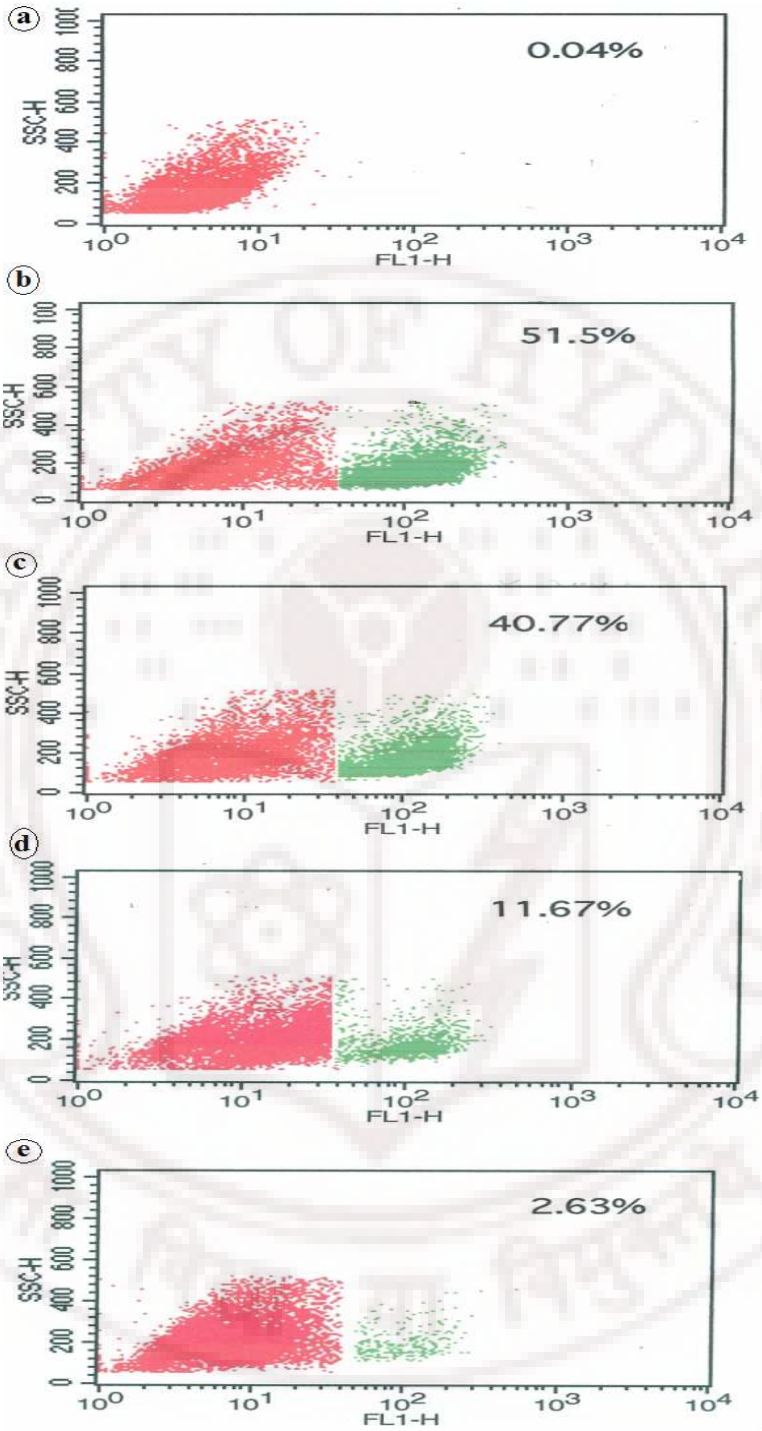
The supernatant from myeloma cells cultured with and without levamisole were harvested at intervals of 24h over a period of 72h. IL-6 was assayed using sandwich ELISA. The standard graph is presented in Fig 4.3. The assay showed linearity in the range of 2- 300 pg/ ml of IL-6. There was a dose and time dependent increase in the concentration of IL-6 in the supernatant at 0.5 and 1.0 mM concentration of levamisole. (Fig 4.4) .

Fig - 4.1 Effect of Levamisole on CD138 expression of myeloma cells using FACS analysis
A. U266B1



Results are those of one experiment representative of three independent experiments. Cell population in red color represents unstained cells. Cell population in green color represents cells stained positive for CD138. a. Isotype control, b. Without levamisole, c. 0.5mM Lev, d. 1.0mM Lev, e. 2.5mM.

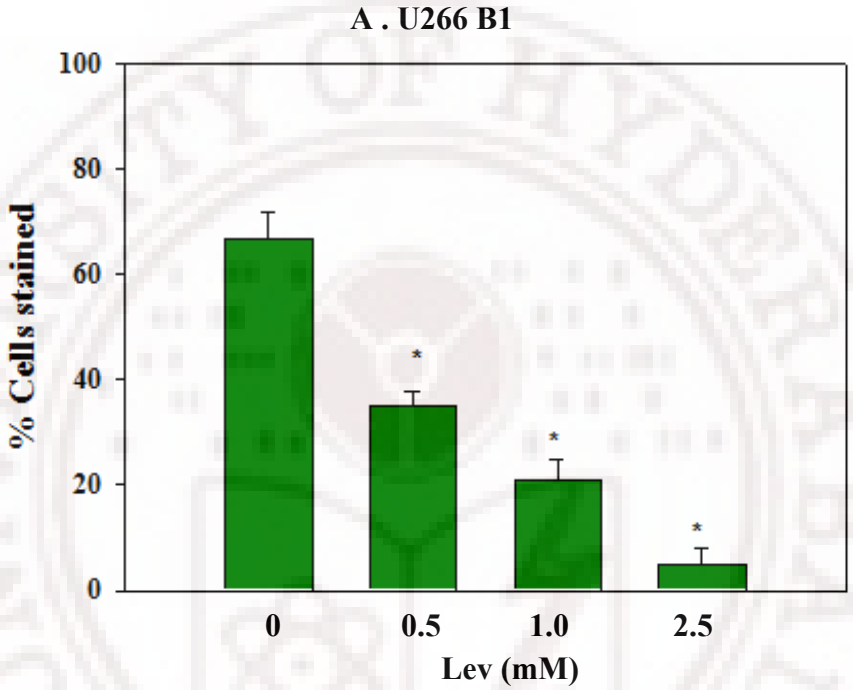
B. RPMI 8226



Results are those of one experiment representative of three independent experiments. Cell population in red color represents unstained cells. Cell population in green color represents cells stained positive for CD138. a. Isotype control, b. Without levamisole, c. 0.5mM Lev, d. 1.0mM Lev, e. 2.5mM

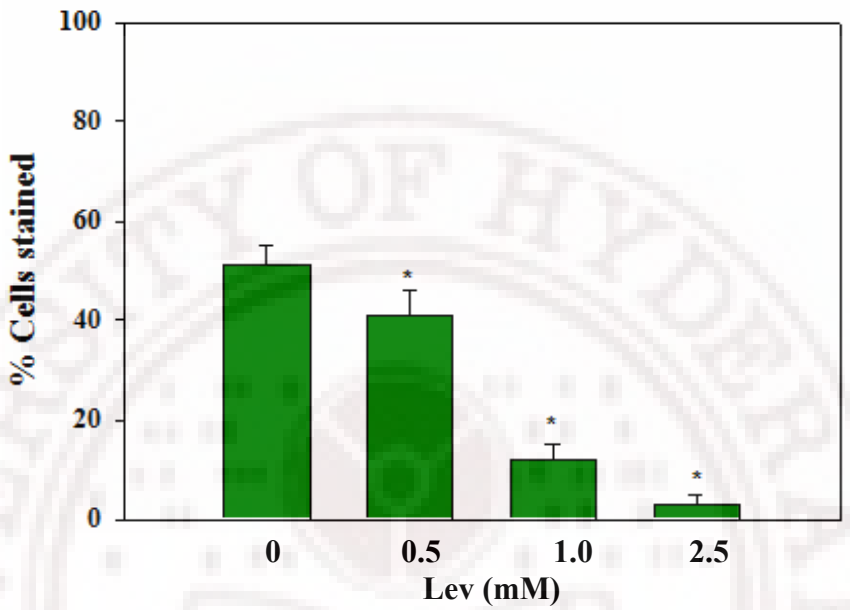
Fig - 4.2

CD 138 expression on levamisole treated myeloma cells



Each value represents the mean ± SEM of three experiments. * p<0.05 , Treated Vs untreated

B. RPMI 8226



Each value represents the mean ± SEM of three experiments. * p<0.05 , Treated Vs untreated

Fig - 4.3

Standard graph of IL 6

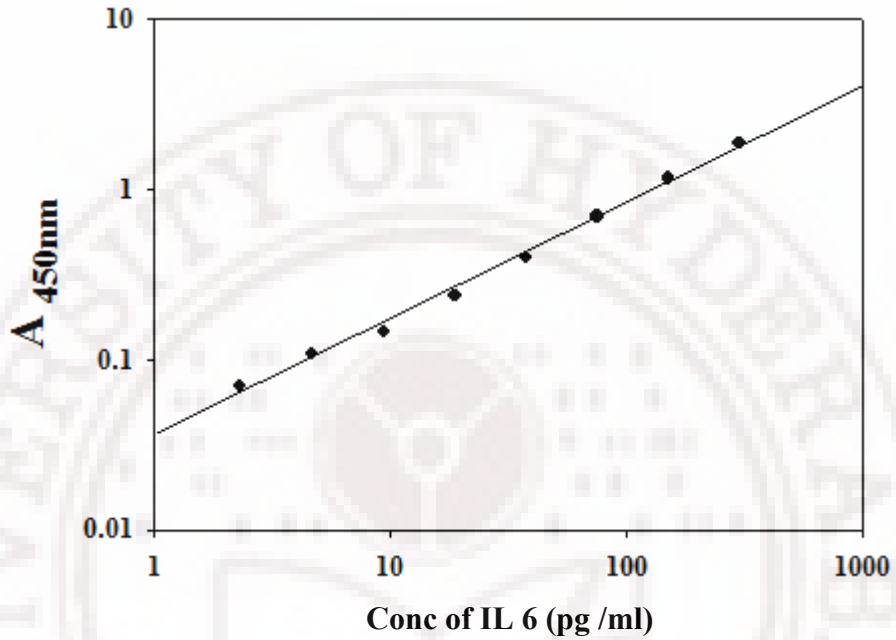
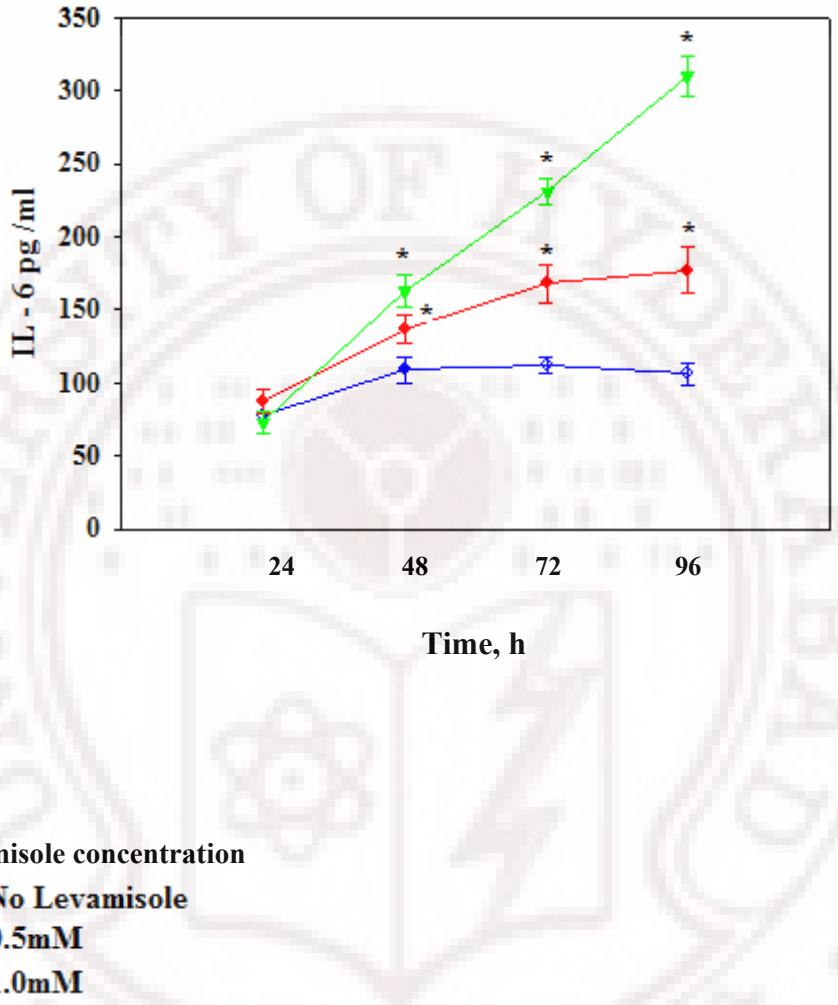


Fig - 4.4

Effect of levamisole on Interleukin -6 secretion by U266 cells



Concentration of IL-6 in the culture supernatant was determined by ELISA. Each value represents the mean ± SEM of three experiments. * $p<0.05$, Treated Vs untreated.

Discussion:

CD138 and IL-6 play a major role in regulating the pathobiology of myeloma. The defining characteristic of the syndecan family is a highly conserved transmembrane and cytoplasmic domain which contains four conserved tyrosines and a variable number of serines / threonines that may serve as phosphorylation sites. Activation leads to phosphorylation of the receptors themselves and initiation of a cascade of phosphorylation events that activate or deactivate a wide variety of other kinases and regulatory molecules, ultimately resulting in changes in cellular behaviour (Hunter, 1995).

In the present study, we observed that on culturing with increasing concentration of levamisole there was a significant decrease in the percentage of $CD138^+$ cells. However even untreated cells were heterogenous in terms of CD138 expression and only 67% of U266 B1 cells were positive for CD138 this observation is in agreement with Mihaela et al 2007. It has been reported earlier that the membrane bound CD138 has a turnover and that the ectodomain of it is shed constitutively by cultured cells (Wijdenes et al 1997, Ihrcke et al 1996, Subramanian et al 1997).

In murine mammary gland cells, NMuMG cells , it has been shown that CD138 is constitutively phosphorylated at low levels and orthovandate inhibits a phosphatase which rapidly dephosphorylates the protein. This inhibition of cellular phosphatase lead to activation of a cytoplasmic kinase that recognizes and phosphorylates CD138, leading to its subsequent shedding from the membrane. The shedding of CD138 is highly regulated by the activation of at least two distinct receptor classes, G protein - coupled and protein tyrosine kinases. The shedding of CD138 has been shown to be increased by tyrosine phosphorylation of its cytoplasmic domain (Reiland et al 1996). Similar findings were reported in myeloma cells (Wijdenes et al 1997).

In the present study, it was found that expression of CD138 in myeloma cell lines RPMI 8226 and U266 B1 was inhibited by levamisole in a dose dependent

manner in both the cell lines. The decreased expression of CD138 in levamisole treated cells could be due to its accelerated shedding from the membrane. We propose that such a shedding of syndecan-1 on myeloma cells might be due to inhibition of APase by levamisole . On inhibition of APase , there is probably reduced dephosphorylation of the receptors resulting in phosphorylation of CD138 and its subsequent shedding from the membrane.

The shedding or loss of CD138 is associated with apoptosis and inhibition of cell growth *in vitro* (Madhav et al 1998). Loss of CD138 may also contribute to the mechanism of induction of apoptosis in myeloma cells and it may be due to loss of interaction of heparan sulfate chains with a cellular receptor or growth factor. The CD138 functions as a receptor or co receptor for different factors including growth factors which are proven to have possible effects on the biology of multiple myeloma. (Sebestyen et al 1997). Earlier it has been reported that in murine B lymphoid cells CD138 expression is regulated by IL-6 via post translational mechanisms (Sneed et al 1994). It was reported that CD138 was lost by primary myeloma cells induced to apoptosis when cultured without IL-6. With dexamethasone, virtually all myeloma cells were apoptotic and had lost CD138. Addition of exogenous IL-6 partially prevented apoptosis in association with an increase of CD138 positive cells.(Michel et al 1998).

IL-6 has multiple effects including regulation of cell growth, differentiation, and maturation, in a wide variety of cell types (Barton 1996). For example, IL-6 secreted by T helper cells stimulates terminal differentiation and growth of B-cells (Altmeyer et al 1997, Morse et al 1997). In addition, IL-6 appears to have broad anti-apoptotic activity in several B cell tumors including multiple myeloma (Chauhan 1997a). IL-6 is a potent survival factor for myeloma cells and probably prevent them from undergoing apoptosis under drug exposure. (Michel et al 1998). It was shown that U266 cells secrete and require IL-6 for growth and survival (Jernberg et al 1991). It was earlier reported that U266 proliferation was inhibited by neutralizing anti-IL-6 mAb or IL-6 antisense oligonucleotides (Simpson et al 1991, Schwab et al 1991, Levy et al 1991).

Here we report that levamisole mediated growth inhibition of myeloma cells in vitro is associated with a loss of CD138 and increased IL-6 secretion. The increased secretion of IL-6 by myeloma cells could be an attempt to protect themselves form apoptosis.

CHAPTER - 5

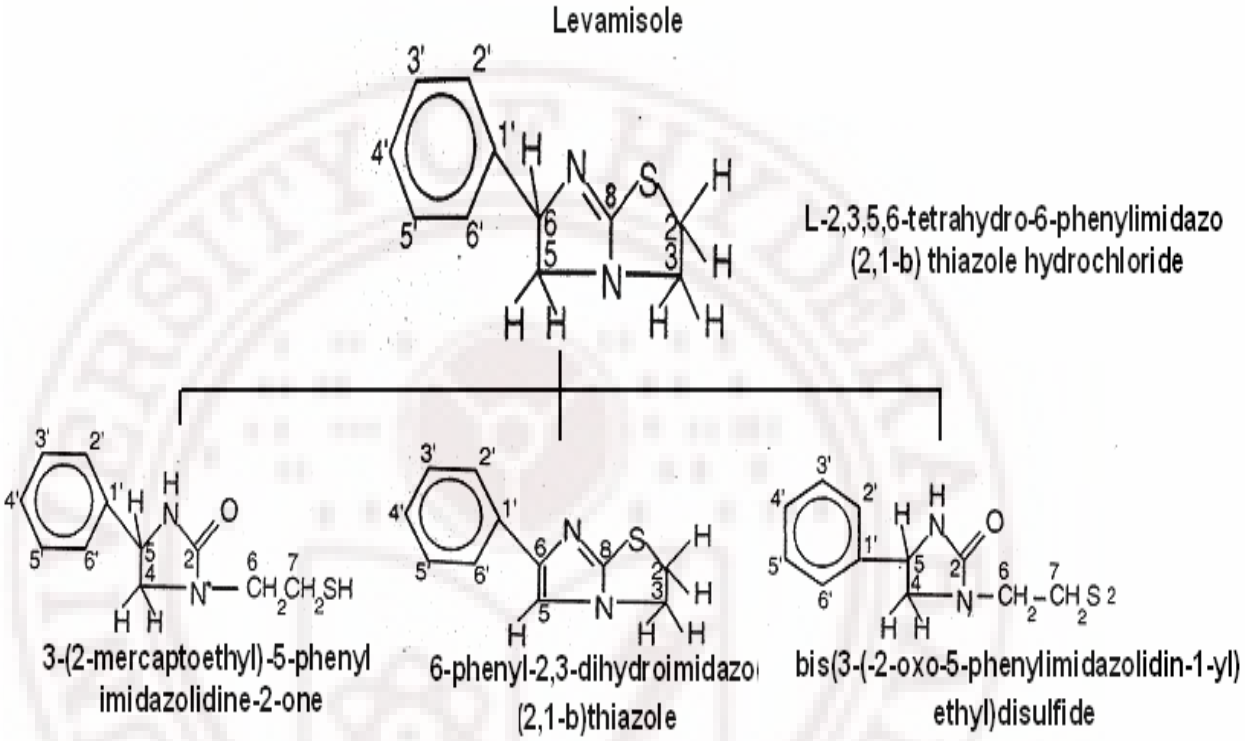
Fate of levamisole in cell culture
And
^{3}H-levamisole binding to murine splenic lymphocytes, normal and malignant human lymphocytes.

In all the experiments described thus far myeloma cells were incubated with varying concentrations of levamisole. Although effect of levamisole has been observed in a dose dependent manner the effective concentration of levamisole to bring about the observed effect is not known. Levamisole, being a freely water soluble compound, it is expected that its entry into the cells through the lipid bilayer will be meagre. Also as fetal calf serum is used for cell culture it is possible that levamisole can bind to the serum proteins. Hence in this chapter it is attempted to determine the fate of levamisole in cell culture by estimating the concentration using HPLC at different culture time periods.

Further the interaction of levamisole with myeloma cells was studied by estimating the binding of ^{3}H-levamisole to whole cells and broken cell preparations.

Levamisole has been shown to bind avidly to proteins. The binding of levamisole to total plasma proteins of 6 animal species was determined in vitro by equilibrium. dialysis. The amount of drug bound to plasma proteins was independent of levamisole concentration. Levamisole was bound to a limited extent to plasma proteins of animal species studied (Sahagun et al 1997). Levamisole has been shown to be decomposed enzymatically / non-enzymatically into three degradation products (Kimberly et al 1991). The structures of the purified products are A) 3-(2-mercaptoethyl)-5-phenyl-imidazolidine-2-one (OMPI) B) 6-phenyl-2,3-dihydroimidazo (2,1-b) thiazole and C) bis(3-(2-oxo-5 phenyl imidazolidine-1-yl) ethyl] disulfide. The decomposition of levamisole has been shown to be temperature and pH dependent. Hence, the free and unaltered levamisole concentration available for interaction with cells could be very low as compared to the final concentration in cell culture as a result of protein binding and degradation. Apart from decomposition, other reason for the high concentration of levamisole needed in cell culture to get the effects could be due to low binding of levamisole to the cells. Some of the metabolites of levamisole which have been characterized are given below.

Structure of Levamisole and its metabolites



Objectives :

1. To determine the stability of levamisole in culture with and without FCS.
2. To determine the amount of levamisole bound to normal B cells, mitogen-activated B
   cells and myeloma cells.

Methods:

1. Analysis of Levamisole by HPLC

A. Cell culture and extraction of levamisole

Myeloma cells, 0.25 X 10^6/ml were cultured with and without FCS and levamisole was added at a concentration of 2.5 mM. At 0, 24, 48, 72h of culture

period, 1 ml cell suspension was taken and centrifuged at 500 g and the supernatant was collected. Levamisole was extracted according to the protocol described earlier (Garcia et al 1990, Howaida & Kemppainen 2003).

Briefly, one ml culture supernatant was taken in a 15 ml polypropylene centrifuge tube and 0.8 ml water was added and vortex mixed. Then, 0.5 ml of 10N sodium hydroxide was added and vortex mixed. To this, 5ml of ethyl ether : n-hexane, 80:20 (v/v) was added and vigorously shaken. The mixture was centrifuged for 5 min at 850 g and the organic layer was separated and dried at room temperature under a stream of nitrogen. The residue was re-dissolved in 1 ml of mobile phase and filtered through a 0.22µm pore sized filter and 20µl was used for HPLC analysis.

B. HPLC analysis

Chemicals and Reagents : Methanol, Water, Ethyl ether, Hexane and Chloroform used were all of HPLC grade and glacial acetic acid, Sodium hydroxide were of analytical reagent grade.

A stock solution of 1 mg/ml levamisole was prepared in methanol. It was stored at -20^{o}C and used.

Chromatography: A HPLC system (Waters, USA) fitted with a 5µm C_{18} (Octadecylsilane), 150 mm x 4.6 mm analytical column (Phenomenex, USA), guard column packed with Perisorb RP-18 (Upchurch Scientific, USA) and with a UV- detector was used. The chromatography was carried out using the following conditions:

Elution : Isocratic, Sample volume : 20ul, Flow rate : 1ml / min.

Mobile phase : 2% Acetic acid in water, methanol (50:50(v/v)) pH adjusted to 7.30 with 10N NaOH solution.

Detection : U.V. detection at wavelength of 225nm.

Levamisole, 5-20 µg was analyzed as standard along with the extracted samples. The concentration in the samples was determined using a graph obtained with vales of standard levamisole .

2. ^{3}H-levamisole binding

Resting & mitogen stimulated murine & human peripheral blood lymphocytes and myeloma cells were used for binding studies.

A.^{3}H-levamisole binding to intact cells

Reagents: Bray's mixture (0.1 **%** Triton-X 100, 4gm PPO , 200mg POPOP, 60gm Naphthalene, 20ml ethyleneglycol, 100ml methanol- volume made up to 1 ltr with 1,4-Dioxan).

Procedure: 1 X 10^{6} cells were taken and washed twice with RPMI-1640 and then resuspended in 100µl of RPMI-1640, ^{3}H-levamisole (50 nmoles, 1,35,000 cpm) was added and incubated at 37^{0}C for 1hr. At the end of incubation, the cells were washed twice with RPMI-1640 and finally the cell pellet was lysed using 100µl of 0.1% TX-100 and counted in Bray's mixture.

B.^{3}H-levamisole binding to lysate

Procedure: 1 X 10^{6} cells were taken and washed twice with RPMI-1640 the cell pellet was then lysed with 0.1% Triton X-100. To 0.10 ml of the cell lysate, ^{3}H-Levamisole (50 nmoles, 1,35,000 cpm) was added and incubated for 1hr at 37^{o}C. Then 10 % TCA was added to a final concentration of 10% and incubated for 1hr at 4^{o}C. The precipitate was pelleted by centrifugation at 5000g for 10min and the pellet was washed twice with 5% TCA and finally with ether. The pellet was air dried and dissolved 100µl of 0.1% TX-100 and counted in Bray's mixture (Moreno-Guzman et al 1998).

Non-specific binding was determined in the presence of a 1000-fold molar excess of unlabelled compound. Non- specific binding was subtracted from the total in order to obtain the specific binding.

Results :

^{3}H-levamisole binding

Tritium labeled levamisole binding assays were performed using unstimulated and mitogen stimulated murine splenic lymphocytes and human peripheral blood lymphocytes. Earlier studies have shown that mitogen-stimulated murine splenic lymphocytes show enhanced APase activity. This experimental system was used for comparative purposes to assess the binding of ^{3}H-levamisole to myeloma cell lines.

LPS was used for mitogenic stimulation in case of murine splenic lymphocytes and PWM was used for human peripheral blood lymphocytes. LPS stimulated murine splenic lymphocytes showed enhanced alkaline phosphatase activity(Fig 5.1). No enhancement of APase activity was observed in human peripheral blood lymphocytes upon mitogenic stimulation (Fig 5.2). However, myeloma cell lines displayed significant APase activity(Fig 5.3).

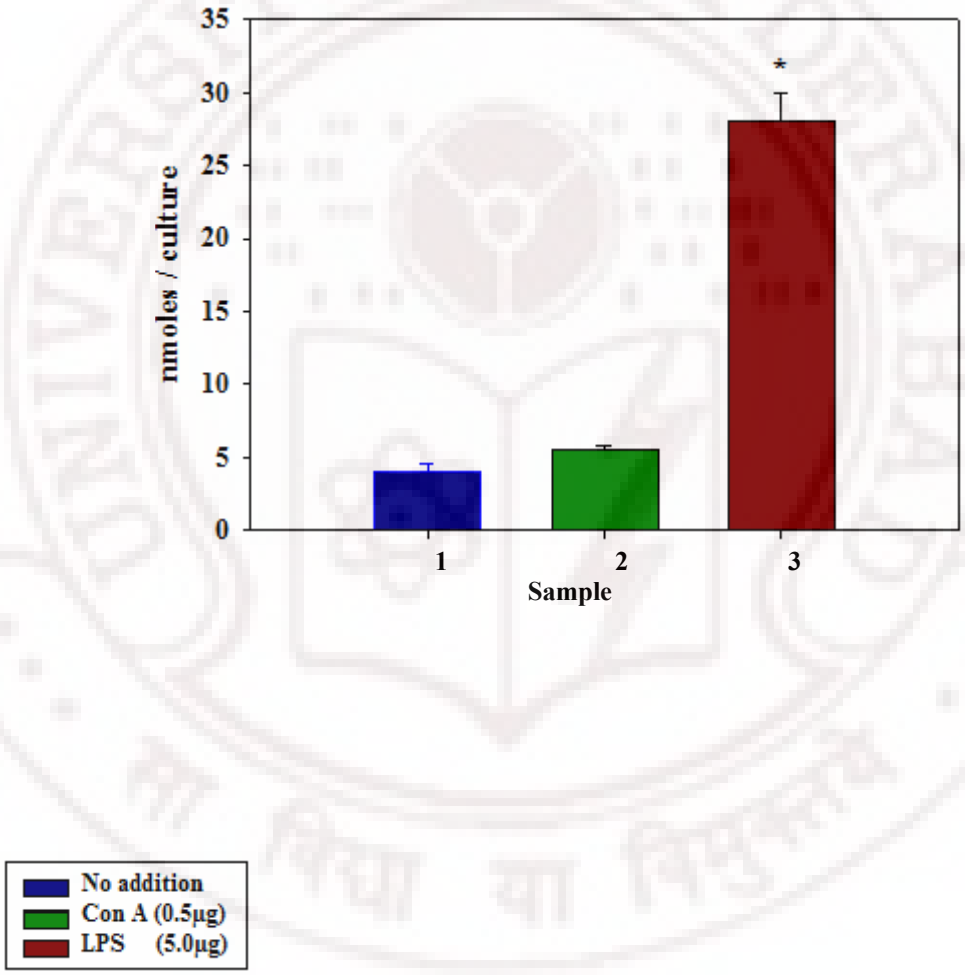
The binding of ^{3}H-levamisole was significantly higher in lysates of LPS stimulated murine splenic lymphocytes as compared to whole cells. (Fig 5.4). The binding of ^{3}H-levamisole to human PBL was minimal in whole cells as well as in lysates and there was no difference between unstimulated and PWM stimulated cells (Fig 5.5). The myeloma cells which express APase activity had significant ^{3}H-levamisole binding (Fig 5.6). In all the cases, ^{3}H-levamisole binding correlated well with the expression of APase activity.

Analysis of Levamisole by HPLC

Levamisole eluted with a retention time of about 6.0 minutes on reverse phase column under the conditions employed. Levamisole ranging from 4-20 µg concentration was used (Fig 5.7 A-D) and a standard graph was calibrated (Fig 5.8). The values used to construct standard graph are presented in Table 5.1. Levamisole was extracted from myeloma cell cultures at various time points 0, 24, 48, 72h and analysed by HPLC. The extracts obtained with RPMI and RPMI + FCS was alnalyzed and the elution profiles

Fig - 5.1

Alkaline Phosphatase activity of mitogen stimulated murine splenic lymphocytes



*** $p < 0.05$ control Vs mitogen**

Fig - 5.2

Alkaline phosphatase activity of Poke Weed Mitogen stimulated normal human peripheral blood cells

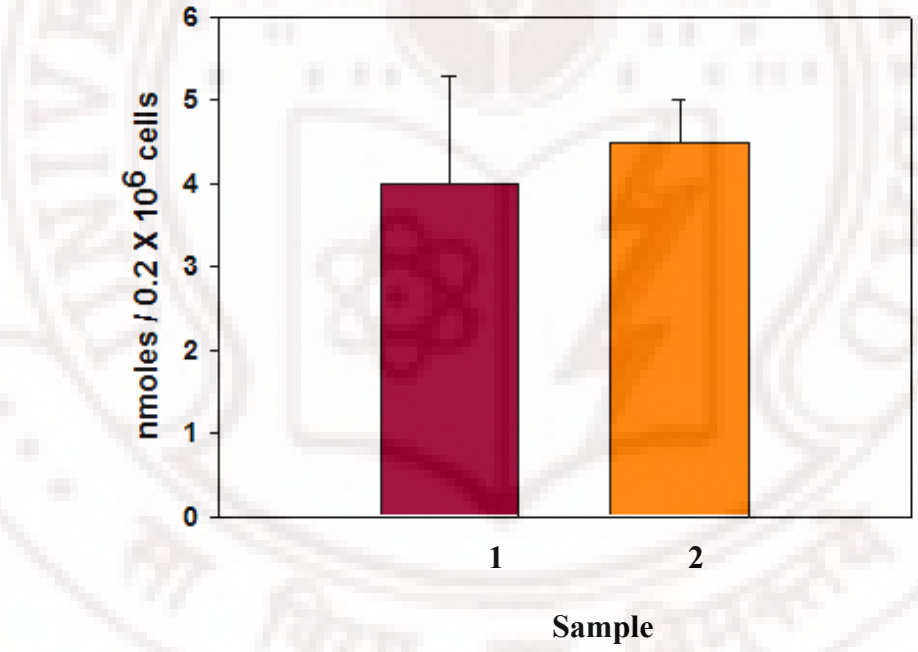


Fig - 5.3

Alkaline phosphatase activity of myeloma cell lines

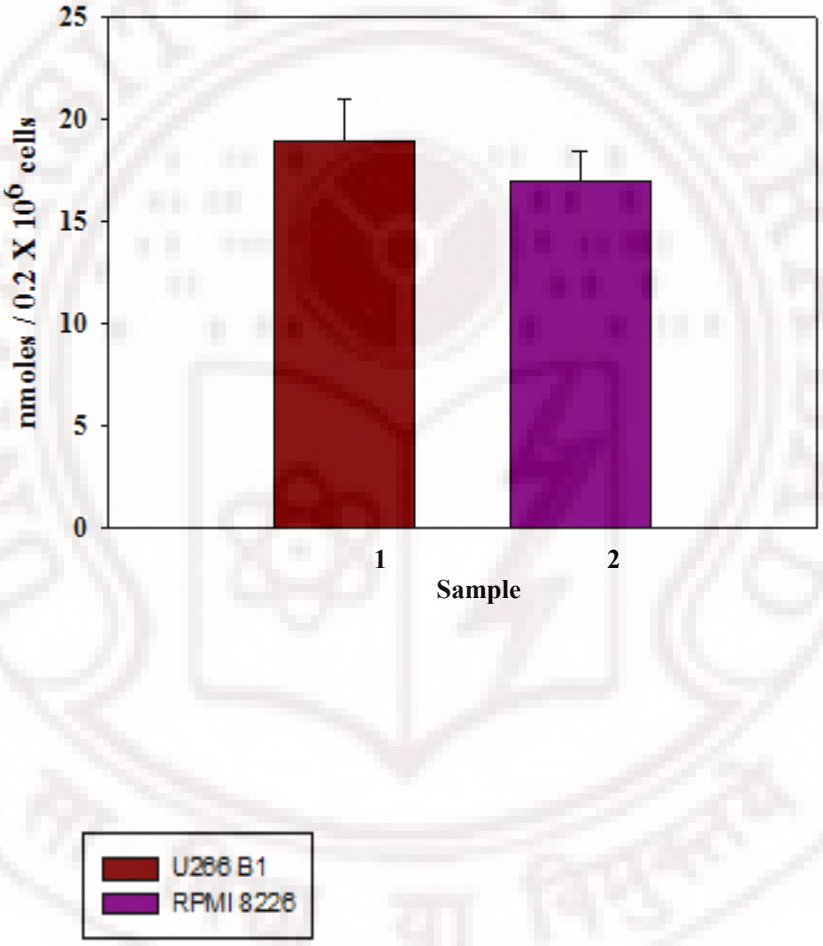
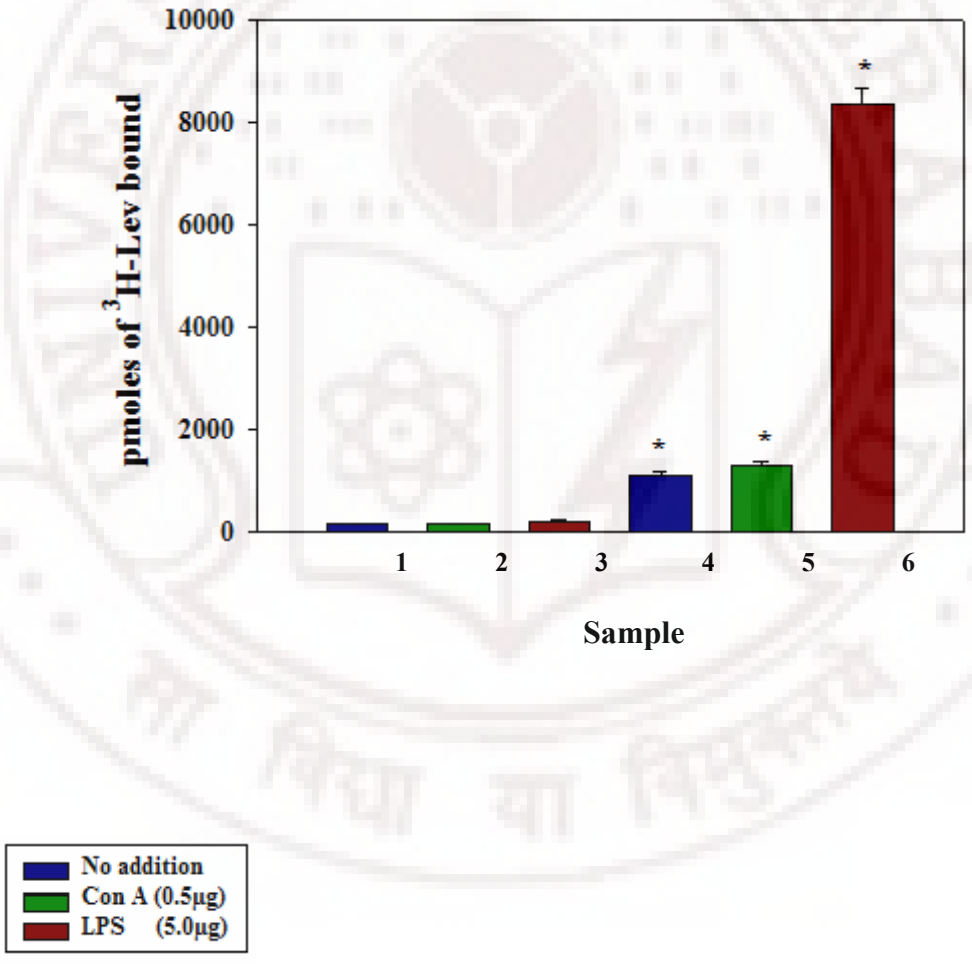


Fig - 5.4

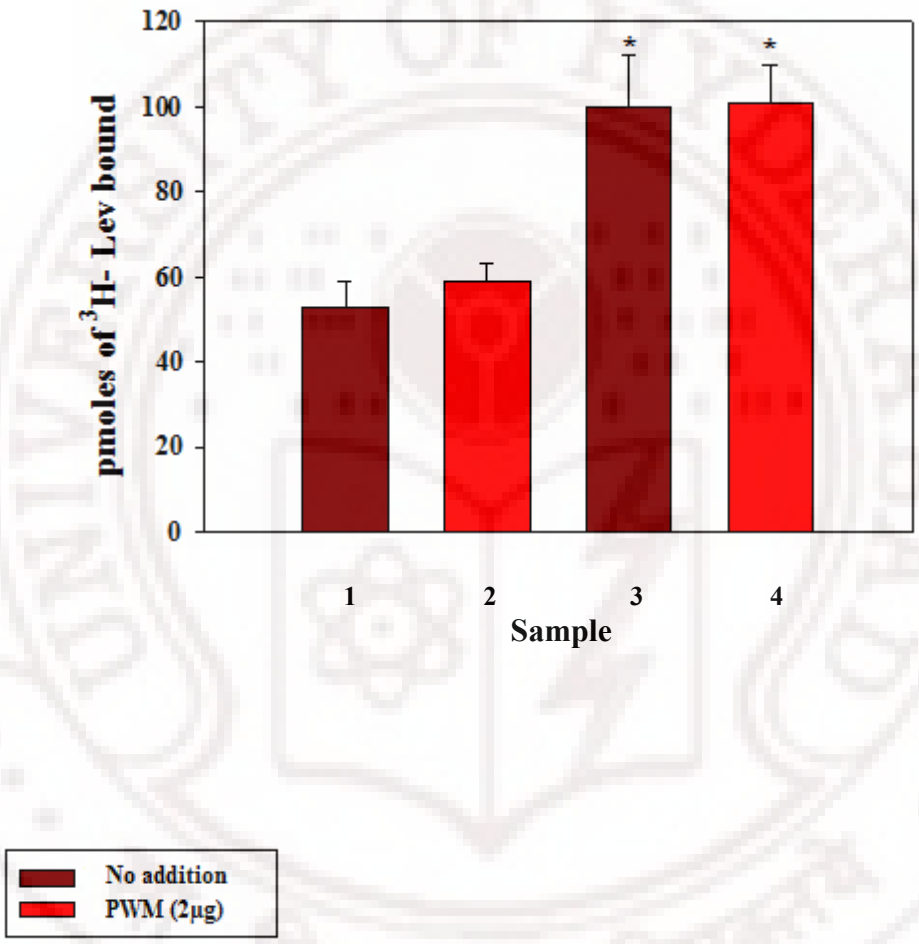
^{3}H-Levamisole binding of mitogen stimulated murine splenic lymphocytes



1-3 : Whole cells 4-6 : Cell lysate
*** $p < 0.05$ lysate Vs whole cells**

Fig - 5.5

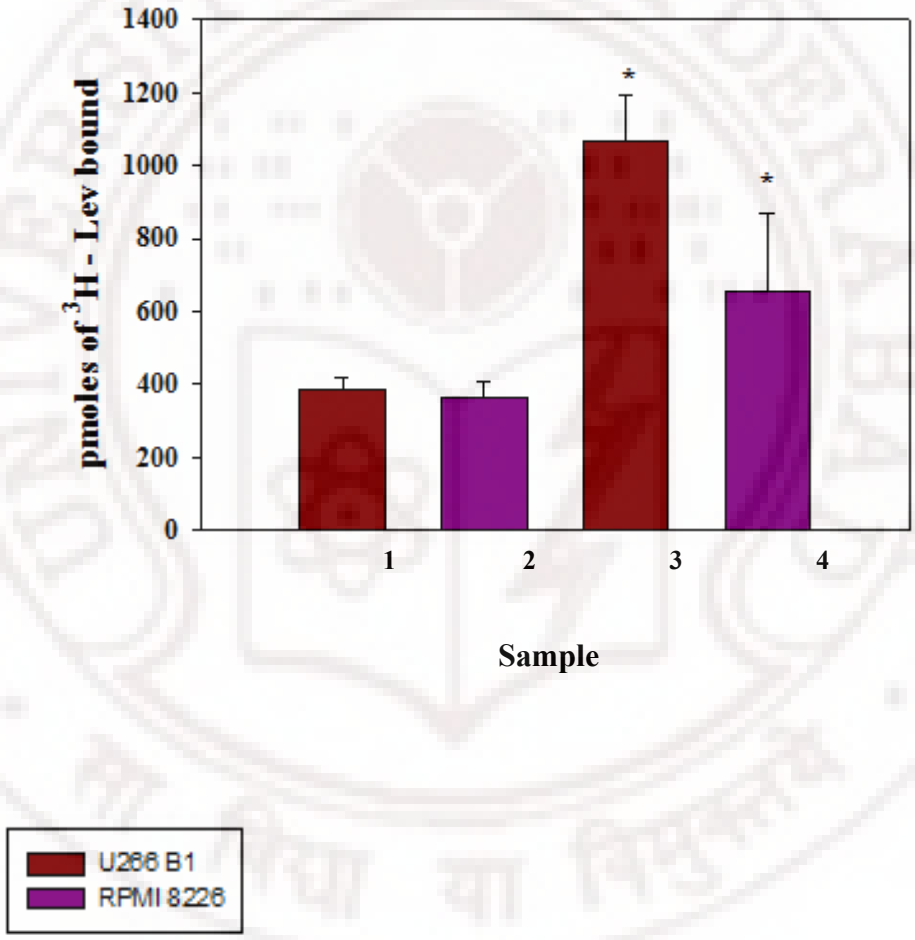
^{3}H-Levamisole binding of of Poke Weed Mitogen stimulated normal human peripheral blood cells



1,2 : Whole cells, 3,4 : Cell lysate
*** $p < 0.05$ lysate Vs whole cells**

Fig - 5.6

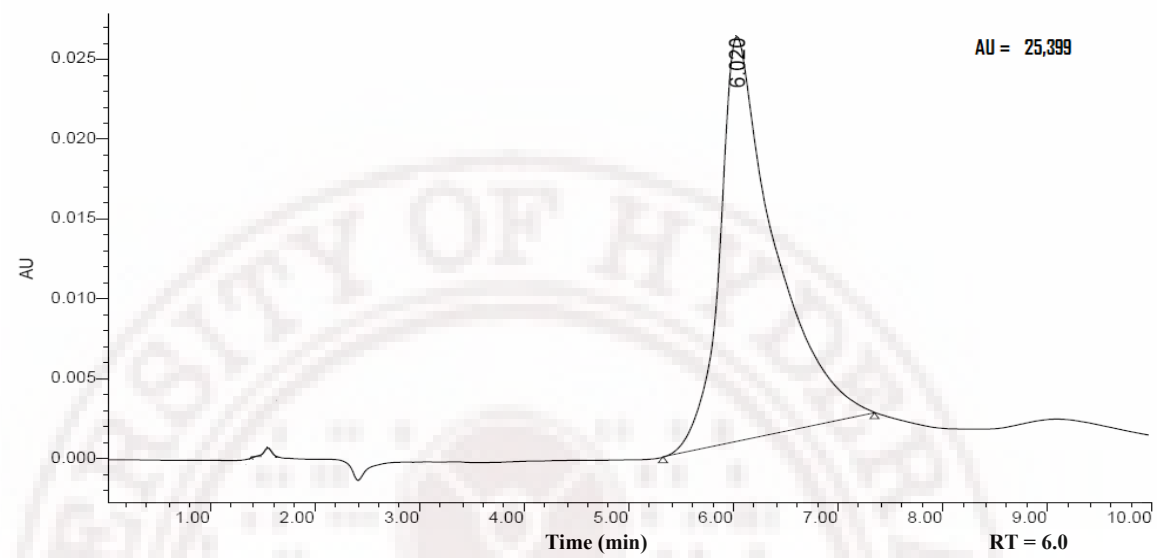
^{3}H-Levamisole binding of myeloma cell lines



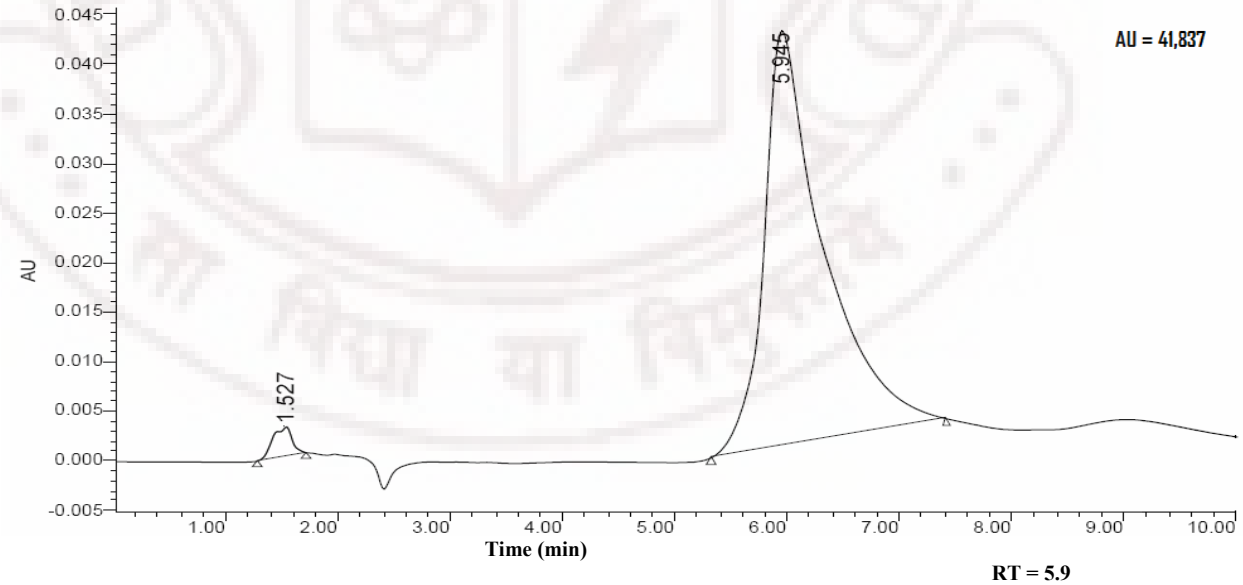
1,2 : Whole cells, 3,4 : Cell lysate
*** $p < 0.05$ lysate Vs whole cells**

Fig: 5.7 : HPLC Elution Profile of Levamisole.

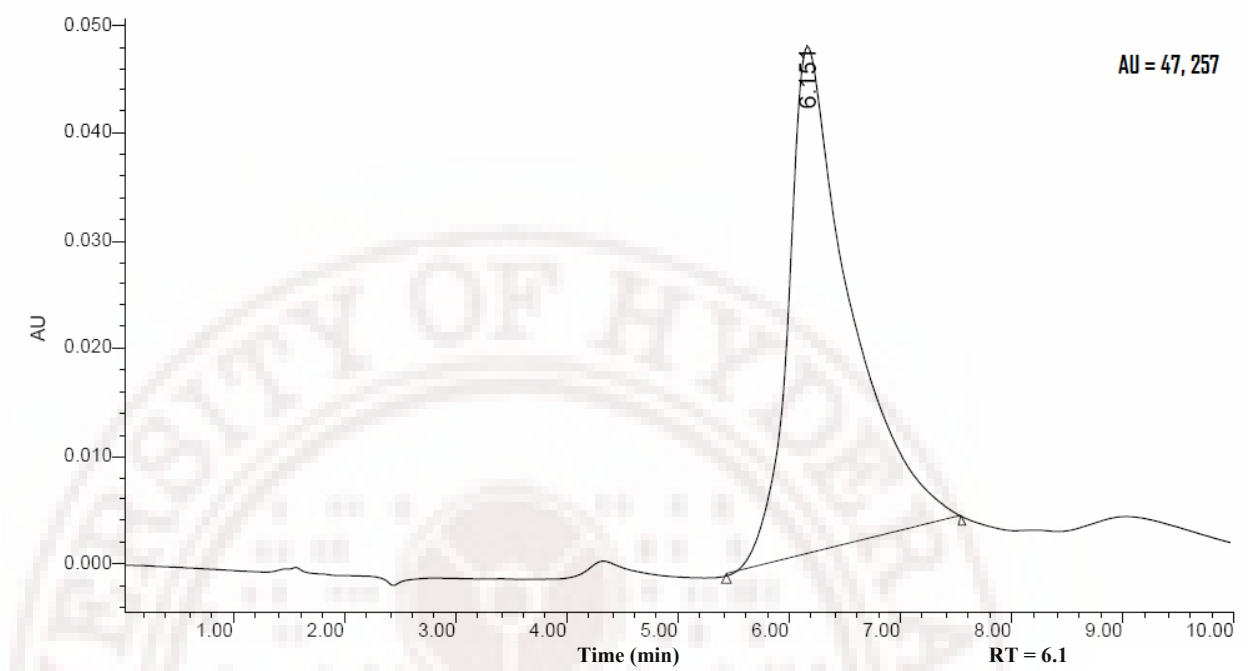
A. 4µg



B . 8µg



C: 12 µg


AU = 47, 257
6.154
AU
0.050
0.040
0.030
0.020
0.010
0.000
1.00
2.00
3.00
4.00
5.00
6.00
7.00
8.00
9.00
10.00
Time (min)
RT = 6.1

D: 20 µg

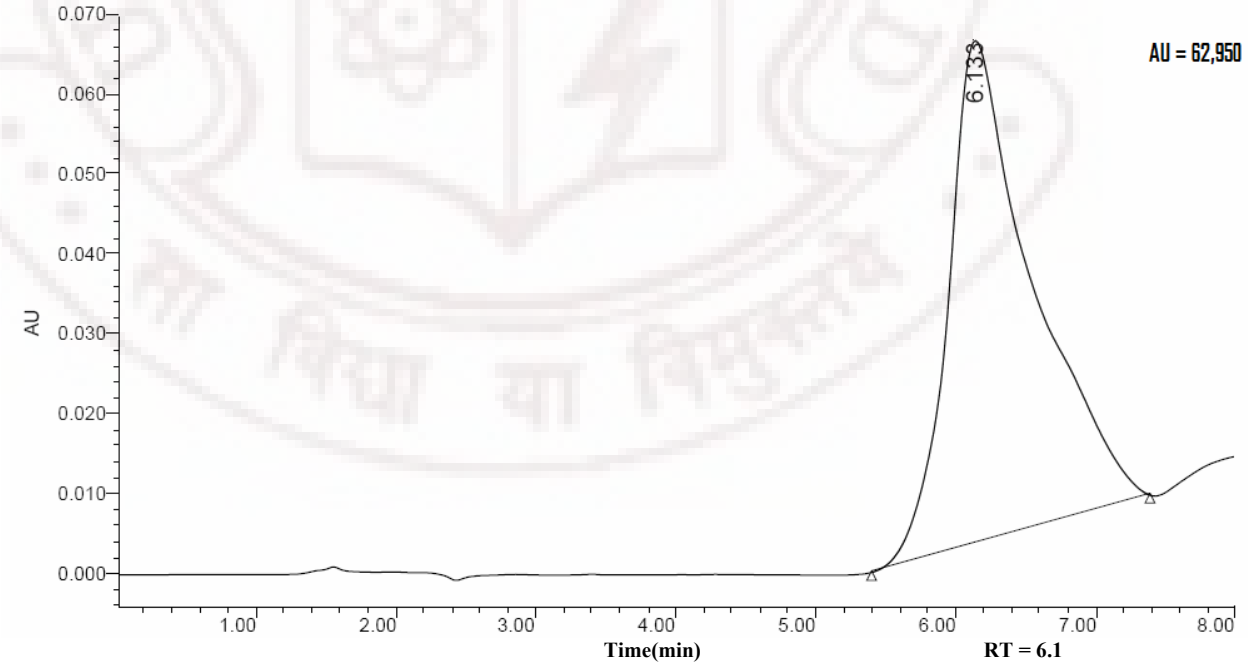
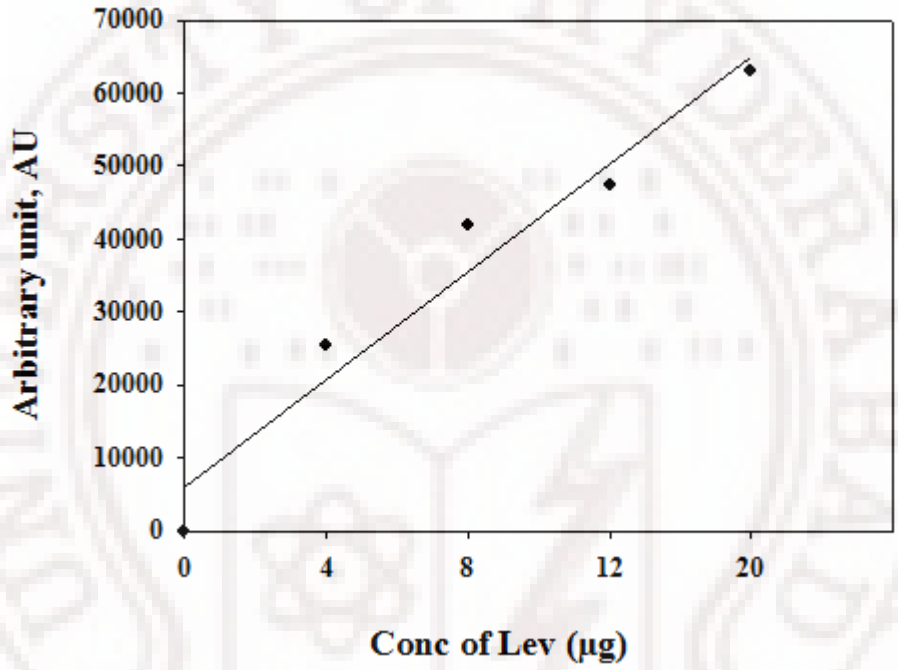

AU = 62,950
6.133
AU
0.070
0.060
0.050
0.040
0.030
0.020
0.010
0.000
1.00
2.00
3.00
4.00
5.00
6.00
7.00
8.00
Time(min)
RT = 6.1

Fig - 5.8

Standard graph of levamisole



The area under the curve is taken and standard graph is plotted.

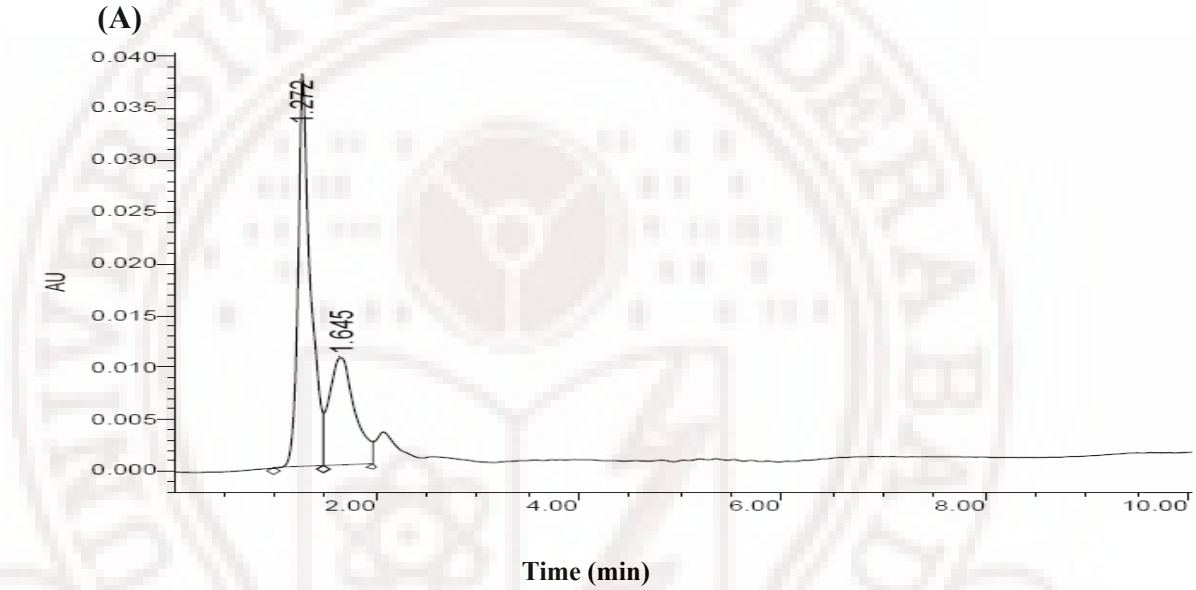
Table - 5.1

Concentration Of Levamisole (µg)	Area under the curve (Arbitrary unit, AU)
4	25399
8	41837
12	47257
20	62950

are presented in Fig. 5.9 A&B. This was carried out to examine whether any substances present either in RPMI or serum have a retention time similar to that of levamisole. The elution profiles of levamisole at various time periods of culture are presented in the Fig 5.10 A-D, Table 5.2. The elution profiles indicated that compounds with retention times different from that of levamisole were obtained with progressive culture time period. The amount of levamisole recovered from the culture supernatants also decreased with time.

Fig - 5.9

HPLC elution Profile of Levamisole in (A) RPMI 1640 and (B) RPMI + FCS



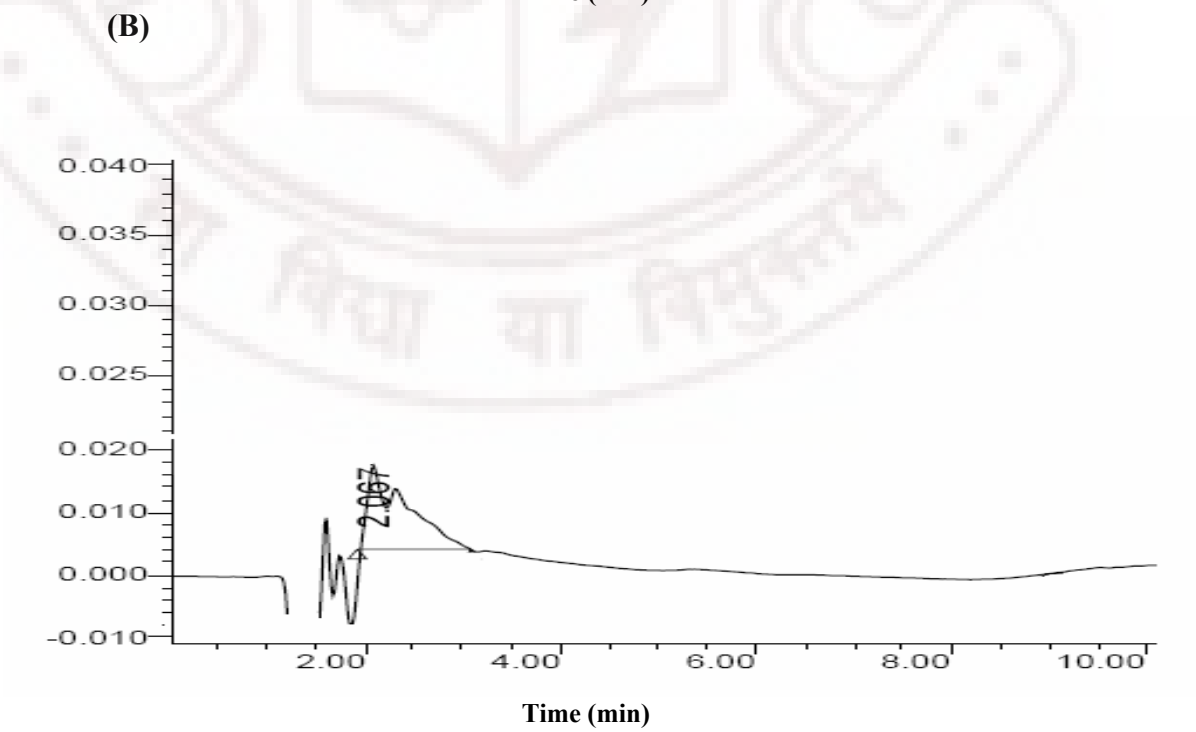
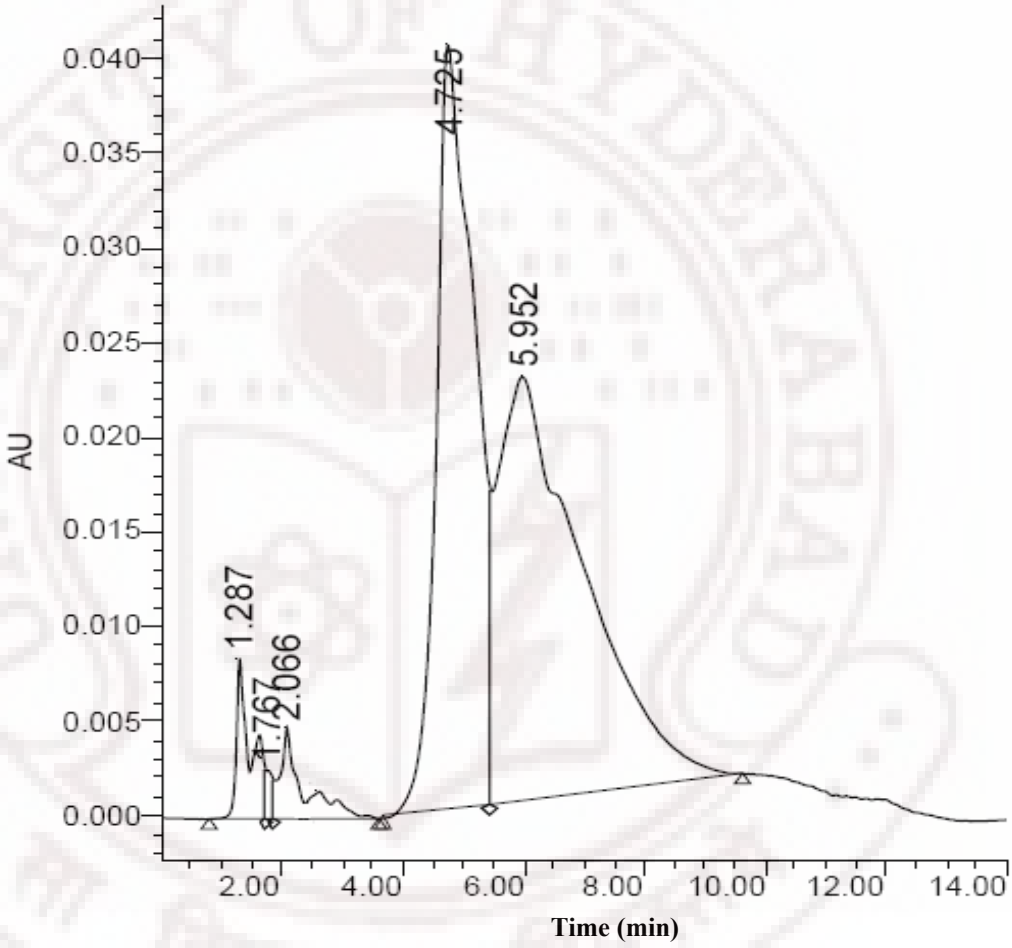
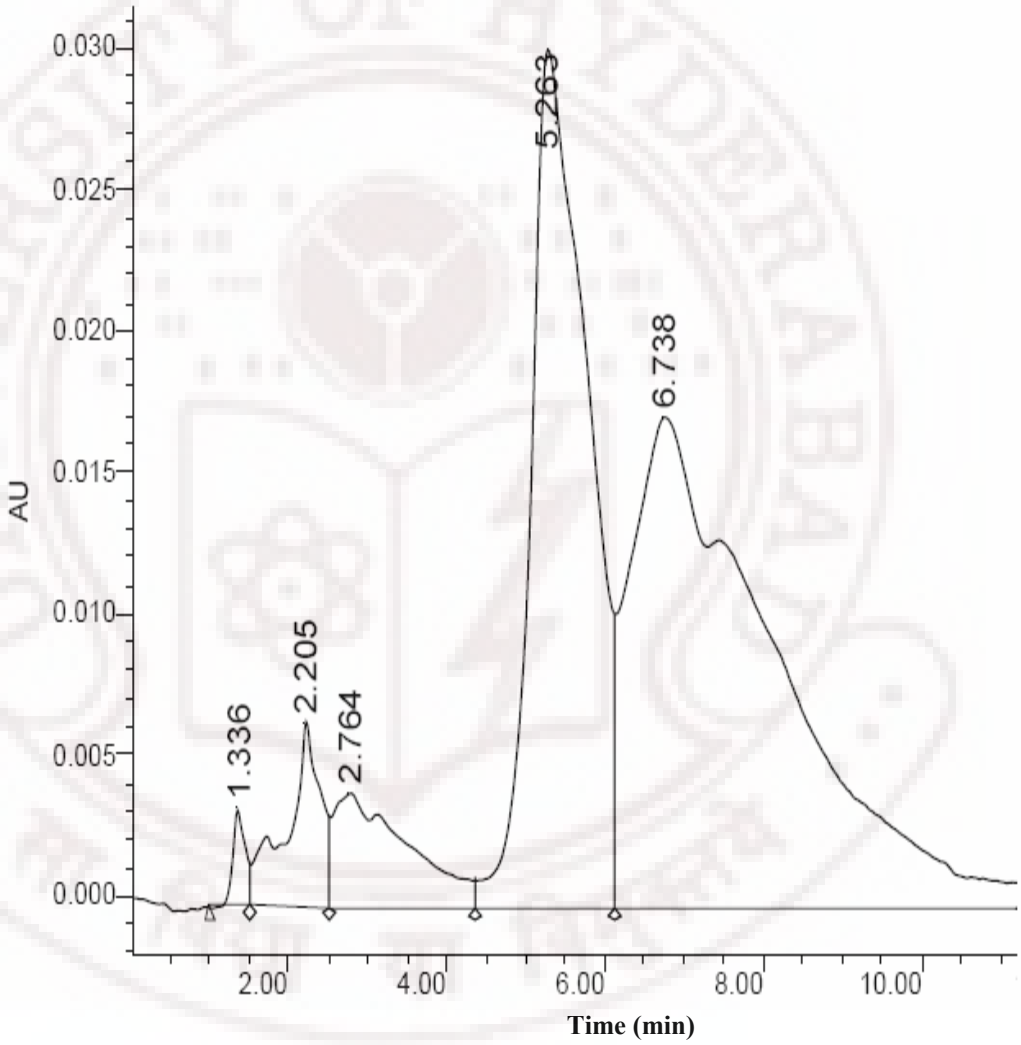


Fig - 5.10

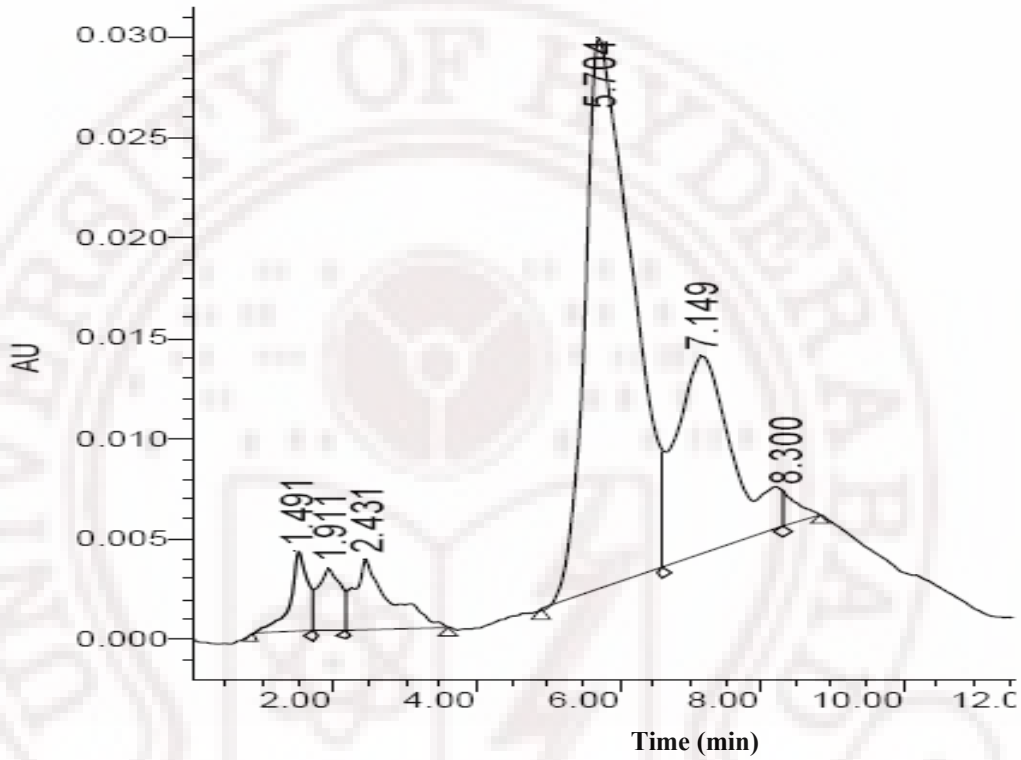
A. HPLC elution profile of levamisole extracted from culture – '0' time of culture period



B. HPLC elution profile of levamisole extracted from culture – 24 hours of culture period



C. HPLC elution profile of levamisole extracted from culture – 48 hours of culture period



D. HPLC elution profile of levamisole extracted from culture – 72 hours of culture period

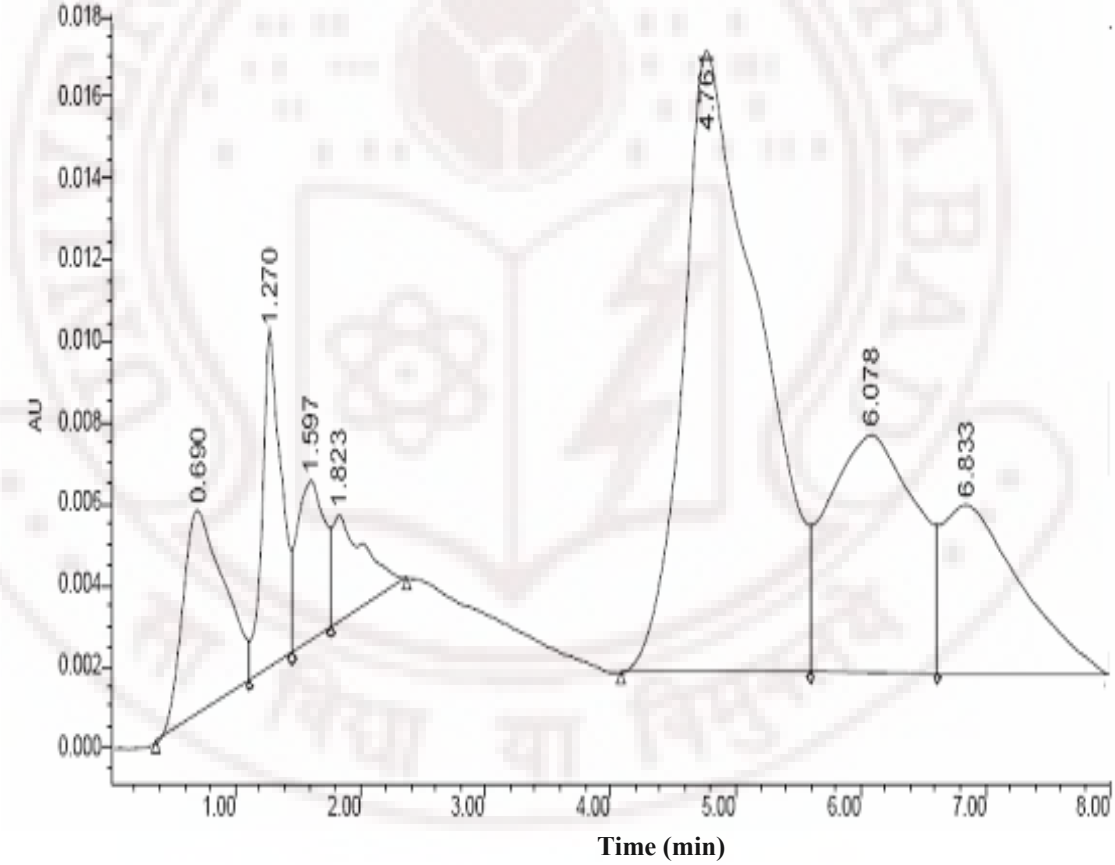


Table - 5.2

Levamisole extracted at various time points and analysed/quantified by HPLC.

(h)	RT	AU	(% recovered)
0	4.7	40,443	100
	5.9	22,415	
24	5.2	30,382	76
	6.7	17,414	
48	5.7	27,533	
	7.1	9,966	62
	8.3	1,797	
72	4.7	15,332	
	6.0	5,898	36
	7.0	1,176	

The values preented are representative of 2 independent experiments.

Discussion:

The results reported in the previous chapters have revealed that levamisole inhibits proliferartive response of multiple myeloma cells probably via inhibition of alkaline phosphatase activity. The concentration of the drug required to get half-maximal inhibition in myeloma cells was shown to be around 1mM. This corresponds to a value of 240 μg/ml.

Drugs are transported mostly as complexes with serum albumin. The binding of levamisole to total plasma proteins of 6 animal species was determined in vitro by equilibrium dialysis and it has been shown to bind avidly to plasma proteins. (Sahagun et al 1997). Levamisole being a basic organic drug has weak lipophilic tendency in an alkaline medium (Neilsen & Rasmussen, 1983). It was reported earlier that levamisole when administered to patients with colorectal carcinoma at a concentration of 100mg/ m^2 three times a day, resulted in 1μg / ml of peak plasma concentration (Reid et al 1998) . The high concentration required in cell culture could be firstly due to low lipophilic nature of levamisole there by resulting in low binding to membrane proteins and secondly due to binding to serum proteins resulting in lower concentration of free drug available to the cells. It is also possible that the drug undergoes transformation under the culture conditions employed.

In order to address this issue, ^{3}H-levamisole was used to estimate its binding to cells. Earlier, ^{3}H- labeled levamisole specific binding assays revealed a specific binding carrier for levamisole in human peripheral lymphocytes and granulocytes. (Ogawa, 1983). The results from the present study of ^{3}H-levamisole binding assays indicate that the binding was significantly higher in lysates of LPS stimulated murine splenic lymphocytes as compared to whole cells. The binding of ^{3}H-levamisole to human PBL was minimal in whole cells as well as in lysates and there was no difference between unstimulated and PWM stimulated cells. The myeloma cells which express APase activity had significant ^{3}H-levamisole binding. In all the cases, ^{3}H-levamisole binding correlated well with the cells expressing APase activity. Enhanced binding in the lysates compared to whole cells could be due to the exposure of putative levamisole binding site (domain) of APase present on cell membrane facing the cytosol.

Earlier studies have reported the effect of temperature and pH on the chemical stability of levamisole where levamisole was shown to degrade rapidly between pH 5 - 8. (Dickinson et al, 1971 a,b & c). Levamisole solution stored at 4 ^{0}C was shown to be stable (Fouad et al 2005). Levamisole stability was assessed when stored at 4 or 37^{0}C and at pH 6, 7, 7.5 and 8. Analysis of the various solutions by high pressure liquid chromatography demonstrated that levamisole degradation occurs during storage in neutral and alkaline conditions to form three products. The formation of the products was accelerated by increasing the temp from 4 to 37^{0}C. The degradation products were purified by preparative high pressure liquid chromatography and their structures determined by spectrometry , IR spectrometry and homo-and heteronuclear two dimensional NMR spectroscopy (Hanson et al, 1991).

Levamisole, used presently in the culture along with cells, medium (RPMI 1640) and fetal calf serum at 37^{0}C up to period of 72h might have affected the stability of levamisole, in order to assess the same HPLC was done. A high-pressure liquid chromatographic with ultraviolet detection methods (HPLC-UV) was used for quantification of levamisole. Calibration curves for levamisole were linear over the range 4-20ug.

HPLC analysis of cell culture supernatants of myeloma cells has shown that products of levamisole appeared with progressive culture period indicating a metabolic transformation. Also the amount of levamisole recovered from the culture supernatants with FCS was lower (less stable) than levamisole extracted from culture samples without FCS. This could be due to additional enzymatic degradation of levamisole in the presence of serum apart from degradation due to temperature. The degradation of levamisole could be one more possible reason for the high concentration of levamisole required to get desirable cytotoxic effect on myeloma cells.

SUMMARY AND CONCLUSIONS

In the present work, the primary objective is to elucidate the role of APase activity in the proliferative and differentiative phenomena of myeloma cells using levamisole as a tool. The mechanism of action of levamisole has also been analyzed in terms of the expression of CD138 and secretion of Interleukin-6 by the myeloma cells.

The salient observations and conclusions are:

1. Alkaline phosphatase activity was not detectable in resting and mitogen-stimulated normal human peripheral blood lymphocytes. When pokeweed mitogen was used, a significant proliferative response was observed with no increase in APase activity. This observation is in contrast to that observed in LPS stimulated murine splenic B lymphocytes wherein enhancement in APase activity occurs concomitant with proliferation.

2. APase activity could be measured in lymphocytes isolated from bone marrow of myeloma patients and in human myeloma cell lines - RPMI 8226 and U266 B1. This observation suggested that in case of human B lymphocytes, APase activity is not expressed in normal human lymphocytes, but only in malignant lymphocytes. Based on this observation, it is proposed that APase activity could be used as a therapeutic target in malignant B cells. Levamisole, an uncompetitive inhibitor of APase activity and an anti-cancer agent which is used in clinical medicine has been chosen for further studies.

3. Initial experiments were carried out using murine lymphocytes to analyse the effect of levamisole. The enhanced expression of APase activity and IgM secretion correlated well with proliferation and differentiation respectively of B cells in mitogen (LPS) stimulated murine splenic lymphocytes. Levamisole inhibited the LPS-induced proliferative response as well as secretion of immunoglobulin in murine splenic B lymphocytes.

4. Incubation of myeloma cell lines, RPMI 8226 and U266 B1 with increasing concentrations of levamisole (0.1- 2.5 mM) in culture for a period of 48-72 hours showed a significant cytostatic effect at low concentrations (up to 0.5 mM) and a potent cytotoxic effect at high concentration (> 1.0 mM). The proliferative response of both the myeloma cell lines was significantly inhibited as assessed by ^{3}H- thymidine incorporation in to DNA as well as by MTT assay.

5. In order to understand the specificity of levamisole effect on myeloma cells, the binding of ^{3}H-levamisole to unstimulated and mitogen-stimulated murine B cells, myeloma cell lines as well as pokeweed mitogen stimulated human peripheral blood lymphocytes was studied. Binding of ^{3}H -levamisole was significantly higher in LPS- stimulated splenic B lymphocytes and multiple myeloma cells (which express APase activity) when compared to PWM- stimulated normal B cells (no APase activity). The binding of ^{3}H-levamisole was more in lysates than in intact cells which suggests that the binding site of the putative target, i.e. APase is less accessible in intact cells.

6. Effect of levamisole on IL- 6 secretion and CD138 expression of myeloma cell lines was also analyzed**.** Levamisole treatment enhanced the secretion of IL-6 and increased the shedding of CD138. The expression of CD138 has been shown to be regulated by activation of at least two receptor sub classes (G-protein coupled and protein tyrosine kinase). It is possible that inhibition of APase probably leads to the activation of these receptors and subsequent shedding of CD138 (syndecan-1) from the cell membrane. On exposure to levamisole, myeloma cells showed an enhanced secretion of IL-6 in to the supernatant. This increased secretion of IL-6 by myeloma cells could be an attempt to protect themselves from undergoing apoptosis.

7. In levamisole treated myeloma cells, DNA fragmentation, enhanced cytosolic Ctochrome C concentration and increase in Caspase 3 activity were observed as compared to untreated cells. These results suggested that the cytotoxic effect of levamisole could be via mitochondrial pathway of apoptosis.

8. The cytotoxic effect of levamisole on myeloma cells was observed to be irreversible.

9. Levamisole has been shown to be degraded in cell culture. HPLC analysis of cell culture supernatants of levamisole treated myeloma cells has shown that products of levamisole appeared with progressive culture period indicating a metabolic transformation. Hence, the concentration of intact levamisole available for interaction with cells could be lower than the concentration added initially. In order to examine the effect of serum, myeloma cells were cultured in the absence of serum and it was found that levamisole extracted from the culture supernatants with FCS is less than in those without FCS. This decrease could also be due to the degradation of levamisole in presence of serum.

10. The effect of levamisole as a cytotoxic adjuvant when used in combination with atorvastatin was analyzed using myeloma cells. It was found that the combination of levamisole with atorvastatin did not show any synergistic cytotoxic effect.

Although APase has been identified and characterized in many malignant conditions, its role in the malignancy per se has not been studied in detail. Presence of APase activity specifically in malignant human B lymphocytes, but not in normal human B cells provided a experimental model to analyse the role of APase in the proliferative and differentiative events of these cells. The results obtained in the present work strongly suggest that APase could be used as potential therapeutic target to control the proliferation of malignant B lymphocytes. The ability of levamisole to inhibit APase activity , growth of human myeloma cells and to induce apoptosis opens up possibilities to design, develop and characterize new compounds as anti-myeloma agents using APase as a molecular target.

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