ROLE OF ALKALINE PHOSPHATASE IN B LYMPHOCYTE ACTIVATION

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By

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I declare to the best of my knowledge that no part of this thesis was earlier submitted for the award, of research degree of any University.

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CONTENTS

LIST OF ABBREVIATIONS

Anti-Ig	Anti-Immunoglobulin
APase	Alkaline phosphatase
β-ME	β- mercaptoethanol
ConA	Concanavalin A
DAG	Diacylglycerol
DEAE cellulose	Diethyl amino ethyl cellulose
DMEM	Dulbecco's modified minimal essential medium
DXS	Dextran sulfate
ELISA	Enzyme linked immunosorbent assay
FCS	Fetal Calf Serum
GCAP	Germ cell APase
GPI	Glycophosphatidyl inositol
HU	Hydroxyurea
kDa	kilodalton
IL	Interleukins
IFA	Incomplete Freund's adjuvant
LPS	Lipopolysaccharide
Lev	Levamisole
PBL	Peripheral Blood Lymphocytes
PHA	Phytohemagglutinin
PKC	Protein Kinase C
PLAP	Placental APase
PMA	Phorbol myristate acetate
p-NP	p- Nitro phenyl phosphate
p-NPP	p-Nitrophenyl phosphate
RPMI	Rosewell Park Memorial Institute

SDS-PAGE		Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Seph.anti-Ig	Sepharose.	anti-immunoglobulin
S Ig		Surface immunoglobulin
TNAP		Tissue non-specific APase
TT		Tetanus Toxoid

CHAPTER 1

INTRODUCTION

Living organisms developed defense mechanisms for their survival against a wide variety of pathogens in the form of innate and acquired immune responses Lymphocytes play an important role in conferring acquired immunity. The two types of lymphocytes — B lymphocytes (Bursa/Bone marrow-derived), T lymphocytes (Thymus-derived) carry out humoral and cell mediated immunity respectively. The mature T and B lymphocytes derived from the primary lymphoid organs circulate through the blood and interact with the antigen mainly in the secondary lymphoid organs The secondary lymphoid organs consist of spleen, lymphnodes and the disseminated lymphoid cells associated with gastro-intestinal, broncho-pulmonary and uro-genital tracts etc, Secondary lymphoid tissues consist of macrophages and dendritic cells that are involved in the processing and presentation of antigen to the lymphocytes.

B lymphocyte activation:

The B lymphocyte is the principal cellular mediator of humoral immunity The resting B lymphocyte is small, quiescent, non-dividing cell with low metabolic activity. It has sparse cytoplasm and a compact nucleus containing mainly heterochromatain. Resting B cells have been shown to be in GO phase of the cell cycle prior to encounter by a specific antigen (Monroe and Cambier1983). They express cell surface IgM and IgD, and possess a different set of cell surface markers compared to the activated B cells. Upon antigenic activation, the cell undergoes several morphological and metabolic changes (Anderson et al 1977). Surface IgM and IgD play a crucial role in the recognition and specific binding of antigen which leads to the activation of B cell (Kehry et al 1980). This activation has been shown to be aided by soluble factors released from other immune cells which in turn help in clonal expansion of the antigen specific B cells (DeFranco 1987).

This was followed by either differentiation of B cells in to plasma cells or in to memory cells.

B cell antigen receptor:

The **B** cell antigen receptor is a multi polypeptide chain assembly involved in antigen recognition and signal transduction (DeFranco 1993). The B cell antigen receptor is composed of monomeric IgM and IgD molecules non-covalently associated with two accessory molecules IgMα and Igβ, heterodimers linked through disulfide linkages (Campbell and Cambier 1990, Chen et al 1990 , Parkhouse 1990). Another receptor called Ig y which is identical in structure with Igβ except for the cytoplasmic tail that is truncated by 30 amino acid residues has also been shown to be present on B cells (Friedrich et al 1993).

The B cell antigen receptor shows two functions. Firstly, it efficiently takes up antigen by endocytosis, thereby allowing it to be processed and presented to helper T cells (Myers 1991). Secondly, the antigen receptor recognizes the antigen and transduces signals across the membrane necessary for B cell activation (DeFranco 1987, Pleiman et al 1994)

Interaction of antigen with B cell antigen receptor:

When an immature B cell encounters an antigen , the binding of antigen to the receptor leads to either clonal deletion or clonal anergy (Burnet 1959, Hartley et al 1991, Goodnow 1992), while binding of an antigen to the receptor of a mature B cell leads to activation and clonal proliferation The pathway followed by a B cell after receptor ligation by an antigen depends mainly on the nature of the antigen, differentiation state of the B cell and the nature of the additional signals provided by the helper T cells. If the

antigen is in multivalent form and complexed with carbohydrate, it induces a strong activation signal that takes the cell to proliferation (Mosier and Subbarao 1982). If the stimulation is with a paucivalent and proteinaceous antigen, the B cell requires additional signals from activated T cell for its complete activation (Monroe and Cambier 1983 , Noelle et al 1983). The cellular activation by these proteinaceous antigens results in anergy unless the secondary signals from the T cells are provided with in certain time after the initial activation. Thus B cell activation requires two signals, -- first signal derived from the ligation of the surface receptors and the second signal provided during the G1 phase of the cell cycle mostly by the T cell derived soluble factors which decide the fate of the B cell to further proliferation and differentiation (Brestcher 1971, Anderson et al 1979, Anderson et al 1980).

Signal transduction through B cell antigen receptor:

B lymphocyte activation is a complex process that occurs in a series of events after the binding of the antigen to its receptor on the cell surface This acts as an initial activation signal which inturn transduces the signal across the plasma membrane in to the cell Then, changes occur in many cellular biochemical pathways required for the sustained activation and progression through the cell cycle. Binding of antigen to the membrane Ig receptor causes the aggregation or cross linking of the surface receptors and leads to conformational changes that trigger B cell activation (Bretscher and Cohn 1970). It has been shown that crosslinking of Ig receptors results in the activation of tyrosine kinases leading to PhospholipaseC y activation by tyrosine phosphorylation. (Dymecki et al 1989, Carter al 1991, Brunswick et et al 1991, Reth 1992, Roifman and Wang 1992). The other events which occur are Phospholipid hydrolysis (Myers et al 1987, Coggeshall

and Cambier 1984, Harris and Cambier 1987), Diacylglycerol generation (Coggeshall and Cambier 1985) , Ca^{24} mobilization (Ransom and Cambier 1986a), Protein kinase C (PKC) activation (Chen et al 1986), membrane depolarization and enhanced MHC class II antigen expression (Monroe and Cambier 1983, Monroe et al 1984, Ransom et al 1986b). After the initial tyrosine kinase activation, B cell antigen receptor signaling pathway has been shown to diverse into three major biochemical mechanisms involving— 1 phosphoinositides , 2. G-proteins and 3 activation of phosphoinositol -3- kinase.

Generation of second messengers by Phosphoinositide breakdown:

The phosphorylated phospholipase Cy catalyses the breakdown of phophatidylinositol 4-5 bis phosphate to inositol 1,4,5 triphosphate (IP3) and diacyl glycerol (DAG) (Berridge and Irvine 1984, Bisterbosch and Klaus 1985, Nishizuka 1992). The water soluble IP3 diffuses in to the cytosol to mobilize Ca^{2+} from endoplasmic reticulum (Braun et al 1979, Pozzan et al 1982, Berridge 1987, Khan et al 1992). The elevated intracellular calcium activates calcium dependent calmodulin kinase II and presumably other calcium dependent enzymes (Fischer et al 1991). The neutral DAG which remains in the plane of the membrane mediates the translocation of calcium — phosphatidylserine and DAG dependent protein serine -threonine kinases (PKC) (Mishack et al 1991). The activated PKC phophorylates a variety of proteins that help in further cellular activation (Burke et al 1989). Thus, elevation of Ca^{2+} also is necessary for the activation of PKC. Anti-Ig mediated cellular activation events have been shown to be physiologically important positive events as they can be achieved by using pharmacological activators of PKC like phorbol esters, calcium ionophores like ionomycin and A23187 that help in calcium influx and initiation of transcription of specific genes.

PMA has been shown to activate B lymphocytes to G1 phase of the cell cycle by activating PKC mediated signal transduction (Hawrylowicz et al 1984) Transient activation of resting B lymphocytes can be achieved by stimulation with ionomycin.

G-Protein mediated activation:

$P21^{ras}$, a low molecular weight G-protein becomes activated with in l-2min after receptor ligation with antigen (Harwood and Cambier 1993). This protein has been reported to be regulated by a GTP exchange protein, Vav (Bustelo and Barbacid 1992) and a GTPase activating protein (Gold et al 1993) The activation PLCy I and PLC γII by the B cell antigen receptor also involves G- protein mediated pathway (Cambier et al 1994).

Activation of PI-3 Kinase :

Antigen receptor stimulation leads to the increase in PI-3 kinase (Cantley et al 1991, Yamanashi et al 1992) and tyrosine phosphorylation mediated activation of PI-3 kinase which inturn acts on phosphatidylinositol (PI), PI-4 phosphate, PI-4,5 bisphosphate, PI-3,4,5,trisphosphate (Auger and Cantley 1991, Tuveson et al 1993) It has been hypothesized that these different phosphates may play a major role as second messengers during the signal transduction.

Signal transduction through Lipopolysaccharide (LPS) receptors:

LPS, a polyclonal B cell activator induces a strong B cell proliferative response with subsequent differentiation, through a complex signal transduction pathway (Dearden and Revillard 1993). The process has been shown to be different from the antigen or anti-Ig mediated signal transduction, since LPS does not bind to sIg receptors (DeFranco 1987). Unlike anti-Ig mediated signal transduction pathway, LPS does not induce IP3 and DAG

production but initiates the translocation of PKC from the cytosol to the cytoplasmic membrane(Chen et al 1986) without Ca^{2+} mobilization (Rosoff and Cantley 1985) Jakway and DeFranco (1986) have shown that LPS mediated activation occurs by activating a Gi type G protein, by binding to GTP as a part of a coupling receptor Activated Gi inhibits adenylate cyclase activity resulting in decreased levels of cyclic AMP (cAMP) which inturn activates B lymphocytes. Whereas, Klaus et al (1987) have reported that LPS mediated signal transduction is not mediated via G protein activation. Eventhough it does not initiate phosphoinositide pathway, there is an involvement of Protein tyrosine kinase (PTK) mediated pathway in B lymphocyte activation (Dearden and Revillard 1993).

PTK72, a cell cycle- specific kinase has been shown to be activated upon activation of B cells with anti-Ig or mitogens. The activation profile of PTK72 in anti-Ig mediated activation has been reported to be biphasic ~ initial activation occurring with in 5min after stimulation and returning to normal levels over a period of 9h , which is not seen in case of LPS mediated activation However, the second increase observed in PTK 72 around 18-30h after the initial stimulation is common for both anti-Ig and LPS mediated activation pathways This second late phase of elevated tyrosine phosphorylation is cell cycle dependent and is an important event that coincides with the major control point in the cell cycle of activated B cells when commitment to proliferation occurs(Melchers and Andersson 1986).

Role of B cell co-receptors in signal transduction:

Several CD antigens modulate the transmembrane signaling mediated by the B cell antigen receptor. CD22, a 135 kDa, B cell restricted glycoprotein (Clark 1993) has been shown to mobilize Ca^{2+} in anti-Ig mediated signaling (Pezzutto et al 1988) CD19/21

complex (Tedder and Engel 1994), CD20 (Tedder et al 1994) and IL-4 receptor are some of the co-receptors that have been shown to modulate B lymphocyte activation CD45 is essential for sIg mediated signaling and is involved in the Ca^{2+} mobilization and $P21^{ras}$ activation that are essential for the activation and proliferation of B lymphocytes

Role of cAMP and cGMP during B lymphocyte activation:

Kammer (1988) has shown that the production and active participation of cAMP and cGMP take place during B lymphocyte activation It has been shown by Sutherland (1972) that these cyclic nucleotides act as second messengers in the signal transduction from the cell surface in to the cell and the nucleus Smith et al (1971)have reported for the first time that cAMP levels raise during the mitogenic activation of lymphocytes Even though cAMP, and its analogues and cAMP activators have been shown to inhibit T cell activation (Novak and Rothenberg 1990), they showed varied effects on B cell activation. It has been shown that the effects mediated by cAMP are biphasic.i.e, early addition and removal of cAMP enhanced antibody generation, whereas prolonged exposure to cAMP inhibited antibody production (Ishizuka et al 1971,Watson et al 1973 Teh and Paetkall 1974). Melmon et al (1974) have shown inhibition of antibody secretion in mixed splenic lymphocytes population, whereas Burchill and Melmon (1981) showed an enhancement of antibody production in B cell enriched population treated with cAMP analogues. Whisler et al (1992) have reported that proliferation induced by the combination of PMA and ionomycin was positively regulated by cAMP Koh et al (1995) implicated an essential positive regulatory role for cAMP during B cell activation. It has been shown that cAMP acts synergistically with LPS in activating the B lymphocyte

proliferative response and alkaline phosphatase activity (Kasyapa and Ramanadham 1995).

Role of lymphokines in B lymphocyte activation:

Lymphokines are the group of cytokines that regulate the activation differentiation and maturation of B and T lymphocytes and activation and migration of inflammatory cells. Cytokines are low molecular weight glycoproteins which regulate the major cellular functions like cell growth, activation, inflammation, immunity, tissue repair and morphogenesis. EL-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 (Jelinek et al 1986). IL-4 stimulates division and differentiation in B cells IL-5 and EL-6 act as B lymphocyte differentiation factors (Hirano et al 1985). In bone marrow, the antigen independent phase of B cell differentiation depends upon cytokines like IL-7 and IL-11 derived from stromal cells (Callard 1990). The cytokines are pleiotropic in their action and the *in vivo* effect observed with some cytokines is not due to the effect of a single cytokine but it is a concerted effect of several cytokines

Tyrosine phosphorylation has been recognized as a key mechanism by which the cytokine receptors induce a variety of intracellular events For example, IL-2 induces strong phosphorylation of PI-3 kinases, Raf (Turner et al 1991), Shc (Burns et al 1993), and Vav (Evans et al 1993) in T cells(Remillard et al 1991, Merida et al 1991). One of the major substrates for IL-4 induced phosphorylation in B cells and mast cells is a 170 kDa protein referred to as 4PS (IL-4 induced phosphotyrosine substrate), which is a distinct form and immunologically related to major Insulin receptor substrate-1 (IRS-1) (Wang et al 1993). The phosphorylated substrates assemble in to a complex that translocates to the nucleus to activate the transcription of specific genes (Keegan et al 1994)

Kinases and phosphatases involved in B lymphocyte activation:

Tyrosine phosphorylation is the major event which occurs soon after the ligation of surface antigen receptors of B lymphocytes catalyzed by various protein tyrosine kinases like P53/p561yn, p56blk, p59fyn and p561ck and PTK72/syk (Gold et al 1990, Campbell and Cambier 1990, Lane et al 1990, Brunswick et al 1991, Burkhardt et al 1991, Yamanashi 1991, Campbell and Sefton 1992, Leprince et al 1992, Li et al 1992). Several serine-threonine kinases have also been shown to be expressed which regulate the transcription of various genes down stream to the phosphorylation event(DeFranco 1992). cAMP-dependent kinases, mitogen activated kinases (MAP kinases), S6 kinase are some of the kinases that are activated and play a crucial role in B lymphocyte activation

Apart from these kinases, 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 that dephosphorylates the phosphorylated proteins at serine-threonine residues, for example, Calcinuerin, 2. tyrosine phosphatase that dephosphorylates at tyrosine residues, for example, CD45. Among the various phosphatases that are expressed upon lymphocyte activation, alkaline phosphatase is expressed specifically in activated B lymphocytes.

Lymphocyte activation studies using mitogens *in vitro:*

Understanding the mechanism of lymphocyte activation has been the pursuit of several workers Mitogens are the 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 Lymphocyte activation by mitogens has been shown to mimic antigen induced B cell activation *in vitro* (Sell and Gell 1965)

Subsets of lymphocytes show differential reactivity to different mitogenic stimuli. Some mitogens activate only certain subsets of B or T cells and some stimulate lymphocytes of certain species only Lectins like Concanavalin A (Con A) stimulate only T lymphocytes. LPS, an endotoxin from Gram-negative bacteria stimulates only B cells. Stimulation of lymphocytes by some mitogens like Phytohemaglutinin (PHA), Pokeweed mitogen (PWM) changes with the species. Moreover, the response of specific stimulators frequently requires the interaction of different subsets of lymphocytes and accessory cells. For example, soluble protein antigens require the help from T lymphocytes for the production of antibodies by B lymphocytes and hence are called T dependent antigens (T dep Ag) (Augustin and Coutinho, 1980)

LPS can induce strong activation of B cells without the help of T cells and is called a T independent antigen (T indep Ag) (Mosier and Subbarao 1982) Stimulants composed of regular repeating units can also induce lymphocyte activation without the aid of T cells (Mond et al 1978). The T indep Ags were further subdivided in to Type 1 and Type 2 based on their effect to induce activation of B cells in *xid* mice (Mosier et al, 1977, Mond et al 1980). T indep Ag type 1 is mitogenic to B cells and they can stimulate not only mature B cells but also neonatal B cells and B cells from *xid* mouse T indep. Ag type 2 is characterized by having large molecular weight with repeating epitopes (Feldmann and Basten 1971, Dintzis et al 1989) and is resistant to degradation *in vivo* It stimulate mature B cells only and is unable to stimulate B cells from neonatal or *xid mice.*

Alkaline phosphatase:

Alkaline phosphatase (APase, EC 3.1 3.1) an orthophosphoric monoester phosphohydrolase having alkaline pH optimum, is a membrane bound metalloenzyme. APases are a group of isoenzymes encoded by at least 4 different gene loci, Tissue non-specific (TNAP), intestinal, placental and germ cell types. Post translational modifications and differential glycosylation of tissue non-specific APase gives rise to tissue specific Apases of liver, bone and kidney Komoda and Sakagishi (1978) hypothesized that the physiological role of the sugar moieties could be in the protection of the enzyme from rapid removal from circulation through binding by the asialoglycoprotein receptors of the liver.

The gene for TNAP is localized on chromosome 4 in mouse (Terao et.al 1988 and 1990) and on the short arm of the chromosome 1 in humans (Swallow et al 1986 Weiss et al 1986) The difference in the localization of TNAP in mice and humans is because of the fact that the distal portion of mouse chromosome 4 and the short arm of human chromosome 1 carry the largest signals of conserved linkage between mice and humans (Lally et al 1978, Nadeaue and Taylor 1984, Lalley and McKusick 1985, Marth et al 1986). Intestinal and placental genes are present closely on chromosome 2 in both mice as well as humans (Weiss et al 1988, Henthorn et al 1988, Knoll et al 1988).

APase is a metalloenzyme containing zinc that is required for the stability and magnesium that is required for catalytic activity (Coleman and Chlebowsky 1979). The enzyme is active between pH 8.2 and 10 7. The enzyme is inactivated under acidic conditions (Fishman and Ghosh 1967). The pH optimum is affected by the type and concentration of the substrate (Ross and Archer 1951, Fedde et al 1988) The different

isoenzymes of APase can be differentiated based on their structure, immunological properties and sensitivity to heat and different inhibitors

Functions of APase:

Like many other glycophosphatidylinositol (GPI) anchored proteins, APase may also be involved in transmembrane signaling 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 and 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.

Biochemical functions of APase:

Three *in vitro* functions have been attributed to human APase--1 .Phosphohydrolysis of organic monoesters of low molecular mass, 2 Phoshotranseferase activity 3 Protein phosphatase activity.

APase has little preference for a particular substrate and will hydrolyze all the phophomonoesters(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 (phosphohydrolysis) or to an organic acceptor alcohol (Phosphotransferase) (McComb et al 1979, Coleman and Gettins 1983) However, phosphoester cleavage is faster if the transfer of phosphate is to an acceptor rather than to water.

Role of APase in embryonic development and cell differentiation:

Millan (1990) postulated that germ cell APase (GCAP) may be able to interact with extracellular matrix proteins and therefore serve as the cell guidance molecule during the

migration of germ cells Ligands involved in directing the cell migration via APase binding might be phosphoproteins representing natural substrates for APase.

Even though the exact physiological role of the enzyme is not clearly understood, based on the above observations it is possible to suggest the following functions to APase:

1. In transport of nutrients across the cell membranes.

2 In ossification of bone (Whyte 1989)

3. As 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 and Stinson 1986).

4. As a phosphotransferase (Herraez et al 1980 and Sarrouilhe et al 1992).

5. In the transport of IgG in placenta Makiya and Stigbrand (1992a and 1992b) have observed that placental APase is involved in the internalization of IgG in HEP2 cell line. Placental APase has potential to act as Fc receptor and the presence of which is necessary to provide the fetus with maternal IgG as an acquired passive immunization during gestation.

6. APase has also been shown to be involved in cell adhesion(Hui et al 1993), proliferation (Fedarko et al 1990) and differentiation (Owen et.al 1990, Andracchi and Korte 1991). The enzyme activity has been shown to be stage specific during embryonic development (McWhinnie and Saunders 1966). 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. Thus, APase with its tissue-specific structure, broad specificity towards its substrates and wide distribution displays its functions depending on the tissue and its requirement.

Several biological components like hormones, intracellular metabolites, vitamins and serum proteins have shown to act as modulators of APase activity expression Apart from these APase has also been shown to shown to be influenced by several of its substrates and other compounds- adrenaline, noradrenaline, ACTH are some of the hormones that are reported to induce APase activity (Moog 1961, Fishman 1974, Herz and Koss 1979) vitamin D(Haussler et al 1980, Majeska and Rodan 1982) and vitamin A are good stimulators of APase activity(Riley and Spearman 1969, Scheibe et al 1991) Serum proteins play a major role in regulating APase activity of cells in culture (Herz and Sevadalian 1971, Sorimachi and Yasumura 1986). Fetuin in fetal calf serum has been shown to reduce the expression of APase activity but calf serum enhances it (Spencer 1970). Several inhibitors like puromycin , actinomycin D (Moog 1974, Moog and Wiemerslage 1981), Colchicine (Ikehera et al 1978) enhance APase activity in culture. Levamisole, an uncompetitive inhibitor of tissue non-specific APase has been shown to inhibit of APase activity of liver, bone and kidney. 5-'bromodeoxyuridine, a thymidine analogue (Koyama and Ono 1971, Hamilton et al 1979), dbtcAMP, an analogue of cAMP (Finestone and Heath 1981), sodium butyrate (Herz and Halver1983) and Prednisolone an analogue of hydrocortisone are some of the inducers of APase activity. APase substrates like phenyl phosphate have been shown to enhance APase activity.

Many different isozyme patterns have been reported in malignancies and renal diseases (De Broe and Van Hoof 1991) APase activity provides the clinician valuable information for diagnosis and follow up of patients during treatment. A change in the level of serum APase has been used as an important diagnostic marker in several disease states. In hepatobiliary disease, rise in serum Type 1 APase has been reported. Bone

isoenzyme has been shown to be elevated in serum in osteomalacia (Morgan et al 1965) Hypophosphatasia is an inborn error of metabolism that is clinically characterized by defective bone mineralisation and biochemically by a deficient serum type 1 APase activity (Henthorn and Whyte 1992) APase activity has been reported to be enhanced in cancer patients (Gordon 1993) Various tumor cell lines like teratocarcinomas and osteosarcomas also show elevated APase activity(Hamilton et al 1979, Benham et al 1981) Reduced APase activity has been reported in cells transfected by Rous sarcoma virus (Bader et al 1978) or Simian sarcoma virus 40 (Chou 1978). 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).

B Lymphocyte APase:

APase activity has shown to be expressed on B lymphocytes upon mitogenic stimulation (Greaves and Janossy 1972). Garcia-Rozas (1982) showed that APase activity is expressed only in activated B lymphocytes, neither resting B lymphocytes nor resting and activated T lymphocytes express APase activity. It has been shown that the enhancement in APase activity correlates with proliferation (Ohno et al 1986) and differentiation (Burg and Feldbush 1989, Marquez et al 1989, Souvannavong et al 1994). It has been reported earlier that expression of APase activity occurs around 8h after mitogenic stimualtion and continues till 120h (Kasyapa and Ramanadham 1992). It has also been shown that APase activity is not expressed in B lymphocytes stimulated with incomplete mitogens (Kasyapa 1996) cAMP has been reported as a positive regulator of

enhancement of APase activity in mitogen stimulated B cells (Kasyapa and Ramanadham 1995) IL-5 has been reported to be a costimulant in increasing the APase activity in dextran sulfate stimulated murine splenic lymphocytes (Souvannavong 1992). It has also been shown that UV irradiation of 7TD1 hybridoma B cells leads to an over expression of APase activity in cycling as well as in apoptotic cells(Souvannavong et al 1997). Also, the B cells from senescent mice showed an age related alteration in apoptosis and activation with a decline in the expression of Apase activity(Souvannavong et al 1998). Binding of specific monclonal antibody to cell surface APase has been reported to modulate antibody production by B cells(Marty and Feldbush 1993). Eventhough much is known about APase the exact physiological role of the B lymphocyte enzyme has not yet been elucidated Earlier it has been hypothesized that APase may be involved in the transport of Ig molecules(Feldbush and Lafrenz 1991) and in phosphorylation / dephosphorylation reactions (Souvannavong et al 1992)

AIM AND SCOPE OF THE PRESENT WORK

Understanding the molecular mechanism of lymphocyte activation gives us new insights in developing potential vaccines towards various infectious diseases Moreover, lymphocytes offer an ideal model system for understanding the various cellular activation events like proliferation, differentiation, maturation, and apoptosis.

APase is one amongst the many enzymes that are triggered during B lymphocyte activation. It has been shown to be an activation and differentiation marker of B lymphocytes as it is specifically expressed only in activated B lymphocytes. Although B lymphocyte APase has been well studied, its physiological role has not been elucidated.

The present study was undertaken with the main objective of characterizing the physiological role of APase in activated B lymphocytes The main objectives are to study--

1. The effect of *invivo* antigen priming on B lymphocyte proliferative response and expression of APase activity,

2 The effect of recombinant interleukins IL-2 and IL-4 on the proliferative response and APase activity of mitogen stimulated splenic lymphocytes,

3. The involvement of alkaline phosphatase activity in mitogen induced proliferative response of murine splenic B lymphocytes — Analysis using levamisole and hydroxyurea,

4. Purification of APase from mouse liver ,YAC-1 cells and preparation of polyclonal antibody to liver APase. Comparison of catalytic properties of liver, YAC-1 and B cell APase,

5 Effect of anti-APase antibody on proliferative response and induction of APase activity in LPS stimulated splenic lymphocytes,

6 Effect of various mitogens on the proliferative response and APase activity of human peripheral blood lymphocytes and screening of human and murine cell lines for APase activity.

CHAPTER 2

MATERIALS AND METHODS

Animals: C57BL6 / J, Swiss albino mice (male and female) of 8-12 weeks old and adult female New Zealand white rabbits were purchased from National Center for Laboratory Animal Sciences, National Institute of Nutrition, Hyderabad The animals were maintained under hygienic conditions in the animal facility of University of Hyderabad until use.

Chemicals: RPMI-1640 medium (with 2mM L-glutamine and 25mM HEPES buffer), Phytohaemagglutinin (PHA), ConcanavalinA (ConA), Lipopolysaccharide (LPS) from E.coli , Dextran sulfate (DXS), Phorbol myristate acetate (PMA), goat anti- mouse IgM (μ chain specific), goat anti - mouse IgG (heavy and light chain specific), histopaque (d= 1.077), goat anti-human IgG (heavy and light chain specific), CNBr -activated sepharose 4B, heparin , p-nitro phenyl phosphate sodium salt , β- naphthyl phosphate , Fast blue RR, Levamisole (Lev) and Hydroxyurea (HU) were purchased from Sigma chemicals Co., USA ^{3}H-Thymidine (specific activity 6.5Ci/mmole) were purchased from BRIT, INDIA Fetal calf serum (FCS) was purchased from Biological Industries, Israel. Protein A from Staphylococcus aureus (SPA) was purchased from Pharmacia, Sweden. Tetanus toxoid (TT) was a generous gift from Biological Evans Ltd, India. Recombinant interleukin 2 (IL-2) and interleukin 4 (IL-4) were purchased from Boehringer Mannheim, Germany. All other chemicals used were of analytical grade. 96 well flat bottomed plates, ELISA plates and tissue culture flasks were purchased from Tarsons , India

Methods:

1.Media preparation:

A. RPMI-1640 Medium:

Powdered RPMI-1640 medium was dissolved in double distilled water(DDW). 100 U/ml of penicillin G and 100 μg/ml of streptomycin and 2g of sodium bicarbonate were

added, pH was adjusted to 7 2 and the volume made up to 1 litre with DDW. This was sterile filtered through 0 22μ membrane filter using Millipore filtration unit.

B Dulbecco's minimal essential medium (DMEM):

Powdered DMEM was dissolved in **DDW** 100 **U/ml** penicillin G and 100 jig/ml streptomycin, 3 6 g of sodium bicarbonate were added and pH was adjusted to **7.2** The volume was made up to 1 litre with distilled **water** The medium was sterile filtered through 0.22 μ membrane filter using Millipore filtration **unit**

2. Isolation of mouse splenic lymphocytes :

Splenic lymphocytes were prepared as described earlier(Zimmerman and Kern 1973).

Complete medium : **RPMI-1640** medium supplemented with 5% FCS and **50uM** β - mercaptoethanol.

Mice were killed under mild ether anesthesia and spleens were dissected and put in RPMI-1640 **medium** Single cell suspension was prepared as follows :

Spleens were placed on a sterile stainless steel mesh connective tissue and fat were removed and **minced** The tissue was teased into the medium using an arterial forceps fitted with a stainless steel brushes. The suspension was allowed to settle for 5min on ice. Large clumps which settled to the bottom were removed The suspension was centrifuged for 400xg for 7min The pellet was resuspended in **10ml RPMI-1640** medium and layered over histopaque (d =1.077) and centrifuged at 600xg for **10min.The** cells at the interface were collected and washed thrice with complete medium and suspended in the same medium.

3. Isolation human peripheral blood lymphocytes (PBL):

Heparin : A stock solution of 10,000 U/ml in 0.9% NaCl

PBL were isolated according to the method of Boyum(1964). Venous blood collected in to heparinized tubes (10U/ml) was diluted 1:1 with saline Diluted blood, 2.5 ml was layered over 1.0 ml histopaque and centrifuged at 400xg for 20min.The cells at the interface were collected and washed thrice with complete medium and suspended in the same medium

4. Isolation of sIg^+ mouse splenic B lymphocytes:

Principle: sIg positive splenic lymphocytes were isolated from mixed lymphocyte population using anti-Ig coated plates to which cells with sIg receptor adhere (B lymphocytes) while those without sIg receptor do not (T lymphocytes) (Mage et al 1977).

Phosphate buffered saline : 0.9% NaCl solution containing 10 mM phosphate buffer, pH 7.2.

Coating of petriplates with goat anti-mouse Ig :

25µg /ml of goat anti- mouse IgG (H & L specific) and goat -anti mouse IgM were used for coating the plates

Petridishes (tissue culture grade) were coated with 25µg/ml of anti-IgG or anti-IgM solution overnight at 4^0C The antibody solution was removed and plates were washed thrice with PBS. Splenic lymphocyte suspension, 6 ml ($4x10^6$ cells/ml) was added to the plates and incubated at 4^0C for 60min with gentle swirling for 30sec for every 30min. After one hour the non-adherent cell population (T cell-enriched) was collected in to a sterile 10ml culture tube and the plates were washed twice with PBS and once with

RPMI-1640 medium and the washes were discarded The adherent cell population (B cell-enriched) was removed in to the medium and both adherent and non -adherent cell population was gently removed using a sterile rubber policeman Both adherent and non-adherent fractions were centrifuged at 400xg and washed with complete medium once and the pellet was suspended in a small volume of complete medium.

5. Lymphocyte counting :

Principle: Gentian Violet stains the lymphocyte nucleus while dilute acetic acid lyses the RBC

Turk's solution (0.01% gentian violet w/v in 3% acetic acid) was added to a small volume of the cell suspension mixed and counted using a hemocytometer The average number of cells in the cell suspension was determined using the formula..

$$\text{No. of cells /ml} = \frac{\text{Average no. of cells/ large square}}{\text{dilution}} \times 10^4$$

6. Determination of cell viability :

Principle Dead cells take up the dye trypan blue while the live cells exclude it ,there by viable cells could be distinguished from a non-viable of dead cells which are stained.

Procedure:

A small volume of the cell suspension was diluted appropriately in Trypan blue solution (0.2 % w/v in 0.9 % NaCl) minimum of 200 cells were counted microscopically using a hemocytometer. The percentage of viable cells were calculated using the formula

$$\% \text{ Viability} = \frac{\text{No. of unstained cells}}{\text{Total no. of cells}} \times 100$$

7. Coupling of anti- immunoglobulins to CNBr -activated Sepharose 4B:

Anti-immunoglobulin was coupled to CNBr -activated Sepharose 4B according to the procedure described earlier(Ramanadham et al 1984).

Principle: Cyanogen bromide reacts with OH groups on Sepharose and converts them into imido carbonate groups which react with the nucleophiles. The activated groups react with primary amine groups of the proteins to form isourea linkages.

Reagents:

coupling buffer: 0.1M Bicarbonate buffer in 0.5M NaCl pH 8 5

1M ethanolamine , pH 8.0

0.1M Acetate buffer in 1M NaC, pH 4.0

0.1M Borate buffer in 1M NaCl, pH 8.0

0 1N HCl

CNBr activated sepharose 4B

Affinity purified anti-IgG or anti- IgM

RPMI-1640

Procedure:

One gram of CNBr activated sepharose 4B was swollen in 0. 1N HCl for 20min and was then washed with large volumes DDW The swollen gel was transferred to 10ml of coupling buffer containing 10mg of affinity purified goat anti-mouse Ig The mixture was kept for end-over mixing for 2h at room temperature. The coupled gel was centrifliged at 1000rpm for a minute and the supernatant was saved The gel was washed with 5ml of coupling buffer to remove unbound protein. The unreacted groups of the gel were

blocked by adding ethanolamine buffer and end over mixing for 1 h at room temperature. The gel was centrifuged and the supernatant was saved. The coupled gel was washed with acetate buffer followed by borate buffer for three cycles Finally, the gel was washed once with 5ml of RPMI-1640 medium, suspended in the same medium and stored at 4°C. All the operations were carried out under sterile conditions and at each step the supernatant was saved The absorbance of all the supernatants was measured at 280 nm and the amount of protein bound to the gel was calculated. At least 85 % of the protein taken for coupling was bound to the gel.

8. Immunization of mice with TT :

C57BL6/J mice were immunized with TT (20μg) in incomplete Freund's adjuvant (IF A) intraperitonially. On the day 15 booster dose was given with 10μg of TT in IF A and the mice were sacrificed two weeks later.

9. Lymphocyte proliferation assay:

1.Mitogens

2.complete medium: RPMI-1640 supplemented with 5% FCS, 50μM

β– mercaptoethanol

3. ^{3}H-thymidine (6 Ci/mmole)

4.Scintillation cocktail: 4g PPO and 200mg POPOP in 1 litre of scintillation grade toluene.

Procedure:

$2x10^{5}$ splenic lymphocytes in triplicate were cultured with and without mitogens or antigens in 200μl of complete medium in 96well flat bottomed microtitre plates cultures were kept at 37^{0}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 harvester The dried filters were transferred into toluene based scintillation cocktail and the radioactivity was measured using Beckman Scintillation counter The results obtained are expressed as cpm/10^6cells

10. Measurement of APase activity :

Principle: p-nitrophenol (p-NP) is released from p-nitrophenyl 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

Reagents:

1. 0.1M bicarbonate buffer containing 2mM $MgCl_2$, pH 9.8
2. 1N NaOH
3. 0 9%NaCl
4. 2.5 mM p-nitrophenyl phosphate in bicarbonate buffer
5. p-nitrophenol

Procedure:

The assay was performed according to the method of Hashimoto and Zubler (1986) with modifications Lymphocyte cultures in 96 well microtitre plates were centrifuged at 2000 rpm for 7min at 4°C. The pellet was washed twice with saline and to the pellet 180 ul of 2.5mM p-NPP in 0.1M bicarbonate buffer was added. Incubation was carried out at 37^{0}C in a humidified incubator. After 30min, the reaction was stopped by the addition of 20ul of 1N NaOH and the absorbance was measured at 405nm against a substrate blank in UV max ELISA reader (Molecular Devices, USA),

The amount of p-NP released was calculated from the standard graph. The results are expressed as nmoles of p-NP released /10^6 cells

11. Cytochemical Staining of Lymphocytes **for** APase activity:

Lymphocytes positive for APase activity were stained according to the method of Tokuda et **al** (1994).

Reagents:

1. 0.1M bicarbonate buffered saline containing 2mM $MgCl_2$ pH 9.8
2. 2.5 mM β -naphthyl phosphate in bicarbonate buffer
3. Fast blue RR ,1 mg/ ml in bicarbonate buffer
4. 0.9%NaCl

Procedure:

Lymphocyte cultures were centrifuged at 400xg and the pellet was washed with saline. To the pellet, β-naphthyl phosphate with fast blue RR in 0.1 M bicarbonate buffered saline was added and incubated for 5 min at 37^0C in a humidified incubator. The cells were washed thrice with saline to remove the excess substrate and the number of cells stained positive for APase were counted using an inverted microscope. At least 300 cells were counted in each sample The number of cells positive for APase are expressed as percentage of the total.

12. Purification of APase from **YAC-1** cells **and** mouse liver:

Purification of APase was carried out according to the procedure of Ikehara et al(1978) with modifications.

Principle: Alkaline phosphatase binds to anion exchangers like DEAE cellulose and the bound enzyme can be eluted using a salt gradient APase was solubilized using

triton X-100. The enzyme eluted from DEAE column was further purified by Sephacryl S-200 column chromatography.

Reagents:

1. lOmM Tris-HCl buffer containing 2mM $MgCl_2$ pH 7.4

2. lOmM Tris- HC1 buffer containing 2mM $MgCl_2$ and 0.5% triton X -100(TX-100), pH 7.4 (TMT buffer)

3. lOmM Tris -HC1 buffer containing 2mM $MgCl_2$ and 0.1% TX-100, pH 8.0

4 NaCl, 1 M

Purification from YAC-1 cells:

Confluent YAC-1 cell suspension was harvested by centrifugation at 500xg for 7min. The cells were washed twice with saline and then suspended in lOmM Tris buffer containing 2mM $MgCl_2$ pH 7.4 The cells were sonicated at half -maximal amplitude for 15sec for 5 cycles with 30sec interval. To the sonicate, TX-100 was added to a final concentration of 0.5 % and centrifuged at 15000xg for 20min.The supernatant was collected and loaded on to DEAE column pre equilibrated with TMT buffer pH 7.4. The bound enzyme was eluted with TMT buffer containing 75mM , 100mM ,150mM, 200mM, 500mM NaCl The absorbance was monitored at 280nm. Enzyme activity in the fractions was estimated as described in materials and methods Fractions with high absorbance were pooled, lyophilized and dialyzed against TMT buffer pH 8.0.This was loaded on to a Sephacryl S-200 column and the column was eluted with TMT buffer. Absorbance of the fractions collected was measured as described above and the fractions with high O.D and enzyme activity were analyzed on sodium dodecylsulfate

polyacrylamide gel electrophoresis (SDS-PAGE) APase was also purified from mouse liver using the same protocol

13. Native PAGE of APase for activity staining :

Reagents:

Stacking gel buffer: 1M Tris -HC1 buffer stock, pH 6 8

Separating gel buffer: 1 5M Tris -HC1 buffer stock, pH 8.8.

Acrylamide - Bis acrylamide stock solution : (30 : 0.9 %), 30g of acrylamide and 0 9 g of Bis-acrylamide, dissolved in DDW and made up to 100 ml .

Electrophoresis buffer : Tris -glycine buffer pH 8 8, 0.75g of Tris base, 3.75g of glycine dissolved in 250 ml of DDW.

Sample buffer: 2x , 0.125 M Tris, 20% w/v glycerol, 0.01% bromphenol blue in DDW and pH adjusted to 7.0.

Substrate: 1mg/ml each of β-naphthyl phosphate and fast blue RR dissolved in 0.1M bicarbonate buffer containing 2 mM $MgCl_2$, pH 9.8.

Procedure:

The samples prepared in 1X sample buffer were loaded on a mini polyacrylamide gel having 3.0% stacking-7.5% separating gels and electrophoresis was carried out at a constant voltage of 60V for 2h at 4°C. One half of the gel was stained for proteins by silver staining and the other half was treated with APase substrate solution. The reaction was terminated by washing the gel with water and transferred to 7% acetic acid for storage.

14. SDS-PAGE under reducing conditions:

SDS-PAGE was performed according to the method of Laemmli (1970).

The samples were treated with 1% β -mercaptoethanol and were run on a 7 5% polyacrylamide gel to determine the sub unit mass of the enzyme Standard protein molecular weight markers-- myosin 205 kDa, β-galactosidase: 116 kDa, phosphorylase b: 97.4 kDa , bovine serum albumin: 66 kDa and egg albumin: 45 kDa were also run on the gel

15. Preparation of antiserum to APase :

Antiserum to liver APase was prepared in rabbits. Partially purified liver APase at a concentration of 1mg/ml was thoroughly mixed with Freund's complete adjuvant and 1.0 ml emulsion was injected intramuscularly. Two booster doses were given with lmg/ml of APase protein in Freund's incomplete adjuvant with an interval of one month between the immunizations. Blood samples were collected from the rabbits by ear vein puncture on the day 20 after the last booster dose. Serum was separated and stored in aliquots at -20^0C until use

16. Western blot analysis:

Reagents:

Electro blotting buffer: 50mM Tris, 40mM glycine in 20% methanol

Blocking buffer : 5% Lactalbumin peptide solution

Tris buffered saline(TBS): 50mM Tris buffer pH 7.4 containing 0.15M NaCl

TBS containing 0.5% tween-20,(TBS-T)

Substrate solution: 0.06% 4-chloro-1-naphthol and 0.1% H_2O_2 in TBS

Goat anti-rabbit IgG-horse radish peroxidase(HRPO) conjugate, (Sigma Chem Co, USA)

Procedure:

SDS-PAGE was carried out using partially purified APase was from mouse liver, activated B lymphocytes and YAC-1 cells The proteins separated on a 7.5% polyacrylamide gel were transferred on to a nitrocellulose membrane filter using a wet transfer apparatus at a constant voltage of 20V for 16h. The filter was washed with TBS and incubated with blocking buffer for 2h at room temperature with constant shaking. Then the membrane was washed thrice with TBS -T and then incubated with 1:4 dilution of rabbit anti-APase antiserum for 3h at room temperature Then the membrane was washed thrice with TBS-T and incubated with 1:2000 dilution of goat anti- rabbit IgG peroxidase conjugate for 1h The membrane was washed 5times with TBS-T and incubated with substrate for 20min in dark. The reaction was stopped by rinsing the membrane thrice with DDW.

17. Protein estimation:

A. Protein estimation was done according to the method of Lowry et al (1976) 1mg/ml of bovine serum albumin was used as the protein standard

B. Protein estimation of samples containing TX-100 was carried out by Bicinchoninic acid method (Smith et al 1985).

Reagents:

1. Reagent A(RA): 8% sodium carbonate, 1.6% sodium hydroxide, 1.6% disodium tartrate and sufficient sodium carbonate was added to adjust the pH to 11.2
2. Reagent B(RB): 4% bicinchoninic acid, disodium salt in deionized water
3. Reagent C(RC): 4 parts of 4% cupric sulphate solution and 100 parts of reagent B
4. working reagent (WR): 1 part of RC and 1 part of RA

Procedure:

The sample or standard was mixed with an equal volume of WR and incubated at 60°C for 1h. The samples were cooled to room temperature and the absorbance was measured at 562 nm. The protein concentration of unknown sample was determined from the standard curve.

CHAPTER 3

ALKALINE PHOSPHATASE ACTIVITY OF *IN VITRO* MITOGEN- STIMULATED AND *IN VIVO* ANTIGEN-PRIMED MUREVE SPLENIC LYMPHOCYTES

The B lymphocyte is the principal cellular mediator of specific humoral immune response to infection Activation of B cells occurs on selection of the appropriate clones by antigen, leading to B cell proliferation and differentiation to antigen specific antibody secreting plasma cells Understanding the molecular mechanism underlying the lymphocyte activation has been pursuit of several investigators (DeFranco 1993, Cambier et al 1994). Prior to the onset of immune response, lymphocytes are quiescent non-dividing cells circulating in the blood and reside in the secondary lymphoid organs Upon encounter with an antigen B lymphocytes are activated to proliferate, expanding the number of such cells and differentiate into plasma cells, secreting large amounts of antibody(DeFranco 1987) Owing to the low magnitude of antigenic stimulation which is difficult to monitor, mitogens, a group of lymphocyte stimulators with broad specificity have been used to study the mechanism of lymphocyte activation *in vitro.* Mitogen induced lymphocyte activation has been suggested to mimic the antigen stimulated lymphocyte activation *in vivo* (Sell and Gell 1965)

Several enzyme systems have been shown to be expressed during the B lymphocyte activation Several protein tyrosine kinases and phosphatases that are essential for B cell activation have been shown to be expressed during B lymphocyte activation(Sefton and Campbell 1991). Phosphate turnover by the active participation of kinases and phosphatases is a crucial event that controls the activation of lymphocytes(Perlmutter et al 1993).

APase is a membrane bound glycoprotein which has been shown to be expressed in B lymphocytes upon activation has been used as marker for B cell activation (Garcia-Rozas et al 1982, Ohno et al 1986, Burg and Felbush 1989, Marquez et al 1989).

In the present set of experiments, the effect of various T and B cell mitogens on lymphocyte proliferative response and expression of APase activity was studied Effect of *in vivo* antigen priming on the enhancement of APase activity of primed splenic lymphocytes was also determined

The objectives are :

1 To study the effect of T cell mitogens ConA, PHA and B cell mitogens LPS, DXS, seph.anti-IgM on proliferative response and APase activity of murine splenic lymphocytes.

2. To study the effect of *in vivo* antigen priming on proliferative response, APase activity and enumeration of the number of APase positive cells by enzyme cytochemical analysis

Methods:

In vivo immunization:

Mice were immunized intraperitonially with 20μg of Tetanus toxoid(TT) in incomplete Freund's adjuvant (IFA) and booster dose was given on day 15 with 10μg of TT in IFA. Immunized mice were sacrificed two weeks later and splenic lymphocytes were prepared as described in materials and methods Control mice were administered with IFA intraperitonially using the immunization protocol mentioned above.

Preparation of lymphocytes:

Lymphocytes from spleen of mice were prepared as described in materials and methods. Highly enriched B cell populations were isolated by Panning technique using petriplates coated with anti-Ig antibodies(Goat anti mouse IgM or IgG) at 4^0C The percentage of Ig^+ cells was always more than 90% in the adherent population

Proliferative response

Splenic lymphocytes from immunized and control mice were cultured with various concentrations of TT Cultures were pulsed with 0.5μCi of ^{3}H-thymidine for the last 24h of 48h culture period and processed as described in materials and methods.

APase activity assay:

APase assay was performed as described in materials and methods

Enzyme cytochemical staining:

Splenic lymphocytes were analyzed for the presence of APase activity as described in materials and methods. Adherent and non-adherent populations prepared from TT stimulated cells cultured for 72h were used for enzyme cytochemical staining along with LPS stimulated cells

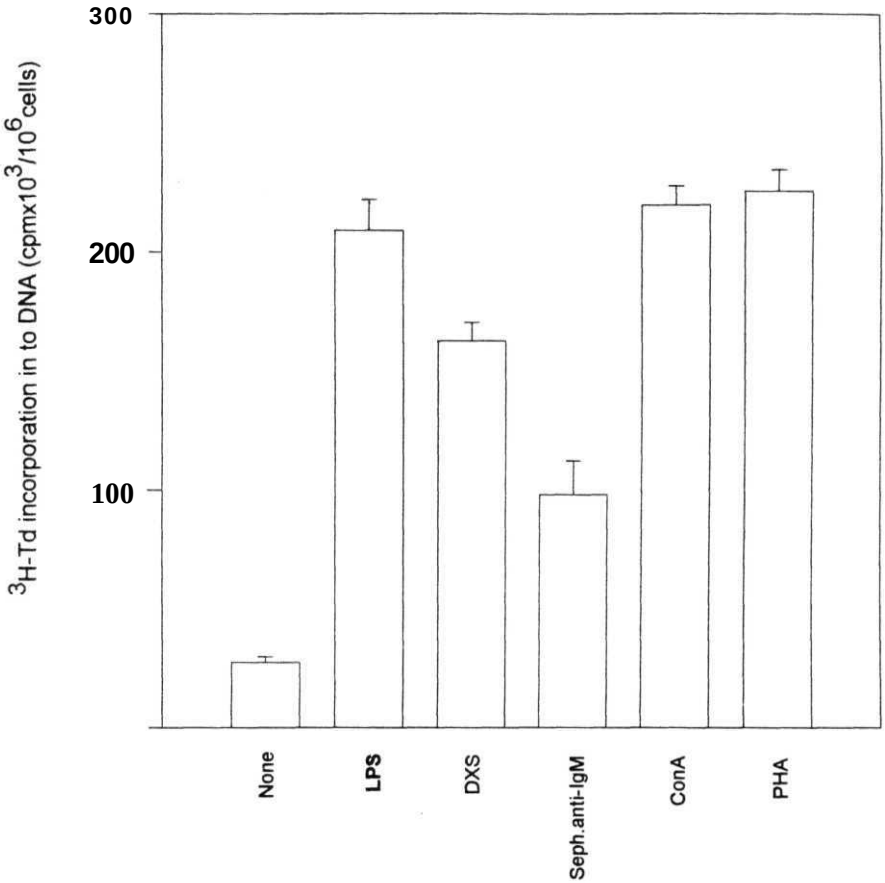
Results:

Effect of various T and B cell mitogens on the proliferative response and APase activity of murine splenic lymphocytes:

Murine splenic lymphocytes were stimulated with two T cell specific mitogens — ConA (2μg/ml), PHA (25μg/ml) and three B cell specific mitogens --LPS(50μg/ml), DXS(50μg/ml) and Seph anti-IgM(5μg/ml) Splenic lymphocytes showed an increase in the proliferative response when stimulated with both T and B cell mitogens compared to controls (Fig 1). However, APase activity enhancement was observed only in the splenic lymphocytes stimulated with B cell mitogens. Splenic lymphocytes stimulated with T cell mitogens did not show any APase activity (Fig 2).

Figure -1

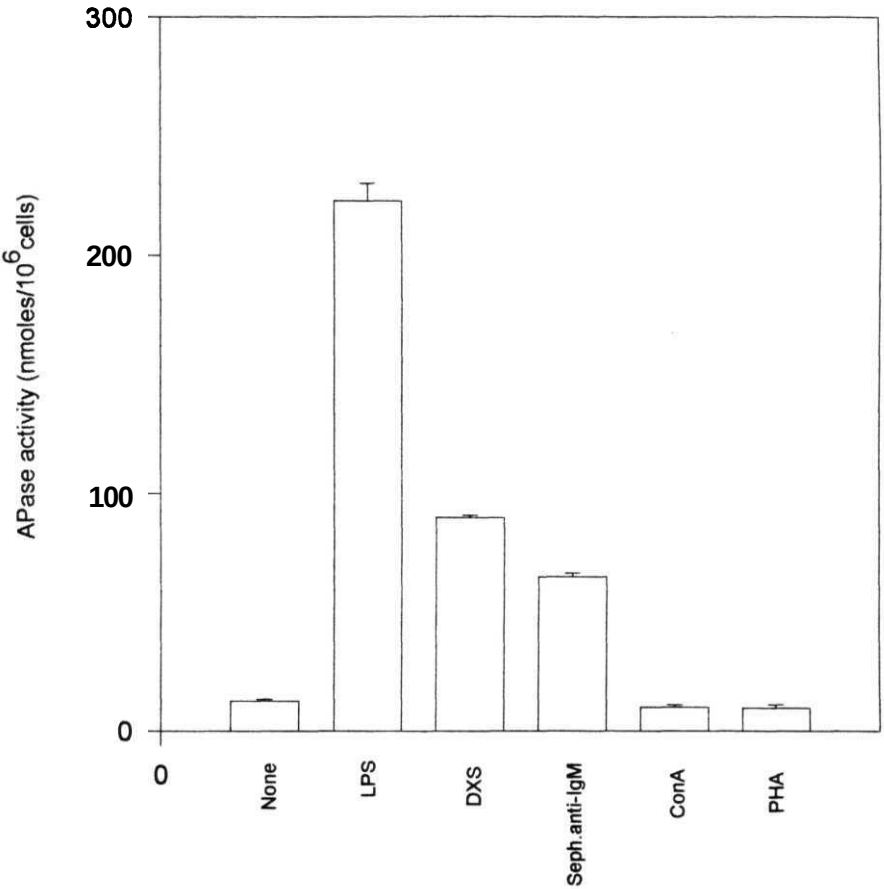
Effect of T and B cell mitogens on proliferative response of murine splenic lymphocytes



Values represented are Mean ±SEM of five experiments.

Figure -2

Effect of T and B cell mitogens on APase activity of murine splenic lymphocytes



Values represented are Mean ±.SEM of five experiments.

Effect of T cell mitogens on the proliferative response and APase activity of murine thymocytes:

Murine thymocytes were cultured with optimal concentrations of two T cell specific mitogens ConA and PHA Thymocytes showed an increased proiiferative response when stimulated with both the mitogens. However, no induction of APase activity was observed in these cells (Table 1).

Enzyme cytochemical staining of lymphocytes:

Enzyme cytochemical analysis of LPS stimulated splenic lymphocytes showed a significant enhancement in the number of APase positive cells compared to controls(Fig 3 a, & b). Whereas, in ConA stimulated splenic lymphocytes and thymocytes the number of APase positive cells detected were insignificant and were similar to that in controls (Fig3c, Fig 4a & b)

Proiiferative response and APase activity of *in vivo* TT primed murine splenic lymphocytes:

Splenic lymphocytes from *in vivo* TT primed mice when cultured *in vitro* showed an increase in the proiiferative response (Fig 5, $p<0$ 001) concomitant with a significant increase in the APase activity (Fig 6, $p<0$ 05). The increase observed was dose dependent and TT specific as there was no increase in the proiiferative response and APase activity of lymphocytes from IFA treated mice.

Enzyme cytochemical analysis of TT primed-lymphocytes:

Enzyme cytochemical staining for APase activity in intact cells stimulated with TT showed a significant increase in the number of APase positive cells compared with controls (Table 2).

Table -1

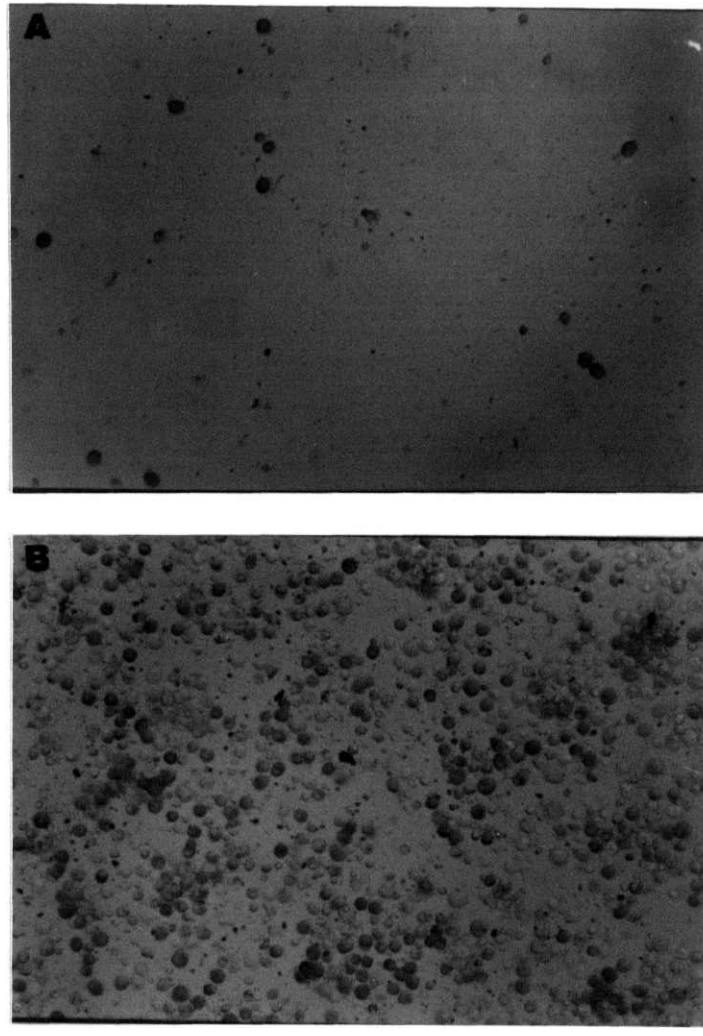
Effect of various mitogens on proliferative response and APase activity of mouse thymocytes.

Addition (μg/ml)	^{3}H-Thymidine incorporation in to DNA	Δ	APase activity	Δ
	(cpm/10^6cells)		(nmoles/10^6cells)	
None	**10,170** ± 1505	—	10 ±0.5	--
ConA				
2.0	2,50,150 ± 1795	**2,39,980**	15 ±1.0	**5**
5.0	2,17,405 ± 2395	2,07,235	14 ±0.5	**4**
PHA				
25.0	2,05,145 ± 1290	1,94,975	12 ±0.5	**2**

Values represented are Mean ± **SEM** of three experiments.

Figure -3

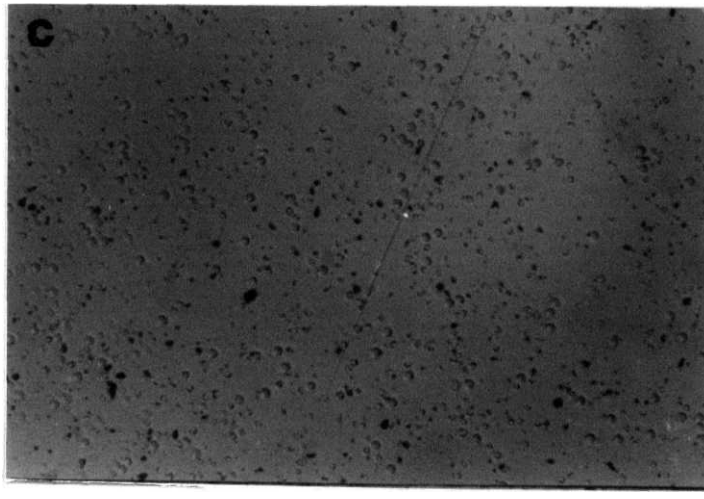
Cytochemical staining of murine splenic lymphocytes for APase activity.



a) control b) LPS stimulated cells, Magnification : 120x.

Figure -3

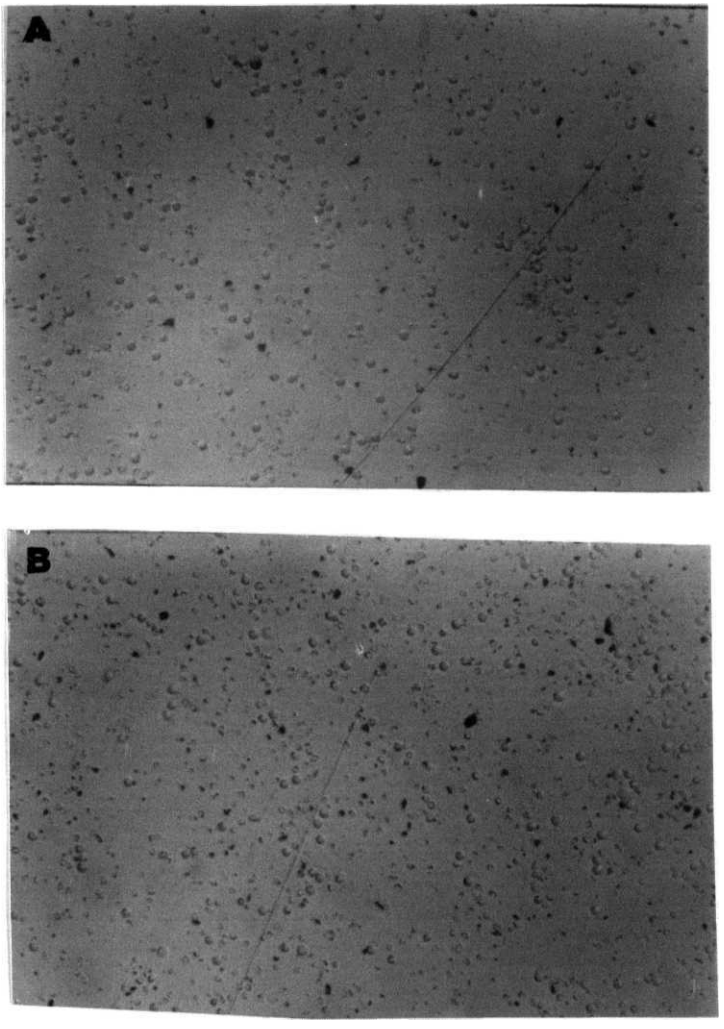
Cytochemical staining of murine splenic lymphocytes for APase activity



c) ConA stimulated cells, Magnification : 120x

Figure -4

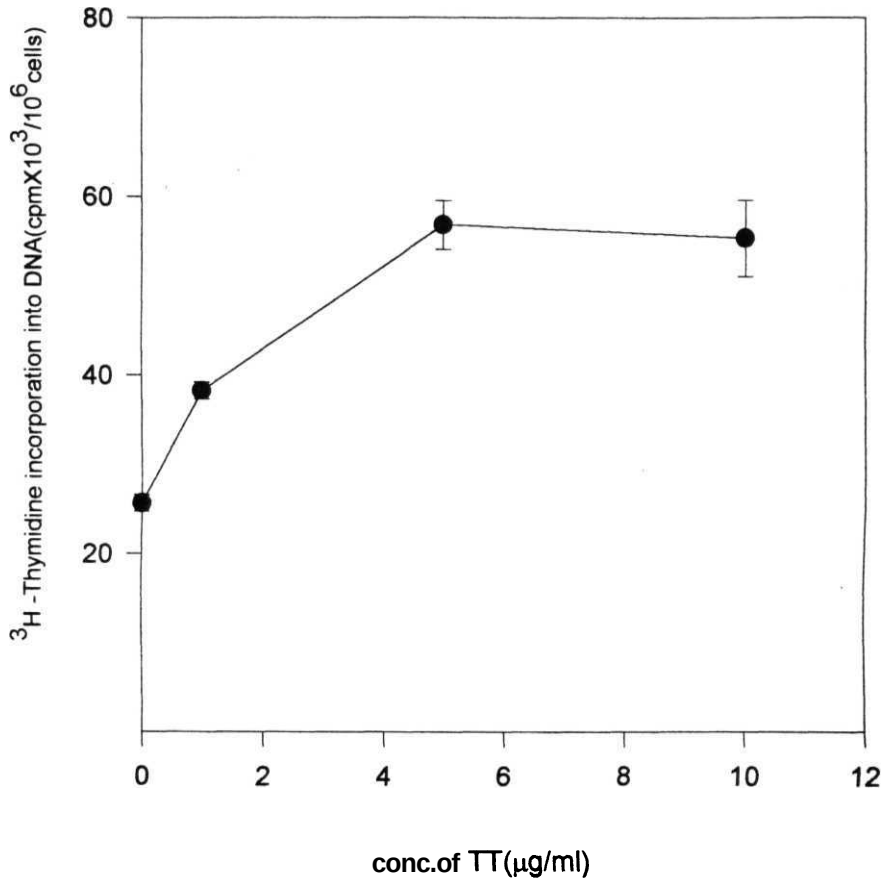
Cytochemical staining of murine thymocytes for APase activity



a) Control b) ConA stimulated, Magnification 120x

Figure -5

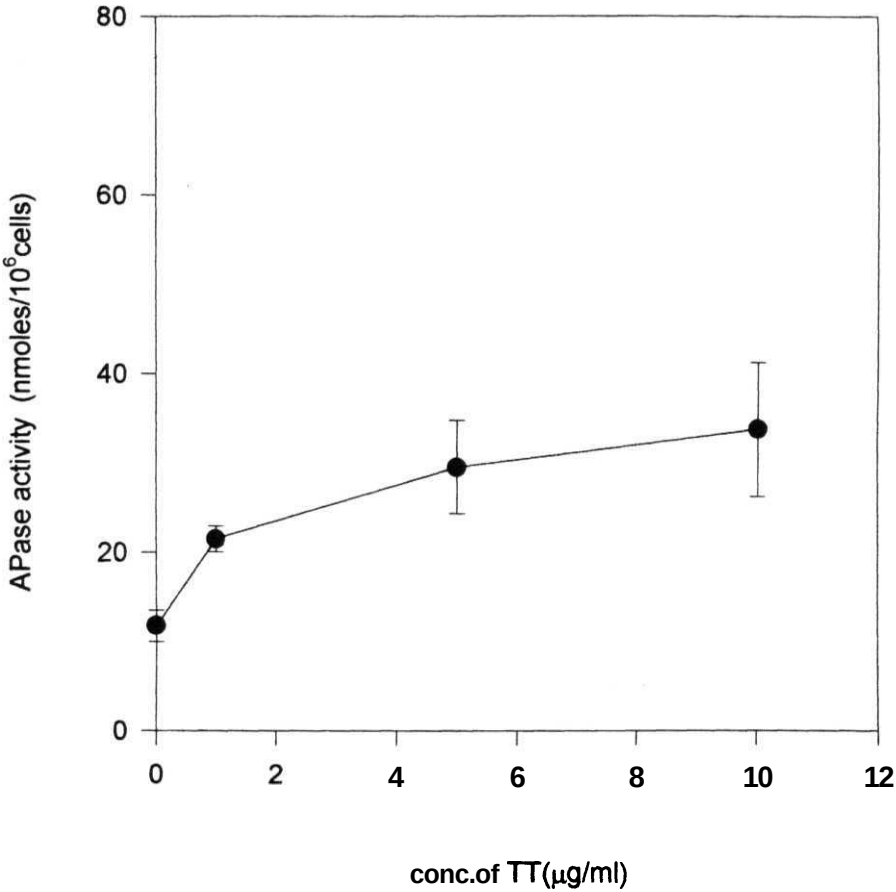
Proliferative response of TT primed splenic lymphocytes



Values represented are Mean ± SEM of five experiments. p< 0.001 at all concentrations of TT vs no addition.

Figure -6

APase activity of TT primed splenic lymphocytes



Values represented are Mean ± SEM of five experiments. p< 0.001 at all concentrations of TT vs no addition

Table -2

Enzyme cytochemical analysis of TT - stimulated lymphocytes

Cell Population	Addition	Conc. (μg/ml)	APase positive cells (%)	Δ
Splenocytes	None		7.0 ± 1.0	
Splenocytes	LPS	50.0	41.0 ± 1.0*	34
Splenocytes	TT	1.0	12.0 ± 1.0*	5
		5.0	17.0 ± 1.0*	10
Adherent Ψ	None	---	4.0 ± 1.0	
	TT	5.0	35.0 ±2.0*	31
Non-adherent Ψ	None	-	3.011.0	
	TT	5.0	5.0 ± 1.0	2

Values presented are Mean 1 SEM of four experiments. ΨAdherent and non- adherent fractions were prepared from splenic lymphocytes stimulated with TT for 72h. *P< 0.001 LPS and TT vs no addition. Atleast 300 cells were counted for each sample.

Discussion:

B cell specific mitogens, LPS, DXS and Seph.anti-IgM induced proliferation concomitant with an enhancement in APase activity in murine splenic lymphocytes On the other hand, T cell specific mitogens ConA and PHA did not induce any APase activity although a significant proliferative response was observed compared with controls. Similarly, ConA and PHA stimulated thymocytes showed a significant enhancement in the proliferative response with no enhancement in APase activity. Earlier it has been shown that APase activity is expressed only by activated B lymphocytes. Neither resting nor activated T lymphocytes express APase activity. Thus, APase activity has been suggested as to be a marker of B cell activation (Garcia-Rozas et al 1982). It has been shown that lymphocyte proliferation is associated with an enhancement of APase activity (Moschbach et al 1986, Kasyapa and Ramanadham 1992). Further, it has been demonstrated that APase activity is induced only in B cells upon mitogenic stimulation and correlates with proliferation (Ohno et al 1986) and differentiation (Burg and Feldbush 1989, Marquez et al 1989).

In vivo antigen priming of mice with TT and culturing of these cells with TT showed an enhancement in proliferative response concomitant with significant increase in APase activity. Like in mitogen stimulated cells, in *in vivo* sensitized splenic lymphocytes paralleling the increase in APase activity, it has been observed that there is an increase in APase positive cells upon enzyme cytochemical staining These results suggest that the increase in the APase activity is due to the increase in the number of APase positive cells upon antigenic/mitogenic stimulation but not just an increase of catalytic activity. Further, the increase in APase positive cells and APase activity was seen in B cell enriched fraction

(sIg positive cells fraction, adherent fraction) but not in T cell enriched fraction (sIg negative cells, non-adherent fraction) These results strongly suggest that APase activity occurs only in antigen activated B cells but not in T cells. Earlier Tokuda et al (1994) have demonstrated that the appearance of APase positive areas in lymph nodes and spleens of immunized animals by enzyme histochemical analysis.

Based on all the above results it is strongly suggested that enhancement of APase activity is a physiological phenomenon occurring in B lymphocytes upon activation with an antigen or mitogen.

CHAPTER 4

EFFECT OF IL-2 AND IL-4 ON PROLIFERATIVE RESPONSE AND APase ACTIVITY OF MITOGEN STIMULATED B LYMPHOCYTES

Upon stimulation with an antigen, B lymphocytes differentiate into immunoglobulin (Ig) secreting plasma cells B lymphocyte activation has been proposed to be mediated by two activation signals - first one from the cross-linking of sIg receptors and second one in the form of a soluble helper factor derived from the activated T cells Interleukins are the group of cytokines that regulate the activation, differentiation and maturation of B and T cells and activation and migration of inflammatory cells IL-2 and IL-4 are T cell derived interleukins that exert their effects on both T and B cells Earlier it has been shown that addition of supernatants of activated T cells enhances the proliferation caused by anti-IgM alone and induces high levels of IgM antibody production in rabbits (Kishimoto et al 1975) and mice (Parker et al 1979) Raincho et al (1993) showed that IL-4 stimulates APase activity and inhibits proliferative response in MG63 human osteosarcoma cells It has been reported that IL-4 stimulates the osteoblast proliferation and induces APase mRNA expression (Nohtomi et al 1994) IL-4 increases the Ia antigen levels on resting B cells and acts as a co-stimulant in the synthesis of DNA in presence of low concentrations of $(Fab)'_2$ or intact anti-Ig molecules (O'Garra et al 1987) IL-4 also acts as a differentiation factor of B cells O'Garra et al (1986) have shown that IL-5 acts as a growth and differentiation factor for pre activated murine B lymphocytes cAMP has been demonstrated to be a synergistic activator of APase activity in LPS stimulated B cells (Kasyapa and Ramanadham 1995). However, the molecular signal responsible for the enhancement of APase has not been elucidated in detail. In the present set of experiments, the role of IL-2 and IL-4 as regulators of APase activity in mitogen stimulated cells has been explored.

The objectives are :

1. The effect of IL-2 and IL-4 on proliferative response and APase activity of LPS , DXS and Seph anti-IgM stimulated splenic lymphocytes,

2 The effect of delayed addition of IL-4 on proliferative response and APase activity of LPS and Seph.anti-IgM stimulated cells, and

3 Effect of IL-2 and IL-4 on proliferative response and APase activity of PMA stimulated lymphocytes.

Methods:

Proliferative response and APase activity were determined as described in materials and methods.

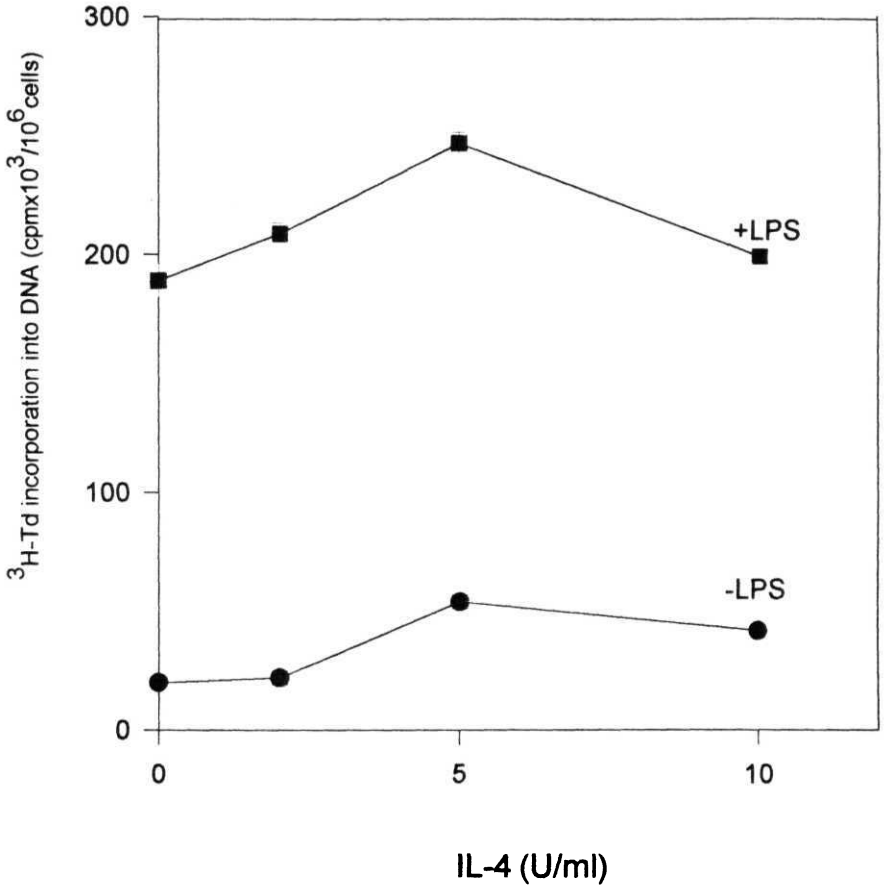
Results:

Effect of varying concentrations of IL-2 and IL-4 on the proliferative response and APase activity of LPS stimulated cells:

Murine splenic lymphocytes were stimulated with B cell mitogens LPS ,DXS, Seph anti-IgM The proliferative response was measured during 24-48h of culture peroid was measured and APase activity was measured at 48h LPS was used at a concentration of 10 μg/ml. There was a significant enhancement in proliferative response concomitant with an enhancement in APase activity in LPS stimulated cells compared to controls To the LPS stimulated cells, IL-4 was added at 2,5, and 10 U/ml of culture The enhancement in proliferative response and APase activity was maximal at 5 U/ml (Fig 7&8) In all further experiments, IL-4 was used at 5 U/ml concentration Similarly EL-2 was used at 2,10, and 20 U/ml of culture(Fig 9&10). At all concentrations used, IL-2 failed to show any effect on proliferative response and APase activity of mitogen stimulated cells.

Figure - 7

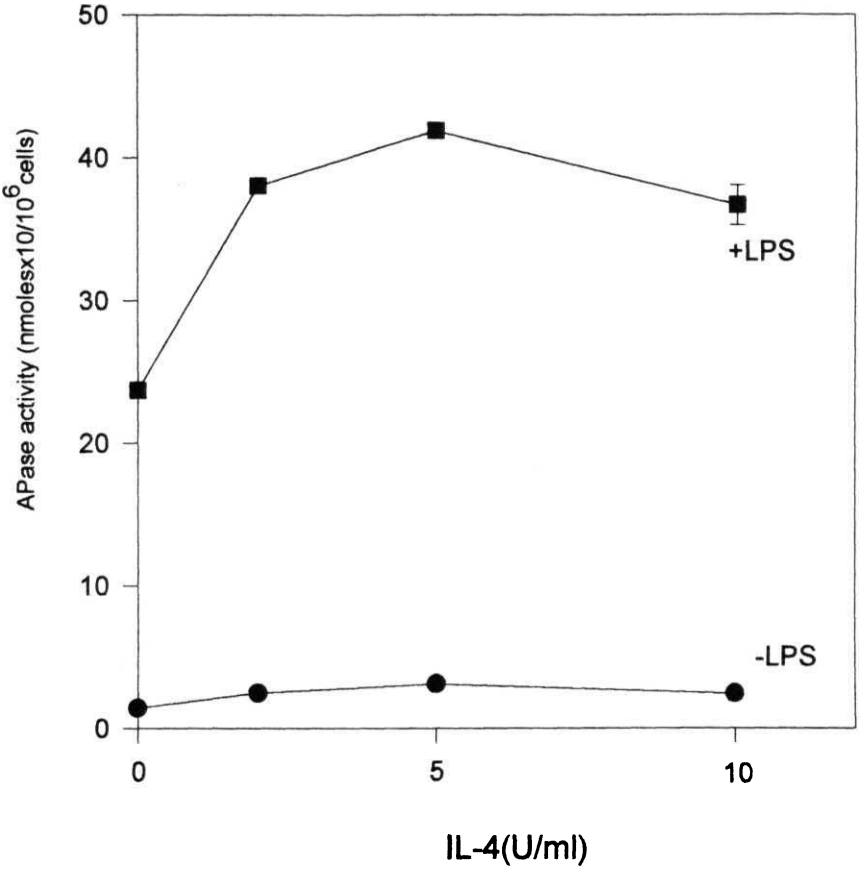
Effect of IL-4 on proliferative response of LPS stimulated splenic lymphocytes



Values represented are Mean ± SEM of four experiments.

Figure - 8

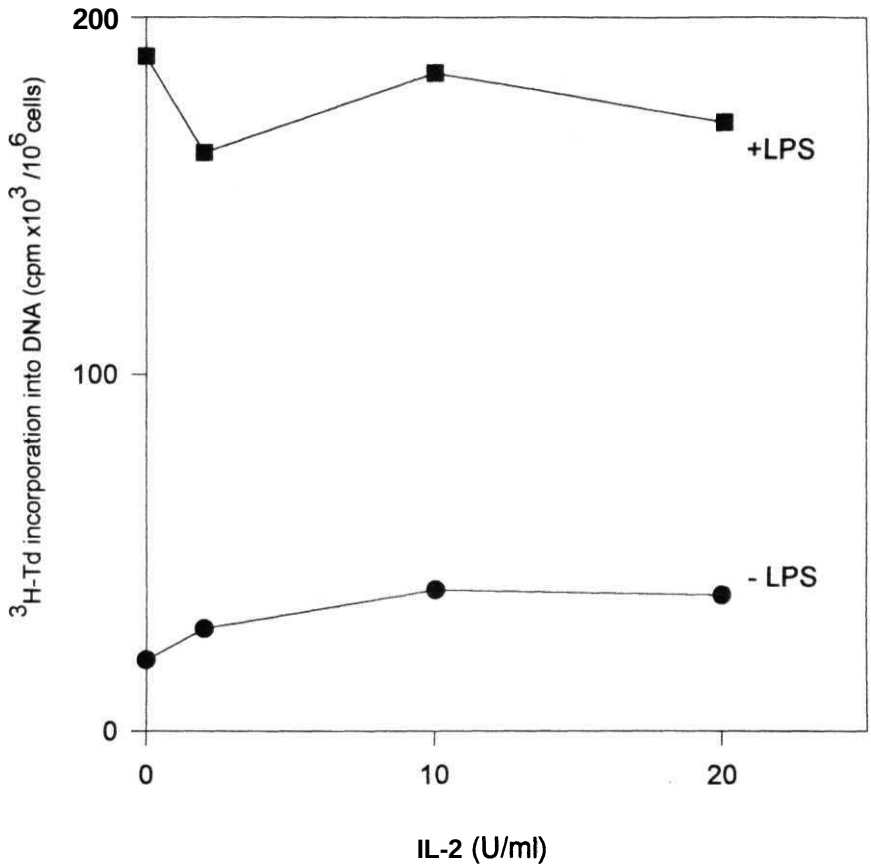
Effect of IL-4 on APase activity of LPS stimulated splenic lymphocytes



Values represented are Mean ± SEM of four experiments.

Figure - 9

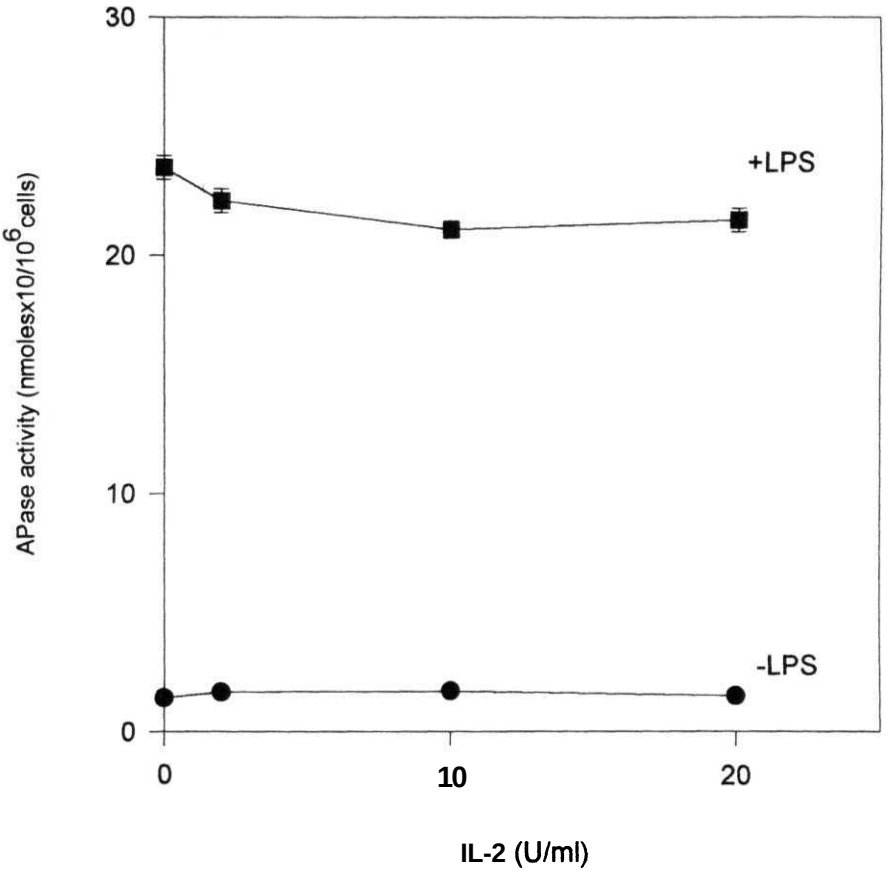
Effect of IL-2 on proliferative response of LPS stimulated splenic lymphocytes



Values represented are Mean ± SEM of four experiments.

Figure - 10

Effect of IL-2 on APase activity of LPS stimulated splenic lymphocytes



Values represented are Mean ± SEM of four experiments.

Effect of IL-4 and IL-2 on proliferative response and APase activity of the LPS stimulated murine splenic lymphocytes:

At optimal concentration of 5 U/ml, IL-4 significantly enhanced the proliferative response and APase activity of LPS stimulated cells (Fig 11& 12 , $p < 0.005$). On the other hand, IL-2 did not have any effect on the proliferative response and APase activity.

Effect of IL-2 and IL-4 on Seph anti-IgM stimulated cells:

Seph. anti-IgM acts as a mitogen to B lymphocytes by cross-linking surface immunoglobulin (sIg) receptors. Inorder to see the effect of IL-4 on Seph. anti-IgM stimulated cells, IL-4 was added at the time of intiation of culture along with Seph anti-IgM. Addition of IL-4 resulted in a significant enhancement in proliferative response and APase activity (Fig. 13 &14, $p < 0.05$). However, addition of IL-2 to Seph.anti-IgM treated cells had no effect either on proliferative response or APase activity.

Effect of delayed addition of IL-4 to LPS and Seph anti-IgM stimulated cells

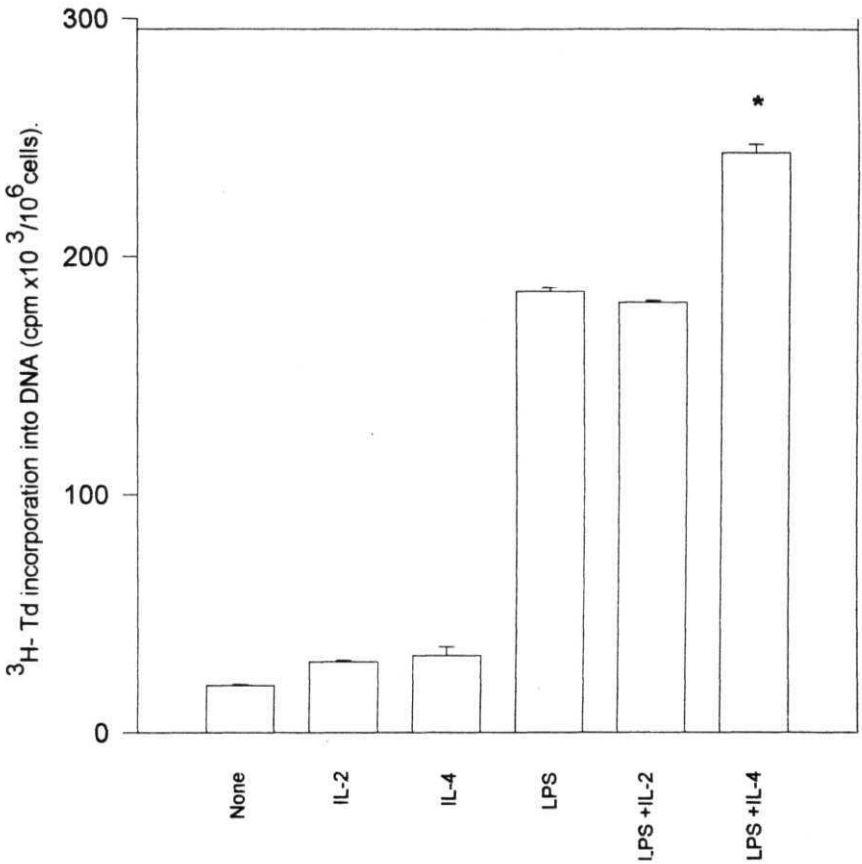
IL-4 was added at 17, 24h (during G1phase) and 48h (during 'S' phase) to mitogen stimulated cells in culture. IL-4 caused a significant increase in the proliferative response and APase activity when added at 17h and 24h. However, addition of EL-4 at 48h did not show any effect on proliferative response or APase activity (Table 3 & 4).

Effect of IL-2 and IL-4 on DXS stimulated lymphocytes

DXS, a type II T independent B cell stimulator was used at 10µg/ml concentration along with 5U/ml IL-4 and the proliferative response and APase activity were measured. IL-4 caused a significant enhancement in the proliferative response of the DXS stimulated lymphocytes (Fig 15, $p < 0.005$). However, there was no enhancement in the APase activity in DXS stimulated cells upon addition of IL-4(Fig 16).

Figure -11

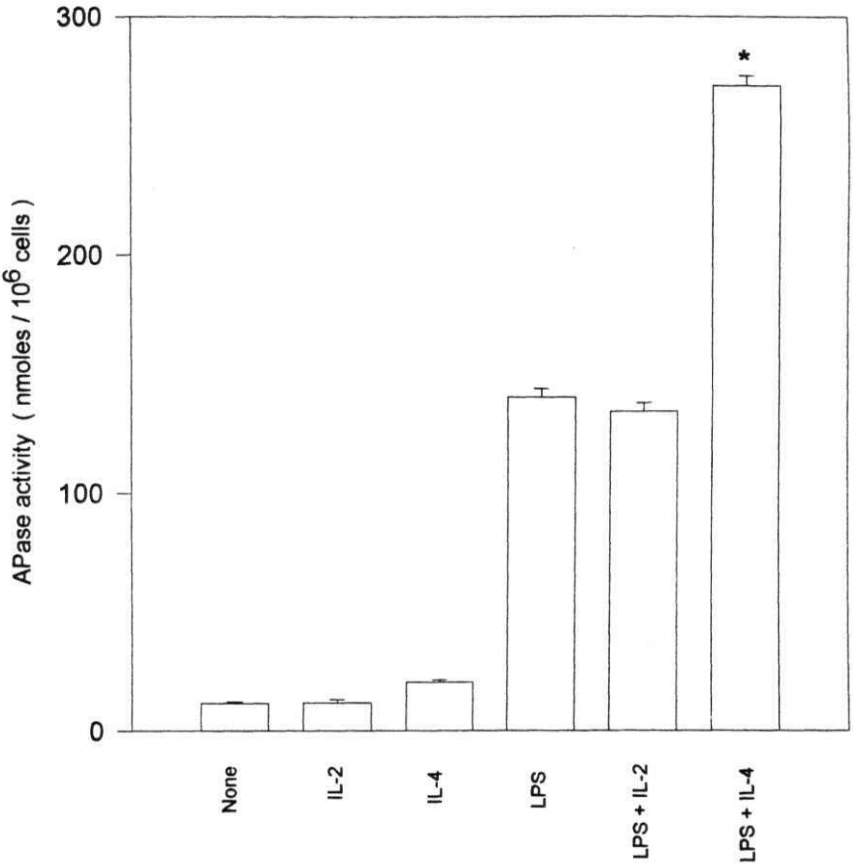
Effect of IL-2 and IL-4 on proliferative response of LPS stimulated splenic lymphocytes



Values represented are Mean ± SEM of four experiments. *p< 0.005 compared with the corresponding mitogenic response.

Figure -12

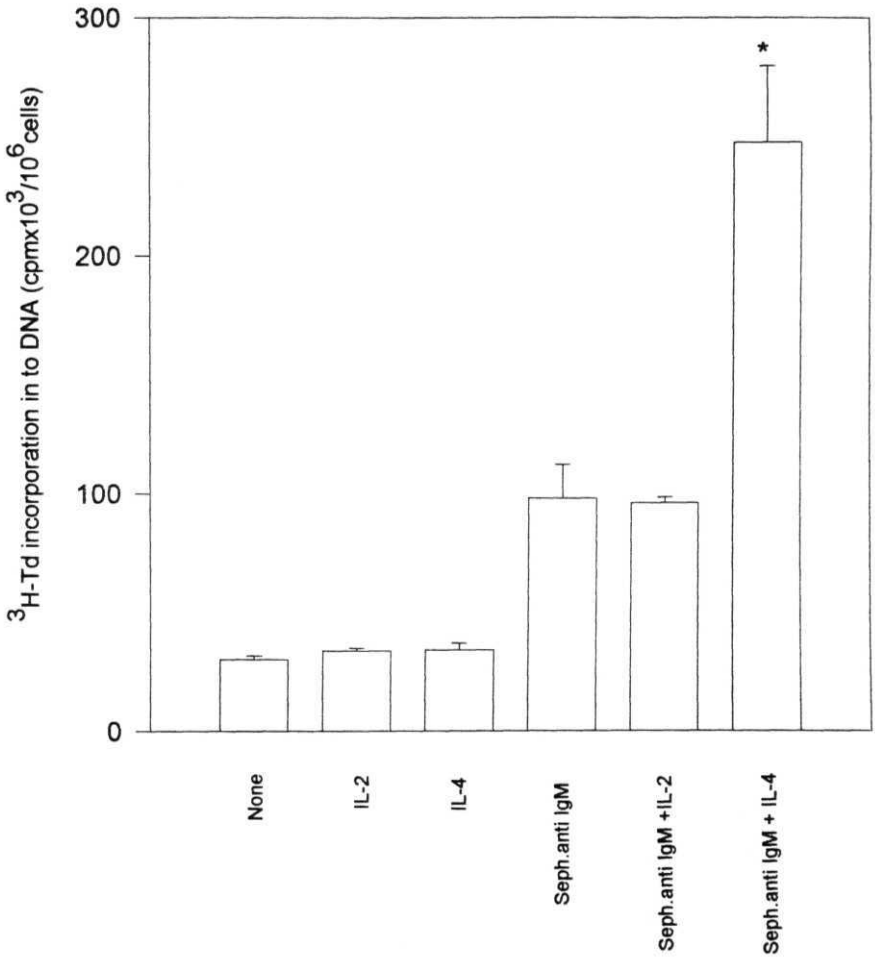
Effect of IL-2 and IL-4 on APase activity of LPS stimulated Splenic lymphocytes



Values represented are Meant SEM of four experiments. * p< 0.005 compared with respective mitogenic response.

Figure -13

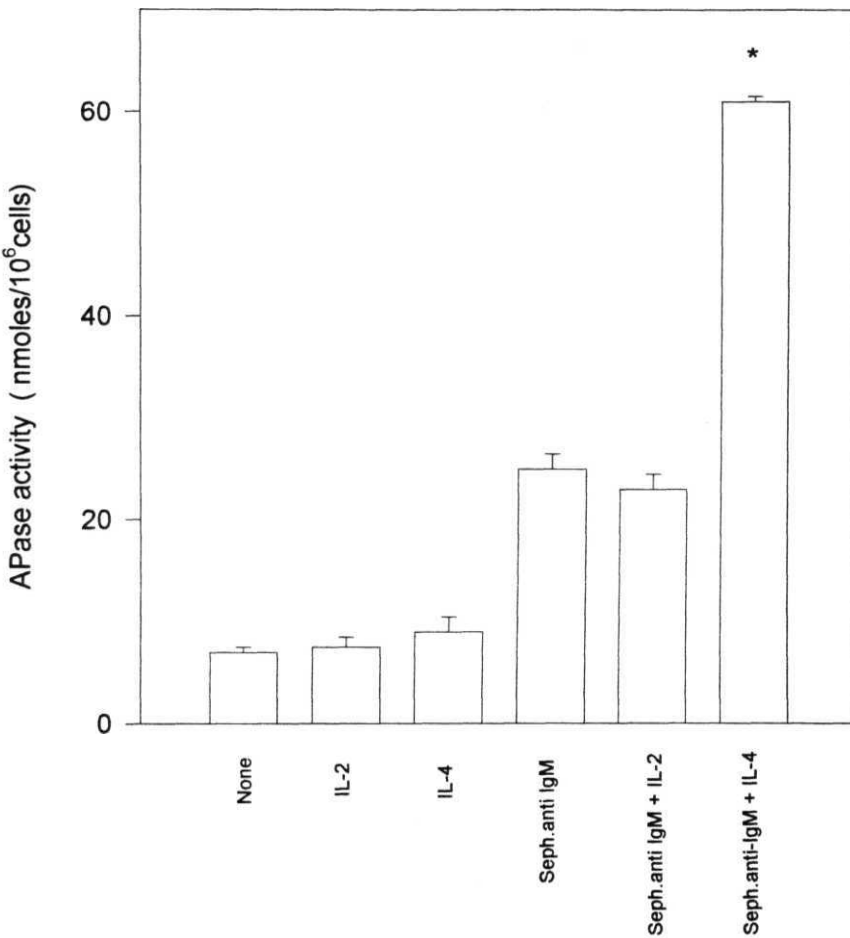
Effect of IL-2 and IL-4 on proliferative response of Seph.anti-IgM stimulated splenic lymphocytes



Values represented are Mean ± SEM of five experiments. * $p < 0.05$ when compared with corresponding mitogenic response.

Figure - 14

Effect of IL-2 and IL-4 on APase activity of Seph.anti-IgM stimulated murine splenic lymphocytes.



Values represented are Mean ±SEM of five experiments. *p< 0.05 when compared with corresponding mitogenic response.

Table -3

Effect of delayed addition of IL-4 on proliferative response and APase activity of LPS stimulated splenic lymphocytes

Addition	^{3}H-Thymidine incorporation into DNA	Δ	APase activity	Δ
	(cpm/ 10^6cells)		(nmoles/ 10^6cells)	
None	16,040 ±1600		12.5 ±0.5	
IL-4	34,065 ±5070	18,025	14.0± 1.0	1.5
LPS	2,17,730 ±10160	2,01,690	208.7 ±2.5	196.2
LPS +IL-4	2,84,620 ± 12160 *	2,68,580	348.0 ± 7.5 *	335.5
IL-4♥	30,285 ±1450	14,245	13.0+1.5	0.5
LPS + IL-4♥	2,75,870 ±8137*	2,59,830	340.5 ± 7.5 *	327.5
IL-4♦	36,220 ±1780	20,440	14.0± 1.0	1.5
LPS+ IL-4♦	2,67,285 ± 8170 *	2,51,245	307.5 ±6.5*	295
IL-4 ♣	33,220 ± 2800	17,180	11.5 ±1.0	
LPS +IL-4 *	2,22,620 ±7160	2,06,580	205.0 ±4.5	

IL-4 added at ♥ 17h ♦ 24 h and ♣ 48 h. Values represented are Mean ± SEM of four experiments. *P < 0.005 when compared with the corresponding mitogenic response.

Table -4

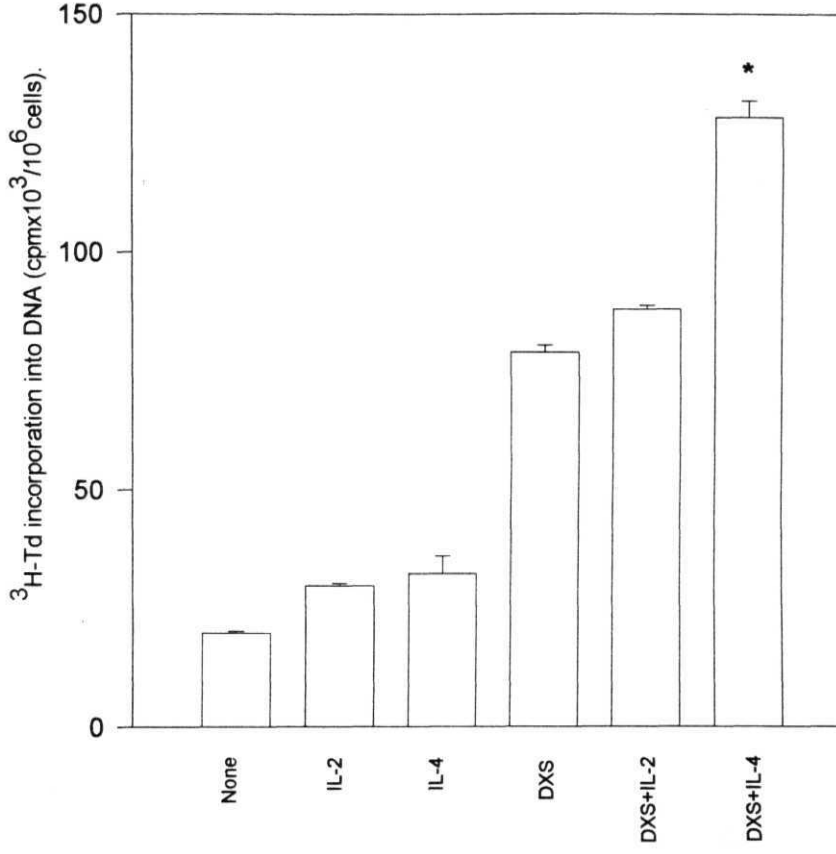
Effect of delayed addition of IL-4 on proliferative response and APase activity of Seph.anti-IgM stimulated splenic lymphocytes

Addition	^{3}H-Thymidine incorporation into DNA	Δ	APase activity	Δ
	(cpm/ 10^6cells)		(nmoles/ 10^6cells)	
None	20,870 ± 2,346		11.0 ± 0.5	—
IL-4	23,050 ± 966	2,180	12.0 ± 1.0	1.0
Seph anti-IgM	37,160 ±1366	16,290	20.0 ± 0.5	9.0
Seph.anti-IgM +IL-4	55,620 ± 2676*	34,750	62.0 ± 0.5*	31.0
IL-4 ♦	22,030 ± 1900	1,160	12 010.5	1.0
Seph.anti-IgM +IL-4♦	68,080 ± 13380*	47,210	66.0 ± 0.5•	35.0
	21, 780 ± 3670	910	11.5 ± 0.5	0.5
Seph anti-IgM + IL-4ψ	57,450 ±1240*	36,580	65.0 ± 10*	34
IL-4• ♣	19,340 ±385	...	11 01 1.0	
Seph.anti-IgM + IL-4•	34,240 ± 2340	13,370	23.0 ± 0.5	12

IL-4 added at • 17 h, ψ 24h and ♣48h. Values represented are Mean 1 SEM of four experiments. • P < 0.05 when compared with the corresponding mitogenic response.

Figure -15

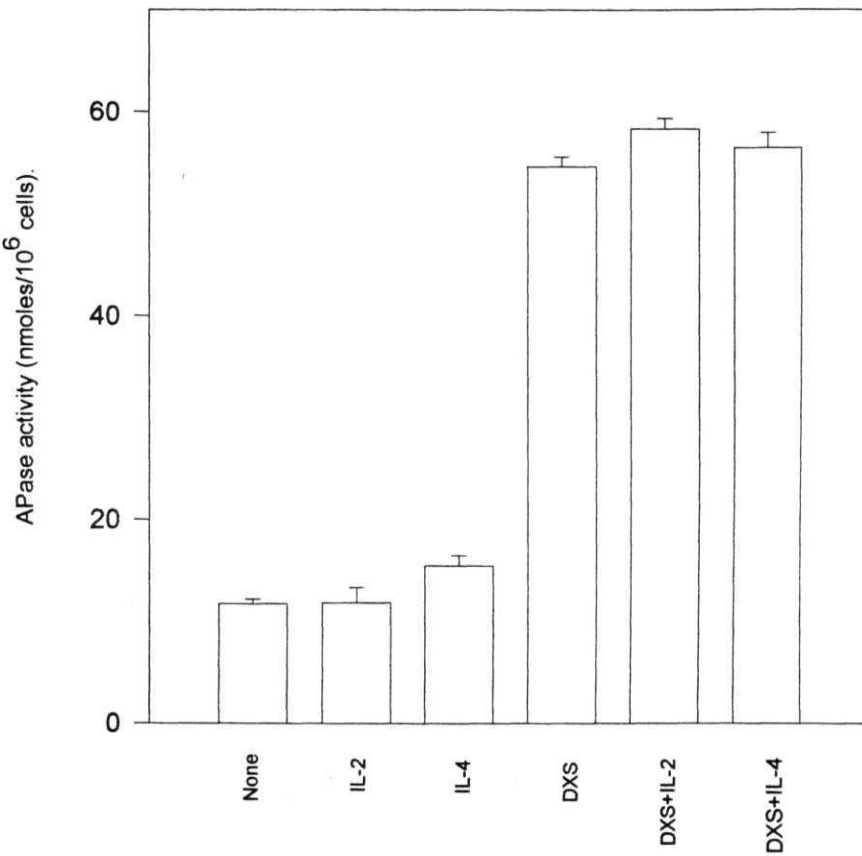
Effect of IL-2 and IL-4 on proliferative response of DXS stimulated splenic lymphocytes.



Values represented are Mean ± SEM of four experiments. * p< 0.005 compared with corresponding mitogenic responses

Figure-16

Effect of IL-2 and IL-4 on APase activity of DXS stimulated splenic lymphocytes



Values represented are Mean ± SEM of four experiments.

Effect of IL-2 and IL-4 on PMA stimulated splenic lymphocytes

PMA is an incomplete mitogen which stimulates the B cells to G1 phase of the cell cycle IL-4 when added to the PMA stimulated cells, induced a significant proliferative response as well as APase activity(Fig 17 & 18a, $p < 0.001$) Whereas, addition of IL-2 to PMA stimulated cells did not induce any proliferation in Gl phase arrested cells On the other hand, addition of IL-2 and IL-4 together to PMA stimulated cells resulted in a significant enhancement in the proliferative response as well as in APase activity (Fig 17 & 18a)

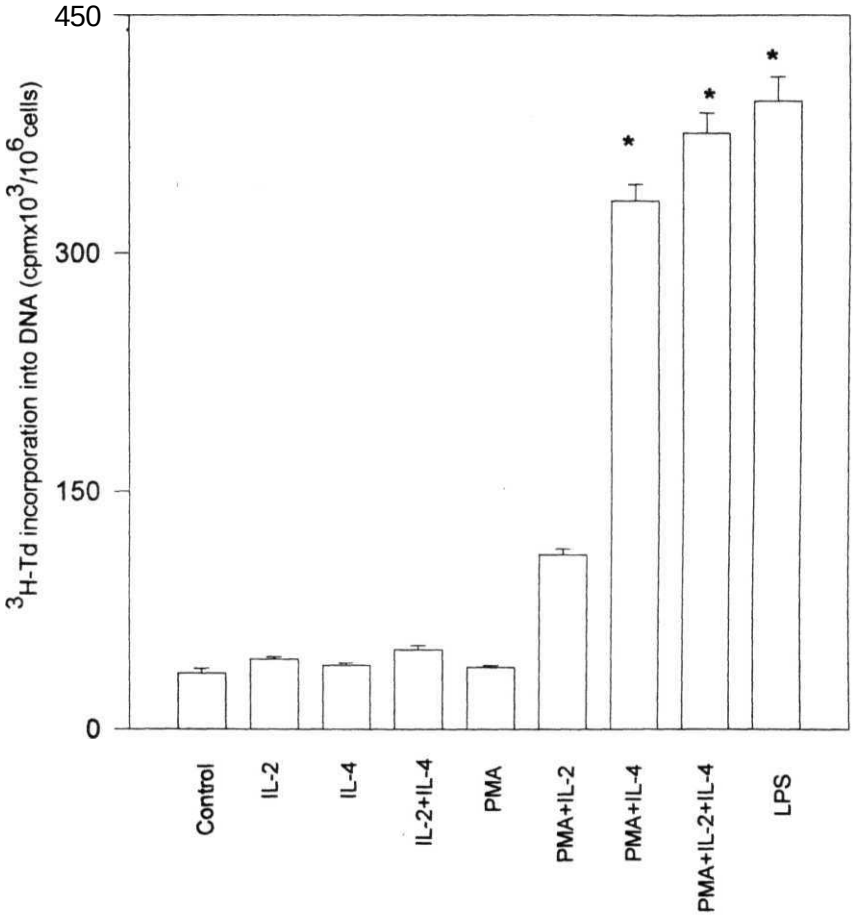
Discussion:

B lymphocyte activation has been suggested to be mediated by two activation signals. The first signal comes from the ligation of the surface immunoglobulin receptor with the antigen and the second one delivered to the activated cell during the Gl phase. The later one is as an interleukin that commits the B cell to proliferation (Bretscher 1971, DeFranco 1987).

Interleukins are a family of molecules that transmit growth and differentiation signals between various types of lymphocytes and thus are the major effectors of immune regulation It has been shown that T cell derived interleukins have no effect on the resting B cells but enhance B cell proliferation induced by low concentration of anti-Ig (Parker et al 1979). The Th2 subset of helper T cells are important regulators of B cell function. Th2 cells secrete IL-4, IL-5 and IL-6 which exert their effects on B cells. IL-4 causes enhancement in the expression of MHC class II antigens on B cell surface (Noelle et al 1984, Hu and Moller 1994). It also enhances the viability and causes increase in the cell volume with in few hours of mitogenic stimulation IL-4 acts together with anti-IgM as a

Figure -17

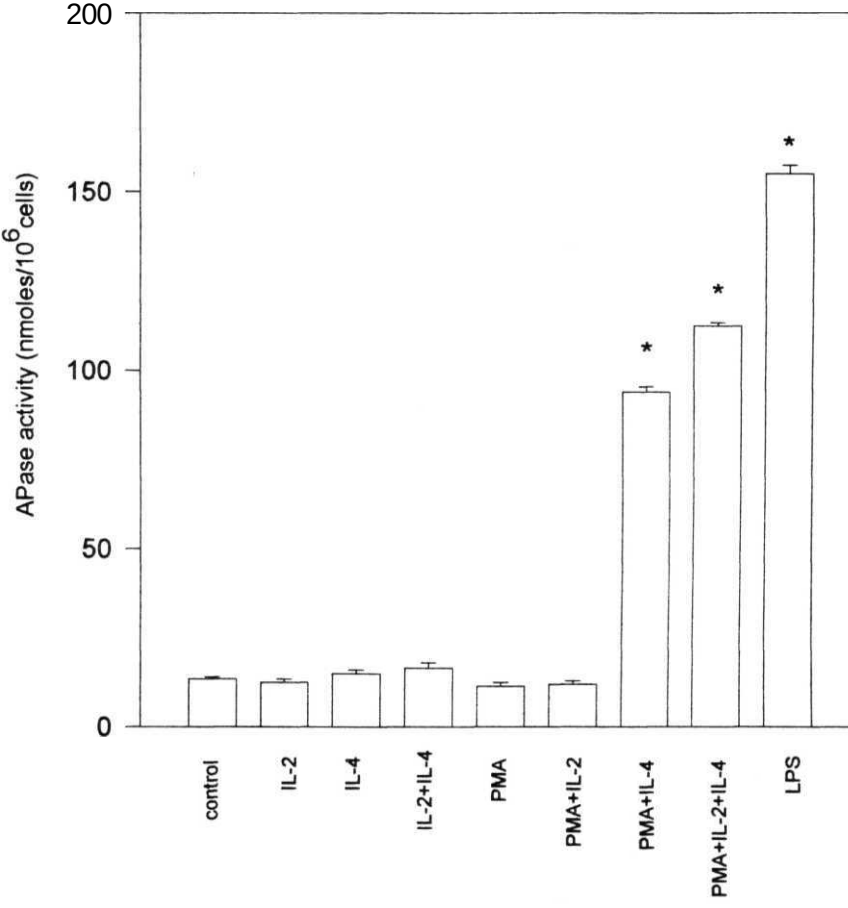
Effect of IL-2 and IL-4 on proliferative response of PMA stimulated splenic lymphocytes



Values represented are Mean± SEM of four experiments. * p< 0.001 compared to PMA stimulated cells.

Figure -18 a

Effect of IL-2 and **IL-4** on APase activity of PMA stimulated splenic lymphocytes



Values represented are **Mean±**SEM of four experiments. * p <0.001 compared with PMA stimulated **cells**.

co-stimulatory factor for the activation of a sub population of B cells (Rabin et al 1985, 1986, Hodgkin et al 1991) IL-4 has been reported to act as a co-stimulant of mitogen activated B cells in G1 phase of the cell cycle(O'Garra et al 1986) It also induces cell adhesion and motility in mitogen stimulated B cells(Clinchy et al 1991, Ellenstrom et al 1995).

In the present studies an enhancement in proliferative response concomitant with an increase in APase activity was observed by the addition of IL-4 in LPS and Seph.anti-IgM stimulated cells. Even though the initial pathway of activation differs in LPS and Seph.anti-IgM mediated stimulation, IL-4 acts as a positive signal during the Gl phase in both cases by enhancing the proliferative response and APase activity. This is further substantiated by the observation that the addition of IL-4 after the Gl phase of the cell cycle had no effect on the proliferative response and APase activity of LPS and Seph.anti-IgM stimulated cells.

On the other hand, in DXS stimulated cells IL-4 enhanced the proliferative response with no effect on APase activity. This could be because in DXS activated cells, IL-4 mediated pathway may not be operational in the stimulation of APase activity. It has been shown earlier that IL-5 acts as a positive signal in inducing APase activity in DXS stimulated cells but not IL-4 (Souvannavong et al 1992)

The cytokine receptors are known to activate intracellular tyrosine kinases Phosphorylation of target molecules by tyrosine kinases is a major intracellular signaling event triggered by cytokine receptors (DeFranco 1993) Although IL-4 is pleiotropic , its most important functions are evoked in B cells. Like many other growth factor receptors

tyrosine phosphorylation of IL-4 receptor facilitates its association with other signaling molecules in the initiation of signal transduction (Keegan et al 1994).

Cross linking of Ig receptors on the surface of B cells leads to the activation of phospholipase C-y which in turn activates the hydrolysis of phosphoinositide bis phosphate and through a series of steps it releases IP3 and DAG IP3 mobilizes Ca^{2+} which in turn activates DAG dependent protein kinase(PKC) These have been considered to be critical signals for B cell activation (Monroe et al 1985, Klaus et al 1987) It has been reported that Phorbol esters which are potent activators of PKC (Niedel et al 1983, Kraft and Anderson 1983) do not provide adequate signals for the murine B cell proliferation These are known as abortive stimulators of B cell activation as the cells are arrested in G1 phase due to the lack of secondary signal In the present experiments, addition of IL-4 and IL-4 along with IL-2 to PMA stimulated cells helped the Gl arrested B cells to progress to 'S' phase and concomitantly express APase activity.

However, IL-2 which is a T cell growth factor and B cell differentiation factor did not show any potentiating effect on proliferation and APase activity of mitogen stimulated splenic lymphocytes

Thus, based on these observations , it is suggested that IL-4 acts as a potent second signal for the induction of APase activity in mitogen stimulated B cells . In PMA stimulated cells, EL-4 provides an accessory signal which is required in the G1 phase for the activated B cell to progress further through cell cycle.

CHAPTER 5

ROLE OF APase ACTIVITY IN PROLIFERATIVE RESPONSE OF MITOGEN STIMULATED B LYMPHOCYTES-ANALYSIS USING LEVAMISOLE AND HYDROXYUREA

Resting B lymphocytes go through three distinct phases of cell cycle upon stimulation with an antigen -early activation (G1 phase), proliferation (S phase) and differentiation (plasma cell stage) These stages can be studied using different mitogens and cell cycle inhibitors Amongst various enzymes that get expressed upon mitogen induced B cell activation, alkaline phosphatase has been shown to be enhanced during the G1 phase of the cell cycle (Kasyapa and Ramanadham 1992).

Levamisole (Lev), the levo isomer of tetramisole (+) -2,3,5,6, tetrahydro 6-phenyl imidazo (2,l,b) thiazole has been used as a nematocidal agent in veterinary practice and as an anti-helminthic drug in man (Thienpont et al 1966, Rollo 1980) Lev and its derivatives have been shown to be potent inhibitors of APase (Vanbelle 1972, Bhargava et al 1977), whereas other phosphatases which include 5'nucleotidase (el Kouni and Cha 1982), acid phosphatase (Borgers 1973), ADPase (Leake et al 1983) and ATPase (LeBel et al 1980) are resistant to inhibition by Lev.

Mammalian alkaline phosphatase of Liver/Bone/Kidney type has been demonstrated to be inhibited uncompetitively by Lev with a Ki of 30uM (Goldstein et al 1980). It has been reported that Lev at 10-100uM concentration enhances T lymphocyte activation (Woods et al 1974, Hadden et al 1975). It has also been shown that Lev enhances ConA induced lymphocyte proliferation (Metaye et al 1989). It has been suggested that Lev has anti-anergic properties and can restore immune response to normalcy in immunocompromised individuals (Janssen 1976, Symoens and Rosenthal 1977). Lev can exert anti-neoplastic effects when used as an adjuvant to conventional anti-neoplastic therapies(Surgery, radiotherapy and chemotherapy) (Amery et al 1981) Long term treatment of rheumatoid arthritis patients with Lev has been shown to inhibit elevated

B cell activity by reducing the levels of IgM and IgG and circulating immune complex concentrations in serum(Symoens et al 1979). Lev induces the differentiation of murine splenic and lymphnode immature T cells into mature functional T cells.

Farley et al (1982) have suggested that APase may be involved in bone formation since APase inhibitors impair the growth of embryonic bone Lev at 2 mM concentration inhibited the proliferation of cultured bone cells with concomitant inhibition in bone APase activity Lev has been reported to affect the metabolism of cyclic GMP and cyclic AMP in lymphocytes (Hadden et al 1975, Lewinski et al 1979). Lev has been shown to enhance lymphocyte proliferation (Lichtenfeld et al 1974, Woods et al 1974) and phagocytosis of macrophages *in vitro* (Lima et al 1974). A number of lymphocyte and macrophage functions *in vivo* have also been shown to be modulated by Lev, probably through the mediation of cyclic GMP (Hadden 1975).

Hydroxyurea(HU), a reversible cell cycle inhibitor which arrests the cells at G1/S boundary (Mitchison 1971) was used to study its effect on the proliferative response and APase activity. The enzyme ribonucleotide reductase which is responsible for the conversion of ribonucleosides to deoxyribonucleosides has been shown to be inhibited by HU (Adams and Lindsay 1966, Thelander and Reichard 1979). It has been shown earlier that HU reversibly inactivates ribonucleotide reductase by reacting with the free radicals (Moore 1969). In PHA stimulated human lymphocytes HU has been shown to inhibit DNA synthesis (Albert and Bluestein 1984). HU has been used as a myelosuppressive agent in advanced phase of chronic myelogenous leukemia as it decreases specifically the intracellular pool of dATP thereby inhibiting the DNA synthesis (Gandhi et al 1998).

In the present experiments Lev and HU were used to study the interdependence of cell cycle progression and APase activity in murine splenic B lymphocytes stimulated with LPS

The objectives are to study:

1. The effect of Lev on proliferative response and APase activity of LPS stimulated splenic lymphocytes,
2. Reversibility of effect of Lev on proliferative response and APase activity,
3 Effect of HU on proliferative response and APase activity of LPS stimulated splenic lymphocytes, and
4. Reversibility of effect HU on proliferative response and APase activity.

Methods:

Proliferative response:

$0.2x10^6$ splenic lymphocytes were cultured with 50µg/ml of LPS as described in materials and methods. Lev was added at concentrations ranging from 250uM to 1mM and HU was added at 100µM to 750uM. In reversibility experiments, Lev and HU were removed by pelleting the cells by centrifugation at 500xg for 7min and washing the cells with RPMI-1640 medium Then fresh complete medium and LPS were added to the cultures Cultures were pulsed at 48h with 0.5µCi of ^3H -Thymidine and were processed at 72h as described in materials and methods.

APase activity:

$0.2x10^6$cells were cultured as described above and APase activity was determined as described in materials and methods

Results:

Effect of Lev on proliferative response:

Lev was added to LPS stimulated splenic lymphocytes at concentrations ranging from 250μM-1 mM LPS stimulated proliferative response was significantly inhibited by Lev (Table 5). The inhibition was concentration dependent and a maximal inhibition was observed at 750μM.

Effect of Lev on APase activity:

APase activity of LPS stimulated cells was significantly inhibited by Lev in a concentration dependent manner The inhibition ranged from 43 % to 98 % and maximal inhibition was observed at 750μM (Table 5)

Reversibility of Lev effect on proliferative response and APase activity:

Removal of Lev from cultures at 24 h completely reversed the inhibition caused by Lev (Table 6). The proliferative response measured by pulsing the cultures during 48-72h showed similar ^{3}H -thymidine incorporation and APase activity when compared with LPS stimulated cells

Effect of HU on proliferative response and APase activity:

HU at concentrations ranging from 100μM to 750μM inhibited ^{3}H -thymidine incorporation by ١٥٪ to ٩٩٪respectively and maximal inhibition was observed at 500μM concentration. Even though the proliferative response was inhibited significantly, there was no inhibition of APase activity at lower concentrations of HU. However, at 500μM and 750uM concentrations of HU there was a minimal inhibition of 20% in APase activity (Table 7).

Table -5

Effect of Levamisole on proliferative response and APase activity of LPS stimulated splenic lymphocytes.

Addition	^{3}H-Thymidine incorporation into DNA	Inhibition	APase activity	Inhibition
	(cpm/10^6cells)		(nmoles/10^6cells)	
None	17,835 ± 1,190	—	115 ± 10	—
LPS	2,20,900 ± 7,840	—	305.0 ±7.0	—
250	12,840 ± 1,015	28	8 0 ±1.5	30.5
500	11,550 ± 850	35	7.0 ± 1.0	40
750	2,850 ± 150	84	2.5 ± 1.0	78
1000	350 ± 15	98	0.5 ±0.2	96
LPS+Lev(μM)				
250	1,50,212 ± 1,950	32	143.0 ±3.5	53
500	1,32,540 ± 1,815	40	82.5 ±1.0	73
750	16,270 ± 890	92.6	14 0± 15	95
1000	15,463 ± 1,505	93	12.0 ±0.5	96

LPS was added at a concentration of 50μg/ml. Values represented are Mean ± SEM of five experiments.

Table -6

Reversibility of the effect of levamisole on proliferative response and APase activity of LPS stimulated splenic lymphocytes.

Addition	^{3}H- Thymidine incorporation in to DNA	Inhibition %	APase activity	Inhibition %
	(cpm/10^6cells)		(nmoles/10^6cells)	
None	20,570 ± 1,340	—	11.5 ± 0.4	—
LPS	2,02,960 ± 15,295	—	306.2 ± 7.0	—
Lev	1,405 ± 205	93.2	3.5 ± 0.4	696
LPS +Lev	6,585 ± 275	96.8	12.0 ± 0.3	96.1
Lev*	25,630 ± 2,030	—	10.4 ± 0.4	8.0
LPS+ Lev*	2,33,205 ± 14,290	—	314.4 ± 4.0	—

Values represented are Mean ± SEM of five experiments Levamisole was used at 750μM concentration . ♣levamisole was removed at 24 h, cells were washed and recultured as described in materials and methods

Table -7

Effect of Hydroxy Urea on proliferative response and APase activity of LPS stimulated splenic lymphocytes.

Addition	^{3}H-Thymidine incorporation in to DNA	Inhibition %	APase activity	Inhibition %
	(cpm/10^6cells)		(nmoles/10^6cells)	
None	17,835 ±1,190	—	11.5 ± 10	—
LPS	2,15,390 ±7,840	—	315.0 ±7.0	—
HU(μM)				
100	13,309 ±1,050	16	10.5 ±1.5	4.5
200	10,140 ±1,530	46	10.6 ±1.0	3.6
500	1500 ± 195	90.5	11.0 ±1.5	---
750	940 ± 155	94	11.3 ±1.0	
LPS +HU (μM)				
100	1,81,350 ±3,940	15.8	319.0 ±10.0	
200	1,14,160 ± 4,750	47	310.0 ± 6.5	2
500	2,800 ± 190	98 7	259.0 ±7.5	18
750	945 ± 75	99 5	250.0 ±6.0	20.8

LPS was added at a concentration of 50μg/ml . Values represented are Mean ± SEM of five experiments

Reversibility of the effect of HU on proliferative response and APase activity:

The inhibitory effects caused by HU were completely reversible when the inhibitor was removed at 24 h of culture (Table 8)

Lymphocyte viability in presence of Lev and HU:

The viability of LPS stimulated cells at 72 h was 97 % On the other hand, in LPS stimulated cells treated with 750μM of Lev or 500μM HU the viability was 94 % and 93% respectively and was not significantly different from that of LPS stimulated cells (Table 9 & 10).

Enzyme cytochemical staining:

Cytochemical staining of LPS stimulated cells using β- naphthyl phosphate and Fast blue RR showed that 40% of the cells were positive for APase activity In Lev treated cells the percentage of positive cells was significantly lower compared to controls (Table 9 &10, $p < 0.001$), where as in HU treated cells, the number of cells stained positive for APase activity was 35%. However, removal of Lev at 24 h showed an increase in the number of positive cells to 42% comparable with LPS.

Effect of Lev and HU on proliferative response and APase activity of YAC-1 cells:

Treatment of YAC-1 cells with Lev showed a minimal inhibition of 35% in proliferative response and 13 % APase activity However, treatment with HU a cell cycle inhibitor, inhibited the proliferative response and APase activity up to 60% in YAC-1 cells (Table 11).

Discussion:

Lev was used to analyze whether a mitogen stimulated B cell can progress through the cell cycle when the APase activity is inhibited. Further, HU was used to study the

Table - 8

Reversibility of the effect of hydroxyurea on proliferative response and APase activity of LPS stimulated splenic lymphocytes

Addition	^{3}H-Thymidine incorporation in to DNA (cpm/10^6cells)	Inhibition %	APase activity (nmoles/10^6cells)	Inhibition %
None	23,870 ± 1340	—	11.5 ±0 4	—
LPS	2,62,960 ± 15,295	—	306.2 ± 7.0	
HU	3,190 ± 505	86.7	9 9 ±0.3	13
LPS+HU	7,040 ± 180	97.3	252.5 ±5.5	18
HU*	27,785 ± 1,405	—	10.5 ±0.2	8
LPS+HU*	2,51,895 ± 7,990	4.2	302.0±6.5	1.8

Values represented are Mean ± SEM of five experimetns HU was added at a concentration of 500μM. ♣HU was removed at 24 h and the cells were washed and recultured as described in materials and methods

Table -9

Effect of Levamisole on viability and enzyme activity staining in LPS stimulated splenic lymphocytes.

Addition	Viability %	Cells stained %
None	88 ± 10	7 ±1.0
LPS	97 ±1.0	40 ±1.0
Lev	83 ±1.0	2 ±1.0
LPS+Lev	96 ±2.0	6 ±1.0
Lev *	87± 1 0	6± 1.0
LPS+Lev*	97 ± 2 0	42 ±1.0

Values represented are Mean ± SEM of three experiments. Levamisole was added at 750μM concentration * levamisole was removed at 24 h and cells were washed and recultured as described in materials and methods

Table -10

Effect of Hydroxy Urea on viability and APase positive cells of LPS stimulated splenic lymphocytes.

Addition	Viability %	Cells stained %
None	88 ± 1.0	7 ±1.0
LPS	97 ± 3.0	40 ±2.0
HU	80 ± 1.0	5 ±1.0
LPS+HU	81 ± 1.0	35 ±1.0
HU •	94 ± 1.0	6± 1.0
LPS+HU*	97 ± 1.0	38 ±1.0

Values represented are Mean ±SEM of three experiments. HU was added at 500μM concentration. •**HU** was removed at 24 h and the cells were washed and **recultured** as described in materials and methods.

Table -11

Effect of levamisole and hydroxyurea on the proliferative response and Apase activity of YAC-1 cells.

Addition	^{3}H- Thymidine incorporation into DNA	Inhibition %	APase activity	Inhibition %
	(cpm/10^{6}cells)		(nmoles/10^{6}cells)	
Control	41,360 ±1492	nil	730 ±2.5	nil
Lev(μM)				
250	35,580 ±405	14	735 ±2.5	-
500	34,985 ±1200	15	728 ±1.8	0.27
750	28,565 ±1280	31	650 ±1.6	11
1000	26,895 ±1076	35	635 ±9.0	13
HU(μM)				
250	19,840 ±360	52	289 ±4.0	60
500	18,865 ±380	54	285 ±3.5	61
750	15,070 ±1050	64	269 ±3.5	63
1000	15,825 ±450	62	258±4.0	67

Values represented are Mean ± SEM of five experiments.

effect of arresting the cells at G1/S boundary on APase activity Splenic lymphocytes showed an increase in proliferative response concomitant with an increase in the APase activity when stimulated with LPS. This enhancement in APase activity is dependent on cell cycle progression of the B cell.

Lev significantly inhibited the APase activity when added to the LPS stimulated cells at the initiation of the culture. In these cells, the proliferative response was also inhibited suggesting that commitment to proliferation requires the enhancement of APase activity. The potent inhibition observed in presence of Lev was not due to cell death as the viability of Lev treated cells was similar to that of the LPS stimulated cells This suggests that the Lev treated cells are metabolically active but are arrested in the G1 phase of the cell cycle. This is further substantiated by the fact that the effect of Lev on the proliferative response and APase activity was completely reversible upon its removal at 24 h from the culture. Lev being an uncompetitive inhibitor, binds only to the enzyme - substrate complex but not to the free enzyme Hence, in the LPS activated B cell, Lev avidly binds to the putative "substrate-APase" complex thereby affecting a crucial physiological function which is required for the progression of the activated B cell through cell cycle Upon removal of Lev, the B cell is able to progress through the cell cycle in presence of LPS and concomitantly enhancement of APase activity was observed. The enzyme activity staining of the cells showed that the number of cells positive for APase were reduced in Lev treated cells compared to LPS stimulated cells This further substantiates that indeed in presence of Lev , the number of B cells expressing APase activity was significantly reduced and the reduction in activity observed was not due to a

kinetic alteration of enzyme activity Thus, these results clearly indicate the requirement of APase for the B cell to enter the proliferative phase

In the second part of the work hydroxyurea, a cell cycle inhibitor, which arrests the ceD at Gl/S boundary was used to study its effect on the proliferation and expression of APase activity HU is a potent inhibitor of ribonucleotide reductase (RR), the enzyme responsible for converting nucleoside diphosphates to deoxynucleoside diphosphates (Adams and Lindsay 1966, Thelander and Reichard 1979) thereby hampering the supply of deoxynucleotides required for DNA synthesis. In the present study, the results obtained clearly showed that hydroxyurea inhibited the proliferative response by 91% but did not inhibit the APase activity But at high concentrations the proliferative response was inhibited by 99% and inhibited the APase activity by 18%. The viability in these cells remained unaffected and the enzyme cytochemical staining shows that the number of cells positive for APase activiity were similar and comparable with that of LPS stimulated cells The inhibition caused by HU was reversible and the cells entered in to 'S' phase.

Treatment of YAC-1 cells with Lev did not inhibit APase activity and proliferative response However, treatment of cells with HU resulted in the inhibition of proliferative response concomitant with a marginal inhibition in APase activity. These results suggest that expression of APase in YAC-1 is constitutive and probably is not cell cycle dependent. Lack of inhibition of proliferative response and APase activity in presence Lev strongly suggests that the membrane APase in these cells in not required for the physiological growth unlike B cells. These results clearly show that APase does not have any role in T cells.

All these results suggest that the expression of APase activity is an important event that is essential for cell cycle progression and APase activity is expressed only in cells which are committed to proliferation Thus, the expression of APase activity is an integral feature of activated B lymphocytes

CHAPTER 6

PURIFICATION OF APase FROM MOUSE LIVER AND YAC-1 CELLS

In most animal species, the major isozymes of APase expressed are tissue non-specific, intestinal and placental In humans, a fourth isozyme derived from germ cells has been described These isozymes differ in their carbohydrate content (Hiwada and Wachsmuth 1974) Tissue non-specific APase(TNAP, Liver /Bone/Kidney) is widely distributed in several animal species and is conserved from bacteria to man with high degree of homology Even though it is distributed in many tissues the physiological role of TNAP has not yet been known. It has been shown earlier that APase activity is expressed only in activated B lymphocytes Neither resting B lymphocytes nor resting and activated T lymphocytes express APase activity (Garcia-Rozas et al 1982) Earlier, it has been shown that B lymphocyte APase is a type 1 TNAP and belong to L/B/K type (Kasyapa 1996). Even though it has been shown that T lymphocytes do not express APase activity, YAC-1, a murine T cell lymphoma constitutively expressed APase activity (Neumann et al 1976). In the present study APase has been purified from mouse liver to prepare a polyclonal antibody in rabbits inorder to study the functions of APase in activated B lymphocytes APase was also purified from YAC-1 cells to compare the catalytic properties with APase from mitogen activated B cells and mouse liver.

In this chapter, the results of the purification and characterization of APase from mouse liver,YAC-1 cells and preparation of a polyclonal antibody to liver APase are presented and discussed.

The following experiments were carried out :

1. Purification of APase from mouse liver and YAC-1 cells,
2. Comparison of catalytic properties of APase from mouse liver and YAC-1 with that of APase from mitogen activated B cells,

3 Preparation of polyclonal antibody to mouse liver APase in rabbits,

4. Characterization of anti-APase antibody in terms of its reactivity towards partially purified mouse liver, B lymphocyte and YAC-1 APase

Methods:

The purification of APase from mouse liver and YAC-1 cells was carried out using the protocols described in materials and methods.

Results:

Purification and characterization of APase from mouse liver and YAC-1:

In case of mouse liver, APase could eluted from DEAE -cellulose column with 125 mM NaCl. The fractions containing APase activity were pooled, acetone precipitated and were chromatographed on a Sephacryl S-200 column The data on partial purification of APase from mouse liver is presented in Table 12. The enzyme could be purified 996 fold with an yield of 27%. APase activity from YAC-1 cells could be eluted with 100 mM NaCl from the DEAE-cellulose column The elution profile of APase from DEAE -cellulose column is presented in Fig 18.b The yield was 27% with 976 fold purification(Table 13). The fractions eluted from DEAE cellulose and Sephacryl column in case of liver APase were electrophoresed on a native polyacrylamide gel. Fig 19 shows the silver stained protein profile (panel A) and enzyme activity staining pattern (panel B) A single protein stained positive for APase activity which corresponded to a faintly stained protein band. The electrophoretic profile of proteins in Sephacryl column eluted fraction of YAC-1 cells is presented in Fig 20. A single protein band was stained for enzyme activity as well as for protein The SDS -PAGE analysis of Sephacryl fraction of liver and YAC-1 showed that the apparent molecular weight of the enzyme was

Table -12

Purification of Alkaline phosphatase from mouse liver

Fraction	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification ratio	yield %
Homogenate	18,000	19,800	1.1	1	100
Butanol Extract	2,600	17,160	6.6	6	86
Ammonium sulfate ppt & DE-52 Chromatography	100	15,150	151.5	138	76.5
Acetone ppt.	50	12,000	240.0	218	60 6
Sephacryl S-200 column	5	5,480	1096	996	27.3

Unit is defined as nmoles of P-nitrophenol released per minute.

Table -13

Purification of APase from YAC-1 , a T cell lymphoma.

	Total protein mg	Total activity U	Specific activity U/mg	purification fold	yeild %
Homogenate	72	2.75	0.038	1	100
Sonicate,TX-100 solubilised supernatant	17.5	1.37	0.078	2.05	49.8
DE-52 column chromato-graphy	0.9	1.185	1.316	35.0	43.0
Sephacryl S-200 column chromatography	0 02	0.742	37.1	976.3	26.9

Unit is nmoles of PNP released /min

Figure -18 b

Elution profile of APase from DEAE column with lOOmM NaCl

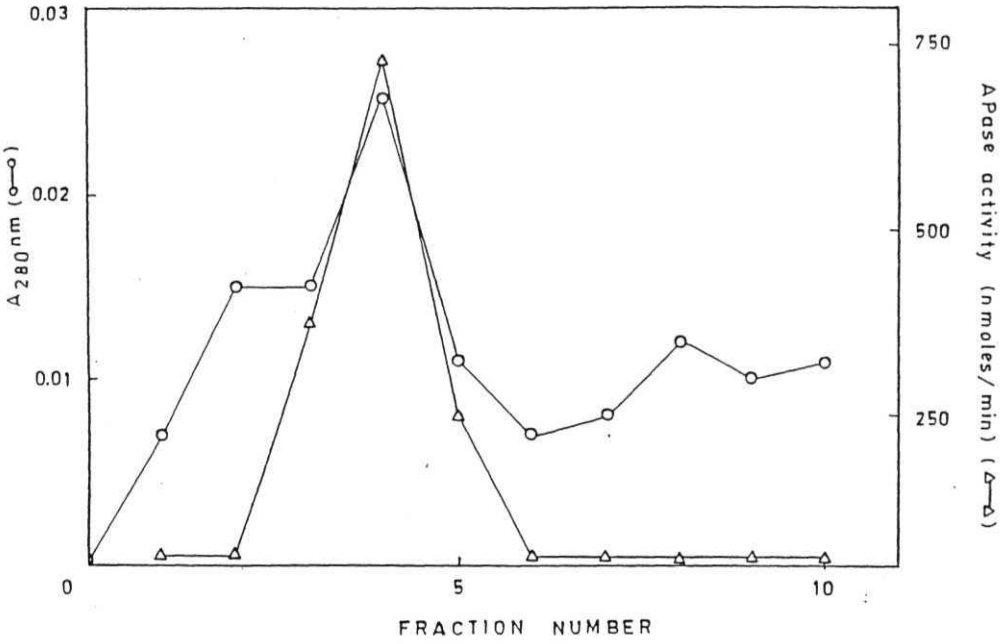
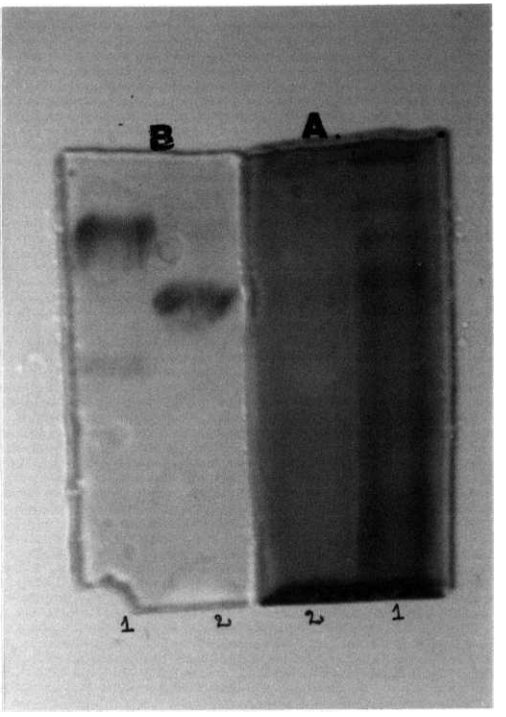


Figure - 19

Enzyme activity staining of Purified mouse liver APase



Panel A : Silver staining of proteins

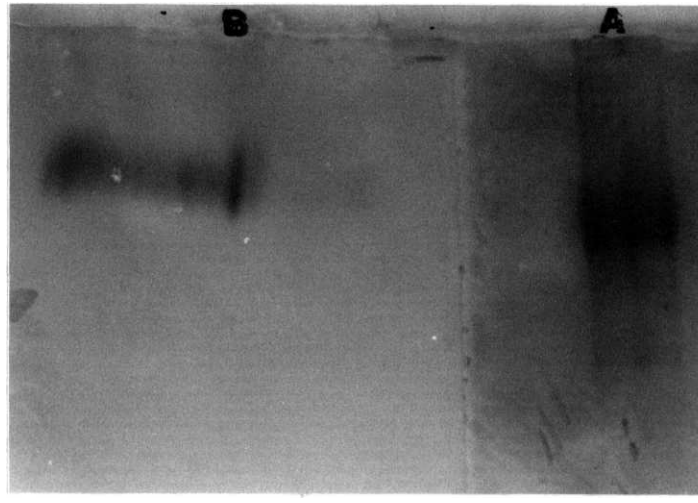
Panel B : Aetivity staining of APase

Lane 1 : Fractions eluted from DE - 52 column

Lane 2 : Fractions eluted from Sephacryl S- 200 column

Figure - 20

Enzyme activity staining of Purified YAC-1 APase



Panel A Silver staining for proteins

Panel B Activity staining for APase

142kD(Fig 21 & 22) The subunit mass of the enzyme was determined by performing the electrophoresis of the sample in presence of β -mercaptoethanol The subunit mass was found to be 72kD suggesting that two identical subunits are present.

The partially purified APase was further characterized using inhibitors, Lev, L-homoarginine and L-histidine The data are presented in Tables 14 & 15 Lev, at 100 uM concentration inhibited the activity of APase from liver, B lymphocytes and YAC-1 cells by 95% The inhibition caused by L-homoarginine and L-histidine was upto 80% in case of liver and B lymphocyte APase and upto 70% in case of YAC-1 cells. Treatment of enzyme preparations at 56^0C for 30 min resulted in inhibition of catalytic activity by 65% in case of mouse liver , 64% in case of YAC-1 cells and 90% in case of B cell APase.

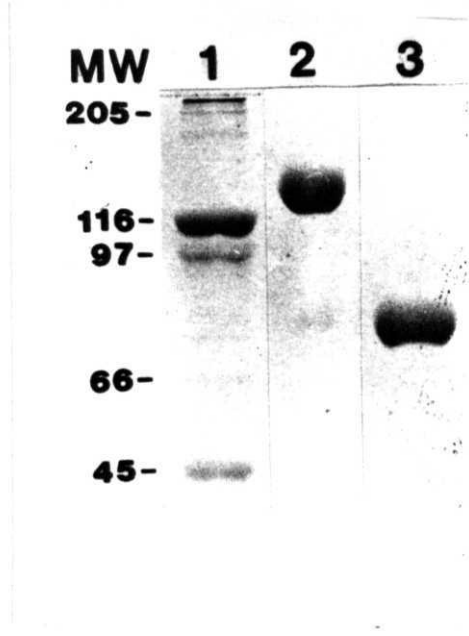
Characterization of anti-APase anti serum:

The partially purified mouse liver APase was injected to rabbits to raise polyclonal antibodies. The antiserum obtained reacted with mouse liver APase and showed a titre of 1:16 by Ouchterlony double immunodifffusion reaction. Western blot analysis showed that the antiserum reacted with APase from mouse liver and B cells However the antiserum failed to recognize APase from YAC-1 cells(Fig 23).

Partially purified mouse liver APase was immunoprecipitated with anti-APase antiserum and the immune precipitate and the supernatant were analyzed for APase activity Data presented in Table 16 shows that negligible amount of APase was present in the immune complex and some of the activity is recovered in the supernatant. A decline in the recovery of APase activity was observed in the supernatant when increasing amount of APase was made to react with a fixed amount of antiserum The loss of APase

Figure - 21

SDS-PAGE of Purified mouse liver APase



Lane 1 : Molecular weight markers

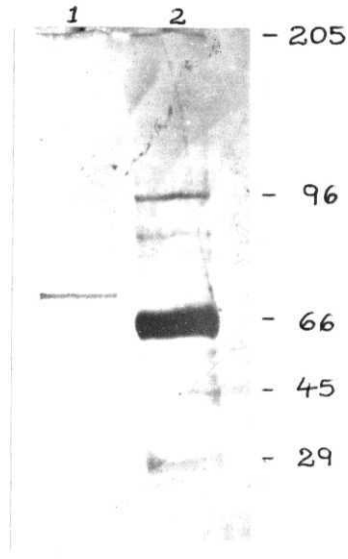
Lane 2 : APase with out p- ME

Lane 3 : APase with β -ME

Electrophoresis was carried out using 7.5% gel and the proteins were stained with comassie brilliant blue.

Figure - 22

SDS-PAGE of purified YAC-1 APase



Lane 1: YAC-1 APase with β- ME.

Lane 2: Molecular Weight markers

Electrophoresis was carried out using 7.5% gel and the proteins were silver stained.

Table -14

Effect of Inhibitors on YAC-1 and B lymphocyte APase.

Addition	YAC-1 (nmoles/10^6cells)	B lymphocyte (nmoles/10^6cells)
Control	450	240.0
Levamisole 40μM	63	48.0
100μM	27	4.5
L-histidine 10mM	130	43.2
L-Homoarginine 10mM	100	33.6
Heat treatment at 56^0c for 30min	162	24 0

Values represented are Mean of five experiments

Table -15

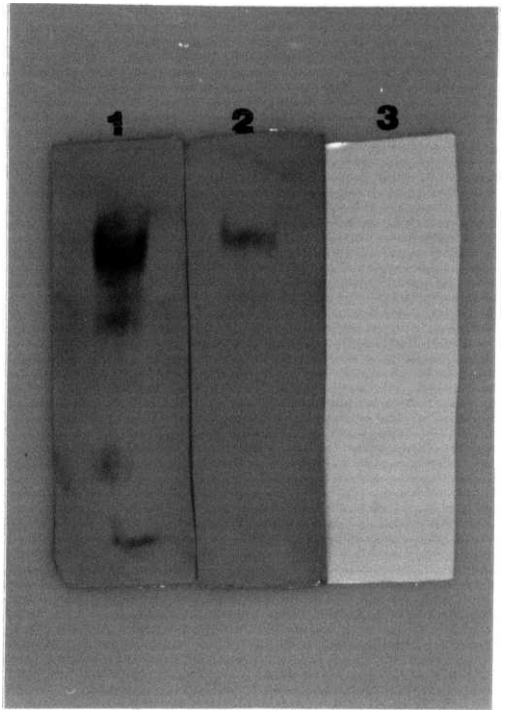
Comparison of properties of YAC-1 APase with partially purified APase from Liver and B lymphocyte

Addition	% Inhibition		
	Liver	YAC-1	B lymphocyte
Control	—	—	—
Lev			
40μM	84	87	80
100μM	95	95	98
L-Homoarginine			
10mM	86	77	86
L-Histidine			
10mM	83	71	82
Heat treatment at 56°c for 30min	65	64	90

Values represented are Mean of five experiments

Figure - 23

Western blot analysis of partially purified APase



Lane 1: Mouse liver APase

Lane 2: B lymphocyte APase

Lane 3: YAC-1 APase

Table -16

Immuno precipitation of APase using anti-APase antiserum

APase	Total activity (U)	Activity recovered		Inhibition %
		supernatant	pellet	
	nmoles of p-NP / min			
Expt.l				
20	1810	1503	22.0	17
30	2715	1824	25.1	33
50	4525	2383	34.3	48
100	9050	4080	59.7	55
Expt.2				
20	1810	1830	—	—

In Expt.l, 100 µl of anti-Apase antiserum and in Expt.2 , 100 µl normal rabbit serum were used .

activity could be due to the presence APase in the immune complex in catalytically inactive form as a result of binding of antibody molecule Thus, the antibody prepared is an inhibitory antibody to liver APase

Discussion:

The yeild and purity of APase obtained in the present experiments is comparable to those reported by Ikehara et al (1978) It has been shown earlier that B lymphocyte APase belongs to the L/B/K type(Kasyapa 1996) YAC-1, a T cell lymphoma constitutively expresses APase activity (Neumann et al 1976). In the present study, characterization of APase from YAC-1 cells showed that it also belong to the L/B/K type isozyme. Data on inhibitory effect of Lev, aminoacids and heat stability studies suggest that APase from liver, B cells and YAC-1 cells are catalytically similar.

The antibody prepared towards liver APase did not react with YAC-1 APase indicating its antigenic non-identity even though it is similar to liver and B cell APase catalytically The anti-liver APase antiserum recognized B cell APase both in Ouchterlony assay and in Western blot anlysis Immunoprecipitation reaction of liver APase with anti-APase antiserum shows that the antibody which is prepared is an inhibitory antibody to type 1 APase Due to the specific interaction of anti- APase antibody with B cell APase, it is possible to use the antibody to study the role of APase in B lymphocyte activation

CHAPTER 7

EFFECT OF ANTI- APase ANTIBODY ON PROLIFERATIVE RESPONSE AND APase ACTIVITY OF MITOGEN STIMULATED SPLENIC LYMPHOCYTES

The role of several glycophosphatidylinositol (GPI) anchored proteins has been determined by treating lymphocytes with mAb towards them (Presky et al 1990) It was shown earlier that GPI anchored proteins are involved in the lymphocyte activation , second messenger generation, and proliferation (Davis et al 1988, Stefanova et al 1991). Alkaline phosphatase is a glycophosphatidylinositol anchored membrane bound metalloenzyme found on the membranes of activated B lymphocytes(Garcia -Rozas et al 1982, Hashimoto and Zubler 1986, Jemmerson and Low 1987 , Noda et al 1987). It has been shown earlier that expression of APase activity correlates more with proliferation (Ohno et al 1986, Kasyapa and Ramanadham 1992) and differentiation (Burg and Feldbush 1989, Marquez et al 1989). It has also been reported that cAMP acts as positive regulator of expression of APase activity (Kasyapa and Ramanadham 1995). It has been shown earlier that APase gets expressed at around 8h after the mitogenic stimulation and increases till 120h (Kasyapa and Ramanadham 1992) Also, enhancement of APase activity occurs only in B lymphocytes committed to proliferation, as incomplete mitogens failed to trigger APase activity(Kasyapa 1996).

In order to study the physiological role of APase in B lymphocyte activation, LPS stimulated lymphocytes were treated with anti-mouse liver APase antibody and the following parameters were determined:

1. Effect of anti-APase antibody on LPS stimulated B lymphocyte proliferation .
2. Effect of anti-APase antibody on the expression of APase activity in LPS stimulated B lymphocytes.
3. Effect of anti-APase antibody on the release of APase into the supernatant of LPS stimulated B cell cultures.

4. Detection of APase-IgM complexes in the culture supernatants of LPS stimulated cells treated with anti-APase antibody.

Methods:

Lymphocyte proliferation:

Lymphocyte proliferation assay was carried out with indicated concentrations of mitogens and antibody as described in materials and methods(chapter 2) In some experiments Anti-APase antibody was added at 17h after the initiation of the culture and were pulsed with 0 5uCi of ^{3}H -thymidine for the last 24 h of the 48h culture period and processed as described in materials and methods.

Release of enzyme into the supernatant of LPS stimulated cultures:

The enzyme released was assayed as follows : Cells were pelleted at 400xg for 7min and the supernatant was collected and centrifuged at 15000xg for 20min to remove the cell debris. The supernatant thus collected was used for estimation of APase activity. Complete medium (RPMI 1640 containing 5% FCS and 50uM β–mercaptoethanol was used as blank along with the substrate blank The enzyme activity was expressed as nmoles of p-NP released /ml/30min

ELISA for the detection of APase and IgM complexes in the supernatant of LPS stimulated splenic lymphocytes:

In order to detect the IgM associated APase, ELISA plates were coated with 2μg/well of goat anti-mouse IgM and the plates were blocked with 5% lactalbumin peptide solution. The plates were washed with Tris-buffered saline, pH 7.4 containing 0.05% tween-20 To the washed wells LPS and control supernatants were added and incubated at 37°C for 2 h To one set of wells anti-mouse IgM -HRPO conjugate(1:2000)

was added and incubated at 37^0C for 1h and the plate was washed thoroughly and the substrate o-phenylene diamine (OPD) was added in 0 1M phosphate-citrate buffer pH 5.0. The plate was incubated for 20min at 37°C and the reaction was stopped by the addition of 20µl of 3N H_2SO_4 and the absorbance was read at 490nm in a ELISA reader.

To the second set of wells, p-NPP was added and incubated at 37^0C for 1h and the reaction was stopped by adding 20µl of 1N NaOH. The absorbance was measured at 405nm and the amount of p-NP released was estimated from the standard graph

Results:

The effect of anti-APase IgG on proliferative response of LPS stimulated splenic lymphocytes:

In LPS stimulated splenic lymphocytes treated with 2 5µg/ml of anti-APase antibody proliferative response was inhibited by 64%. At 5µg/ml concentration of the antibody the inhibition observed was 97%. The inhibition by anti-APase antibody was specific to LPS stimulated cells, as there was no inhibition in control cells as well as in cells treated with normal rabbit IgG and LPS(Table 17)

Effect of anti -APase antibody on the expression of APase activity in LPS stimulated cells:

Control and LPS stimulated splenic lymphocytes were treated with anti-APase IgG at 2.5 and 5µg/ml and normal rabbit IgG at 5µg/ml concentration. In anti-APase antibody treated LPS cells there was a significant inhibition of APase activity, whereas in normal rabbit IgG treated cells APase activity was similar to that of LPS stimulated lymphocytes (Table 17)

Table -17

Effect of anti -APase antibody on proliferative response and APase activity of LPS stimulated splenic lymphocytes.

Addition (μg/ml)	^{3}H-Thymidine incorporation into DNA (cpm/10^6cells)	Inhibition %	APase activity (nmoles/10^6cells)	Inhibition %
None	14,695 ± 495	---	11.0 ± 1.0	
Rabbit IgG				
5.0	14,235 ± 190	3	10.5 ± 0.5	4.5
Anti-APase IgG				
2.5	13,895 ± 290	6	129 ± 1.0	—
5.0	13,975 ± 785	5	9.5 ± 1.0	14
LPS	2,68,765 ± 7,895	---	276.7 ± 4.0	---
LPS+Rabbit IgG	2,62,720 ± 6,970	3	258.6 ± 4.5	7
LPS+Anti-APase IgG				
2.5	90,030 ± 4,875	64.2	143.8 ± 3.5	48
5.0	8,365 ± 175	96.7	19.5 + 1.0	93

Values represented are Mean ± SEM of four experiments.

Effect of anti-APase IgG on proliferative response of ConA stimulated splenic lymphocytes

The proliferative response of ConA stimulated cells remains unaffected when anti-APase antibody was added to ConA stimulated cells at a concentration of 5µg/ml (Table 18).

Delayed addition of Anti-APase antibody on the proliferative response and APase activity:

APase antibody was added to LPS stimulated cultures at 17h after the initiation of the culture Delayed addition of antibody resulted in less inhibition caused by the antibody in proliferative response and APase activity of LPS stimulated splenic lymphocytes compared to the addition at the intitiation of culture(Table 19)

Release of enzyme in to the supernatant of the LPS stimulated cultures:

APase activity could be detected in the 20,000xg supernatant of LPS stimulated cells starting from 48h and increased continuously till 120h of culture(Table 20). However, in anti-APase antibody treated, LPS stimulated cells the release of enzyme in to the supernatant was insignificant.

ELISA for the detection of IgM associated APase:

The results represented in Fig 24 shows the association of APase with IgM. The APase activity increased with increase in the concentration of supernatant added to the wells coated with anti mouse IgM. Similarly, the amount of IgM bound also increased with increase in the volume of supernatant added to the anti-IgM coated wells indicating that LPS stimulated cells were in the differentiated state, actively secreting IgM (Fig 25).

Table-18

Effect of anti-APase antibody on proliferative response and APase activity of ConA stimulated splenic lymphocytes.

Addition	^{3}H-Thymidine incorporation into DNA	Inhibition %	APase activity	Inhibition %
(μg/ml)	(cpm/10^{6}cells)		(nmoles/10^{6}cells)	
None	37,875 ± 4,940	—	10.5 ± 1.0	
Anti-APase IgG				
5.0	35,400 ± 3,755	6.5	10.3 ± 10	
ConA				
2.0	4,23,800 ± 33,590	—	13.4 ± 2.0	
ConA + Anti-APase IgG				
5.0	4,09,855 ± 11,915	3.4	12.711.5	——

Values represented are Mean 1 SEM of triplicate cultures of a representative experiment.

Table -19

Effect of delayed addition of anti-APase antibody on proliferative response and APase activity of LPS stimulated splenic lymphocytes.

Addition	^{3}H-Thymidine incorporation into DNA	Inhibition %	APase activity	Inhibition %
(μg/ml)	(cpm/10^6cells)		(nmoles/10^6cells)	
None	16,360 ± 1,465	—	10 75 ± 1.75	---
LPS	2,31,965 ± 10,865	—	249.7 ± 7.0	
Anti-APase IgG*				
2.5	17,470 ± 1,750	—	9.6 ±1.0	10
5.0	15,285 ± 1,565	7	9.4 ±1.5	13
LPS + Anti-APase IgG				
2.5	1,05,705 ± 7,560	54.5	130 ± 3.5	48
5.0	13,785 ± 1,815	94	26 1 1.7	90
Anti-APase IgG				
2.5	15,765 ± 1,360	4	10 ±1.5	
5.0	15,301 1 1,015	6.5	9.9 ± 1.0	8
LPS + Anti-APase IgG*				
2 5	1,68,665 ± 16,075	27.3	198 1 ± 28	20.7
5.0	1,71,860 ± 10,765	26	203.7 ± 3.5	18.4

Anti-APase IgG was added at ♣24 h of culture Values represented are Mean ± SEM of four experiments.

Table -20

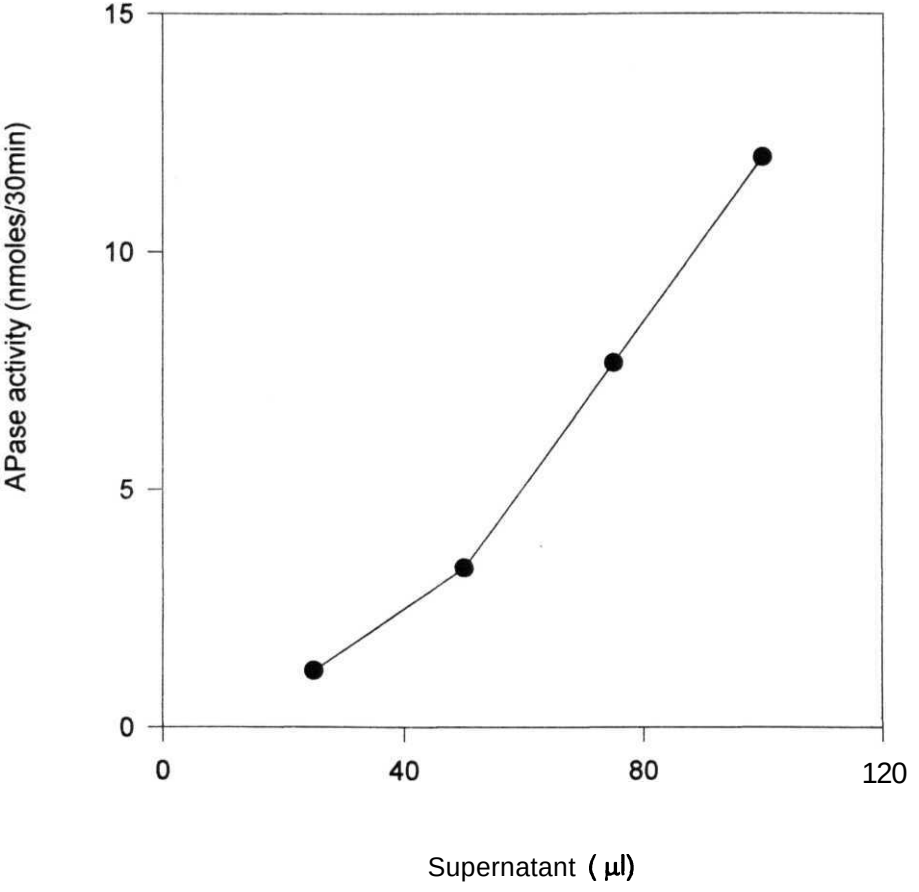
Release of APase in to the culture supernatant of LPS stimulated lymphocytes and YAC-1 cells

Culture period	Splenic lymphocytes		YAC-1 cells	
	cell associated	supernatant	cell associated	supernatant
h	nmoles/10^6cells	nmoles/ml	nmoles/10^6cells	nmoles/ml
0	4	nil	403 9	nil
24	125	nil	403 0	nil
48	240	15	405.0	nil
72	268	52	N.D	N.D.
96	279	136		
120	290	190		

Data presented is representative of 5 similar experiments. Values presented are mean of duplicates N D : Not done

Figure -24

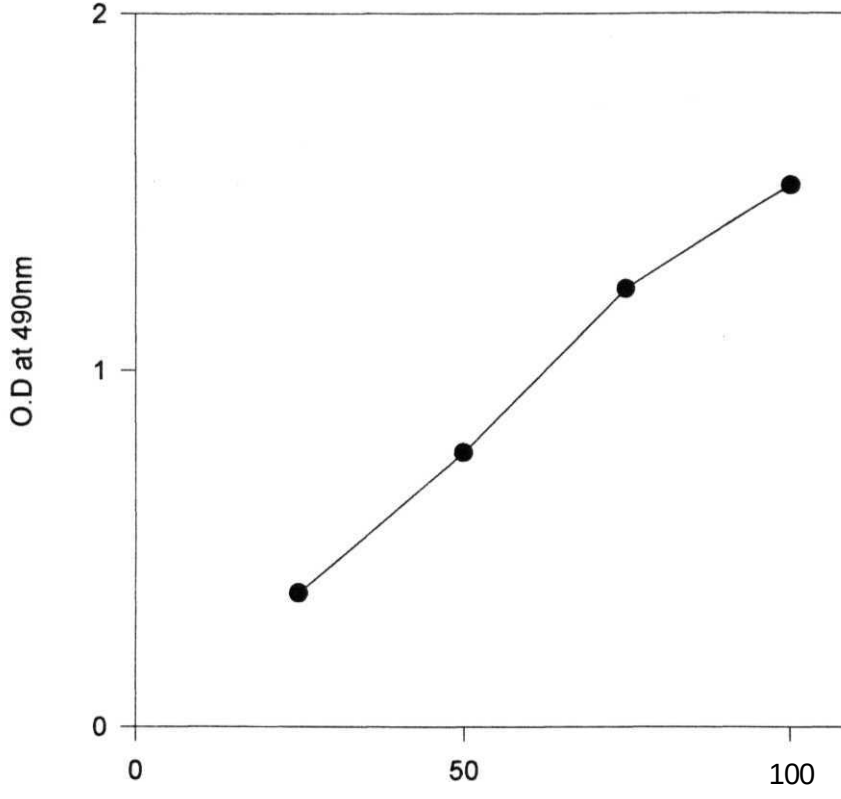
ELISA for detection of IgM bound APase in the supernatant of LPS stimulated splenic lymphocytes



Values represented are Mean of five experiments.

Figure -25

ELISA for detection of IgM released in to the supernatant of LPS stimulated splenic lymphocytes



Values represented are Mean of five experiments

Viability and enzyme cytochemical staining

Table 21 shows the viability of LPS treated cells in the absence and presence of Anti-APase antibody The viability in Anti-APase antibody treated cells remained unaffected and was similar to LPS stimulated cells However, cytochemical staining of these cells showed that the percentage of cells stained positive in case of Anti-APase treated cells was only 3% compared to LPS stimulated cells which was 43%.

Discussion:

Alkaline phosphatase is a GPI anchored membrane bound protein expressed only on activated B lymphocytes Treatment of mitogen stimulated cells with antibody to alkaline phosphatase resulted in the inhibition of proliferation and APase activity. Further, delayed addition of antibody resulted in reduction in the inhibition Feldbush and Lafrenz (1991) have shown that the level of APase mRNA increased during the mitogenic stimulation and preceded the peak transcriptional activity of μ heavy chain gene. It has also been shown that APase gets released in to the surrounding milieu of mitogen stimulated B cells (Marty and Feldbush 1993). A variety of plasma membrane proteins have been shown to be anchored through GPI (Low 1989). Some of these are involved in cell adhesion and regulation of complement system, but physiological function of most of them remains unknown (Horejsi 1991). Earlier it has been demonstrated that binding of a ligand or antibody to GPI anchored cell surface proteins results in activation of leukocytes (Stefanova et al 1991)

The mechanism of stimulatory signal transduction by the GPI anchored membrane proteins has been suggested to be through the protein tyrosine kinase activation(Stefanova et al 1991). In cells stimulated with specific monoclonal antibodies to cell surface

Table -21

Effect of anti-APase antibody on viability and enzyme activity staining of LPS stimulated splenic lymphocytes

Addition	viability %	APase positive cells %
None	87 ±1.0	6.0 ±1.0
LPS	98 ± 1.0	43 ± 1.0
Anti-APase IgG	83 ± 1.0	2 + 1.0
LPS + anti-APase IgG	96 ± 1.0	3 ± 1.0

Values represented are Mean ± SEM of five experiments.

antigens, in addition to tyrosine phosphorylation, calcium mobilization and/or PI turnover have been implicated in the later events of activation such as proliferation (Nakashima et al 1991, Snapper et al 1991 Korty et al 1991, Stefanova et al 1991, Korczek et al 1991). Treatment of LPS stimulated cells with anti-APase antibody resulted in the inhibition in the release of APase into the culture supernatant , where as in LPS stimulated cells the enzyme was released from the cells along with secretory IgM Earlier Marty and Feldbush (1993) have shown that treatment of LPS stimulated cells with anti-APase monoclonal antibody (mAb) resulted in decrease in APase activity with out affecting the proliferative response. Also treatment of cells with anti-APase mAb resulted in increase of IgM secretion. Pezzi et al (1991) reported that murine B cell APase associates with the cytoskeleton which correlates with changes in the phosphorylation of several cytoskeletal proteins. In rat and human liver plasma membranes APase has been shown to be a major protein phosphatase(Chan and Stinson 1986, Mataye et al 1989). It was hypothesized that APase can function as a cellular phosphatase in dephosphorylation events(Pezzi et al 1991, Roifman and Wang 1991, Stanley 1990). APase has been shown to play a role in the internalization of Ig molecules in placental epithelial cells (Makiya and Stigbrand 1992a & 1992b)

Based on the above results it is suggested that APase is an important phosphatase which has a significant role in B lymphocyte activation Further, it may be involved in the transport of Ig molecules as it is released in to the supernatant of mitogen activated B lymphocytes in complex with IgM.

CHAPTER 8

ALKALINE PHOSPHATASE ACTIVITY AS A MARKER OF B LYMPHOID MALIGNANCY

Alkaline phosphatase has a wide but unequal tissue distribution in mammals In rodents, B lymphocytes upon stimulation with mitogen or antigen have been shown to express APase activity (Burg and Feldbush 1989, Kasyapa 1996). However, mitogenic stimulation of PBL from rabbits and humans failed to induce APase activity (Kasyapa 1996). Differential expression of APase in activated B cells from the above mentioned mammals could be due to the probable differences in the signaling pathways that are operational following mitogenic activation.

Expression of APase activity has been considered as one of the many identifiable markers of malignancy (Millan 1992) Various tumors and tumor cell lines such as teratocarcinomas, choriocarcinomas and oesteosarcomas express APase activity (Van Hoof and De Broe 1994, Millan and Fishman 1995) Tumorogenicity has been correlated with high levels of APase expression (Latham and Stanbridge 1990).

Increased levels of APase activity has been reported in neutrophils in chronic myeloproliferative diseases (Rosenblum and Petzold 1975). On the other hand, neutrophil APase activity is significantly decreased in chronic myelogenous leukemia and chronic myelomonocytic leukemia (McComb et al 1979) 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).

In the present study, B and T cell lines of murine and human origin were screened for the presence of APase activity in order to explore the possibility of using APase as a marker of B lymphoid malignancy.

The objectives of the present work are:

1. To study the effect of various T and B cell mitogens on proliferative response and

APase activity of human PBL.

2. To screen various human and murine cell lines and hybridomas for the presence of APase activity.

Methods:

The T , B cell lines and hybridomas were procured either from American Type Culture Collection, Rockville, MD,USA or from National Centre for cell Science, Pune, India They were grown in appropriate culture media and were used for assay of APase activity as described in materials and methods

Results:

Mitogen induced proliferative response and APase activity of human PBL:

Human PBL were cultured with and without various T and B cell mitogens for 72 hours A significant increase in the proliferative response was observed with T cell mitogens PHA and staphylococcal aureus protein A and B cell mitogen Seph.anti-IgM (Fig 26). However, no enhancement of APase activity was detectable(Fig 27).

Screening of Cell lines:

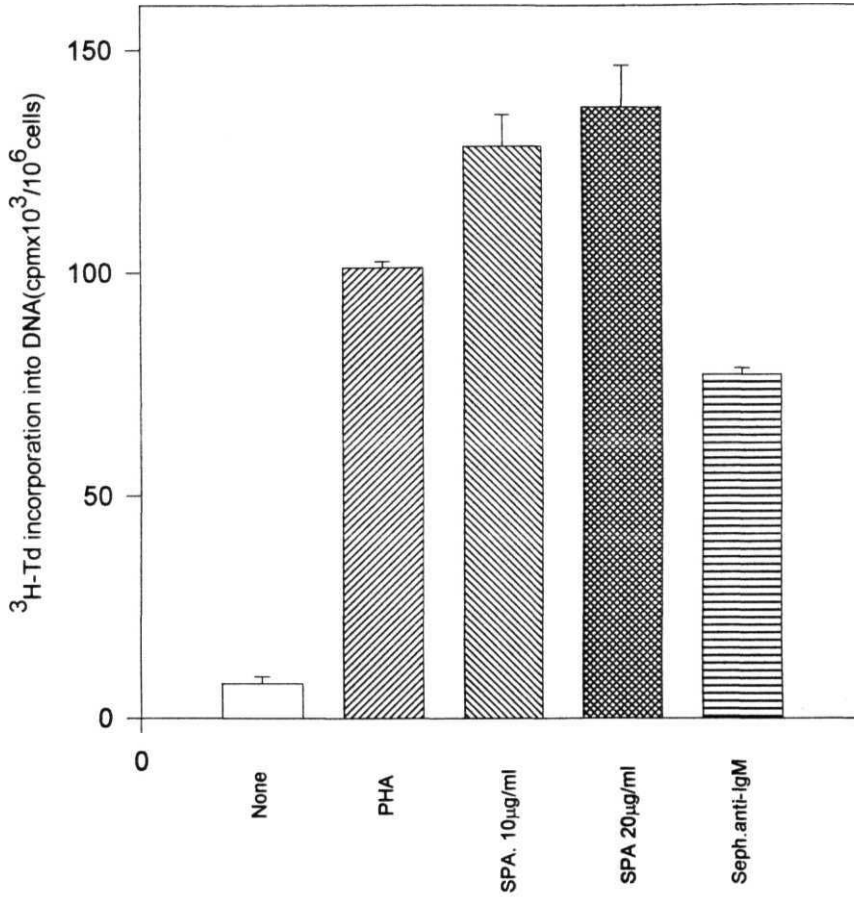
Of the various T and B cell lines screened, significant level of APase activity was observed in all B cell lines T cell lines with the exception of YAC-1 (a murine lymphoma) showed very low level of APase activity. The hybridomas OKT-4 and OKT-8 expressed APase activity in high amounts (Table 22).

Discussion:

Human peripheral blood lymphocytes stimulated with various T and B cell mitogens did not show expression of APase activity, even though there was a significant enhancement in the proliferative response. It has been shown earlier that use of serum

Figure -26

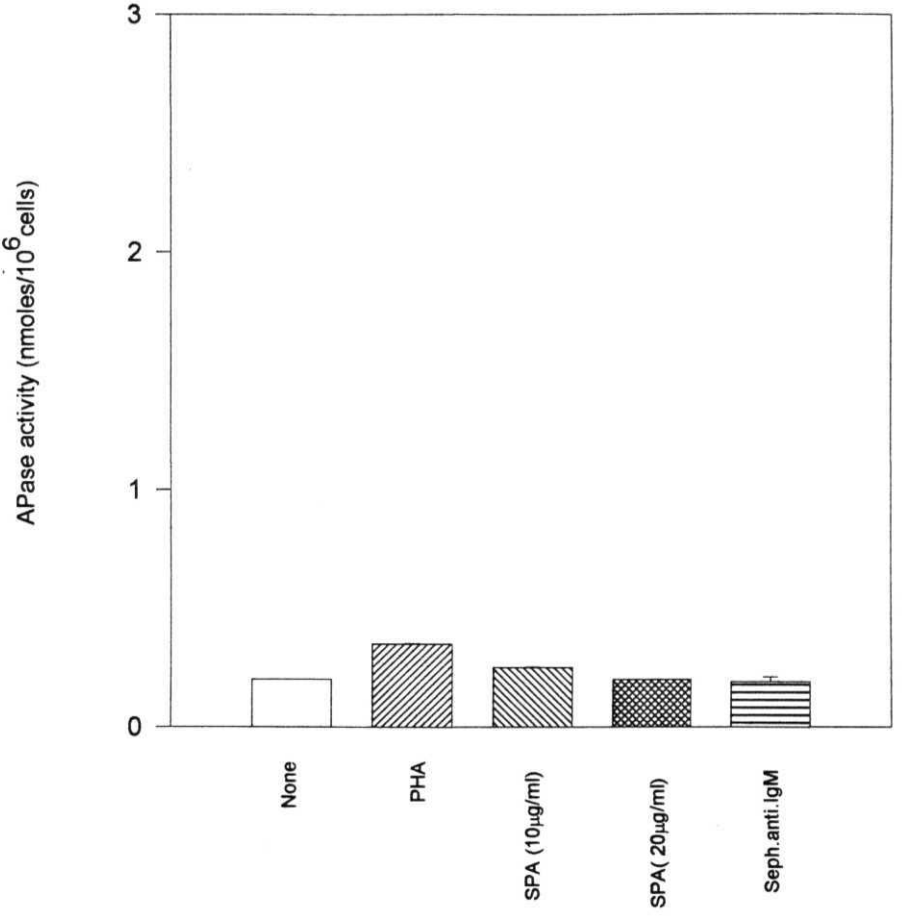
Effect of various mitogens on proliferative response of Human peripheral blood lymphocytes



Values represented are Mean ± SEM of five experiments

Figure -27

Effect of mitogens on APase activity of Human peripheral blood lymphocytes



Values represented are Mean± SEM of five experiments.

Table -22

Screening of cell lines for APase activity

Name	origin	APase activity (nmoles/10^6cells)
T cell lines		
YAC-1	Murine	205.0
PM-1	Human	10.8
SupT-1	"	12.0
Jurkat	"	16.0
CRIT	"	103
B cell lines		
HS-Sultana	Human	90.0
Daudi	"	60 0
Hybridomas		
OKT-4	Munne	268 5
OKT-8	"	295 0

Values represented are Mean of triplicates.

APase levels as diagnostic marker for cellular dysfunction and malignancies Elevation of serum APase levels have been reported in hepatic obstruction, hepatoma and in osteosarcoma (Kay 1930, Franseen and McLean 1935) Jacobs and Haskell (1991) have defined that a perfect tumor marker would be one that is produced solely by the tumor and secreted in measurable amounts in body fluids The marker should also be - 1) measurable in presence of cancer only (useful in screening), 2) in quantitative amounts in body fluids in order to reflect the bulk of tumor and 3) a useful prognostic indicator of the disease While at present no such ideal marker exists, eventhough a number of useful tumor markers are available. Amongst the APase isozymes , placental APase(PLAP) and germ cell APase(GCAP) come closest to the definition given above and therefore have been evaluated extensively in various malignant conditions. Tissue non-specific APase due to its ubiquitous nature fails to serve as a marker of primary malignancy and its elevation is often taken as a confirmatory finding.

Increased PLAP activity has frequently been found in serum samples from ovarian cancer patients and testicular cancer patients(Vergote et al 1987, De Broe et al 1988, Lange et al 1982) Plasma TNAP level(particularly heat labile bone derived fraction) has long been recognized as an indicator of oesteoblastic activity (Lung et al 1993). Oesteosarcomas display high serum TNAP levels and these levels have been shown to be higher in metastatic disease than in patients with localized disease (Bacci et al 1993). Leukocyte APase (TNAP isozyme) has been shown to serve as a useful marker in cases of advanced lung cancer (Walach and Gur 1993)

Different mechanisms suggested for the enhanced APase expression in tumor cells have been :

1. Functional involvement of APase isozymes in tumourogenesis,

2. Representing one crucial factor in a multi factorial etiology,

3. A close linkage of APase gene with disease susceptibility gene,

4. Simultaneously deregulation with the disease susceptibility gene, and

5. Result of random chromosomal aberrations

Cox and MacLeod (1961) have shown that corticosteroids added to the growth medium of HeLa cells increased the APase activity by de novo synthesis of APase protein (Hamilton and Sussman 1981, Hanford et al 1981)

Humans peripheral blood B lymphocytes do not express APase activity even after mitogenic activation. However, the various cell lines tested in the present study as well as earlier reports show that human B cell lines and leukemic B cells express APase activity but not T cell lines and T cell leukemias . These results strongly suggest that APase activity could be used as an important marker for screening malignancies of B lymphoid lineage in humans.

SUMMARY AND CONCLUSIONS

Murine splenic lymphocytes expressed alkaline phosphatase activity upon stimulation with B cell mitogens, LPS, DXS and Seph anti-IgM However, APase activity was not observed in splenic lymphocytes stimulated with T cell mitogens ConA and PHA The enzyme activity was barely detectable in resting or unstimulated B lymphocytes Both resting and activated T lymphocytes also do not express APase activity In mitogen stimulated cells, enhancement in APase activity was observed in cells committed to proliferation during G1 phase of the cell cycle *In vivo* immunization of mice with an antigen, Tetanus toxoid (TT) showed an enhancement in B lymphocyte proliferation concomitant with an enhancement in APase activity, when cultured *in vitro* with TT. The number of cells stained positive for APase activity was also increased indicating that enhancement of APase activity was due to the enhancement in the number of APase positive cells but not due to mere increase in the catalytic activity of the enzyme. The results of these experiments suggested that expression of APase activity in activated B lymphocytes is a physiological phenomenon

Addition of IL-4 to mitogen stimulated splenic lymphocytes showed an enhancement in the proliferative response and APase activity. In DXS stimulated cells, addition of IL-4 resulted in increase in proliferative response without any enhancement in APase activity. The enhancement in the proliferative response and APase activity was specific to IL-4 when added during Gl phase APase activity was not expressed in lymphocytes stimulated of with PMA which arrests them in Gl phase. Addition of IL-4 to these cells resulted in the progression of cells in to 'S' phase and also resulted in significant increase of APase activity. Addition of EL-2 to mitogen stimulated cells had no effect on proliferative response as well as APase activity. These results suggested that

IL-4 may be acting as a requisite signal for the expression of APase activity in activated B lymphocytes committed to proliferation.

Treatment of LPS stimulated cells with Levamisole, an inhibitor of type 1 APase resulted in inhibition of proliferative response and APase activity without affecting the cell viability. At 750uM concentration, Lev reversibly inhibited the proliferative response and APase activity in LPS stimulated cells The number of cells stained positive for APase activity in Lev treated cells was also significantly decreased compared to LPS stimulated cells Hydroxyurea, a cell cycle inhibitor which arrest the cells at G1/S boundary, inhibited proliferation in LPS stimulated splenic lymphocytes However, APase activity remained unaffected in these cells Whereas, treatment of YAC-1 cells with Lev resulted in only 20-30% inhibition in proliferation and very marginal inhibition in APase activity. On the other hand HU inhibited the proliferation up to 60% in YAC-1 cells concomitant with an inhibition in APase activity.

The above observations show that the expression of APase is not dependent on cell cycle progression in malignant YAC-1 cells . On the other hand, APase expressed in mitogen stimulated B cells plays a crucial role in cell cycle progression.

B lymphocyte APase belongs to Type 1 APase isozyme category and resembles liver APase in its properties. Hence, in order to use it as an antigen, APase was purified from mouse liver and characterized APase was also purified from YAC-1 cells, a T cell lymphoma which expresses APase activity constitutively The partially purified APase from YAC-1 was compared with B cell APase in terms of heat stability, inhibition by aminoacids, L-homoarginine, L-histidine and the uncompetitive inhibitor, Lev. Antibody to mouse liver APase was prepared in rabbits. The antibody reacted with partially purified

mouse liver APase and B lymphocyte APase but did not cross-react with partially purified YAC-1 APase This antibody was characterized further and was used for studying the role of APase in activated B lymphocytes.

Treatment of LPS stimulated splenic lymphocytes with anti-APase antibody resulted in inhibition in proliferative response and APase activity up to 94% and 90% respectively The viability in these cells was not affected Delayed addition of anti-APase antibody at 24h after initiation of culture resulted in a significant reduction in the inhibitory effect on proliferation and APase acivity. On the other hand, addition of anti-APase antibody to ConA stimulated splenic lymphocytes did not show any significant change in proliferation APase was released in to the culture supernatant of mitogen stimulated splenic lymphocytes However, no release of APase was observed in case of YAC-1 cells Using ELISA, it was found that IgM and APase get released in to the supernatant as a complex Also treatment of LPS stimulated cells with anti-APase antibody resulted in inhibition of release of APase in to the culture supernatant. These results suggest that APase plays a very important role in the proliferation and differentiation of mitogen stimulated B lymphocytes

Stimulation of human peripheral blood lymphocytes with PHA, ConA, Seph. anti-human IgM and Staphylococcal protein A resulted in enhancement of proliferative response with no significant increase in APase activity. Screening of various T and B cell lines of murine and human origin and hybridomas showed that only B cell lines and hybridomas express APase activity APase activity was not detectable in T cell lines with the exception of YAC-1. Based on these results it is suggested that APase activity can be

used as a potential marker for the identification of lymphoid malignancies of B cell lineage

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PUBLICATIONS AND ABSTRACTS

FROM THE THESIS

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Immunology Letters 61 (1998) 175-178

Immunology Letters

Alkaline phosphatase activity is expressed in murine splenic B-lymphocytes sensitized in vivo with Tetanus toxoid

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Abstract

Alkaline phosphatase (APase) activity and proliferative response to Tetanus toxoid (TT) were measured in murine splenic lymphocytes immunized in vivo with TT. APase activity was enhanced in TT-stimulated B-lymphocytes concomitant with an increase in the proliferative response in a dose-dependent manner. Cytochemical staining for APase using β-naphthyl phosphate also showed an increase in APase positive cells in TT-stimulated lymphocyte population. The results suggest that membrane APase expression is a physiological phenomenon occuring in antigen-stimulated B-lymphocytes.

Keywords: Alkaline phosphatase; B-lymphocytes; Tetanus toxoid; in vivo sensitization

1. Introduction

Alkaline phosphatase (APase, E.C.3.1.3.1) is expressed on the membranes of mitogen-stimulated lymphocytes and has been suggested as a marker of B-lymphocyte activation and differentiation [1 5]. APase activity is not detectable in resting B-lymphocytes and in resting and mitogen-stimulated T-lymphocytes. Burg and Feldbush [2] reported that enhancement of APase activity correlated closely with B-cell differentiation rather than proliferation, and precedes maximal immunoglobulin (Ig) secretion. Mitogens, a group of polyclonal stimulators have been used in vitro to study the mechanism of lymphocyte activation. Mitogen induced lymphocyte activation has been suggested to mimic the antigen-stimulated lymphocyte activation in vivo [6]. Enhancement of APase activity in mitogen-activated B-cells occurs around 8 h after mitogenic stimulation and shows a continuous increase until 120 h [5]. cAMP has been shown to be a positive regulator of APase activity in mitogen-stimulated B-cells [7]. However, APase activity could not be induced in B-cells stimulated with incomplete mitogens [8]. In order to examine whether lymphocyte activation in vivo, following antigenic stimulation, results in enhancement of APase activity of sensitized lymphocytes, proliferative response and APase activity were determined in splenic lymphocytes obtained from mice immunized with Tetanus toxoid (TT).

2. Materials and methods

2.1. Mice

Male C57BL/6J mice of 8-12 weeks of age, obtained from the National Institute of Nutrition, Hyderabad, were used in all the experiments.

2.2. Chemicals

RPMI-1640 medium, penicillin G, *p*-nitrophenyl phosphate sodium salt, lipopolysaccharide (LPS, from

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Table 1
Proliferative response and APase activity of mitogen-stimulated splenic lymphocytes

Mitogen	Concentration (μg/ml)	[3H]thymidine incorporation (cpm/10^6 cells)	APase activity (nmol/10^6 cells)
None		25 670 ± 980	11.7 ±1.5
LPS	50.0	227 340 ± 8905	206.2 ±8.5
Con A	2.0	220 000 ± 8040	10.0 ± 1.0

Values presented are mean + S.E.M. of four experiments. [3H]thymidinc incorporation and APase activity were measured at 48 h as described in Section 2.

E. coli), concanavalin A (Con A), goat anti-mouse Ig G (H and L specific), goat anti-mouse IgM (//-chain specific), histopaque {*d*= 1.077) and incomplete Freund's adjuvant (IFA) were purchased from Sigma, St. Louis, MO. TT was a generous gift from Biological Evans, Hyderabad. [3H]thymidine (specific activity 6.5 Ci/mmol) was purchased from BRIT, India.

2.3. Immunization

Mice were immunized intraperitoneally with 20 //g of TT in IFA and a booster dose was given on day 15 with 10 μg of TT in IFA. They were sacrificed 2 weeks later.

2.4. Lymphocyte preparation

Mice were sacrificed under mild ether anaesthesia and the spleens were dissected and put in RPMI-1640 medium. The single cell suspension was prepared as described earlier [9]. The lymphocyte-rich population was prepared by density gradient centrifugation on histopaque. The cells at the interface were collected and washed three times with RPMI-1640 medium containing 5% FCS and 50 μM 2-mercaptoethanol (complete medium). Highly enriched B-cells were isolated by 'panning', using plates coated with anti-Ig antibodies (goat anti-mouse IgM or IgG) at 40°C [5]. The percentage of Ig + cells was always more than 90% in the adherent population. Adherent and non-adherent fractions were prepared using splenocytes stimulated with TT for 72 h.

2.5. Proliferative response

Cells (2 x 10^5) in 0.2 ml complete medium were cultured in 96-well flat-bottomed microtitre plates (Tarsons, India) with different concentrations of TT or mitogen. Lymphocyte cultures were pulsed with 0.5 μCi of [3H]thymidine for the last 24 h of a 48-h culture period. The cultures were processed using a Skatron automatic cell harvester. Radioactivity was measured using a Beckman Scintillation counter. The results were expressed as cpm/10^6 cells.

2.6. APase assay

APase activity was measured using 2.5 mM *p*-nitrophenyl phosphate as substrate in 0.1 M bicarbonate buffer pH 9.8 containing 2 mM $MgCl_2$ as described earlier [5]. In brief, lymphocyte cultures were centrifuged at 400 x *g* for 5 min and the supernatant was removed. The cell pellet was washed once with saline and to the cell pellet 180 μl of substrate solution was added; the microtitre plate was then incubated at 37°C in a humidified incubator. The reaction was stopped by the addition of 20 μl of 1 N NaOH and the absorbance was measured at 405 nm using a UV max ELISA reader. The enzyme activity was expressed as nmoles of *p*-nitrophenol released/10^6 cells/30 min.

2.7. Enzyme cytochemical staining

Splenic lymphocytes were stained for APase positive cells by incubating cells with β-naphthyl phosphate as substrate and fast blue RR in 0.1 M bicarbonate buffered saline for 5 rain at 37°C. The excess stain was removed by washing the cells with saline. At least 300 cells were counted in each sample using an inverted microscope at a magnification x 60 and was expressed as percentage of total cells.

2.8. Statistical analysis

The data were analysed for statistical significance using Student's /-test.

3. Results

Spleinc lymphocytes showed an increase in the proliferative response when stimulated with T- and B-cell mitogens like Con A and LPS when compared with controls. However, an increase in APase activity was observed only in splenic lymphocytes stimulated with B-cell mitogen LPS, but not in those stimulated with T-cell mitogen, Con A (Table 1). Enzyme cytochemical analysis of LPS stimulated splenic lymphocytes also showed a significant increase in APase positive cells as compared with controls ($P < 0.001$, Table 2). However,

Table 2
Enzyme cytochemical analysis of TT-stimulated lymphocytes

Cell population	Addition	Concentraton (μg/ml)	APase positive cells (%)	A
Splenocytes	None	-	7.0 ± 1.0	
Splenocytes	**LPS**	50.0	41.0 ±1.0*	34
Splenocytes	TT	1.0	12.0 ±1.0*	5
Splenocytes		5.0	17.0 ±1.0*	10
Adherent[a]	**None**	-	4.0 ± 1.0	
Adherent	TT	5.0	35.0 ± 2.0*	31
Non-adherent"	**None**	-	3.0 ± 1.0	
Non-adherent	TT	5.0	5.0 ±1.0	2

Values presented are mean + S.E.M. of four experiments. Lymphocytes were stained for APase activity as described in Section 2.
[a] Adherent and non-adherent fractions were prepared from splenic lymphocytes stimulated with TT for 72 h.
* $P < 0.001$, LPS and TT vs. no addition. At least 300 cells were counted for each sample.

no APase positive cells were detectable in Con A-stimulated splenic lymphocytes or thymocytes. These results confirm our earlier observation that enhancement of APase activity occurs only in B-cells committed to proliferation.

Splenic lymphocytes from in vivo immunized mice when cultured with TT in vitro showed a significant increase in proliferative response (Fig. 1, $P < 0.001$) concomitant with an increase in the APase activity (Fig. 2, $P < 0.05$). The increase observed was dose-dependent and TT-specific, as no increase in proliferative response and APase activity was seen in splenic lymphocytes from IFA-treated mice (data not shown). Enzyme cytochemical staining for APase activity in intact cells stimulated with LPS and TT showed a significant increase in the number of APase positive cells compared with controls ($P < 0.001$, Table 2). Further, adherent (B-cell enriched) and non-adherent (T-cell enriched) fractions were prepared using splenic lymphocytes stimulated with TT for 72 h and were analysed for APase activity by cytochemical staining. APase positive cells were predominantly present in the adherent fraction as compared to non-adherent fraction ($P < 0.001$, Table 2).

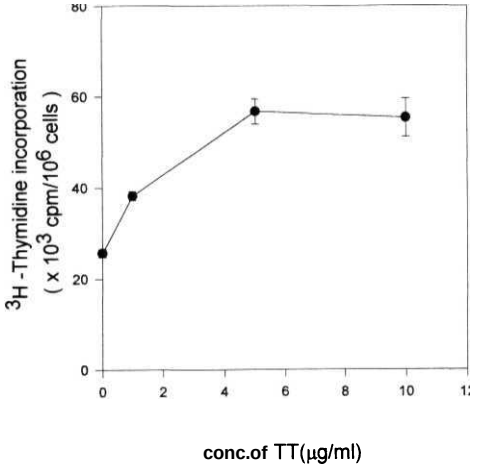

Fig. 1. Proliferative response of TT-primed splenic lymphocytes. Splenic lymphocytes from TT-primed mice were cultured with TT as indicated. The cultures were pulsed with 0.5 μCi of [3H]thymidinc for the last 24 h of 48-h culture as described in Section 2. The data presented are mean + S.E.M. of five experiments. $P < 0.001$, at all concentrations of TT vs. no addition.

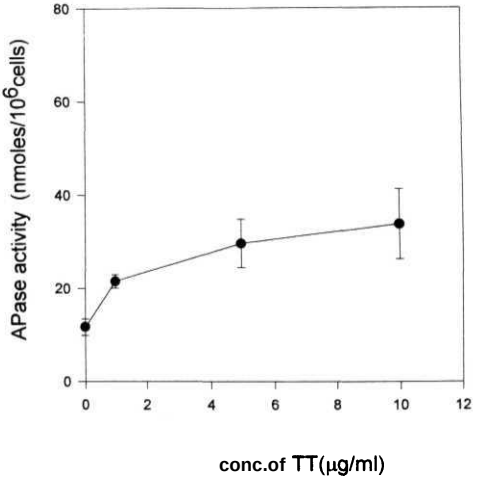

Fig. 2. Alkaline phosphatase activity of TT-primed splenic-lymphocytes. Alkaline phosphatase activity was measured at 48 h as described in Section 2. The data presented are mean + S.E.M. of five experiments. $P < 0.05$, at all concentrations of TT vs. no addition.

4. Discussion

In the present study, TT, a T-dependent antigen was chosen for in vivo immunization. In vivo antigenic stimulation resulted in a significant enhancement of APase activity which is accompanied by an increase in the proliferative response.

Earlier, it has been shown that mitogen-induced B-lymphocyte proliferation is associated with an enhancement of APase activity [4,5]. Further, it has been demonstrated that APase activity is induced only in B-cells upon mitogenic stimulation of B-cell enriched and T-cell enriched fractions of murine splenic lymphocytes [8]. Hence, the induction of APase activity occurs only in B-lymphocytes but not in T-lymphocytes. In in vivo sensitized splenic lymphocytes, paralleling the increase in the APase activity it has been observed that there is an increase in the APase positive cells upon enzyme cytochemical staining using /i-naphthyl phosphate. The enhancement in APase activity as well as increase in APase positive cells could be seen in B-cell enriched fraction but not in the T-cell enriched fraction. These results strongly suggest that the expression of APase activity occurs only in antigen activated B-cells but not in T-cells. Earlier, Tokuda et al. [10] have demonstrated the appearance of APase positive areas in the lymph nodes and spleens of immunized animals by enzyme histochemical analysis.

Although an increase in the expression of APase in activated B-lymphocytes has been demonstrated, its physiological role has not yet been understood. Based on the probable functions of APase in the other tissues, it has been suggested that increase in APase activity is an integral feature of B-cell activation and differentiation and perhaps aids in the metabolite transport to fulfil the demands of the growing cell [11]. It could also be involved in the Ig transport in B-lymphocytes as it is shown to be complexed and secreted out along with IgM [12]. In view of its pleiotropic role, the enhancement of APase activity appears to be a physiological phenomenon in activated B-lymphocytes upon antigenic stimulation. Further experiments are in progress on immunobiology of APase in activated B-cells.

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ABSTRACTS

XXIII
ANNUAL CONFERENCE OF
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SEMINAR ON
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&

SYMPOSIUM ON
"THE IMPORTANCE OF MEDICINAL PLANTS IN
IMMUNOLOGY & BIOTECHNOLOGY"

IA-89 PURIFICATION AND CHARACTERISATION OF ALKALINE PHOSPHATASE FROM *YAC-1,* A T CELL LYMPHOMA

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YAC-1, a murine T cell lymphoma constitutively expresses high alkaline phosphatase (APase) activity. APase has been partially purified from *YAC-1* cells by multi-step protocol involving sonication, Triton X-1000 solubilization, DEAE-cellulose column chromatography, acetone precipitation and gel filtration on Sephacryl S-200. Enzyme activity was estimated using p-Nitrophenly phosphate as a substrate at pH 9.8. the apparent molecular weight of the partially purified enzyme was determined using native polyacrylamide gel electrophoresis (PAGE) and activity staining on Gel. The subunit molecular mass was determined by SDS-PAGE. Isozyme characterisation has been carried out in terms of heat stability, inhibition by amino acids and using levamisole, an uncompetitive inhibitor. Immunoiogical cross-reactivity of the partially purified APase was checked with antibody to rat liver APase. The results of the above experiments will be presented and discussed.

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ABSTRACTS

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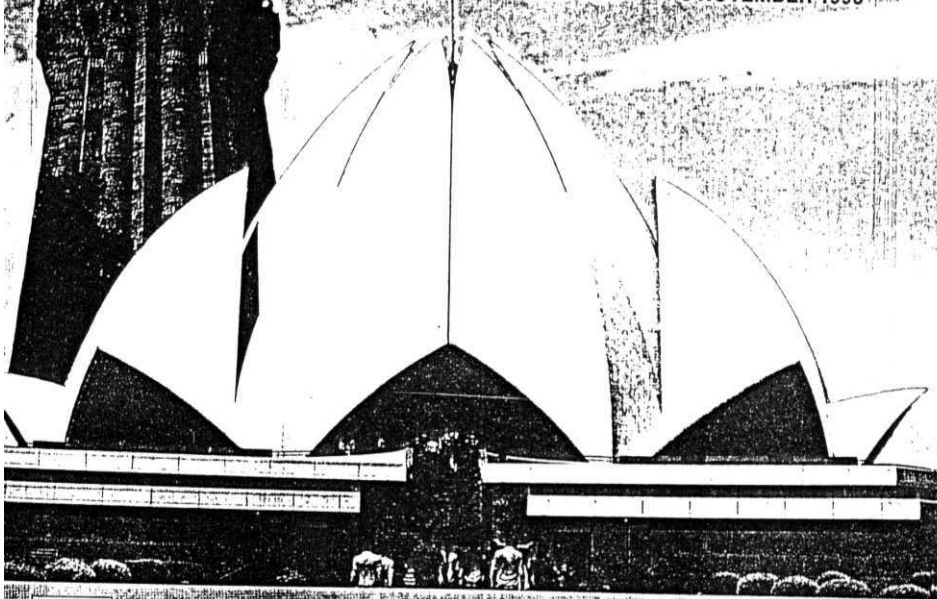

Hogrefe & Huber Publishers

1998
Supplement 1

REGULATION OF B LYMPHOCYTE SPECIFIC ALKALINE PHOSPHATASE ACTIVITY BY IL-2 AND IL-4. K.Padmaja and M.Ramanadham. Univ.of Hyderabad , Hyderabad - 500046, INDIA.

Alkaline Phosphatase (APase, E.C.3.1.3.1) is a membrane bound metalloenzyme expressed by mitogen -stimulated B lymphocytes and is suggested to be a marker of B lymphocyte activation and differentiation. APase is not dctectable in resting B lymphocytes or in resting and mitogen -activated T lymphocytes. cAMP has been shown to be a positive regulator of APase activity in mitogen -stimulated B lymphocytes . As mitogen -activated B cells require interleukins for their cell cycle progression , the effect of reoombinant IL-2 and EL-4 on the enhancement of APase activity of mitogen -stimulated murine splenic B lymphocytes was studied.The addition of IL-4 to LPS-stimulated and Sepharose anti-Ig (Seph.anti-Ig) stimulated splenic lymphocytes showed an enhancement of APase activity concomittant with an increase in prolirerative response , However, in dextran sulfate (DXS) stimulated B cells, an increase in the proliferative response was observed with no enhancement in the APase activity. Addition of IL-2 to LPS, Seph.anti-Ig and DXS stimulated B lymphocytes had no effect on the proliferative response and APase activity.The results strongly suggest that EL-4 acts as a potent second signal in the induction of APase activity in mitogen- stimulated B lymphocytes.