IN VITRO IMMUNIZATION USING IMMOBILIZED IMMUNOGENS

A Thesis Submitted for the degree of Doctor of Philosophy

by

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DECLARATION

I, N. SAI GEETA declare that the work presented in my thesis has been carried out by me under the supervision of Dr. M Ramanadham, and has not been submitted for any degree of any other University.

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CERTIFICATE

This is to certify that **N. SAI GEETA** has carried out the research work embodied in the present thesis entitled " **IN VITRO IMMUNIZATION USING IMMOBILIZED IMMUNOGENS** " under my supervision and guidance for the full period prescribed under the Ph.D ordinance of this University. I recommend her thesis for the submission for the degree of Doctor of Philosophy of this University.

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CONTENTS

ABBREVIATIONS

AFC	Antibody forming cell
BCDF	B cell differentiation factor
BCGF	B cell growth factor
Con A	Concanavalin A
CFA	Complete Freund's adjuvant
ELISA	Enzyme linked immunosorbent assay
FCS	Fetal calf serum
FIPA	Filter immunoplaque assay
FITC	Fluorescein Isothiocynate
HRPO	Horse radish peroxidase
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
Leu-Leu-OMe	Leucyl-Leucine-O-Methyl ester
MDP	Muramyl dipeptide
OVA	Ovalbumin
PBS	Phosphate buffered saline
PBL	Peripheral blood lymphocytes
PMA	Phorbol-12-myristate-13-acetate
POPOP	l-4-Bis[2-(5-Phenyloxazolyl) benzene]
PPO	2,5-Diphenyloxazole
PWM	Pokeweed mitogen
SDS-PAGE	SDS-Polyacrylamide gel electrophoresis
sIg	Surface immunoglobulin
SRBC	Sheep red blood cells
TT	Tetanus toxoid

CHAPTER -I

GENERAL INTRODUCTION

Acquired immune system is characterized by recognition of self from non-self, specificity and memory. It comprises of the major cell types T and B lymphocytes and monocytes (macrophages). Lymphocytes are made up of enormous number of diverse clones, each distinguished by the ability to recognize only one or a few antigens. B lymphocytes and T lymphocytes differentiate into immunologically competent cells in bone marrow and thymus respectively.

Essential function of the acquired immune system is to defend the living organism against infections. A foreign molecule which elicits an immune response is called an immunogen and the specific effector molecule that is produced in response to an immunogen is called an antibody. The immune response to an antigen can either be antibody-mediated (humoral) or cell-mediated immune response.

Cell-mediated response is a self surveillance system designed to detect and eliminate the abnormal cells or cells perceived as abnormal. Cell-mediated immune response is brought about by T lymphocytes. In these reactions the triggering event is the recognition of an antigenic structure on a target cell or a processed antigen on the antigen presenting cell by a T cell. The T cell recognizes the processed antigens in association with the MHC antigens. The T cell responds by secreting lymphokines which stimulate the macrophages to release substances that cause inflammation and the destruction of the target cell or they respond directly by destroying only those cells whose surface antigen they recognize.

The humoral response involves protection against the non-cell associated invasions. It operates via the antigen recognition, proliferation of antigen-specific B lymphocytes and their subsequent maturation to antibody (immunoglobulin) secreting cells. This process is regulated by a number of different soluble mediators called cytokines.

B cells have an inbuilt unique capacity to recognize and respond selectively to immunogenic substances due to the presence of antigenic receptors on their cell surface. These antigenic receptors are indigenously produced surface immunoglobulins (sIg). The major classes of sIg seen on the mature B cells are IgM and IgD. Other markers present on the surface of the B cells are complement receptors, class II MHC products etc. The function of B cells primarily is to respond to the antigen by proliferation and differentiation into antibody secreting cells. Upon stimulation with antigen, appropriate B cell clones get activated and undergo mitosis. In suitable micro-environment some of the descendants follow plasma cell sequence, synthesizing and liberating, first IgM and later IgG antibodies. Other descendants take the form of memory cells, which persist as carriers of immunological memory (Sprent, 1977).

Antigen specific activation of B cells :

It occurs in three sequential steps, *viz*., activation, proliferation and differentiation. These three steps can experimentally be analyzed separately (Noelle *et al*., 1984; DeFranco *et al,* 1987).

Activation :

Antigen binds to the B cells expressing one of the diverse sets of sIg receptors. Antigen binding causes antigen specific receptors on B cells to become crosslinked, an arrangement supposed to trigger cell activation (Bretscher and Cohn, 1970). B cell activation has been examined by looking at the early events such as changes in membrane phospholipid metabolism, calcium influx, membrane protein phosphorylation, and intracellular pH etc., which occur within minutes after ligand-receptor interaction (Grupp and Harmony, 1985). Further progression of activated cells has been monitored by events such as, expression of MHC class II antigens, IL-2 receptors *etc.,* which occur within few hours after antigenic stimulation (Freedman *et al*., 1984; Kehrl *et al*., 1984; Tedder *et al.,* 1985).

Proliferation :

Stimulated cells proliferate and provide a mechanism whereby the antigen specific cells can be expanded to permit the generation of an effective antibody response. It has been shown that differentiation occurs during proliferation as an ongoing proliferation requiring process, rather than as an actively cycling one (Jelinek and Lipsky, 1985). The proliferation of Ig secreting cells after initial differentiation increases the magnitude of the immune response and it may also be important in promoting heavy chain isotype switching (Cebra *et al*., 1984). Cell proliferation is usually monitored by measuring the incorporation of ^{3}H-thymidine into DNA.

Differentiation :

The final step in B cell life cycle is differentiation and maturation into plasma cell. The progression from activated B-lymphocyte is accompanied by a gradual loss of surface Ig with the conversion from synthesis of membrane type to secretary Ig molecules. B cell differentiation is measured in terms of the number of antibody-secreting cells.

Role of T_h cells and cytokines :

The process of B lymphocyte activation is regulated by T cells. There are two major subsets of T cells, T helper cells (T_h)and cytotoxic T cells (T_c), characterized by structurally related cell surface glycoproteins designated as CD4 and CD8 respectively. Mature T cells express only CD4 or CD8 whereas, immature T cells can express both (double positive) or neither (double negative). There are two classes of T_h cells T_h1 and T_h2 cells expressing the CD4 surface antigen. Both of them recognize the epitopes of the processed antigen on the antigen presenting cells and respond by secreting mediators called lymphokines. T_h2 cells are principally helper T cells for B cells *i.e.,* their interaction with the B cells promotes B cell proliferation and secretion of Ig. The helper effect is exerted via the lymphokines IL-4, IL-5 and IL-6 secreted by them. T_h1 cells do not

produce the above said lymphokines but instead secrete others such as, y-interferon (IFN-γ) which promotes inflammatory response and T cell growth factor, IL-2.

IL-4 stimulates a B cell before it enters the cell cycle (Rabin *et al.*, 1985), and causes enhanced expression of a number of cell surface markers which include MHC class II and CD23 as well as an increase in the cell volume (DeFranco *et al.*, 1987). IL-1 and Tumor necrosis factor (TNF) have also been reported as enhancing factors for B cell activation (Mahoney *et al.*, 1985; March *et al.*, 1985).

B cell growth and proliferation are brought about by application of two independent signals (Hamblin *et al.*, 1987) a polyclonal activator *e.g.*, Staphylococcus aureus Cowan strain I (SAC) in human or anti-Ig in mouse, followed by a potential growth factor. The growth factors reported to cause proliferation are IL-2, and IL-4, (Hamblin *et al.*, 1987; Gordon and Guy, 1987). Mouse IL-5 also stimulates proliferation, but, human IL-5 fails to do so (Sanderson *et al.*, 1988).

Most of the factors which stimulate B cell proliferation have been reported to also induce B cell differentiation (Gordon and Guy, 1987). In addition IL-6 has been reported to be a differentiation factor for human B cells, without causing stimulation of growth (Hirano *et al.*, 1986).

Antibody response *in* *vivo* :

When an animal is immunized *in vivo* by the administration of sufficient amount of antigen for the first time, it will respond by forming antibodies. This is called primary response which is characterized by the appearance of predominantly IgM antibodies after a lag period, followed by a peak response and decline of antibody titre in plasma. Upon subsequent administration of the same antigen, the animal will show a secondary response which is characterized by a rapid and higher response with predominantly IgG antibodies compared to the primary response. After the peak has been reached there is a slow decrease in the

Ig titre in the plasma. Moreover, antibodies produced during secondary response are of a different isotype and have a higher affinity than those in the primary response.

when an animal is immunized *in vivo* with a specific antigen, antibodies produced are directed against many epitopes present on that antigen and are produced by several clones of B cells and hence are polyclonal antibodies. They have a range of affinities and show cross reactivity and hence their use in immunotherapy is also limited. To circumvent the problems encountered with the polyclonal antibodies, highly specific antibodies called monoclonal antibodies are produced which are directed against a single epitope on the antigen. These antibodies are produced by hybridomas which are formed by the fusion of antibody forming cells with an appropriate tumor B cell line. Monoclonal antibodies produced by a given hybridoma are all identical interms of Ig class, affinity and specificity for a given epitope.

Preparation of monoclonal antibodies is often limited by failure to stimulate adequate number of antigen-specific B lymphocytes during *in vivo* immunization procedure. This failure may be due to tolerance (antigen-specific non-responsiveness) or to an antigen hierarchy response (selective responsiveness to one or a few components of the immunogen preparation) rather than to a lack of antigen reactive precursor cells. Following immunization with low levels of antigen, animals with high serum titers of specific antibodies may fail to yield desired hybridomas because of low frequency of sensitized cells in the lymphocyte population used for fusion. Immune response is not elicited towards autoantigens and evolutionarily conserved antigens *in vivo.* Also, only very few antigens can be used for immunization of humans *in vivo.* To circumvent these problems, *in vitro* immunization has been developed.

In vitro immunization :

In vitro immunization is defined as a primary antigen-specific activation of cultured B cells resulting in clonal expansion and maturation of specific antibody producing lymphocytes.

Production of antibodies *in vitro* :

Primary antigen-specific stimulation of dissociated mouse spleen cells *in vitro* was first demonstrated by Mishell and Dutton (Mishell and Dutton, 1966, 1967) and Marbrook, (1967), using Sheep red blood cells as immunogen and the antibody response was measured using Jerne's hemolytic plaque assay. This was used to study the factors that control and regulate the cellular events in the immune response. Response to certain antigens (thymus-dependent) was found to require cooperative interactions between two classes of lymphocytes, thymus-derived T lymphocytes and bone marrow-derived B lymphocytes (Claman *et al.*, 1966).

Development of *in vitro* immunization :

The description of different lymphocyte populations in the 70's and the increasing knowledge and availability of cytokines in the 80's have led to the development of immunization. Since then, a number of reports have been published describing monoclonal antibodies produced against a variety of antigens using immunization of mouse spleen cells.

Hybridoma technology for the production of monoclonal antibodies:

The revolutionary new technology for monoclonal antibody production by hybridization of spleen cells from an immune mouse with murine myeloma cells was first reported by Kohler and Milstein (1975). The first successful attempt to produce monoclonal antibodies using *in vitro* immunized spleen cells was reported in 1978 (Hengartner *et al.*, 1978). Two years later, a T cell dependent protein antigen was used as immunogen (Luben and Moller, 1980). First report on

production of human monoclonal antibodies derived from *in vitro* immunization against SRBC appeared in 1984 (Strike *et al.,* 1984).

Human monoclonal antibody technology is expected to be a valuable tool in diagnostics and therapeutics, as well as to increase our knowledge of the B cell repertoire in health and disease (Dorfmann, 1985; Larrick and Bourla, 1986, Carson and Freimark, 1986; James and Bell, 1987). Antibody-dependent immunotherapy based on mouse monoclonal antibodies has shown a low therapeutic efficacy against tumours in a majority of clinical studies. One of the major reasons for this is the hunian immune response against the foreign proteins *i.e.,* the production of human anti-mouse antibodies (HAMA). Production of HAMA and the subsequent reduced efficacy of the monoclonal antibody is considered to be most serious obstacle to human immunotherapy based on xenogenically derived monoclonal antibodies. Human, monoclonal antibodies have been produced against a variety of antigens using lymphocytes from patients sensitized *in vivo.* This dependence on *in vivo* immunized lymphocytes severely limits the number of human monoclonal antibody specificities that can be obtained. Very few antigens (Killed microorganisms, blood group or histocompatability antigens) can be used for deliberate *in vivo* immunization of humans due to ethical and legal considerations. Thus the development of *in vitro* immunization would overcome this serious limitation in human hybridoma technology and allows the production of human monoclonal antibodies against therapeutically valuable antigens *e.g.,* antigenic phenotype of malignant human cells, polymorphic epitopes on histocompatability antigens and other determinants which the mouse system neglects (Borrebaeck, 1988; Effros *et al.,* 1986).

Cytokines involved in the antigen-specific activation of B cells in an *in vitro* culture system :

The *in vitro* culture system adopted by Dutton (1967) and Fishman (1969) using mouse lymphocytes, and those by Mann and Falk, (1972) and Hoffmann *et al.,* (1973) using human cells allowed the demonstration of the participation of several cell types in the elaboration of humoral response. In addition, it was soon discovered that many cellular interactions were mediated through soluble factors produced by lymphocytes (lymphokines) and monocytes (monokines).

The first evidence that the helper cell activity can be mediated by soluble molecules was demonstrated in 1971, again in Dutton's laboratory (Dutton *et al,* 1971). The T cell-derived factors were produced by bidirectional mixed lymphocyte culture and the harvested supernatant acted on B cells in the absence of T cells. These initial results were confirmed by Schimple and Wecker (1972) and this supernatant was soon found to contain other factors (Dutton, 1975; Schimple and Wecker, 1975). Later Luben and Moller (1980) demonstrated that soluble factors in conditioned medium of cultured thymocytes from ten day old mice could replace the immune T cell requirement for initiation of primary humoral response in culture. The factor was called T cell replacing factor (TRF).

When a T-dependent antigen is added to a culture of dissociated spleen cells, it does not evoke a significant antigen-specific antibody forming cell response and hence appreciable amount of monoclonal antibodies could not be produced from hybridomas prepared from these cells (Borrebaeck, 1984). The response to T dependent antigen requires co-operation between helper T cells, accessory cells and B cells in an MHC restricted fashion (Jones and Janeway, 1981; Julius *et al.*, 1982). The initial event in B cell activation process is the interaction between surface immunoglobulin (sIg) and the antigen. Crosslinking of sIg by a multivalent antigen is followed by a second signal provided by the T cells which leads to the proliferative response.

Resting B cells can also be activated in an MHC-unrestricted manner by antisera to surface IgM or by antigen stimulated T cell lines and syngenic antigen presenting cells (DeFranco *et al,* 1984). Lanzavecchia (1985) has shown that B cells function as antigen presenting cells by internalization, processing and presentation of the antigen to T cells in an MHC unrestricted manner. Abbas (1988) has suggested that the major role of cell surface Ig receptors was to bind antigen at limiting concentrations for the presentation to T cells, which in turn get stimulated to secrete lymphokines. Specific B cells utilize the lymphokines for proliferation and differentiation.

Effect of supplementation of cytokines such as IL-4 (Swain *et al,* 1983), B cell differentiation factor (BCDF) (Nakanishi *et al,* 1983; Kishimoto *et al.,* 1984), interferon-y, IL-1 and IL-2 has been studied using *in vitro* immunized cells. Supernatant from a culture of resting thymocytes (Luben and Moller, 1980; Herbst and Braun, 1981; Van Ness *et al.,* 1984) or from a mixed lymphocyte culture (MLC) has been used as a source of cytokines (Miner *et al.*, 1981; Borrebaeck 1983; Pardue *et al.*, 1983; Moller *et al,* 1986). These supernatants contained IL-2, allogeneic helper factors and yet undefined molecular entities acting directly on B cells (Moller *et al,* 1986). Supplementation with MLC supematants derived from splenocytes evoked a better AFC response compared to the superaatants derived from young thymocytes (Ossendrop *et al,* 1986; Glad *et al,* 1988). Spleen cells contain more Ia positive cells than the thymocytes which probably results in a higher lymphokine production. The low activity of the lymphokines derived from the thymocyte superaatants is also partly due to their active resorption by thymocytes themselves.

Addition of supematants from Phorbol myristate acetate (PMA) stimulated EL-4 cell line (Borrebaeck and Moller, 1986) which contains cytokines such as IL-2 (50-100U/ml) (Farrar *et al,* 1980), BCGF (Swain *et al,* 1983) BCDF-μ (Isaksson *et al,* 1982) and BCDF-y (Vitetta *et al,* 1984) has been shown to

supernatant (Helleman *et al.,* 1988) The optimal ratio of supernatants derived from MLC and EL-4 cultures used for supplementing the *in vitro* immunization systems was (33 :25%). EL-4 derived lymphokines had the ability to support the *in vitro* immunization 2 - 3 times better than MLC derived lymphokines (Borrebaeck and Moller, 1986). Using this *in vitro* immunization system, monoclonal antibodies could be produced against autologous antigens such as purified mouse albumin, mouse haemoglobin and mouse serum proteins and other weak immunogens.

Tht requirement of cytokines for *in vitro* immunization and clonal expansion of human PBL has been investigated by Danielsson and co-workers (1987). According to them, the PBL had to be separated into B cells, T cells and accessory cells (A cells) to antigen-specifically activate the peripheral B cells. Unseperated cells could not be activated by an antigen. A recombination of purified accessory cells, B cells and T cells at a ratio 0.25 : 1 : 2 respectively, has been shown to give 100 - 200 specific plaque forming cells/10^6 B cells. The effect of recombinant IL-2 and IFN-γ together with B cell differentiation factor (BCDF) and the supernatant from PWM-stimulated irradiated T cells (sPWM-T) were tested using this combination of T, B and A cells. The presence of sPWM-T was shown to be crucial for antigen-specific activation of peripheral B cells. The addition of exogenous IL-2 further increased the number of plaque forming cells whereas IL-1 and BCDF had no effect (Danielsson *et al.,* 1987).

Polyclonal B cell activators in *in vitro* immunization :

The type of antigens that can activate B cells polyclonally, irrespective of clonal specificity of the Ig receptor, are called polyclonal activators. Polyclonal B cell activators in presence of antigen can serve as second signals to stimulate specific responses (Claman, 1975). LPS, PWM and Con A stimulate specific PFC responses to SRBC in T cell depleted cultures. LPS in the presence of antigen can induce specific B cell responses in athymic (nude) mice (Watson *et al.,* 1973).

Methanolic extract of Bacillus Calmette Guerin (BCG) stimulates antigen-specific T-dependent primary responses in unprimed mouse spleen and blood cells in culture (Ben-Efraim and Diamantstein, 1975). A water soluble mitogen of *Nocardia* (Bona *et al.,* 1979), PWM etc., can activate human B cells and stimulate non-specific and specific responses. Strike *et al.,* (1984) developed a simple *in vitro* immunization system to produce human monoclonal antibodies against human prostatic acid phosphatase using an allogeneic culture of spleen cells in presence of PWM and antigen. PWM has been reported to be essential for *in vitro* stimulation *ie.,* induction of a secondary immune response in culture. Human monoclonal antibodies against several different viral antigens have been produced using PWM as an adjuvant using cells from *in vivo* pre-sensitized patients (Sugano *et al*., 1987; Matsumoto *et al*., 1986).

TECHNICAL ASPECTS OF IN VITRO IMMUNIZATION

Development of plaque assay for B cell differentiation :

An assay for the determination of the number of antibody secreting cells was first introduced by Jerne and Nordin (1963). This haemolytic palque assay and Cunningham's plaque assay (Cunningham, 1965) was based on a complement-mediated lysis of antigen-coated SRBC that surround antibody secreting cells, as a result of which a clear zone of plaque appears in the red cell cover. Antigen can be coupled to the erythrocytes but the technique has its own limitations, such as instability of antigen coated SRBC, inconsistent lysability of different batches of SRBC and uncertainty about the antibody type involved in the formation of direct plaques (Wortis *et al.,* 1969; Jerne *et a!.,* 1974).

An assay capable of screening several hundreds of samples in a rapid and reproducible way was developed by Moller and Borrebaeck,(1985) and is called Filter immuno plaque assay (FIPA). It is based on ELISA principle and detects only B cells, secreting higher amounts of antibody compared to the hemolytic

plaque assay which needs fewer antibodies to initiate a complement dependent cellular lysis. This is considered as an advantage since B cells that are optimized for fusion should be actively secreting Ig. However, caution should be observed when evaluating the assay since quality variation between different batches of nitrocellulose have been shown to drastically affect the non-specific binding of protein and cell debris, which could make the interpretation of the data difficult (Moller and Borrebaeck, 1985).

Dose of immunogen

The dose of immunogen used to elicit an immune response in an *in vitro* immunization system shows variation over a wide range. At the lower end 1 ng/ml sperm whale myoglobin was sufficient to elicit an immune response (Borrebaeck, 1984) and 100 µg/ml for porcine insulin was at the higher end (Borrebaeck, 1983). However, the antigen dose that gives an optimal response has to be determined for each antigen in the *in vitro* immunization system used.

Effect of serum source and concentration :

The source and the concentration of serum is a critical parameter. Fetal calf serum (FCS) was used first in *in vitro* immunization system described by Mishell and Dutton (1966, 1967) and Marbrook (1967, 1968). Several other investigators have since used FCS with satisfactory results (Click *et al.,* 1972; Luben and Mohler, 1980; Sethi and Brandis, 1981; Boss, 1984). However, variations between batches of FCS were reported to critically influence the outcome, measured as the yield of hybridomas (Rathjen and Underwood, 1985). This may be due to variations in the levels of endotoxins and hormones. A low concentration of rabbit serum (1 - 3%) is more advantageous than using high serum concentrations (15 - 20%) as it reduces the effects of serum proteins on B cell activation (Miner *et al.,* 1981; Borrebaeck, 1983; Pardue *et a!.,* 1983; Borrebaeck and Moller, 1986). Other investigators have developed completely serum-free conditions (Borrebaeck, 1984; Ossendrop *et al.,* 1986) or an initial (7 - 9 h) serum-free culture followed by

addition of FCS (Van Ness *et al,* 1984) for the immunization of spleen cells *in vitro.*

In case of human *in vitro* immunization systems some investigators found human serum to be necessary (Ho *et al,* 1985; Hoffmann and Hirst, 1985, Bieber and Teng, 1987; Jonak *et al.,* 1988; Larrick *et al.*, 1988), whereas others found FCS to be necessary (Strike *et al.*, 1984; Lagace and Brodeur, 1985; Jocot-Guillarmod, 1988; Kozbor, 1988; Pollock and d'Apice, 1988; Mc Roberts *et al,* 1988).

Effect of cell density and duration of culture :

A high concentration spleen cells (6 - 10 x 10^6 cells/ml) was reported to be required during cell culture by most investigators (Luben and Mohler, 1980; Van Ness *et al,* 1984; Rathjen and Underwood, 1985; ossendrop *et al,* 1986; Moller and Borrebaeck, 1988)

The duration of the *in vitro* immunization varies widely between different protocols : 3 days (Ossendrop *et al,* 1986), 4 days (Luben and Moller, 1980), 5 days (Mishell and Dutton, 1967; Click *et al,* 1972; Borrebaeck, 1983; Pardue, *et al* 1983; Borrebaeck and Moller, 1986; Brams *et al,* 1987) 5 - 6 days (Rathjen and Underwood, 1985) and 5 - 8 days (Van Ness *et al,* 1984). A limiting factor for the duration of the culture is the cell recovery versus the clonal expansion of the desired antibody-producing cells. The cell recovery in an *in vitro* immunization system normally decreases rapidly after day 5 of the culture period.

Cell source and strain differences :

An early report by Cbck and co-workers (1972) stated that the magnitude of the immune response *in vitro* depends on the mouse strain used as the spleen cell source, C57BL/6J was the highest responder, BALB/c intermediate and CBA gave the lowest response to the antigen SRBC. This did not correlate with the *in vivo* response, which was in reverse order. Schreier and Nordin (1977) reported that spleen cells show a negligible variation in response in the *in vitro* immunization.

status of the mouse in an *in vitro* immunization system as compared to the *in vivo* response. Presently the most commonly used cell source for *in vitro* immunization is splenic lymphocytes of BALB/c mouse.

Source of B lymphocytes :

Human lymphocytes can be obtained from different lymphoid compartments *eg.,* spleen, tonsils, lymph nodes, bone marrow or Peripheral blood. However, the most readily available lymphoid compartment is by far the peripheral blood. Compared to the other sources which are to be obtained by surgery, peripheral blood easily lends itself to repeated sampling from the same individual. Peripheral blood lymphocytes (PBL) have however, performed poorly in *in vitro* immunizations for the production of monoclonal antibodies (Olsson *et al.,* 1983; Lagace and Brodeur, 1985; Teng *et al,* 1985; Ho *et al.,* 1987). The reasons for this might be that (a) the unfavourable ratio of T versus B cells (Borrebaeck, 1986), and (b) the circulating B cells are arrested in a phase in which additional stimuli are required to induce antigen - specific clonal activation (Luzzati *et al.,* 1977). They are subjected to cell mediated suppressive restraint as compared to B cells in spleen and lymph nodes, since the role of PBL is not to act as an activation site but rather a transport system connecting both central and peripheral lymphoid organs. The unfavourable ratio of B cells to T cells in PBL population is compensated by the depletion of suppressor T cells and by the addition of proper differentiation and growth factors such as T cell growth factor (Brodeur *et al.,* 1987) B cell growth factor (Matsumoto *et al.,* 1986) interleukins (Delfraissy *et al.,* 1988; Luzzati *et al.,* 1989) and mitogens (Teng *et al.*, 1985) to the culture system.

Several experimental approaches have been followed to obtain *in vitro* immunized human B cells. The relevant B cells can be enriched either by depletion of the cell population which brings about suppressive effect eg., cytotoxic cells, a subset of T cells ($CD8^+$) and/or by enriching or stimulating the

cytotoxic cells, a subset of T cells (CD8) and/or by enriching or stimulating the lymphocyte population with antigens in combination with B cell stimulating factors or mitogens. The most widely used methods are depletion of suppressor T cells (Teng *et al.,* 1985; Ho *et al.,* 1985) and depletion of lysosome rich cell population from PBL by treatment with lysosomotropic agent, Leucyl Leucine-O-methyl ester (Ohlin *et al.*, 1989; Borrebaeck, 1989).

Removal of suppressor activity using a lysosomotropic agent :

A number of different cell types for example $CD8^+$ or $CD8^+/CD11^+$ T cells, large granular lymphocytes, monocytes etc., have been shown to be involved in the suppression of antigen-specific activation of PBL. A lysosomotropic ester of Leucine or its dipeptide Leucyl-leucine (Thiele and Lipsky, 1985) was used to deplete the cells that bring about a suppressive effect on the antigen-specific activation of PBL.

Upon removal of the lysosome-rich sub-populations of human PBL by treatment with the lysosomotropic methyl ester of Leucine (Leu-OMe), the remaining cells respond antigen-specifically during an *in vitro* immunization (Borrebaeck *et al.,* 1988). Unfractionated human PBL treated with Leu-OMe could be immunized *in vitro* with higher efficiency compared with separated B cells, thus eliminating several laborious cell separation steps.

The cytotoxic effect of Leu-OMe on cytotoxic cells such as NK cells is not due to the lysosomotropic property of Leu-OMe but is due to its monocyte derived lysosomal condensation product Leucyl-Leucine O-methyl ester (Thiele and Lipsky, 1985). The amino acid ester is a product of condensation by lysosomal proteinase 1 which is present in freshly prepared monocytes. Hence the depletion of the lysosome-rich *in vitro* immunized cells have been used in somatic cell hybridization and antigen-specific human-human or human-mouse hybridomas have successfully been constructed.

Amplification of *in vitro* immune response :

Efforts have been made to increase the number of antigen specific plaque forming cells by the addition of immune potentiators, such as bacterial peptidoglycan derivatives (Ellouz *et ai,* 1974; Boss, 1984). The adjuvant muramyl dipeptide (MDP) significantly increased the number of PFC when added to an *in vitro* immunization supported by supernatants from MLC and EL-4 (Moller and Borrebaeck, 1988). This potentiation could be obtained only with T cell dependent antigens (Glad *et ai,* 1988).

A simple *in vitro* immunization system based on sole addition of MDP was also reported to give a significantly higher number of glutamic acid decarboxylase-specific monoclonal antibodies compared with those produced by *in vivo* immunization using same antigen (Boss, 1984). The effect of adjuvant peptide is of great interest in these systems since they seem to enhance the *in vitro* immune response without simultaneous enhancement of mitogenicity in cultured lymphocytes. (Specter *et ai,* 1977). Another potentiator, dextran sulphate was reported to increase the yield of specific hybridomas producing monoclonal antibodies (Schilling, 1986).

Attempts have been made to enhance immunogenicity by presenting the antigen on cell or adsorbed to solid phase. A procedure developed by Van Ness *et ai,* (1984) utilized fumed silica (pore size 0.007μm) to which the antigen is adsorbed. The spleen cells were primed in the absence of serum with antigen-silica complex in the presence of peritoneal macrophages. Aggregates of lymphocytes were induced which were believed to increase the cell to cell contact and hence enhanced antigen binding was achieved. Eddy *et al.,* (1985) used a glycolipid glass conjugate in *in vitro* immunization system. The advantages of this are 1. use of small quantities of antigen, 2. glass beads remain at the bottom of the flask in contact with the spleen cells, and 3. the antigens can easily be removed from the culture. This system allows the control of temporal, quantitative and steric

contact with thc spleen cells, and 3. the antigens can easily be removed from the culture. This system allows the control of temporal, quantitative and steric parameters of antigen presentation in *in vitro* environment. Antigen immobilized on nitrocellulose has been tried with extremely limited amounts of antigen present in complex mixtures (Gratecos *et al.,* 1987).

Advantages of *in vitro* immunization :

Human monoclonal antibodies have a very high therapeutic value. Since only a few antigens can be used for immunizing humans *in vivo, in vitro* immunization is very crucial for human hybridoma technology. The advantages of *in vitro* immunization, as compared to those *in vivo* are as follows :

1. The immunization takes 5 days *in vitro* instead of usually several weeks *in vivo*..

2. The normal cellular control of the immune response to self antigens and phylogenitically conserved antigens does not function *in vitro* (Borrebaeck and Moller, 1986; Borrebaeck *et al.,* 1987), resulting in elicitation of strong responses against evolutionarily conserved immunogens which normally do not evoke an immune response *in vivo.* This opens up the possibility of producing personalized human monoclonal antibodies against homologous structures or other weak immunogenic structures that do not elicit an *in vivo* immune response. Using *in vitro* immunization technique monoclonal antibodies can be produced against alloantigens (antigens from the same species) *eg.,* tumor associated antigens which are valuable for identifying the antigenic phenotype of malignant cells. A different repertoire of antibody specificites in the resultant monoclonal antibodies will be obtained when immunized across the xenogenic barrier.

3. Extremely low amounts of immunogen are needed.

4. The number of antibody producing cells per million spleen cells is significantly higher *in vitro* due to preferential survival of *in vitro* activated cells.

5. Every step of *in vitro* immunization can be monitored during the entire stimulation period and suitable lymphokines can be added.

Quality of monoclonal antibodies derived from *in vitro* immunization :

Important qualitative parameters of the monoclonal antibodies are isotype, specifity and affinity constant.

The majority of monoclonal antibodies produced from *in vitro* immunizations have the IgM isotype. The percentage of IgG secreting hybridomas in the total hybridomas obtained from cells immunized *in vitro* using a variety of antigens is normally 10 - 25 % (Borrebaeck, 1987). The isotype switching from IgM to IgG is dependent on the physical state of antigen and the type of lymphokines present during the *in vitro* immunization, rather than the duration of incubation.

In human *in vitro* immunizations, monoclonal antibodies of IgM isotype only were reported. However, IgG hybridomas were reported with fusion partners of human origin (Strike *et al.*, 1984; Hulette *et al.,* 1987; Terashima *et al,* 1987) or heteromyeloma origin (Danielsson *et al,* 1987). The combination of EBV-transformation and fusion yielded 25% IgG-secreting hybridomas (Yamura *et al,* 1988). No reports exist that compare the monoclonal antibodies derived from *in vitro* and *in vivo* immunizations.

The monoclonal antibodies derived from *in vitro* immunization do not exhibit a broader pattern of cross-reactivity as compared with the those derived from *in vivo* immunizations (Ohlin *et al.,* 1989). However, the cross-reactivity pattern of antibodies derived from *in vitro* immunizations were similar to those derived from *in vivo* immunizations (Buchman *et al.,* 1985). The antibodies produced from *in vitro* immunized cells exhibited a much broader repertoire of specificities than antibodies derived from *in vivo* immunized cells (Morrison *et al.,* 1985).

The affinity maturation that takes place *in vivo* during an immunization process is due to antigenic selection of high affinity B cells and to a somatic mutation *In vitro* however, antigenic selection can contribute to high affinity antibodies. The immunization time is normally 4 - 6 days which is too short for somatic mutation to contribute significantly to the yield of high affinity antibodies. When high doses of antigen were used, most of the antibodies formed were of low affinity. With low doses of antigen in the *in vitro* immunization system monoclonal antibodies with similar affinity constants were obtained as in the *in vivo* immunizations. This antigen dependent selection *in vitro* can be used to modulate the antibody affinities and that selection of structural variants is obtained by the combination of *in vitro* and *in vivo* immunizations. This combination selected B cell mutants producing IgG with even higher affinity as compared to only *in vivo* immunizations (Wallen and Borrebaeck, 1991).

AIM AND SCOPE OF THE PRESENT WORK :

The main objective of the work presented in the thesis is preparation and characterization of immobilized antigens and to study their efficacy over the soluble form of the same antigen in the *in vitro* immunization system of murine splenic lymphocytes and human peripheral blood lymphocytes.

A soluble antigen is processed by the antigen presenting cells and is then presented to the T lymphocytes which in turn help the relevant B lymphocytes for antibody secretion. Whereas the immobilized antigens activate B lymphocytes directly by crosslinking the membrane receptors for extended duration. The sequence of patching, capping and endocytosis of surface Ig induced by soluble antigens does not occur in case of immobilized antigens since they can not be internalized (Parker *et al.,* 1979).

Earlier, it has been shown that sepharose coupled anti-immunoglobulin (sepharose-anti-lg) acts as a potent stimulator of B lymphocytes as compared to the soluble anti-Ig (Parker *et al.,* 1979; Ramanadham *et al.,* 1984). In presence of sepharose-anti-lg, B lymphocytes could be driven to differentiation (antibody secretion), whereas soluble anti-Ig could induce only a proliferative response (Parker *et al.,* 1979). As anti-Ig induced triggering of B lymphocytes is supposed to mimic the antigenic stimulation via the crosslinking of membrane receptors, attempts have been made to see if the immobilized immunogens could serve as better stimulants in the *in vitro* immunization system. The efficacy of immobilized and soluble forms of the antigen to stimulate B lymphocytes *in* vitro was determined in terms of induction of antigen-specific antibody forming cell response.

CHAPTER - II

MATERIALS AND METHODS

Animals

Balb/c female mice 8-12 weeks old bred in the animal facility of National Institute of Nutrition, Hyderabad, were used in all experiments.

Chemicals :

RPMI 1640 with 2mM glutamine and 25mM HEPES, Fetal calf serum (FCS), Penicillin G, Leucyl-Leucine-O-methyl hydrobromide, goat anti-mouse IgG (γ-chain specific)-horse radish peroxidase conjugate, and goat anti-mouse IgM (μ-chain specific)-horse radish peroxidase conjugate were purchased from Sigma Chemical Co. USA. 4-chloro-1-napthol was purchased from Fluka Chemie, Switzerland. Nitrocellulose membrane and membrane filters (0.22 μ and 0.45 (i) were purchased from Millipore, U.S.A. Dynabeads M 450, (uncoated), were from Dynal, Norway. Streptomycin was from Sarabhai Chemicals, India. CNBr-activated sepharose 4B was obtained from Pharmacia fine chemicals, U.S.A. Tetanus toxid (TT) was obtained from Sera and Vaccines division, Biological Evans Ltd., India. All other chemicals used were of analytical grade.

Coupling of TT to CNBr-activated sepharose-4B ;

Principle :

Cyanogen bromide reacts with hydroxyl groups on sepharose and converts them to imido carbonate groups which react with neucleophiles. The activated groups react with primary amino groups of the ligand to form stable isourea linkages.

Reagents :

1. Coupling buffer : 0.1 M bicarbonate buffer, pH 8.4 with 0.5 M NaCl.

2. Ethanolamine: 1 M , pH 8.0.
3. Acetate buffer : 0.1 M acetate buffer, pH 4.0 with 1 M Nacl
4. Borate buffer : 0.1 M borate buffer, pH 8.0 with 1 M Nacl.
5. Phosphate buffer saline (PBS) : 0.01 M phosphate buffer containing 0.9% NaCl, pH 7.2.
6. CNBr-activated Sepharose 4B (Pharmacia, Sweden).
7. Tetanus toxoid, 5 mg/ml.

Procedure :

All operations of coupling were carried out under sterile conditions. 1 g of CNBr-activated sepharose-4B was washed with 100 ml of 1 mM HC1 followed by large volumes of double distilled water. The swollen gel was taken into 5 ml of coupling buffer containing 10 mg of TT. The mixture was rotated end-over-end overnight at 4 °C. The gel was washed with 2.5 ml coupling buffer to remove unbound TT. Supernatant was saved, and the gel was treated with 10 ml ethanolamine at room temperature for 1 h by end over rotation. This is to block the remaining reactive groups. Then three washing cycles were carried out to remove non-covalently adsorbed protein to the gel, each cycle consisting of acid wash with acetate buffer followed by alkaline wash with borate buffer. Finally the gel was suspended in sterile PBS and stored at 4 °C.

Absorbance of the supernatants saved at each of the above steps was measured at 280 nm to calculate the amount of unbound protein. The amount of TT bound to the gel was expressed as the percentage of the initial quantity of protein used for coupling. Around 80% of the protein was coupled to the gel. Concentration of sepharose bound antigen was calculated based on the amount of protein and volume of the gel taken and expressed as jigs/ml of the gel.

Iodination of TT using Iodobeads :

Principle :

Iodobeads are essentially immobilized chloroamine-T molecules on non-porous polystyrene beads. Iodobeads oxidatively produce an electrophilic iodine species (I^+) from sodium iodide. This allows the radioactive iodine to react with tyrosine residues present in the protein.

Materials and reagents :

1. Iodobeads, Pierce Chemical Co.,USA.
2. Na ^{125}I, activity - 76.6 mCi/ml, BRIT, India.
3. Sephadex G - 25 mini column
4. Sodium iodide
5. PBS, 10 mM, pH 7.2

Procedure :

Iodobeads were washed before use twice in PBS using approximately 1 ml per bead in a small beaker, and blotted dry on filter paper. 5 mg of TT in 0.5 ml PBS was taken in screw-capped reaction tube along with 0.5 mCi of $Na^{125}I$ The iodination reaction was initiated by the addition of one iodobead and allowed to proceed for 15 min at room temperature. The reaction was terminated by transfer of the protein solution with a pasteur pipette to a fresh tube leaving behind the bead. Carrier iodide, 0.5 mol of NaI was added to the protein solution, to facilitate removal of unreacted radioiodide.

Iodinated protein was separated from unreacted iodide by passing the solution through a sephadex G-25 mini column. A small aliquot of the iodinated protein is directly counted in a Gamma Counter, (ECIL, India), to determine the specific activity.

Determination of stability **of antigen on** the bead :

Radiolabelled TT was coupled to CNBr-activated sepharose-4B by the procedure described earlier. About 78% of the protein used for coupling was bound to the gel.

Stability of TT on the sepharose bead was checked at 4 °C and 37 °C. The supernatant from sepharose-^{125}I-TT stored at 4 °C was collected periodically and counted in the Gamma counter and the sepharose-^{125}I-TT was resuspended in fresh PBS. Supernatants were collected on days 10, 20 and 30.

Sepharose- ^{125}I-TT, was suspended in complete medium and in *in vitro* immunization culture with unprimed spleen cells for 5 days at concentration used earlier. From day 1 to 5 triplicate tubes from both sets of cultures (with cells and without cells) were taken and the supernatants were collected and counted in a Gamma counter, to check the release of ^{125}I-TT from the sepharose-4B matrix.

Autoradiography of sepharose- ^{125}I-TT :

Principle

Autoradiography allows the visual localization of the radiolabelled protein on the surface of the bead, by exposure to an X-ray film.

Materials and reagents :

1. Microscopic slides
2. Double-sided adhesive tape
3. Dark room with safety light
4. X-ray film, Eastman -Kodak Ltd.
5. Kodak rapid fixer
7. X-ray film cassette with intensifier screen.

Procedure :

Sepharose-^{125}I-TT beads, suspended in PBS were loaded into a chamber made of two microscopic slides using the double sided adhesive tape. The chamber was sealed after loading the labelled beads, and was exposed to an X-ray film at -70 °C overnight in a cassette with an intensifier screen. The X-ray film was developed and viewed under a microscope to see the ^{125}I-TT distribution on the bead.

Preparation of **antiserum** against **TT** ;

One Lf unit of alum adsorbed TT (Behring Pharma, India) in 0.25 ml PBS was injected into each of the rear foot pads of a Newzeland white rabbit. First booster of 0.5 Lf unit was given after one month. A second booster dose of same concentration was given 15 days later. The rabbit was bled 15 days after the second booster and the serum was collected.

Purification of IgG from rabbit anti-TT antiserum :

Materials and reagents :

1. Saturated ammonium sulfate

2 DE-52, (Whatman Corp U.K.)

3. Dialysis tubing

4Phosphate buffer 10 mM pH 7.2

5. Phosphate buffer 17.5 mM pH 6.3

Procedure :

To 11 ml of the antiserum, saturated ammonium sulfate was added (slowly with stirring), until 40% saturation was reached. Precipitate was allowed to form for several hours by incubating the tube in an ice bucket overnight Then the precipitate was centrifuged and dissolved in a minimal amount of double distilled

water and dialysed against 200 volumes of phosphate buffer pH 7.2 for 36 h changing the buffer 3 times. The salt free fraction was loaded on DE-52 column, equilibrated with 10 mM phosphate buffer pH 7.2, and the IgG was eluted with 17.5 mM phosphate buffer pH 6.3. 1 ml fractions were collected, OD at 280 nm was read for all the fractions. The fractions with high OD were pooled and lyophilized. The specificity of the antiserum was checked by Ouchterlony double immuno diffusion technique.

Interaction of anti-TT antibody with TT linked to sepharose bead :

Materials and reagents :

1. Sepharose-TT
2. Anti-TT antibody IgG fraction (rabbit) (7.5mg/ml) in 10 mM PBS pH 7.2.
3. Goat anti-Rabbit IgG-horse radish peroxidase conjugate, Sigma Chemical Co. USA.
4. Substrate solution : 3 mg of 4-chloro-1-naphthol in 1 ml methanol was added to 5 ml PBS. H_2O_2 was added to the above solution before use to a final concentration of 0.003%.

Procedure :

A small aliquot of sepharose-TT was incubated with 20 µl of anti-TT antibody for 30 min at 4 °C. Then it was washed 4 times with 10 mM PBS, and was incubated with 50 µl of horse radish peroxidase conjugated goat anti-rabbit IgG (1:500 dilution in PBS) for 1 h at 4 °C. Unreacted antibody-enzyme conjugate was removed by washing the gel 5 times with PBS. Colour was developed by adding the substrate solution. The deep purple coloured antigen-antibody complex on the surface of the bead was visualized under a microscope. Sepharose-TT treated with non-immune rabbit serum was used as control.

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Preparation of anti-TT antibody FITC conjugate :

Materials and reagents :

1. Bicarbonate buffered saline (BBS) 0.05 M pH 9.2
2. BBS, 0.05 M pH 8.5
3. Tetanus toxoid
4. Anti-TT antibody (7.5 mg/ml)
5. Fluorescein Isothiocynate (FITC) from Sigma Chemical Co. USA.

Procedure :

Anti-TT antibody was first dialysed at 4 °C against BBS, pH 8.5 for 4-5 h, followed by dialysis against BBS pH 9.2 for 2 h. The sample was next dialysed against a solution of 100 µg FITC per ml BBS, pH 9.2 for 14 - 16 h. The reaction was terminated by changing the dialysis buffer to pH 7.0. The sample was dialysed extensively against this buffer to remove the uncoupled free FITC. Fluorescein : protein ratio was determined according to Wells *et al* (1966).

Binding of Fluorescein conjugated anti-TT antibody to sepharose-TT :

Sepharose-TT, 5 µg in 100 µl was incubated with 10 ul of anti-TT antibody-FITC conjugate for 30 min. The unbound antibody was washed off with PBS and the antigen-antibody complex on the bead was visualized under a microscope. Sepharose-OVA was also reacted with anti-TT antibody-FITC as a control.

Coupling of TT to Dynabeads :

Materials and reagents :

1. Dynabeads M-450
2. Magnetic stand

3 Prewashing and coating buffer : 0 1 M phosphate buffer pH 8 5

4.0.01 PBS with 0 1% BSA pH 7 4

Procedure :

The Dynabeads were resuspended well by shaking the vial thoroughly and required volume of beads were pipetted into a suitable tube. The tube was then placed on a magnetic stand for 60 seconds, and the fluid was pipetted whilst the beads remain undisturbed. The tube was removed from the magnet, ample volume of prewashing buffer was added and the beads were resuspended and the washing step was repeated thrice. 3-5 μg TT per mg beads was taken in coating buffer and the washed beads were suspended in it at a concentration of 40-50 mg/ml. The Dynabeads and TT were incubated for 24 h at 4 °C, on a rotary mixer to prevent settling of beads during incubation. The supernatant was removed by placing the tube on the magnet and the coated beads were washed three times, the last time overnight at 4 °C, using the buffer containing 1% BSA. The amount of protein bound to the Dynabeads was quantified and stored at 4 °C in 0.02% sodium azide.

Quantification of the protein bound to Dynabeads :

1. **^{125}I-TT Coupling to Dynabeads** :

^{125}I-TT was coupled to the Dynabeads as described above. The coated beads were washed and separated on a magnet and the supernatant was collected in to a clean tube. The beads were resuspended in PBS containing 1% BSA. The beads and the supernatant were counted in a γ-counter.

$$\% \text{ binding of TT to Dynabeads} = \frac{\text{Radioactivity bound to the bead (cpm)}}{\text{Total radioactivity of TT taken for coupling}} \times 100$$

2. ELISA :

Principle :

ELISA is an immunological assay used to detect solid phase antigen-antibody reactions The assay utilizes an enzyme conjugated to immunoglobulin that specifically binds the immunoglobulin in the antigen-antibody complex. The enzyme then converts the substrate to product which is detectable visually or spectrophotometrically.

Reagents :

1. 96 well ELISA plates (Tarsons, India)
2. Rabbit anti-TT antibody, (working dilution 1: 300 dilution)
3. 10 mM Phosphate buffer containing 0.9% NaCl (PBS) pH 7.2 and with 0.05% Tween-20
4. Blocking solution, 5% Lactalbumin solution in PBS with 0.05% Tween-20
5. 10 mM Carbonate-Bicarbonate buffer pH 9.4
6. Tetanus toxoid (1 mg/ml)
7. Substrate solution, o-Phenylenediamine dihydrochloride, 5 mg tablet was dissolved in 12.5 ml of 0.05 M phosphate-citrate buffer pH 5 and H_2O_2 was added to a final concentration of 0.003% just before use.
8. 8.Dynabeads-TT

Procedure :

TT was diluted with bicarbonate buffer and was added to microtitre plate wells ranging from 25-500 ng/well. The plates were coated overnight at 4 °C. The unoccupied sites were blocked by filling the wells with blocking solution. Blocking was done for 1 h followed by 3 washes with PBS-Tween and 4 washes with PBS. For quantification of TT on Dynabeads blocked wells were used. 50 µl of rabbit anti-TT antibody (1 : 250 dilution) was added into the TT coated wells as well as wells layered with Dynabeads-TT and was incubated at room temperature of 2 h. Unbound antibody was removed by extensive washing with PBS-Tween

and PBS. Horse radish peroxidase conjugated goat anti-rabbit IgG (1:500 dilution in PBS) was added to all the wells and was incubated for 1 h. After washing off the unreacted antibody, 50 µl of substrate solution was added. The reaction was terminated after 15 min by the addition of 3 N HCl The plate was read at 490 nm in an ELISA reader.

In vivo **immunization** :

Mice were injected with 50 µg of soluble TT emulsified with CFA and immobilized TT intra-peritoneally. Saline CFA was injected as control for CFA-TT and uncoated sepharose beads were injected as control for immobilized TT.

Isolation of murine splenic lymphocytes :

Reagents :

l.RPMI 1640 : The powdered medium was dissolved in distilled water, sodium bicarbonate 2 g, Penicillin G (100 U/ml) and Streptomycin (100 jag/ml) were added, pH adjusted to 7.2 and the solution was made upto llitre with distilled water. The medium was sterile filtered through 0.45 µ membrane filter using Millipore filtration unit and stored at 4 °C.

2. Complete medium : RPMI 1640 containing 5% FCS and 50 µM β-mercaptoethanol.

Procedure :

Mouse was killed under mild ether anaesthesia and spleen was quickly removed and put in RPMI 1640. The spleen was cleansed free of connective tissue and fat and was minced. Then the tissue was teased in the complete medium on a stainless steel mesh using arterial forceps fitted with steel brushes. The cell suspension collected in the petridish was allowed to settle for 2 min at room temperature and large clumps which settle to the bottom were discarded, the suspension was centrifuged at 400x g for 7 min and the supernatant was discarded.

The cell pellet was suspended in 6 ml of the complete medium and centrifuged Totally three washes were done with complete medium. The pellet after the third wash was suspended in 2 ml of the medium containing 10% FCS and stored on ice until use.

Cell counting :

Principle :

Gentian violet stains the lymphocyte nucleus while dilute acetic acid helps in lysis of RBC.

Procedure :

A small volume of cell suspension was diluted with Turk's solution (0.01% of gentian violet, (w/v), in 3% v/v, acetic acid) and mixed throughly. Cells were counted in each of the four large squares (WBC counting areas) of a hemocytometer. The average number of cells per large square was calculated and the total number of cells in the suspension was determined using the formula

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

Determination of viability by Trypan blue dye exclusion :

Principle :

Viable cells exclude the dye while non-viable cells take up the dye there by fostering a visual distinction between viable and blue stained non-viable cells.

Procedure :

The cell suspension was appropriately diluted in trypan blue solution (0.2%, w/v, in saline). A total number of 200 cells which included stained cells were counted microscopically using a hemocytometer. The percentage of viable cells was calculated using the formula

% viable cells = No of unstained cells X 100
Total no. of cells

Proliferation assay of TT primed splenic lymphocytes using soluble and sepharose TT :

Principle :

Antigens activate the primed lymphocytes to proliferation, when all the growth requirements are provided. The proliferative response is measured by ^{3}(H)-thymidine incorporation into DNA.

Reagents :

1. Complete medium : RPMI 1640 with 5% FCS and 50 μm β-mercaptoethanol.
2. Antigens : Soluble TT and sepharose TT.
3. Scintillation cocktail : 4 g PPO and 200 mg POPOP in one litre of sciltillation-grade toluene.

Procedure :

Cell cultures containing 2 x 10^5 primed splenic lymphocytes in 0.2 ml culture medium were kept in 96 well flat bottomed sterile microtitre plates (Tarsons, India). TT in soluble and sepharose-linked forms was added to the cultures at the indicated concentrations.

Cultures with and without antigens were kept in triplicate and incubated at 37 °C in a humidified incubator with 95% air and 5% CO_2 atmosphere. The cultures were pulsed with 0.5 |iCi of ^{3}H-thymidine per well for the last 18 h of 96 h culture period and were processed on to glass micro fibre filters using Skatron cell harvester. Dried filters were transferred to the scintillation cocktail and the radioactivity was measured using Beckman Liquid Scintillation counter. The results are expressed as cpm/10^6 cells.

***In vitro* immunization :**

Unprimed splenic lymphocytes were immunized *in vitro* at a concentration of 2 x 10^6 cells/ml medium with 10% FCS and 50 μM β-mercaptoethanol in 12 x 75 mm screw capped tubes. The cells were cultured with different concentrations of soluble and sepharose-TT for 5 days in a humidified incubator with 5% CO_2 at 37 °C. cells.

Filter immuno plaque assay (FIPA) :

Principle :

FIPA is based on enzyme immuno assay principle. The technique utilizes antigen adsorbed on to nitrocelluose membranes. Upon addition of cell suspension, antibodies secreted by *in vitro* immunized lymphocytes bind to the immobilized antigen. Then the membranes are incubated with horse radish peroxidase-conjugated anti-mouse (or anti-human) antibodies Substrate is added, and the product of enzyme-substrate reaction which is insoluble appears as a blue plaque,which is scored as one antibody forming cell.

Materials and reagents :

1. 24 well plates (Laxbro, India)
2. Nitrocellulose membrane (15 mm diameter)
3. Phosphate buffered saline pH 7.2 (PBS) : 0.9% NaCl solution containing 50 mM Phosphate buffer pH 7.2.
4. TT 0.1 mg/ml PBS.
5. PBS-Tween 20 : PBS containing 0.05% Tween-20
6. Blocking solution : 5% skimmed milk powder in PBS was boiled for 15 min and then cooled immediately by transferring into an ice bath This process was repeated thrice, and then the solution was filtered through Whatman no. 42 filter paper.
7. Substrate solution : 3 mg of 4-chloro-1-naphthol in 1 ml methanol was added to 5 ml PBS containing 0.003% H_2O_2 .

Procedure :

Briefly, nitrocellulose membranes were coated with 0.1 mg/ml solution of TT in 24 well plate for 3 h. Then the filters were treated with blocking solution to block the unoccupied sites by Hot-blott technique (Janardana Sarma, 1988). *In vitro* immunized lymphocytes suspended in RPMI 1640 containing 1% FCS were put on the nitrocellulose membranes in wells at a concentration of 1 x 10^6 cells/well. Incubation was carried out at 37 °C for 3 h in a humidified incubator. Then the filters were washed 5 times with PBS and were incubated with horse radish peroxidase labelled goat anti-mouse IgM or anti-human IgM(1:800 dilution) for 1hr. After washing the membrane 7 times with PBS-tween 20 and PBS, plaques were developed by the substrate solution. The filters were rinsed after 15 min, dried and the blue plaques were counted microscopically under low magnification (Fig. - **1).**

Maintenance of EL-4.IL-2 cell line :

EL 4.IL-2, a subline of EL 4, a murine lymphoma cell line derived from a C57Bl/6 mouse. These cells were stimulated with Phorbol-12-myristate-13-acetate (PMA). IL-2 produced is biochemically and biologically indistinguishable from normal spleen cell derived IL-2.

Freezing **and** storage of **the** cells :

EL 4.IL-2 cell line was obtained from NFATCC, Pune, India. 2 - 4 x 10^6 cells /ml of DMEM containing 15% horse serum and 15% dimethyl sulfoxide (DMSO) were taken in sterile plastic vials and were frozen at -70 °C overnight and then transferred to liquid nitrogen for further use.

Revival and sub culture ;

Cells were taken out from liquid nitrogen and thawed quickly using 37 °C water bath. They were washed with warm DMEM supplemented with 10% horse serum for atleast 3 times to remove DMSO. Cultures were maintained in 24 well

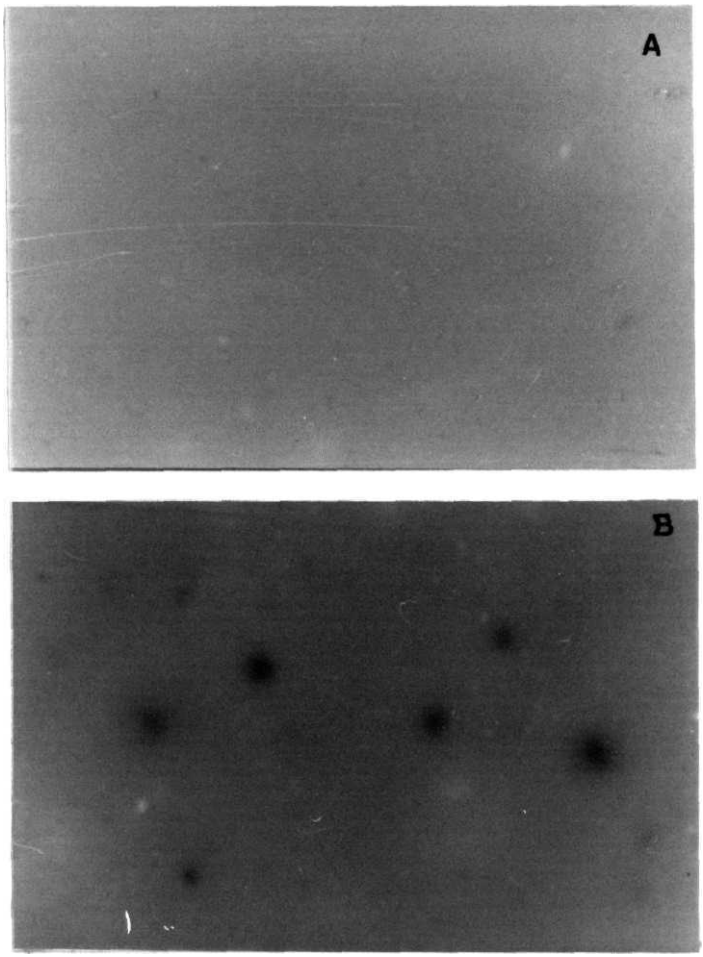

Figure 1 Filter Immuno plaque assay using *in vitro* immunized murine splenic lymphocytes. The nitrocellulose membrane was coated with
A. 0.1 mg/ml BSA.
B. 0.1 mg/ml TT.
Magnification : 63 x

plates (Laxbro, India) at a concentration of 2 x 10^5 cells/well in 1 ml of DMEM with 10% horse serum. Cells were subcultured every 48 h.

Stimulation of EL **4.IL-2 with** PMA for **IL-2** production :

2 x 10^6 cells/ml EL 4.IL-2 cells were stimulated with 20 **ng/ml** PMA in the presence of 1% horse serum for 24 h. The culture supernatant was collected at the end of 24 h and sterile filtered through 0.22 μ membrane filter of low protein binding capacity. The supernatant was aliquoted and stored at -70 °C.

Preparation of **Con.** A stimulated splenic lymphocyte **supernatant :**

Wistar rat splenic lymphocytes were cultured in bulk with soluble and sepharose-Con A at a concentration of 2.5 μM /ml. Cell concentration was adjusted to 4 x 10^6 cells/ml of complete medium with 50 μM β-mercaptoethanol and 8 ml of this cell suspension was taken into a siliconized glass tissue culture bottle, and were cultured at 37 °C in a CO_2 incubator. The supernatant was collected 24 h after stimulation. The soluble Con A supernatant was passed through α-methyl mannoside-sepharose column to deplete Con A. Sepharose Con A was seperated from **the** supernatant by passing through a glass wool column. Finally the supernatant was filter sterilized through a low protein binding 0.22 μ membrane and stored at -70 °C until use.

Preparation **of T cell enriched population from** splenic lymphocytes by **panning technique:**

Principle :

Splenic lymphocytes can be separated into T cell enriched and B cell enriched populations using **anti-Ig** coated plates, B cells with **sIg** receptors adhere to the plates while those without do not.

Materials and reagents :

1. Sterile plastic petridishes 90 mm (Tarsons India).
2. 10 mM Phosphate buffer containing 0.9% NaCl (PBS), pH 7.4
3. Goat anti-mouse IgG (Sigma Chemical Co., USA.) 25 μg/ml in PBS
4. RPMI 1640 supplemented with 5% FCS

Procedure :

Eight ml of anti-Ig solution was poured in petridishes and were kept overnight at 4 °C. Anti-Ig solution was removed from the plates and the plates were washed three times with PBS. 5 ml of spleen cell suspension at a concentration of 4 x 10^6 cells/ml was added to the petridishes and were incubated at 4 °C. The plates were gently swirled for 30 sec twice during the incubation period. After 1 h the non-adherent, T cell rich population was collected into sterile culture tubes and washed twice with the medium. Cell counting was done and the suspension stored on ice until use.

Assay of Interleukin-2 activity in con A-stimulated rat splenic lymphocyte supernatants and EL 4.IL-2 supernatants :

Principle :

Resting T cells upon stimulation with a mitogen get activated in 16 h and these activated T cells proliferate when supplemented with IL-2. Proliferative response is measured in terms of ^{3}H-thymidine incorporation into DNA.

Procedure :

2 x 10^5 T cells were cultured with and without 2.5 μg/ml of sepharose-Con A for 16 h at 37 °C in a CO_2 incubator. After 16 h, the cultures were depleted of the mitogen by passing through a mini-glass wool column. The activated T cells were then supplemented with soluble and sepharose Con A-stimulated splenic lymphocyte supernatant at various concentrations ranging from 0.25 - 40% for 32 h and were pulsed with 0.5 μCi ^{3}H-thymidine for the last 4 h of the culture period.

Control cultures were maintained without the addition of Con-A stimulated splenic lymphocyte supernatants. The cultures were processed at the end of 48 h using a Skatron cell harvester and the radioactivity was counted as described earlier.

Effect of Con A-stimulated splenic lymphocyte supernatant and PMA stimulated EL 4.Il-2 supernatant on AFC response of *in vitro* immunized splenic lymphocytes :

Using soluble and sepharose-TT as antigens, *in vitro* immunization cultures were setup. The culture medium was supplemented with various concentrations of PMA-stimulated EL 4.IL-2 supernatant or Con-A stimulated rat splenocyte supernatant. Control cultures without the supernatant and with antigen were setup. The AFC response was determined by FIPA on day 5.

Isolation of human peripheral blood lymphocytes :

Reagents :

1. RPMI-1640
2. FCS, Sigma Chemical Co. USA
3. Histopaque, density = 1.007 ± 0.001, Sigma Chemical Co. USA

Procedure :

Blood collections and TT immunizations were performed with the prior consent of the donors. Blood was drawn 2 weeks after TT immunization for isolation of TT primed human PBL. Unprimed PBL were collected from normal healthy donors, who were not immunized with TT for the last 3-4 years.

Twenty to forty ml of blood was collected from normal healthy adults and the cells were isolated by centrifugation over Histopaque density gradient according to Boyum (1968). 2.5 ml of Heparinised blood was mixed with equal volume of physiological saline and was layered carefully over 3 ml of Histopaque

in a centrifuge tube. Centrifugation was done for 30-40 min at 18 - 20 °C at 400x g. Lymphocytes which sediment at the interphase during centrifugation, were carefully removed with a pasteur pipette. The cell suspension was then transferred to RPMI-1640 containing 10% FCS. The cells were sedimented by centrifugation at 400x g, for 10 min and washed in RPMI-1640 containing 10% FCS and stored on ice until further use.

Treatment of human PBL and murine splenic lymphocytes with Leucyl-Leucine-O-methyl ester (Leu-Leu-OMe):

Principle :

L-Leucyl-Leucine methyl ester irreversibly eliminates lysosome-rich cytotoxic cells. The peptide ester diffuses into the lysosomes, gets metabolized to free amino acid and alcohol causing secondary swelling and rupture of the lysosomes.

Materials and reagents :

1. RPMI-1640 medium, serum free.
2. L-Leucyl-Leucine methyl ester hydrobromide (10X stock solution) : 0.85 mg/ml of Leu-Leu-OMe was dissolved in serum free cell culture medium immediately before use. This 10X stock was sterilized by filtration through 0.22 μ filter.

Procedure :

Cells were suspended in RPMI-1640 with 2% FCS at a concentration of 1 x 10^7 cells per ml. Leu-Leu-OMe was added to the cell suspension to a final concentration of 250 μM, and incubated for 15 min at room temperature. After the treatment, the cell suspension was diluted 1:2 or more in the medium, and centrifuged at 400x g for 10 min. The supernatant was removed and the cells were washed twice in medium containing *2%* (v/v) FCS. The cells must be handled

with maximum amount of care by avoiding any pipetting of the cell population in order to prevent formation of DNA clots Finally the Leu-Leu-OMe treated cell population was suspended in complete culture medium containing 10% serum. The cell yield is normally 65%, although it may vary between 40-90% depending on the individual buffy coat used as a source of peripheral blood mononuclear cells.

CHAPTER - III

PREPARATION AND CHARACTERIZATION OF IMMOBILIZED ANTIGENS

TT and OVA are immobilized by covalently linking them to two different solid supports ~ CNBr-activated sepharose 4B and immunomagnetic beads (Dynabeads). The immobilized antigens are characterized by studying their a) distribution on the solid matrix, b) stability and c) antibody binding capacity.

RESULTS :

Homogeneity of immunogens :

Protein was estimated in the TT preparation by Lowry's method, and it was electrophoresed on a SDS-polyacrylamide gel along with five molecular weight marker proteins (M.W range 29KD-116KD). The M.W of TT was determined based on its migration in comparison with the standard marker proteins. The M.W of TT was found to be 150 KD and the preparation contained no other protein.

Ovalbumin was also electrophoresed on SDS-polyacrylamide gel and the gel was silver stained. There was no other major protein contaminant, and 99% of the protein was constituted by OVA.

Coupling of TT and OVA to sepharose 4B :

Sepharose 4B is beaded agarose matrix characterized by negligible non-specific adsorption. Its pH stability is in the range of 4 - 9. Ligands can be covalently coupled to sepharose 4B upon activation with cyanogen bromide (CNBr).

CNBr-activated sepharose 4B enables proteins and other ligands containing primary amino groups to be immobilized quickly, easily and safely. CNBr-activated sepharose 4B has cyclic and acyclic imidocarbonates as active groups

which react with the amino groups belonging to the ligand, to form isourea linkages. TT and OVA were coupled to sepharose 4B as described in the methods.

Quantification of TT bound to the sepharose **4B gel :**

TT bound to sepharose gel was quantified by using radio iodinated TT (^{125}I-TT). ^{125}I-TT corresponding to 30 x 10^6 cpm was used for coupling to the gel. 7.6 x 10^6 cpm was recovered in the wash supernatant obtained during coupling. Based on this, it was calculated that 78% of TT used was bound to the gel. It has been reported earlier that proteins are often coupled with an efficiency of 80 - 95% (Ghetie *et ah.* 1978; Au and Varon, 1979). Percentage of TT bound to the gel was calculated as described in the methods.

Distribution of TT on the sepharose bead :

^{125}I-TT covalently coupled to sepharose gel was exposed to the X-ray film (Fig. 2). The autoradiogram shows uniform distribution of TT on the surface of the sepharose beads.

Stability of sepharose-TT at 4 °C **and 37** °C :

Table - 1A shows the release of TT from sepharose beads into the supernatant when stored at 4 °C. Negligible amount was released from the sepharose beads over a period of one month. Hence, sepharose-TT is very stable when stored at 4 °C.

An important consideration in experiments with immobilized antigens is to demonstrate unequivocally that the effects observed were not due to soluble antigen released from the insoluble matrix. Although TT was coupled to sepharose through covalent linkages and was thoroughly washed before use, there is a possibility that bound TT could be released during the cell culture. Enzymatic activity of lymphocytes (for e.g., galactosidase, protease) might release the TT

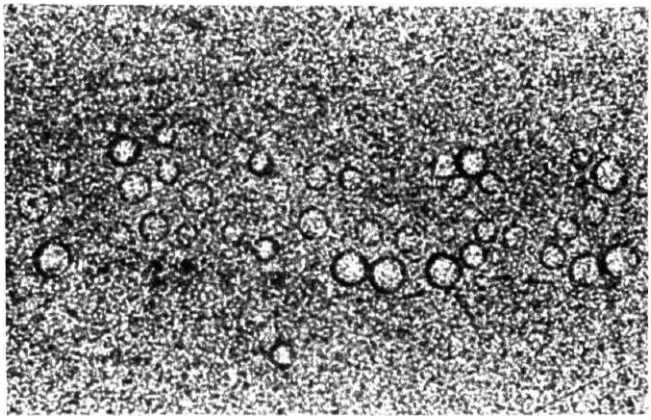

Figure 2 Autoradiogram showing the distribution of TT on the surface of sepharose bead. Magnification : 160 x

Table -1A **Release of ^{125}I-TT from sepharose-^{125}I-TT upon storage at 4 °C.**

Day	^{125}I-TT in the supernatant (cpm)	Release as % of total
10	12,856	0.250
20	8,950	0.200
30	6,875	0.175

0.5 ml of sepharose-^{125}I-TT (7.8 x 10^6 cpm) was stored in PBS at 4 °C and ^{12} 1-TT from released into the supernatant was monitere(The radioactivity is suitably corrected using isotope decay factor.

Table -1B **Release of ^{125}I-TT from sepharose-^{125}ITT in cell culture at 37 °C .**

Day	% Release	
	Medium	Medium + cells
1	2.80	2.31
2	2.00	3.08
3	1.85	3.30
4	2.30	2.45
5	2.20	2.30

The radioactivity is suitably corrected using the isotope decay factor and calculated as percentage of initaial activity (60,000 ± 4,000 cpm) The values presented are mean ± SEM of triplicates.

which could then interact directly with the cells or through the culture medium (Greaves and Bauminger, 1972).

In order to check the stability of immobilized TT during the culture period, sepharose-TT was incubated with and without cells at 37 °C. Only about 2-3% of TT was released from the sepharose beads during a 5 day culture period (Table - 1B). Hence more than 97 % of the TT remained in the immobilized state when it is being presented to cells in culture.

Distribution of TT on sepharose beads of various sizes :

To study the distribution of TT coupled to sepharose beads, FITC conjugated anti-TT antibody was used. When sepharose-TT was reacted with FITC labeled anti-TT antibody a brighter fluorescence was observed on the bigger beads (80μ diameter) as they have many active sites compared to the smaller beads (Fig. 3).

Antibody binding capacity of sepharose-TT :

The procedure adopted for covalently coupling TT to sepharose involves random linking *i.e.,* TT gets coupled to the bead through all the free amino groups irrespective of their location. If the coupling involves the residues located in close proximity to the epitopes, it can lead to substantial masking of the epitopes. The coupling can also result in a significant conformational change in the protein (Matson and Little, 1988) Hence, it is essential to study the antibody binding capacity of immobilized TT.

Rabbit anti-TT antibody reaction with sepharose-TT was visualized by using a anti-rabbit IgG-horse radish peroxidase conjugate. Fig. 4B shows TT-anti-TT antibody reaction on the sepharose bead. The deep purple coloured patches correspond to the antigen-antibody complex formed on the bead. Fig. 4A is the control for this reaction, where sepharose-TT is reacted with a non-specific

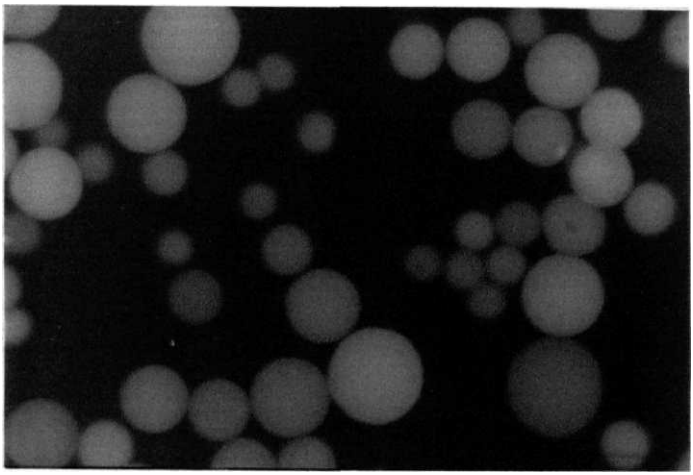

Figure 3 Distribution of TT on various sizes of sepharose beads. Sepharose-TT reacted with anti-TT antibody-FITC conjugate Magnification : 250 x

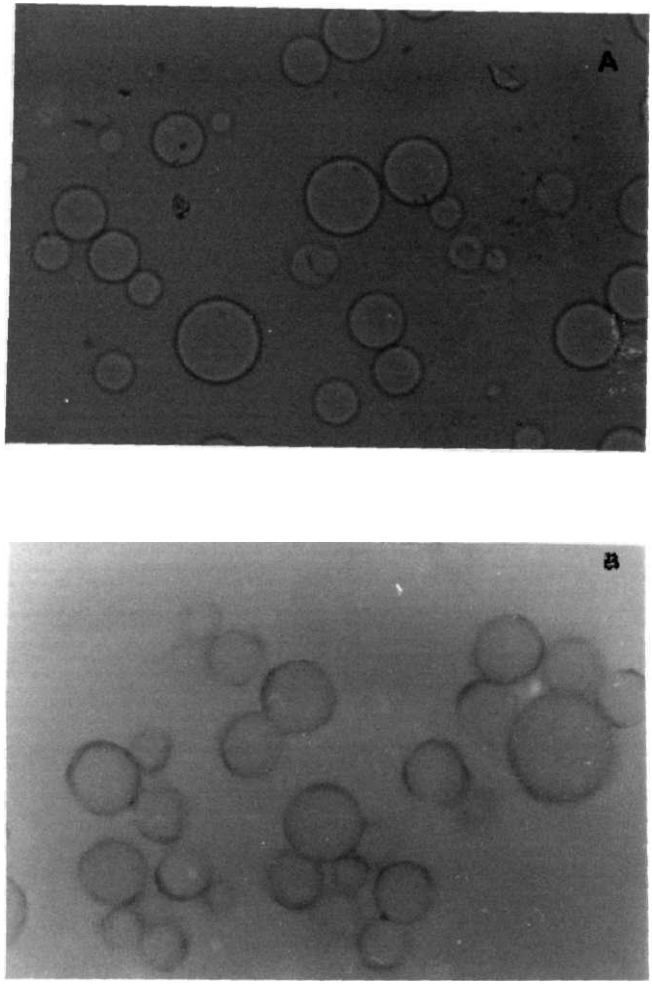

Figure 4 Interaction of anti-TT antibody with TT immobilized on sepharose beads detected by enzyme-linked secondary antibody conjugate.
A. Sepharose-TT with normal rabbit IgG.
B. Sepharose-TT with IgG fraction of rabbit anti-TT antibody.
The enzyme reaction was carried out as described under methods.
Magnification 250 x

antibody (rabbit anti-mouse IgG) From this data it is evident that the epitopes of TT immobilized on sepharose are well exposed and are available for interaction with anti-TT antibody and probably in turn with antigenic receptors on B lymphocytes.

Dynabeads M-450 are uniform, (4.5 μ diameter) magnetizable, (magnetic susceptibility ~ 10^{-2} cgs units) polystyrene beads, with a surface area (1-4 m^2/g) suitable for coating of antibodies or other ligands. Figure 17 shows Dynabeads at 250 x magnification. TT was immobilized on the Dynabeads as described in the methods. About 90% of the TT used was bound to the Dynabeads.

Quantification of TT bound to Dynabeads :

1. Coupling radio iodinated TT to Dynabeads :

^{125}I-TT corresponding to 1.125 x 10^6 cpm was coupled to 10 mg Dynabeads. 1.00 x 10^6 cpm (90 %) of ^{125}I-TT was bound to the Dynabeads. Percentage of ^{125}I-TT bound to the Dynabeads was calculated as described in the methods, taking into account the isotope decay factor.

2. ELISA :

Concentration of TT bound to the Dynabeads was estimated by solid phase ELISA. Fig. 5 shows the standard graph of ELISA of tetanus toxoid. Table - 2 shows the concentration of TT immobilized on Dynabeads. The concentration values are obtained by extrapolating the absorbance values on TT standard graph. 320 ng of TT is immobilized on 1 x 10^6 beads.

Stability of TT immobilized on Dynabeads :

Table - 3A shows the TT released from the beads at 4 °C. About 3% of ^{125}I-TT was released into the supernatant over a period of one month when stored at 4 ^{0}C.

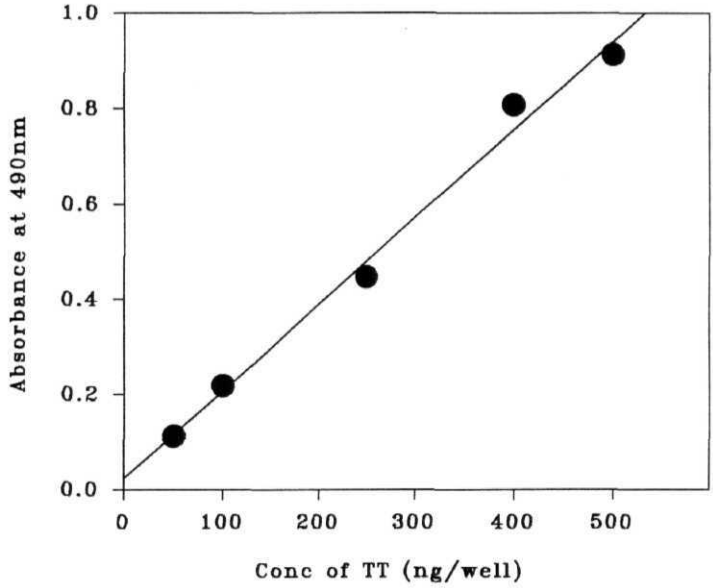

Figure - 5 ELISA of Tetanus toxoid.

Values presented are Mean ± SEM of triplicates of three experiments.

Table -2 **Quantification of TT immobilized on `Dynabeads' by solid phase ELISA.**

No. of beads x 10^6	Absorbance (490nm)	Conc. of TT (ng)
0.25	0.162 + 0.042	76
0.50	0.344 ±0.076	176
0.75	0.432 ± 0.085	217
1.00	0.605 ± 0.090	320

Values presented are mean ± SEM of 3 experiments.

Table - 3A **Release of ^{125}I-TT coupled Dynabeads at 4 °C .**

Day	% Release
10	2.2
20	2.8
30	3.1

Percentage of ^{125}I-TT released into the supernatant is calculated as described earlier.

Table -3B **Release of TT immobilized on Dynabeads when incubated in culture conditions.**

Day	Absorbance at 490nm	Conc. of TT (ng)	% Release
0	0.354 ±0.008	176	---
1	0.352 ± 0.005	173	1.7
3	0.348 ±0.008	167	5.1
5	0.348 ± 0.006	167	5.1

0.5 x 10^6 TT-Dynabeads were used in all the cultures .
Concentration of TT values are obtained from extrapolating the absorbance values in the TT standard graph.
Values presented are mean ± SEM of triplicates.

Table - 3B shows the TT released into the supernatant when Dynabeads-TT was incubated at 37°C under culture conditions. The release was quantified by ELISA. 0.5 x 10^6 beads were incubated with cells in complete medium for 5 days and TT released into the supernatant was quantified by solid phase ELISA at different time points of the culture period. About 95% of the TT immobilized on Dynabeads remained bound to the beads.

DISCUSSION :

Immobilization is a generic term used to describe the retention of a biologically active substance on a support material. The immobilized complex takes the physical characteristics of the support material while retaining its basic biochemical and immunological activity, thus improving the handling properties and the efficiency with which it is used in the bioconvertions. In the present study two T-dependent antigens are immobilized to study their immunogenicity in the *in vitro* immunization system of murine splenic lymphocytes and human peripheral blood lymphocytes. The goal of preparing the immobilized antigens by covalently coupling to the solid phase support is to retain the antigen on the solid phase support without adversely affecting the immune interaction, *i.e.,* to capture the antigenic receptors on the B lymphocytes. A variety of solid supports have been used for immobilization of antigens and mitogens *viz.,* Polyacrylamide beads (Parker *et al.*, 1979); Polystyrene beads (Gippert and Lipsky, 1988); glass beads (Eddy *et al.*, 1985); nitrocellulose membranes (Gratecos *et al.,* 1987); sepharose beads (Ramanadham *et al.*, 1984); latex particles (Wu *et al,* 1991) and immunomagnetic beads (Hawke *et al.,* 1992).

The advantages of using immobilized antigens in the *in vitro* immunization system are :

1. Retention of antigen on the support through out the culture **period** :

The immobilized antigens are available to interact with the lymphocytes through out the culture period, unlike the soluble antigens which are processed and internalized by the antigen presenting cells. The long lasting availability of the antigen permits better interaction with the cells resulting in a strong and sustained activation signal.

2. **High concentration of the antigen** :

Confining the antigen on the solid support inevitably increases the local concentration of the antigen, thus the overall accessibility of the epitopes per unit volume of the culture is consequently higher than for the homogenous soluble antigen cultures.

3. Use **of low** doses of antigen :

Low doses of antigen can be used upon immobilization, since the concentration of antigen per unit volume is higher as compared to the soluble antigen. Low doses antigen can therefore generate an effective response as compared to the same antigen in the soluble form.

4. Separation of the antigen from the culture system :

The separation of soluble antigen from an aqueous culture system is a difficult process. Moreover the free antigen is metabolized and **degraded**, whereas immobilized antigens an be very easily separated from the culture system and hence, the antigen contact time can be varied in the culture system and can be conveniently manipulated according to the experimental requirements.

5. Reusability of the antigen :

Since the immobilized antigens are retained throughout the culture period and can be separated very easily from the cultures **system**, they can be retrieved and reused. Antigens which are very expensive and those available in low concentrations can be effectively used by immobilizing them on a solid matrix.

Limitations of using immobilized antigens in the *in vitro* immunization system :

The process of immobilization inevitably involves handling and processing of the antigen. This invariably results in certain loss of the immune function due to orientation (masking of the epitopes) and accessibility (epitopes inaccessible to the receptors due to steric hindrance) effects. Sometimes the epitopes are occluded from binding to the cell surface receptors due to the steric effects and also due to the inappropriate geometry of the culture vessel.

Two solid phase supports have been used for immobilization in the present study

1. CNBr-activated sepharose 4B and
2. Immunomagnetic beads (Dynabeads) having an iron particle coated with polystyrene.

CNBr-activated sepharose 4B is a organic solid support with a large surface area for coupling the ligands. It is characterized by good chemical, mechanical and thermal stability, suitable shape and particle size, resistance to microbial attack and regenerability. Hence it can be conveniently reused in the *in vitro* immunization system. Previous reports have used mitogens and antigens immobilized on sepharose beads, to stimulate lymphocytes *in vitro* (Greaves and Bauminger, 1972; Parker *et ah,* 1979; Ramanadham *et ah,* 1983; Williams *et ah,* 1985 and Gathuru *et ah,* 1991).

Immunomagnetic beads `Dynabeads' are non-porous supports, which have a extremely low surface area and therefore the available area for protein coupling is very limited. In order to increase the protein coating, very fine particles (4.5μ) are used. However, non-porous carriers provide some advantages, since the antigen used for coupling is immobilized only on the external surface of the carrier and is therefore, totally available for contact with the surrounding environment. These immunomagnetic beads have been used for selecting high affinity antibody

secreting B cell hybridomas and delivering antigen to specific T cells (Ossendrop *et.al* 1989; Hawke *et al*., 1992).

The antigens are immobilized on the above mentioned supports using standardized coupling procedures which are described earlier. In case of CNBr-activated sepharose 4B, the coupling of the antigen was monitored by following the amount of the protein in the coupling solution and in the washes by monitoring the optical density at 280 nm. The percentage of the antigen conjugated was determined by this process and was also cross checked by ^{125}I labeled TT. The antigen uptake was monitored by counting directly the sepharose-^{125}I-TT conjugate in the Gamma counter. This method is most commonly used for the quantification of ligand coupled to sepharose beads (Greaves and Bauminger, 1972; Parker, 1979; Nilsson *et al*., 1987). The exact quantification of the antigen immobilized on the solid support is very crucial for the comparison of responses with antigen in the soluble form.

The antigen binding to the Dynabeads is estimated by coupling ^{125}I-TT and also by a very sensitive solid phase ELISA, by which nanogram quantities of protein bound to the gel could be quantitated.

The distribution of the antigen on the sepharose bead was studied because it is a porous matrix, having some internal surface area to which ligands can bind. If the antigen gets coupled to the internal surface it will not be exposed to the external environment. The autoradiogram shows that almost all the protein bound is on the external surface and is readily available for interacting with the lymphocytes. Stability of the immobilized antigens has to be monitored during the immobilization, storage and more importantly during the operational conditions. The stability of the antigen during the immobilization is taken care of by keeping the stirring rate of the reaction mixture very low and prevent foaming, and also by maintaining low temperatures during coupling to prevent inactivation of the antigen.

In general, the immobilized antigens can be stored in a similar way as the soluble antigens. The storage of an immobilized antigen in solution is often better than that of a soluble antigen in identical solution due to the high local concentration of the protein on the gel structure. The percentage release of ^{125}I-TT from the sepharose beads and Dynabeads over a period of one month shows clearly that the TT immobilized on sepharose beads and Dynabeads is very stable when stored at 4 °C.

Operational stability is the most important property of the immobilized antigen and it has no real equivalent with soluble antigens. Assessment of the stability of the immobilized antigens under the real operational conditions is needed to fully characterize them, and for comparison with soluble antigen. The operational stability of sepharose-TT was determined by monitoring the release of ^{125}I-TT from the sepharose beads incubated at 37 °C with lymphocyte population in a humidified CO_2 incubator. The ^{125}I-TT release over a culture period of 5 days shows that sepharose-TT is very stable under the operational conditions. The TT released from the Dynabeads was monitored by means of solid phase ELISA, which shows that > 95 % of the TT remained bound to the Dynabeads through out the culture period.

Which ever method of immobilization is selected the final product must be characterized in terms of its ability to bind to the specific antigenic receptors. The protocol used for coupling does not involve any specific orientation of the antigen towards the solid support, therefore it is a random coupling process. In order to ascertain that the epitopes are exposed and are not occluded during the coupling process, the antibody binding capacity of sepharose-TT has been studied. From the sepharose-TT and anti-TT antibody reaction which was visualized by a colour reaction, it is clearly evident that the epitopes of the TT immobilized on the sepharose beads are exposed and are available for interaction with the antigenic receptors.

Since the sepharose matrix is made of varying size of beads, the distribution of TT on different sizes of beads was studied. The antigen distribution visualized by sepharose-TT and anti-TT antibody-FITC conjugate, shows that the big beads (>60 μ) take up more amount of TT as compared to the smaller beads since, they have more surface area and hence more number of active sites for ligand binding.

From these studies using sepharose-TT and Dynabeads-TT, it can be said that the antigens immobilized on sepharose and Dynabeads can effectively be used in the *in vitro* culture systems.

CHAPTER - IV

IN VITRO AND IN VIVO IMMUNIZATION OF MURINE SPLENIC LYMPHOCYTES USING IMMOBILIZED IMMUNOGENS

A. *IN VIVO* IMMUNIZATION USING SOLUBLE AND SEPHAROSE-TT :

Immunogenicity of TT in soluble and sepharose linked forms :

In order to ascertain that TT in soluble as well as in sepharose-linked forms can effectively interact with the B lymphocytes, we have studied their effect on proliferative response of TT sensitized splenic lymphocytes derived from TT immunized mice. It is observed that both soluble and sepharose-TT could induce a significant proliferative response in TT sensitized lymphocytes. In presence of sepharose-TT, the proliferative response was significantly higher than soluble TT. ($P < 0.02$) (Table - 4).

Immunogenicity of sepharose-TT *in vivo* :

To assess the potency of immobilized antigens *in vivo,* the mice were injected with soluble TT, soluble TT in complete Freund's adjuvant (CFA-TT) and Sepharose-TT. Saline, CFA-saline and uncoated sepharose beads were injected as controls. Primed lymphocytes were isolated from the immunized mice on day 7, and were stimulated with different concentrations of TT in soluble and sepharose-linked form. Splenic lymphocytes from sepharose-TT primed splenic mice evoked a similar proliferative response when treated *in vitro* with soluble and sepharose-TT. Whereas, lymphocytes from soluble TT primed mice showed a higher proliferative response with sepharose-TT, as compared with soluble TT ($P < 0.005$ **Fig.-6).**

Table - 4 **Proliferative response of murine splenic lymphocytes to soluble and sepharose-TT.**

Addition to culture (μg/ml)	^{3}H-thymidine incorporation into DNA (cpm/10^{6} cells)	A cpm
None	11,395 ± 690	
Sepharose beads(25μl)	11,520 ± 690	125
Sepharose beads (25μl) + soluble TT 0.50	14,455 ± 485	3,060
Soluble TT		
0.10	13,680 ± 755	2,285
0.50	14,920 ± 695	3,525
100	16,950 ± 1,155	5,555
2.00	16,310 ± .980	4,915
Sepharose-TT		
0.10	14,125 ± 705	2,730
0.50	15,795 ± 955	4,400
100	24,930 ± 1,535*	11,535
2.00	23,695 ± 1,075*	10,300

Values presented are mean 1 SEM of 3 experiments.
•P < 0.02 (soluble v/s sepharose-TT)

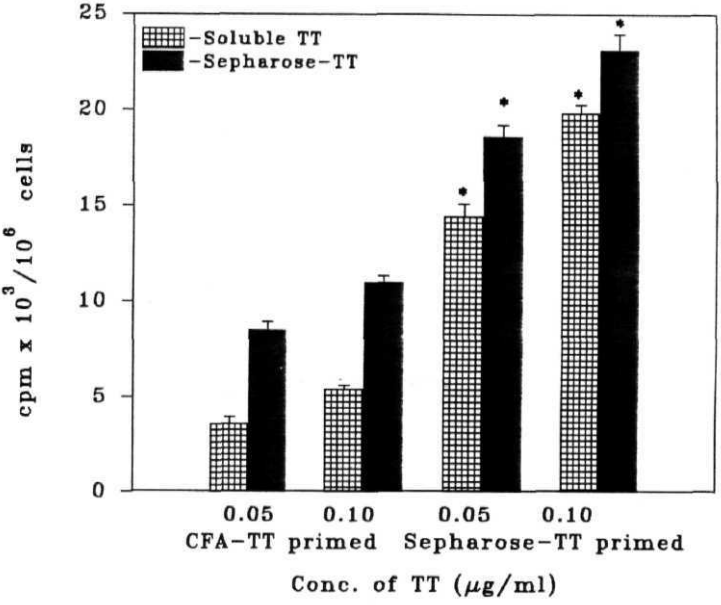

Figure - 6 Proliferative response of TT-primed murine splenic lymphocytes to soluble and sepharose-TT.

Values presented are Mean i SEM of 3 experiments.

• P < 0.005 (CFA-TT v/s Sepharose-TT primed)

Primary AFC response was also determined on day 7. The primed cells after isolation were directly put on TT coated nitrocellulose membranes and the AFC response was determined by F1PA. BSA coated nitrocellulose membranes were used as controls. Sepharose-TT primed splenic lymphocytes showed a higher AFC response as compared with the CFA-TT primed cells (Fig. - 7).

To determine the secondary AFC response, TT primed cells were cultured with immunogens, soluble and sepharose-TT, and the IgG-AFC response was determined by FIPA. Sepharose-TT primed cells showed a similar AFC response when stimulated with soluble and sepharose-TT. Whereas in CFA-TT primed lymphocytes, a higher AFC response was seen with sepharose-TT as compared with soluble TT (Fig.8). Further, when stimulated with soluble TT the AFC response was higher in sepharose-TT primed cells as compared with CFA-TT primed cells ($P < 0.005$). These observations suggest that sepharose-TT is a potent immunogen when injected ***in*** *vivo,* compared with CFA-TT.

B. *IN VITRO* IMMUNIZATION :

TT was used as a model antigen for studying the efficacy of immobilized form as compared to the soluble form. Fig. - 9 shows the interaction of murine splenic lymphocytes with sepharose-TT.

Dose response relationship of soluble and sepharose-TT :

Unprimed splenic lymphocytes from 8-10 week-old Balb/c mice were immunized in culture with different concentrations of soluble and sepharose-TT to study the dose-response relationship in an *in vitro* immunization system. After 5 days of antigen stimulation, the splenocytes were tested for their ability to secrete antigen-specific antibodies by FIPA (Fig. - 10). Sepharose-TT treated cultures showed a significantly higher IgM-AFC response as compared with the soluble TT

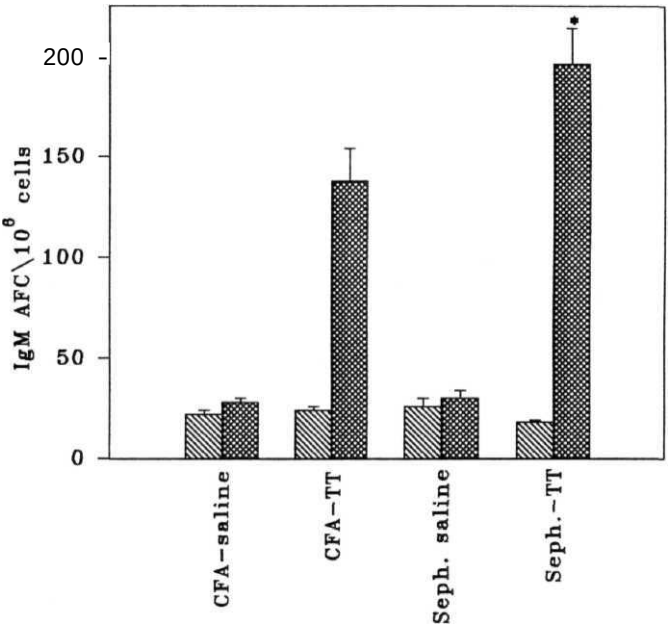

Figure - 7 AFC response of TT-primed murine splenic lymphocytes to soluble and sepharose-TT.

-BSA coated nitrocellulose membranes - **Non-specific**
-TT coated nitrocellulose membranes - **specific**

Values presented are Mean ± SEM of 3 experiments
•**P** < 0.05

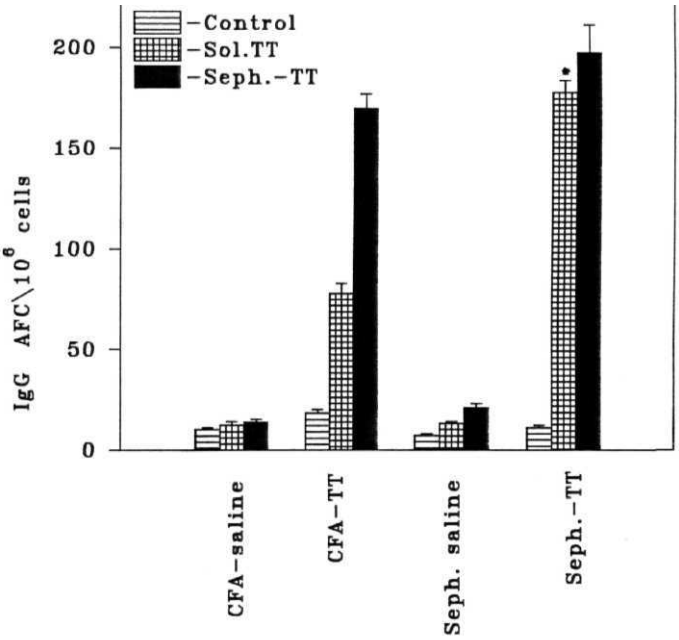

Figure - 8 AFC response of TT-primed murine splenic lymphocytes - Secondary response.

Soluble and Sepharose-TT were used at a conc. of 5μg/ml.
Values presented are Mean ± SEM of 3 experiments
• P < 0.005 (CFA-TT v/s Sepharose-TT primed)

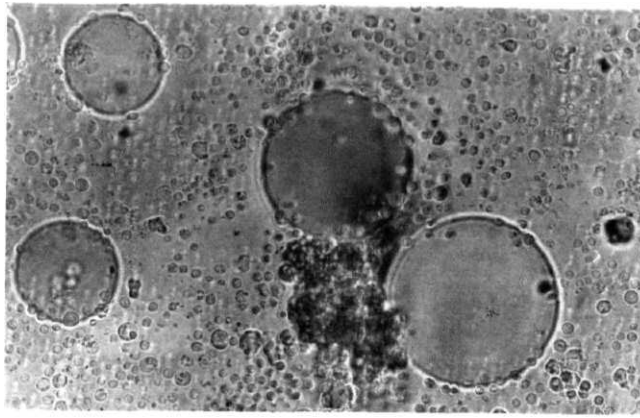

Figure 9 Binding of murine splenic lymphocytes to sepharose-TT in culture. Magnification : 250 x

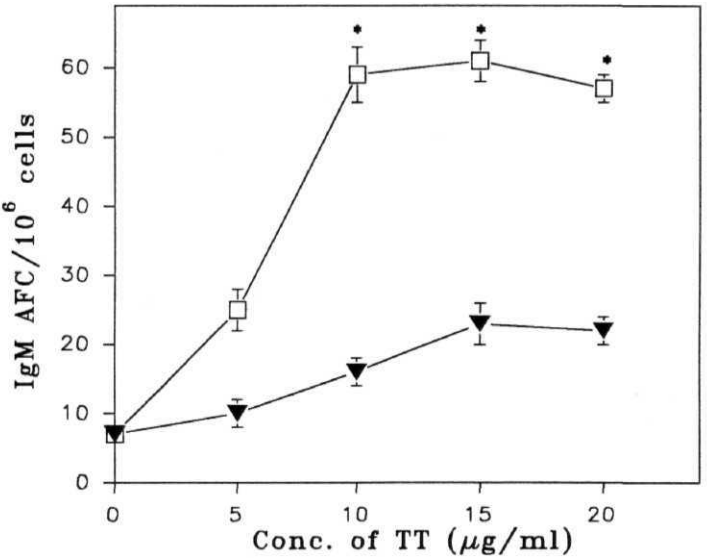

Figure - 10 AFC response of murine splenic lymphocytes immunized ***in*** *vitro* **with** Soluble and sepharose–TT.

▼ – soluble TT
□ – **sepharose–TT**

Values presented are Mean ± SEM of 5 experiments

• P < 0.001

treated cultures ($P < 0.001$). IgG secreting plaques were undectable when enumerated using anti mouse-IgG HRPO conjugate. Hence, in all further *in vitro* immunization experiments only IgM-AFC were measured.

Time course of the AFC response :

Kinetics for the optimal antigen stimulation period was investigated by determining the number of AFC on days 4,5,6 and 7. A two fold increase in the number of AFC was observed on day 5 as compared to day 4 with sepharose-TT (Fig. - 11).

Effect of serum concentration on AFC response :

Effect of serum concentration on AFC response to soluble and sepharose-TT was investigated using different concentrations of serum from 0% to 20% (v/v) in the culture medium. Maximal AFC response was obtained with 10% v/v serum concentration in the culture. At concentrations above 10%, no further increase in AFC response was observed (Fig. - 12).

Effect of Leu-Leu-OMe on AFC response :

Treatment with lysosomotropic agents causes depletion of lysosome-rich cell populations, viz., monocytes, large granular lymphocytes, cytotoxic T cells and a subset of $CD8^+$ T suppressor cells from splenic lymphocyte preparations. When Leu-Leu-OMe treated cells were immunized *in vitro* with soluble and sepharose-TT, the AFC response increased by twofold in soluble TT treated cells as compared with Leu-Leu-OMe untreated controls. However, in cells stimulated with sepharose-TT there was no increase in the number of AFC upon Leu-Leu-OMe treatment (Fig. - 13).

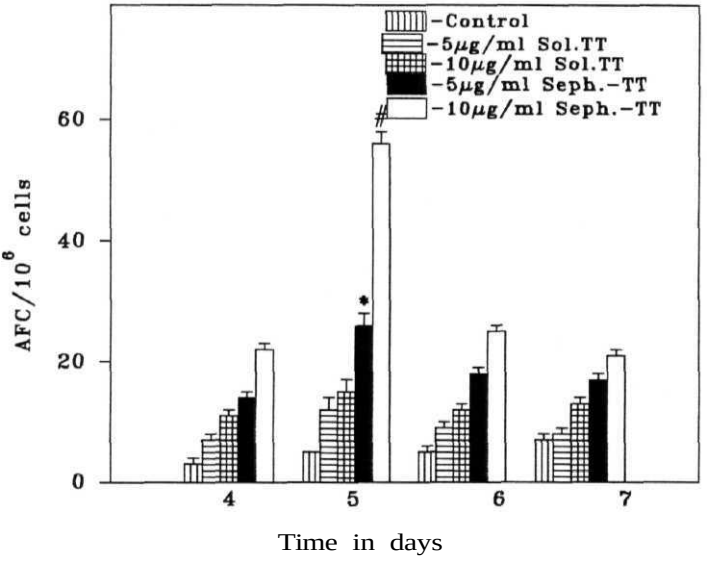

Figure —11 Time course of AFC response of *in vitro* immunized murine splenic lymphocytes

Values presented are Mean ± SEM of triplicate cultures of 5 experiments.
• P < 0.03 # P < 0.001

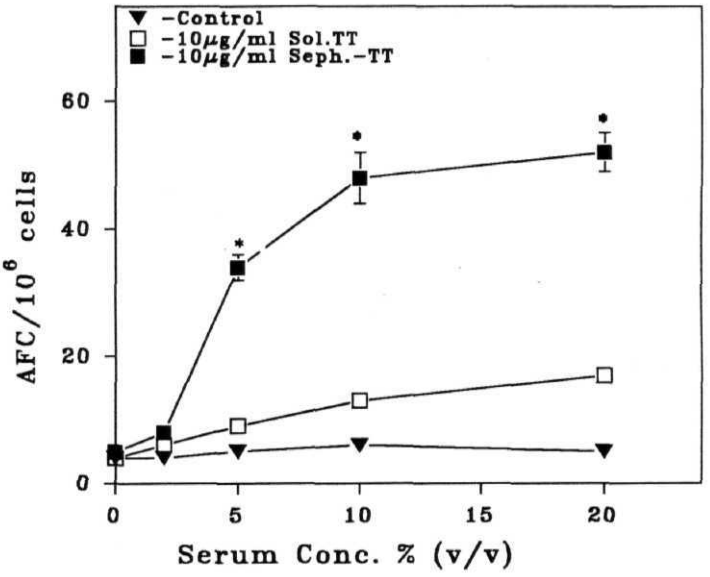

Figure - 12 Effect of serum concentration on AFC response of *in vitro* immunized murine splenic lymphocytes.

Values presented are mean ± SEM of 5 experiments.
• P < 0.001, Soluble vs sepharose-TT.

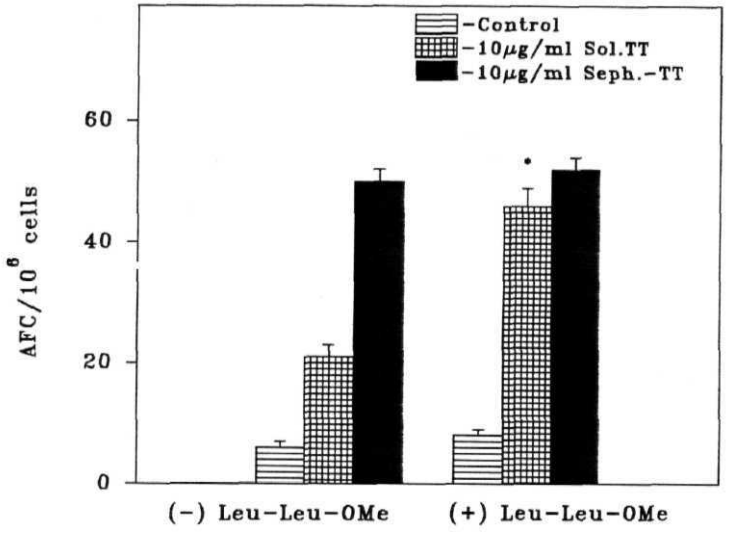

Figure 13 Effect of Leu-Leu-OMe on AFC response of *in vitro* immunized murine splenic lymphocytes.

Concentration of Leucyl-Leucine methyl ester : 250 μM. Values presented are Mean ± SEM of triplicate cultures of 3 experiments. * $P < 0.01$

AFC **response in RPMI-1640 medium and CO_2 free medium** :

CO_2 free medium has a unique phosphate based buffering system which allows cell cultures to thrive at stable pH without CO_2. It can be used in closed as well as open systems, and also eliminates the cytotoxic effects associated with the use of Hepes buffer. The AFC response of *in vitro* immunized murine splenic lymphocytes in CO_2 free medium and in RPMI-1640 medium (bicarbonate buffered medium) was compared. The AFC response was similar in cell cultures grown in RPMI-1640 medium and CO_2 free medium and sepharose-TT was more potent than soluble TT (Table - 5).

Effect of antigen density on the sepharose bead on AFC response :

The density of TT on the sepharose bead was altered during the coupling process. Unprimed splenic lymphocytes were cultured for 5 days with sepharose-TT of different antigen densities (2 mg/ml, 5 mg/ml and 10 mg/ml gel). 2 mg and 5 mgTT/ml of sepharose gel gave an optimal AFC response. (Table - 6). In all the experiments 5 mgTT/ml sepharose beads were used. When 2 mgTT/ml beads were used, at higher concentrations of TT in culture overcrowding of beads resulted in lower AFC response.

Effect of bead size on the AFC response :

The sepharose-TT beads were separated using a 50 μ nylon mesh. The AFC response was higher in 50 - 80 μ sepharose-TT treated cultures as compared to 20 - 50 μ sepharose-TT treated cultures (Table - 7).

Effect of geometry of culture vessel on AFC response :

In vitro immunization of splenic lymphocytes using sepharose-TT was performed in different types of culture vessels, and the AFC response was determined on day 5 using FIPA. AFC response was similar in cultures grown in

Table -5 AFC response of *in vitro* immunized **murine** splenic lymphocytes in RPMI-1640 and CO_2 five medium.

Conc. of TT (μg/ml)		AFC/10^6 cells RPMI-1640	CO_2 -free medium
Control		6 ± 1	8 ± 1
Soluble TT	5	18 ±1	15 ±2
	10	26 ± 2	29 ± 3
Sepharose-TT	5	39* ± 3	35*±2
	10	56*± 2	54*± 4

Values presented are mean ± SEM of 4 experiments.
P < 0.01 (soluble v/s sepharose-TT)

Table - 6 **Effect of antigen density on the sepharose bead on AFC response *of in vitro* immunized murine splenic lymphocytes.**

Density of TT on the sepharose beads (mg/ml)	AFC/10^6 cells
2	54 + 3
5	53 ± 5
10	34 ± 1

Sepharose-TT was used at an concentration (10μg/ml) in the culture. Values presented are mean ± SEM of 5 experiments.

Table - 7 **Effect of bead size on the AFC response of *in vitro* immunized murine splenic lymphocytes**

Conc. of TT (μg/ml)	AFC/10^6 cells		
	Total beads (20M - 80μ)	Small beads (20μ - 50μ)	Big beads (50M - 80μ)
5	37 ± 1	20 ± 1	67 ± 1
10	53 + 2	35 + 4	76 ± 2

Sepharose beads were seperated using a nylon mesh with a pore size of 50μ Control values are substracted from the AFC response values. Values presented are mean ± SEM of 5 experiments.

tissue culture flasks, 24 well plates and Tissue culture tubes (12 x 75mm dia) (Table - 8).

Effect of cytokine supplementation :

In order to study the effect of supplementation of cytokines on *in vitro* immunization, PMA-stimulated, EL-4.IL-2 supernatant and soluble and sepharose-Con A-stimulated rat splenic lymphocyte supernatants were used. These supernatants are obtained as described in the methods.

Assay **of IL-2 (T cell growth factor) activity in the supernatants** :

The activity of the supernatants was assayed in terms of the proliferative response of T cell blasts obtained by stimulation of enriched T cells with sub-optimal concentration of sepharose-Con A. To arrive at a sub-optimal concentration of Con A, enriched T cells were treated with different concentrations of soluble and sepharose-Con A (Fig - 14). Soluble Con A evoked a maximal proliferative response at 5 μg/ml followed by a decline at higher concentrations, whereas sepharose-Con A showed a proliferative response from 5 μg/ml and increased upto 40 μg/ml. At higher concentrations, soluble Con A brings about clumping of cells whereas this was not observed in case of sepharose-Con A.

Fig. -15 shows the proliferative response of sepharose-Con A blasts to soluble and sepharose-Con A-stimulated rat splenic lymphocyte supernatants. The T cell growth factor activity in soluble Con A supernatant was maximal at 2.5 % and thereafter, there was a steep decline in the activity. Whereas, in case of sepharose-Con A stimulated supernatant, maximal activity was obtained at 2.5 % and showed a plateau till 40 %. PMA-stimulated EL-4.IL-2 supernatant showed a maximal activity at 10 % and the activity declined slowly thereafter (Fig. - 16).

Table - 8 **Effect of geometry of culture vessel on AFC response of *in vitro* immunized murine splenic lymphocytes.**

Type of culture vessel	AFC/10^6 cells		
	Control	Soluble TT	Sepharose-TT
Tissue culture tube (12 x 75 mm)	4 + 1	22 ± 1	58 ± 3
24 well plate	6 ± 1	18 ± 3	56 ± 1
Tissue culture flask (25 cm^2)	8 ± 1	32 ± 2	65 ± 5

Values presented are mean + SEM of 3 experiments.
Concentration of soluble TT and sepharose-TT = 10μg/ml.

Table - 9 **Effect of PMA-stimulated EL-4.IL-2 supernatant on AFC response of *in vitro* immunized murine splenic lymphocytes.**

EL-4.IL-2 supernatant	AFC/10^6 cells		
	Control	Soluble TT	Sepharose-TT
None	3 ± 1	23 ± 3	58 + 3
10%	6 + 1	49* ± 3	55 ± 5
20%	6 ± 2	57* ± 4	61 ± 7

Values presented are mean ± SEM of 4 experiments
Concentration of soluble and sepharose-TT = 10μg/ml.
*P<0.001

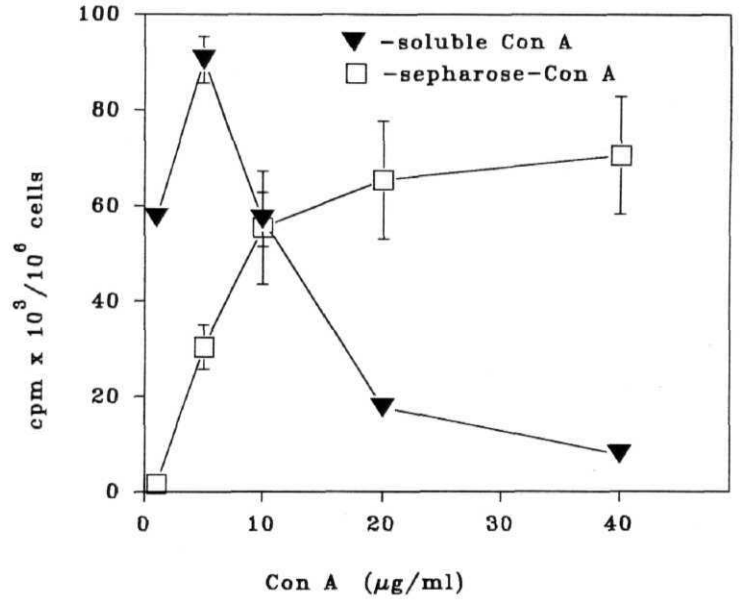

Figure - 14 Proliferative response of murine T cells to soluble and seph.-Con A.

Values presented are Mean ± SEM of 5 experiments

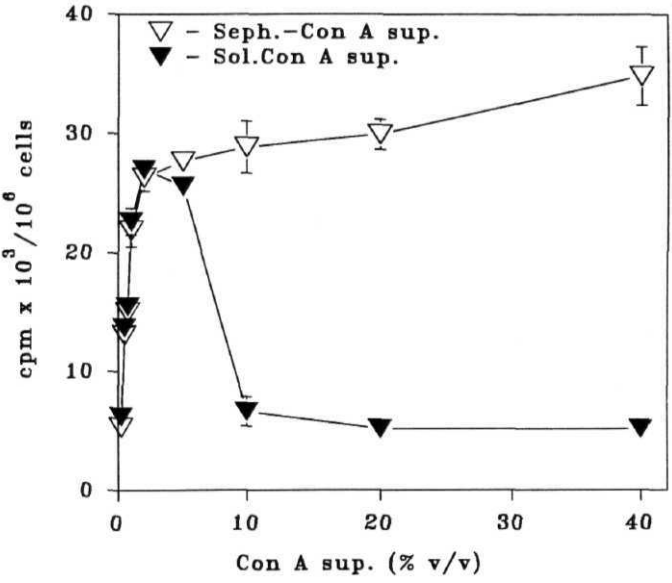

Figure — 15 Proliferative response of Seph – Con A blasts to Con A stimulated rat splenocyte supernatants.

Values presented are Mean ± SEM of 4 experiments.

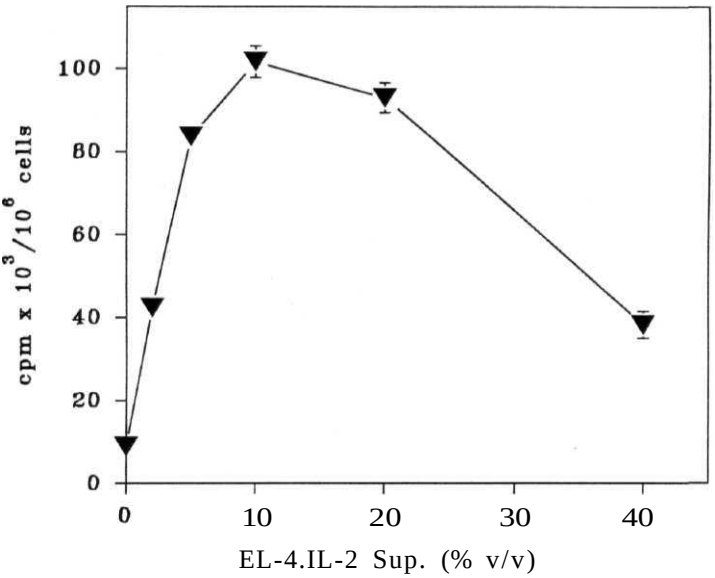

Figure-16 Proliferative response of T cell blasts to PMA stimulated **EL-4.IL-2** supernatants.

Values **presented are Mean ± SEM of 5 experiments**

Supernatant supplementation in *in vitro* **immunization** :

Upon supplementing the *in vitro* immunization cultures with PMA-stimulated EL-4.IL-2 supernatant, there was a significant enhancement in the AFC response in cultures treated with soluble TT. But, in sepharose-TT treated cultures no enhancement in AFC response was observed as compared to unsupplemented cultures (Table - 9).

Similarly, when *in vitro* immunized cells were supplemented with Con A-stimulated rat splenic lymphocyte supernatant, the AFC response increased in the soluble TT treated cultures, whereas the AFC response remained unaltered in the sepharose-TT treated cultures (Table- 10). Upon supplementing the soluble and sepharose-TT treated cultures with Sepharose-Con A supernatant, the AFC response increased significantly in both soluble and sepharose-TT treated cultures unlike the soluble Con A stimulated supernatant.

In vitro **immunization using antigens immobilized on Dynabeads** :

TT and OVA were used as model antigens. The antigens immolilized on Dynabeads were used at different concentrations in the *in vitro* immunization cultures. Cultures with soluble antigens were also kept for comparison. Fig. 17 shows the Dynabeads (250x magnification) and interaction of Dynabeads with murine splenic lymphocytes.

The antigens immobilized on Dynabeads could evoke significant AFC response at a concentration range of 320-640ng. The AFC response evoked by Dynabead-linked antigens at nanogram concentrations was higher then that evoked by soluble antigens used at microgram concentrations. The antigen-linked Dynabeads were easily seperated from the culture using a magnet and were reused for *in vitro* immunization. A similar AFC response was observed with the reused Dynabead-linked antigens as compared to Dynabead-linked antigens used for the first time (Table - 11,12). Upon supplementing the Dynabeads-TT cultures with

Table - 10 **Effect of soluble and sepharose-Con A supernatants on AFC response *of in vitro* immunized m** urine **splenic lymphocytes.**

Addition	AFC/10" cells				
	- supernatant	Soluble Con A sup.		Sepharose-Con A sup.	
		5%	20%	5%	20%
None	4 ± 1	5 ± 0	3 ± 1	6 ± 1	4 ± 1
Soluble TT	13 ± 1	26** ± 2	17 ± 2	23* ± 3	33** ± 2
Sepharose-TT	32 + 2	34 ± 5	33 ± 4	45* ± 1	58" ± 4

Concentration of soluble TT and sepharose-TT =5μg/ml.
Values presented are mean ± SEM of 3 experiments.
*p<0.005 **P < 0.001

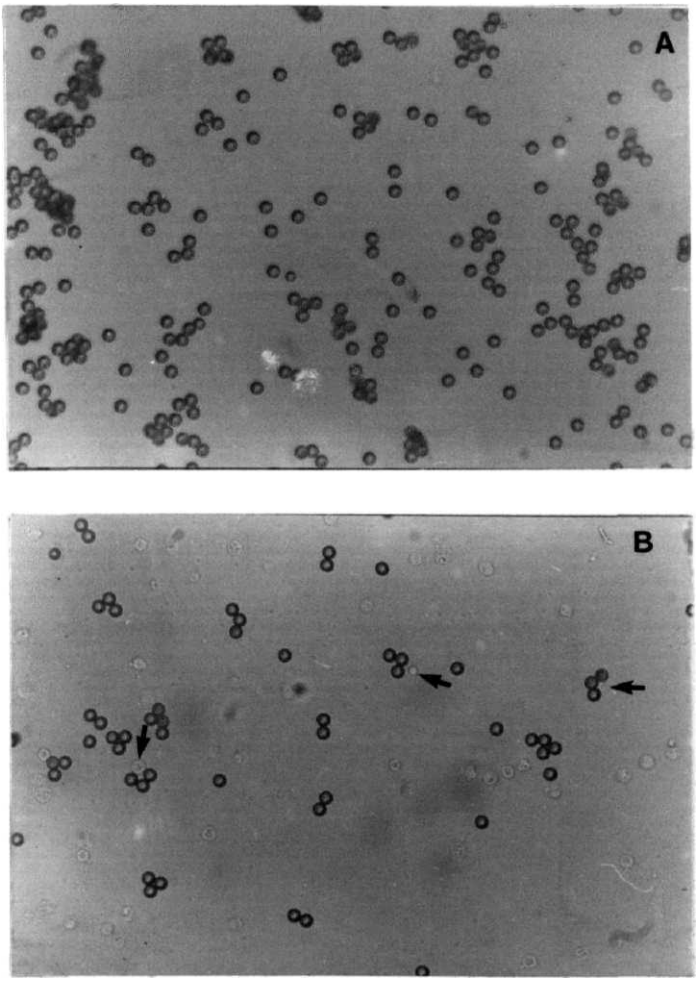

Figure 17 A. Uncoated Dynabeads
B. Binding of Dynabeads-TT in culture with murine splenic lymphocytes. Cells are indicated with an arrow.
Magnification : 250 x

Table -11 AFC response of murine splenic lymphocytes immunized *in vitro* with soluble TT and TT coupled to Dynabeads .

Antigen	Concentration (μg/ml)	Cells : Beads	AFC / 10^6 cells
None			6 ±1
Soluble TT	1.00		12 + 2
Dynabeads-BSA	0.32	2 :1	7 ±1
Dynabeads-TT			
0.05 x 10^6	0.016	0.4:1	8+1
0.50 x 10^6	0.160	4: 1	14 ± 2
1.00 x 10^6	0.320	2 : 1	26*±2
2.00 x 10^6	0.640	1:1	25*±3
Used Dynabeads-TT	0.320	2 : 1	23*± 2

Concentration of TT per 1 x 10^6 beads = 320 ng
Values presented are mean ± SEM of 3 experiments
*P < 0.05 (soluble TT v/s Dynabead-TT)

Table - 12- **AFC response of unprimed murine splenic lymphocytes immunized *in vitro* with soluble OVA and OVA coupled to Dynabeads.**

Antigen	Concentration (μg/ml)	AFC/10^6 cells
None		3 ± 1
Soluble OVA	5.00	11 ± 1
Dynabeads-BSA	0.32	8 ± 1
Dynabeads-OVA	0.32	25 ± 2*
Used Dynabeads-OVA	0.32	23 ± 1 *

Dynabeads and cells were kept at a ratio of 1 : 1.
Values presented are mean ± SEM of 3 experiments.
* $P < 0.001$

Table - 13 **Effect of PMA stimulated EL-4.IL2 supernatant on AFC response of murine splenic lymphocytes immunized *in vitro* with Dynabeads-TT.**

Addition to culture (μg/ml)		AFC/10^6 cells	
		- supernatant	+ supernatant (20%)
None		4 ± 2	3 ± 1
soluble TT	5.00	16 ± 3	33* ± 5
Dynabeads-TT	0.16	14 ± 1	31* ± 2
Dynabeads-TT	0.32	22 ± 1	44* ± 4

Values presented are mean ± SEM of 3 experiments.
*P < 0.005

PMA-stimulated EL-4.IL-2 supernatant, the AFC response increased by two fold (Table- 13).

DISCUSSION :

Tetanus toxoid is a potent immunogen. It is used as a model antigen in the present study in soluble and sepharose-linked forms. The efficacy of immobilized TT in comparison to soluble TT was studied using mouse splenic lymphocytes immunized *in vivo* and *in vitro.* Immobilization of anti-Ig antibody or antigen on sepharose beads increases the valency and renders them more potent activators (Sieckmann *et al* ., 1978; Parker *et al* ., 1979; Pure *et al* ., 1980; Mond *et al* ., 1983). These multivalent antigens are good model systems to study B cell activation.

The TT preparation used in these experiments was checked for its immunogenicity in both soluble and sepharose-linked forms using *in vitro* primed murine splenic lymphocytes. The significant proliferative response of TT primed splenic lymphocytes to soluble and sepharose-TT shows that TT can effectively interact with B lymphocytes in soluble and immobilized states.

Sepharose-TT proved to be a potent immunogen when administered *in vivo.* Primed splenic lymphocytes from sepharose-TT immunized mice showed a higher proliferative response as compared with CFA-TT primed lymphocytes. These results suggest that the sepharose-TT has an adjuvant effect and can interact with more number of B lymphocytes *in vivo,* since it is not easily metabolized or degraded.

Data on the primary AFC response of TT immunized mice also suggests that sepharose-TT stimulates more number of B cells to differentiation and Ig secretion as compared to soluble TT. The data on IgG AFC response was obtained by stimulating the primed splenic lymphocytes *in vitro,* with soluble and

sepharose-TT. Soluble TT primed lymphocytes showed a higher AFC response with sepharose-TT, compared with soluble TT. Whereas sepharose-TT primed lymphocytes showed almost similar AFC response when stimulated with soluble and sepharose-TT. This shows that sepharose-TT probably drives more number of B cells into the memory pool. Upon restimulation the memory cells enter the cell cycle to undergo proliferation and differentiation to Ig secreting cells.

After ascertaining that TT is immunogenic in both soluble and sepharose-linked forms, it was used in the *in vitro* immunization system to study the efficacy of immobilized antigens and to arrive at the optimal conditions to obtain a maximal AFC response.

Sepharose-TT evoked a significantly higher AFC response at all concentrations as compared to soluble TT. From these results it is evident that sepharose-TT acts as a potent immunogen in the murine *in vitro* immunization system. There are a few earlier reports where the antigen is used in the immobilized form adsorbed to different solid supports *e.g.*, fumed silica beads (Van Ness *et al.*, 1984), Glass beads (Eddy *et al.,* 1985), nitrocellulose membrane (Gratecos *et al.,* 1987). In all these reports the immobilized antigens induced a better AFC response as compared with their soluble counterparts. In all the previous reports, the *in vitro* immunization cultures were supported by the exogenous addition of the lymphokines.

A five day antigen stimulation period, was found to be optimal in the present experiments using soluble-TT and sepharose-TT in accordance with the earlier reports. (Mishell and Dutton, 1967; Click *et al.,* 1972; Borrebaeck, 1993; Pardue *et al.,* 1983; Borrebaeck and Moller, 1986; Brams *et al,* 1987) and sepharose-linked antigens (Gathuru *et al.,* 1991).

Another important variable in *in vitro* immunization system is the source and concentration of serum. Activation of B cells by serum proteins is usually obtained during *in vitro* immunization (Borrebaeck and Moller, 1986) although

this can be avoided by performing the entire *in vitro* immunization under completely serum-free conditions (Ossendrop *et al.,* 1986, Schneider, 1989) or an initial (7 - 9 h) serum-free culture followed by addition of FCS (Van Ness *et al.,* 1984). It has been reported earlier that when FCS was used, batch to batch variation critically influenced the yield of hybridomas (Rathjen and Underwood, 1985). This has been ascribed to variations in the level of endotoxins and hormones. Sometimes the use of FCS resulted in high yield of antigen-non-specific hybridomas. But in several cases it has been used satisfactorily in *in vitro* immunization (Luben and Mohler, 1980; Sethi *et al.,* 1981; Boss, 1984; Van Ness *et al.*, 1984). FCS has been used as the serum source in the present study and the optimal concentration in culture was found to be 10 %. A further increase in the serum concentration did not influence the AFC response significantly. A complete serum-free medium may not support the AFC response in presence of immobilized antigens.

Treatment of murine splenic lymphocyte population with the lysosomotropic agent Leu-Leu-OMe, removes macrophages and other susceptible cell types which bring about a suppressive effect on the AFC response, with no adverse effects on B and T_h cells (Thiele *et al.,* 1987). In the present study, AFC response to sepharose-TT stimulation was same in both Leu-Leu-OMe treated and untreated lymphocyte cultures. Whereas, in soluble TT stimulated cultures there is an enhancement in the AFC response in Leu-Leu-OMe treated cultures. These results suggest that the stimulation of B lymphocytes by sepharose-TT is : 1. not influenced by the presence of suppressor cell population, 2. does not involve antigen processing and presentation by macrophages to the T_h cells, 3. is not due to the stimulation of phagocytes (macrophages) by sepharose beads. hence the activation of B cells by immobilized TT is probably, predominently, T cell dependent.

Some of the physical characteristics of sepharosc-TT which influence the AFC response in an //; *vitro* immunization system were studied The density of antigen on sepharose bead is an important parameter to be considered while using immobilized antigens. A density of 5 mg antigen/ml sepharose beads was optimal and could evoke a concentration dependent increase in the AFC response. With low and high density of antigen on the bead the AFC response was observed to be low. This may be due to the overcrowding of the beads and inaccessability of antigen to the cells due to the steric effects.

When sepharose-TT beads in the size range of 50 - 80 \x were used for immunization *in vitro,* the AFC response obtained was higher as compared to the immunization with the whole beads or small beads. This could be due to the fact that the bigger beads have more active sites and hence the local concentration of the antigen on the bead is higher as compared with the small beads. The immobilized antigens are supposed to act as polymeric antigen (GTeaves and Bauminger, 1972). So the bigger beads will have more number of repeating antigen molecules and hence, an effective crosslinking with the membrane receptors of B cells is achieved.

The AFC response of sepharose-TT stimulated cultures was similar in all the three types of culture vessels used. This indicated that the interaction of cells with the immobilized antigen is independent of the geometry of the culture vessel.

Cytokines play an important role in different stages of B cell activation. The cytokines derived from of PMA stimulated EL-4.IL-2 cell line supernatant and soluble and sepharose-Con A stimulated rat splenocyte supernatant were used. The EL-4.IL-2 derived supernatant was shown to contain B cell growth and differentiation inducing factors (BSF-1, BCDF) (Howard *et al.,* 1982), IL-2 (Farrar *et al.,* 1980) and factors with isotype regulatory effects *e.g.,* BCDF u (Isaksson *et al.,* 1982) and BCDF y (Vitetta *et al.,* 1984). Supernatant of rat Con A stimulated splenic lymphocytes have been shown to be rich in T cell growth factor (Gillis *et*

al., 1978). Supernatant obtained from sepharose-Con A stimulated rat splenic lymphocytes were also used due to the ease of its preparation and potential reuse of immobilized Con A

Before testing these cytokines in the *in vitro* immunization system, they were assayed for their ability to stimulate T cell proliferation An assay system was setup using sepharose-Con A blasts obtained by treating enriched T cells with sub-mitogenic dose of the mitogen for 16 h. These blasts were then stimulated with EL-4.IL-2 and Con A supernatants and their IL-2 activity was determined in terms of proliferative response.

The optimal concentration of EL-4.IL-2 supernatant to support T cell proliferation was found to be 10% (v/v of the culture medium). Soluble Con A supernatant showed peak activity at 2.5% and the activity declined very steeply by 10 %. Whereas the sepharose-Con A supernatant also showed a maximal activity at 2.5 % and thereafter showed a plateau in the activity till 40 %. Hence,the soluble and sepharose-Con A supernatants show different patterns of activity in terms of proliferative response of T cells. Both the supernatants show an almost equal activity at 2.5 % suggesting that, they are quantitatively similar in terms of the T cell growth factor activity. However, they differ qualitatively, as can be seen from their effect on T cell proliferation at higher concentrations. Upon supplementation in *in vitro* immunization, only sepharose-Con A supernatant could enhance the AFC response in sepharose-TT cultures. In sepharose-Con A supernatant additional factors could be present as sepharose-Con A might activate B cells in addition to T cells (Parker, 1975). It has been reported by Justement *et al*., (1989), that lymphokine elaboration occured in response to sIg crosslinking by immobilized anti-Ig, but not by whole anti-Ig in soluble form in A20.1 B cell lymphoma. The lymphokines elaborated are Il-2 and a factor which induces la expression by resting B cells.

These supernatants derived from EL-4, Con A, and sepharose-Con A stimulated lymphocytes were used in the *in vitro* immunization The EL-4 and Con A supernatants did not have any effect on AFC response of sepharose-TT treated cultures. However, in case of soluble TT treated cultures there was an increase in the AFC response. The enhancement in AFC response was not observed when Con A supernatant was supplemented at 20 % concentration (inhibitory concentration). Sepharose-Con A supernatant showed an enhancement in the AFC response in both soluble and sepharose-TT treated cultures. EL-4 derived supernatants were used in the *in vitro* immunization system at 25% concentration in association with allogeneic helper factors (Danielsson *et al* 1986) and MLC-derived lymphokines (Borrebaeck and Moller, 1986).

From the above findings it can be said that Sepharose-Con A supernatant has some additional factor(s) which is responsible for the enhancement of the AFC response in sepharose-TT treated cultures. Since it is probable that phytomitogens immobilized on sepharose can activate both T and B cells, the additional factor may probably be of B cell origin. In this context their is evidence to show that normal and transformed B cells produce lymphokines in response to polyclonal B cell activators (Jurgensen *et al.*, 1986; Muraguchi *et al.*, 1986; Walker and Leemhuis, 1987; Walker *et al.*, 1988). Multiple lymphokines are produced by a B cell line A20.1, within 3-4 h after surface Ig crosslinking by immobilized anti-Ig (Justement *et al.*, 1989). Therefore it is likely that the crucial requirement of lymphokines for the growth and differentiation of B cells is circumvented by the use of immobilized antigens which activate the B cells by an effective crosslinking of membrane Ig receptors. As the activation of B cells by sepharose-TT does not require exogenous addition of T helper factors it can be considered to be truely T-independent

Immunomagnetic beads Dynabeads' were used as another solid support for immobilization of antigens. These are uniform sized beads of 4.5 μ diameter,

almost equivalent to the cell size. A single cell can interact with more than one Dynabead unlike the interaction of cells with sepharose-antigens where many cells interact with a single sepharose bead. Very small amounts of antigens (3-5 μg/mg beads) can be immobilized on these beads. When nanogram quantities of antigens immobilized on Dynabeads were used in the *in vitro* immunization system, they could evoke a significantly higher AFC response as compared to the same antigen in the soluble form. Previous studies reported Dynabeads, for selection of high affinity B cell hybridomas and for delivering antigen to specific T cells (Ossendrop *et al.,* 1989; Horton *et al.,* 1989; Hawke *et al.,* 1992). The Dynabeads are extensively used for separation of cells and sub-cellular components (Lea *et al.,* 1988; Hansel *et al.*, 1990; Schwinzer *et al.,* 1992).

Since these beads are magnetic in nature, they could be easily separated from cell culture and could be reused effectively to obtain an AFC response, similar to that evoked by the antigen used for the first time. The Dynabead-antigen cultures when supplemented with EL-4.IL-2 supernatant, an enhancement in the AFC response was observed, unlike the sepharose-antigen stimulated cultures. Probably, T cell help is required when immobilized antigens are used at very low (ng) concentrations. From these findings it can be said that significant AFC response can be evoked with nanogram concentrations of antigens when immobilized on Dynabeads. The AFC response can further be amplified by supplementing these cultures with lymphokines derived from EL-4.IL-2 thymoma cell line.

In the present study, significantly higher AFC response could be achieved using immobilized form of TT as compared to its soluble form. No addition of exogenous lymphokines to the culture medium was neccessary for the induction of enhanced AFC response using immobilized TT. This is in contrast to the previous reports using the soluble form of immunogens where *in vitro* AFC response could

be induced only upon addition of interleukin-rich supernatants to the culture (Danielsson *et al.,* 1987).

The mechanism by which immobilized antigens induce higher response *in vitro* compared to soluble form of the same antigen is probably as follows : It is likely that the immobilized antigens deliver a strong and sustained signal to the lymphocyte via the surface receptors due to their enhanced crosslinking ability. Immobilization of antigen on the surface of the bead creates a matrix of Ugands at a fixed density which interact directly with the sIg receptors leading to activation of B cells. The continued interaction of antigen with the cells allows continuous and repetetive signaling, unlike the soluble antigens where high concentrations of antigen are required to achieve a threshold number of crosslinked antigen molecules to mediate B cell activation. Sepharose linked antigens may be acting as T-independent polymeric antigens due to the presence of many identical antigen molecules which are fixed on the matrix.

B cells, under appropriate conditions respond to antigen-mediated sIg crosslinking by producing IL-2 and a factor which induces Ia expression. This provides a basis for an autocrine and paracrine regulatory network. First, IL-2 could act to potentiate T cell activation and at the same time regulate B cell proliferation and differentiation (Justement *et al.*, 1989). IL-2 stimulates B cell proliferation (Almerigogna *et al* 1985) and also supports the generation of plaque forming cells (Swain *et al.,* 1982). Therefore, IL-2 secretion by B cells could enhance the antibody response through effects on both B and T cells. The production of a factor which regulates the Ia expression could promote cognate T : B interaction via increased expression of Ia which would enhance the ability of B cells to present the antigen to MHC restricted Th cells. Additionally, the la-inducing activity described in A20.1 supernatant (Justement *et al.,* 1989) may exert additional effects on B cells analogous to IL-4 by enhancing proliferation and differentiation (Paul and Ohara, 1987).

Upon supplementation with EL-4.IL-2 supernatant, a significant enhancement of AFC response can be seen only in soluble TT treated cultures and not in sepharose-TT treated cultures. This further suggests that the enhanced AFC response in presence of sepharose-TT is more likely to be independent of helper factors derived from T cells.

Our data, suggests that the degree to which an antigen can crosslink sIg may be a critical factor in influencing its immunogenicity (Mond *et al*., 1979; Pure *et al* 1980). This may also influence the T cell dependency or independency of an antigen. The fact that immobilized antigens can efficiently crosslink sIg may be the cause for apperent T cell independent response, or a different type of T cell help compared with the soluble antigen.

CHAPTER - V

IN VITRO AND *IN VIVO* IMMUNIZATION OF HUMAN PERIPHERAL BLOOD LYMPHOCYTES USING IMMOBILIZED IMMUNOGENS

A. *IN VIVO* IMMUNIZATION :

In order to ascertain that TT can interact with human PBL in soluble and sepharose-linked forms, TT primed human PBL were stimulated *in vitro* with soluble and sepharose-TT. Both soluble and sepharose-TT could evoke a significant proliferative response. Sepharose-TT could evoke a significantly higher proliferative response as compared to the TT in soluble form ($P < 0.05$) (Table - 14).

The secondary AFC response was determined by stimulating the TT primed human PBL with soluble and sepharose-TT *in vitro* for 5 days. The AFC response was significantly higher in cultures treated with sepharose-TT as compared to soluble TT (Table - 15).

B. *IN VITRO* IMMUNIZATION :

Dose-response relationship of soluble antigens and sepharose-linked antigens :

TT and OVA were used in the soluble and sepharose-linked forms in the human *in vitro* immunization system.

Unprimed human PBL were stimulated *in vitro* with different concentrations of soluble and sepharose-linked antigens (TT and OVA). A significantly higher antigen-specific AFC response was observed in cultures treated with sepharose-linked antigens as compared to the cultures treated with their soluble counterparts (Fig. - 18 & 19).

Table - 14 **Proliferative response of TT primed human ?BL to soluble TT and sepharose-TT**

Conc. of TT (ng/ml)	cpm/10^6 cells			
	Soluble TT	Acpm	Sepharose-TT	Acpm
0	1,570 ± 200			
	10,975 ± 2,550	9,405	19,980* ± 1,560	18,410
25	15,290 ± 4,285	13,720	31,425** ± 2,050	29,855
50	25,250 ± 2,950	23,680	30,520 ± 2,600	28,950

^{3}H-thymidine incorporation was measured between 72 - 96 h. Values presented are mean ± SEM of triplicate cultures from a representative experiment out of 3 experiments.
*P<0.01 **P < 0.05.

Table - 15 **AFC response of TT primed human PBL**

Conc. of TT (ng/ml)	IgG AFC response /10^6 cells	
	Soluble TT	Sepharose-TT
0	6 ± 1	6 ± 1
25	31 ± 1	72 + 3
50	68 ± 1	146* ± 5
500	96 ±6	212** ± 8

TT primed human PBL were stimulated *in vitro* with soluble and sepharose-TT. AFC were enumerated on day 5 by FIPA.
Values represented are mean ± SEM of 3 experiments.
*P < 0.005 **P < 0.001.

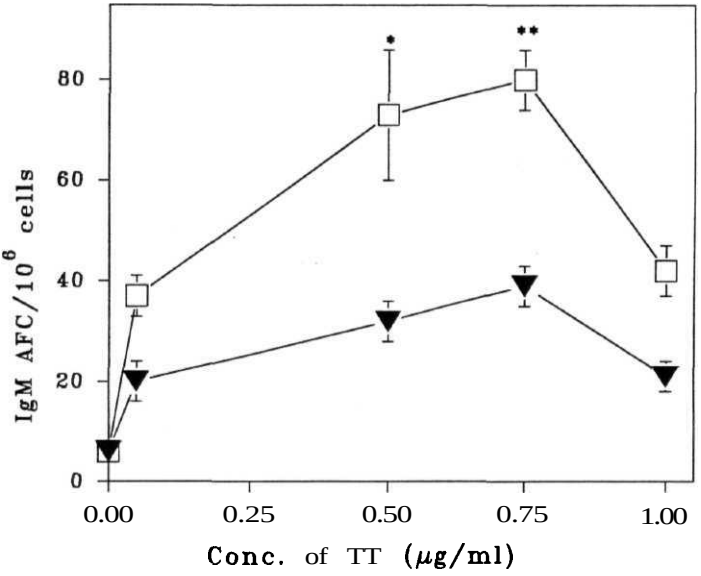

Figure - 18 AFC response of unprimed human PBL immunized *in vitro* with soluble and Sepharose-TT

▼ – Soluble TT
□ – **Sepharose–TT**
Values represented are Mean ± SEM of 4 experiments.
* $P < 0.01$ •• $P < 0.005$

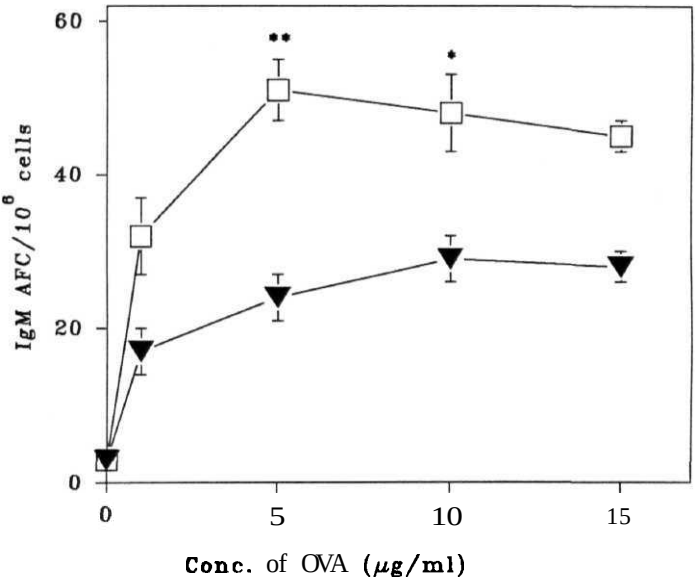

Figure - 19 AFC response of human PBL immunized *in vitro* with soluble and sepharose-OVA

▼ - soluble OVA

□ - sepharose-OVA

Values represented are Mean ± SEM of 5 experiments.

• $P < 0.05$ •• $P < 0.005$

Kinetics of the *in vitro* immune response :

AFC response from day 4 to day 7 was studied for determining the optimal antigen stimulation period. Cultures stimulated with sepharose-linked antigens evoked a maximal AFC response on day 5, whereas in case of cultures stimulated with soluble antigens, the AFC response was maximal on day 6, followed by a decrease on day 7 (Fig. - 20 & 21).

Effect of serum source and concentration on the AFC response :

In vitro immunization cultures were setup with different concentrations (0 - 20 % v/v) of FCS in the culture medium. The AFC response was maximal at 10 % FCS concentration. Further increase in the serum concentration did not show significant enhancement in the AFC response (Fig. - 22 & 23).

A comparative study was performed using rabbit serum, horse serum, FCS and human AB serum, in the *in vitro* immunization cultures. FCS and AB serum supplemented cultures elicited a similar AFC response, which was significantly higher as compared to the response evoked by horse serum and rabbit serum (Fig. - 24).

Effect of Leucyl leucine methyl ester treatment on AFC **response** :

Unseparated human PBL are stimulated *in vitro* with a soluble T dependent antigen, elicit a very poor antigen-specific AFC response due to the presence of $CD8^+$ T cells and cytotoxic/suppressor cells (Danielsson *et al.*, 1987). Therefore, human PBL were treated with a lysosomotropic agent Leu-Leu-OMe which depletes the lysosome rich cells *viz.*, alloantigen-specific cytotoxic T cells, monocytes, NK cells, large granular lymphocytes and a subset of $CD8^+$ T cells (Thiele and Lipsky, 1986). The treatment had no adverse affects on B lymphocytes, $CD4^+$ T cells, endothelial cells and fibroblasts. When the PBL treated with Leu-Leu-OMe were stimulated with soluble and sepharose-linked

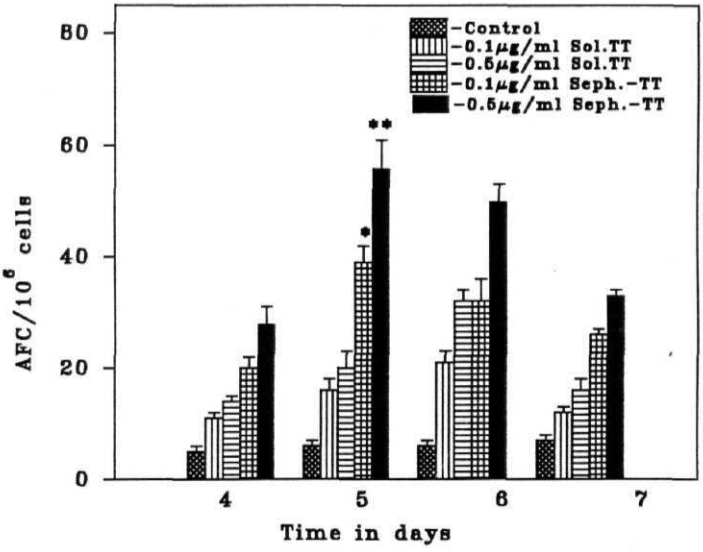

Figure - 20 Time course of AFC response of *in vitro* immunized human PBL

Values presented are Mean ± SEM of 3 experiments.
• $P < 0.05$ ** $P < 0.01$

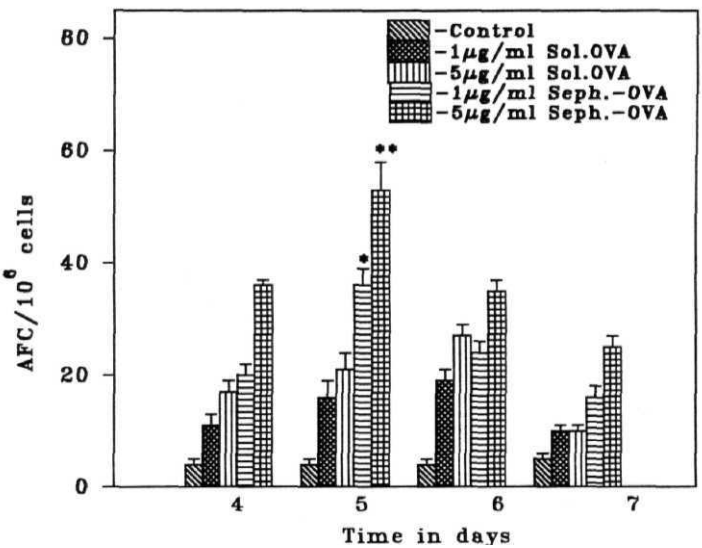

Figure - 21 Time course of AFC response of *in vitro* immunized human PBL

Values presented are Mean ± SEM of 4 experiments.
• $P < 0.05$ •• $P < 0.01$

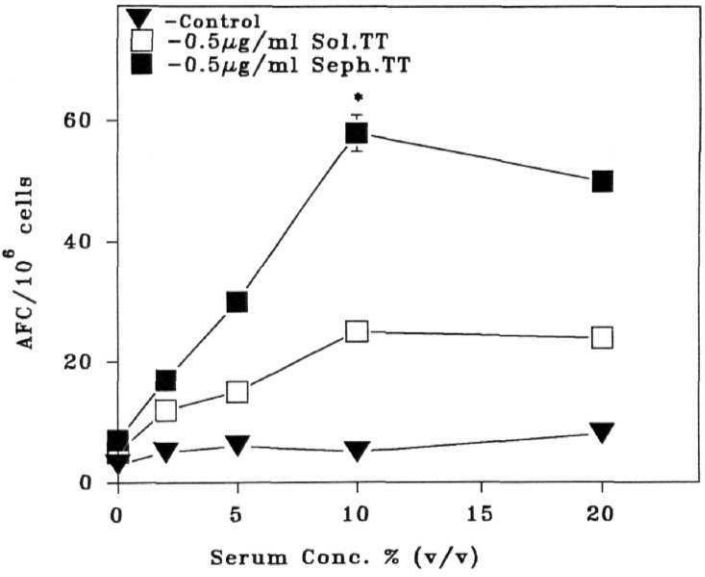

Figure — 22 Effect of serum conc. on AFC response of in *vitro* immunized human PBL

Values presented are Mean ± SEM of 6 experiments.

• P< 0.001

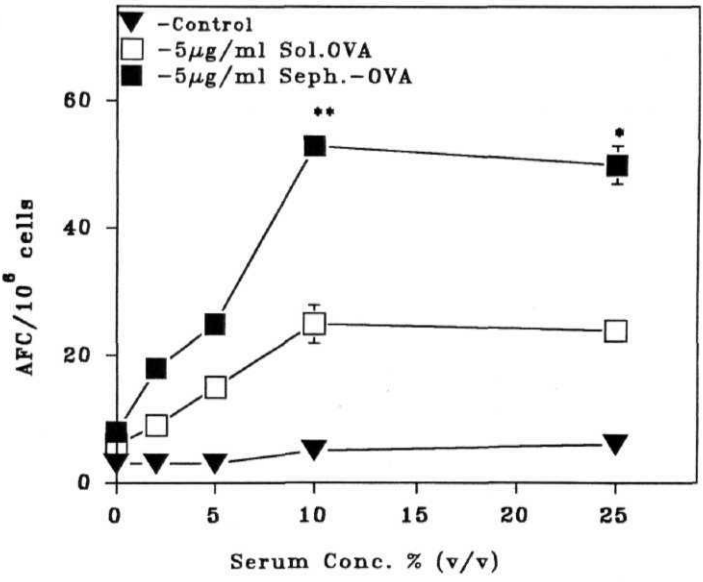

Figure - 23 Effect of serum **Conc.** on AFC response of *in vitro* immunized human PBL

Values presented are Mean ± SEM of 4 experiments.
• P < 0.01 •• P< 0.005

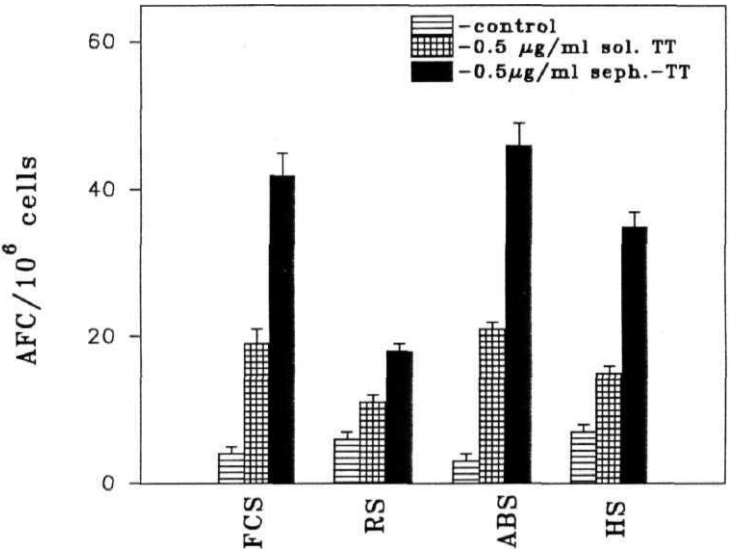

Figure — 24 AFC response of human PBL immunized *in vitro* with soluble and sepharose-TT comparison using different sera

All the sera are used at a concentration of 10% (v/v) in the culture medium. FCS - Fetal calf serum, RS - Rabbit serum AB — AB serum, HS — Horse serum.
Values presented are mean ± SEM of 4 experiments.

antigens, the soluble antigen stimulated cultures showed two fold increase in the AFC response as compared with Leu-Leu-OMe untreated cultures. However, there was no enhancement in AFC response in sepharose-TT or sepharose-OVA treated cultures (Fig. - 25 & 26).

DISCUSSION :

The present experiments clearly demonstrate that unprimed human PBL can be reproducibly sensitized *in vitro,* using immobilized antigens, providing a good source of human B cells secreting specific antibodies. Immunization of human B cells has been shown to be influenced by a number of factors (Borrebaeck, 1987). We have investigated four parameters which influence AFC response induction, to determine the optimal conditions.

The *in vitro* immunization protocols for human PBL reported previously used small quantities of antigen, lymphokines and a non-specific lymphocyte activator, Staphylococcus aureus Cowan 1 protein, (Hoffman, 1980;) PWM (Strike *et al.,* 1984); or adjutant peptides (MDP) (Jacot-Guillarmod, 1988) in culture. It was found that the B cell response was blocked by suppressor T cells and different methods were adopted to remove the suppressor activity, like passing the PBL over a sephadex G-10 column (Hoffman *et al.,* 1982) or by treating the PBL with cimetidine which selectively depletes the PBL of suppressor cells bearing the type 2 histamine receptors (Cavagnaro and Osband, 1983). Danielsson *et al.,* (1987) fractionated the PBL for immunization *in vitro* to obtain an significant AFC response. The antigen-specific activation of PBL was shown to be drastically down regulated by cytolytic cells such as large granular lymphocytes, cytotoxic and suppressor T cells (Borrebaeck *et al.,* 1987). Normal PBL, with out any further fractionation do not respond antigen-specifically when cultured in the presence of antigen for 6 - 7 days (Danielsson *et al.,* 1987). However, in the

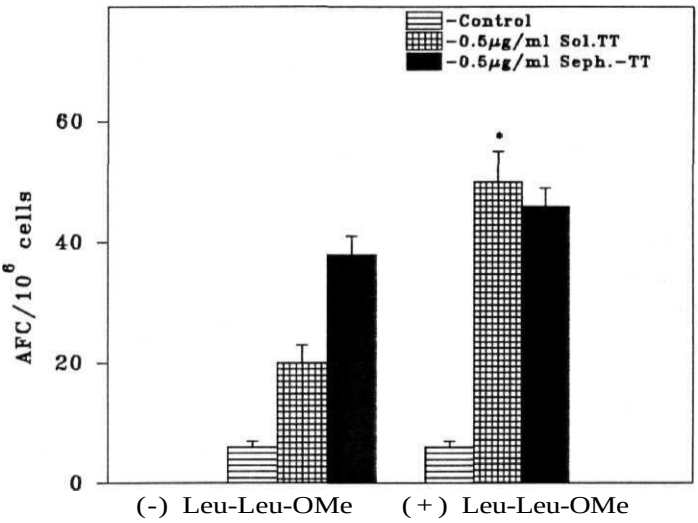

Figure — 25 Effect of Leu **-Leu -OMe** on AFC response of *in vitro* immunized human PBL

Concentration of Leucyl - **Leucine** methyl ester used = 250
Values presented are Mean ± SEM of 6 experiments.
$P < 0.001$

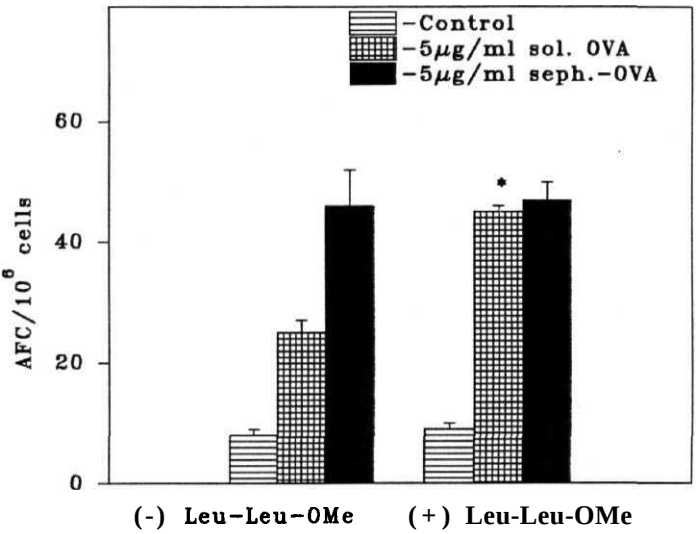

Figure - 26 Effect of Leu-Leu-OMe on AFC response of *in vitro* immunized human PBL

Concentration of Leucyl-leucine methyl ester =250 μM
Values plotted are Mean ± SEM of triplicate cultures of 3 experiments. • **P< 0 001**

present study, a significant antigen-specific AFC response could be evoked by treating the unfractionated PBL with antigen immobilized on sepharose beads, without the supplementation of any exogenous lymphokines.

The kinetics of *in vitro* immune response to the soluble antigen was similar to those in other studies reported earlier using human lymphocytes (Strike *et al.*, 1984; Jacot-Gillarmod, 1988). The peak AFC response occurred on day 6 although the initial response appeared on day 4. However, PBL immunized with the immobilized antigens evoked a maximal response on day 5.

The source and concentration of the serum is another important parameter during *in vitro* immunization of human PBL. Some investigators have demonstrated the critical importance and sometimes the inhibitory effect of sera in the generation of *in vitro* AFC response both in animal and human cells (Mishell and Dutton, 1967; Cavagnaro and Osband, 1983; Van Ness *et al.,* 1984). Earlier there have been controversial reports on the use of serum from different sources. In the human system few investigators have reported that the presence of FCS was critical for the successful *in vitro* generation of specific antibodies (Strike *et al.,* 1984; Lagace and Brodeur, 1985; Jacot-Guillarmod, 1988; Kozbor, 1988; Pollack and d'Apice, 1988; Mc Roberts *et al.,* 1988). However, some investigators reported that human AB serum is necessary (Ho *et al.,* 1985, Hoffman and Hirst, 1985, Bieber and Teng, 1987; Jonak *et al.*, 1988; Larrick *et a!.,* 1988). Schreier and Nordin, (1977); used a combination of FCS and 2% horse serum to obtain antigen-specific response *in vitro.* Rabbit serum was also used at a concentration 1 - 3 % which has been suggested to reduce the effect of serum proteins on B cell activation (Miner *et al.,* 1981; Borrebaeck, 1983; Pardue *et al.,* 1983; Borrebaeck and Moller, 1988). *In vitro* stimulation has also been performed using serum-free conditions to avoid activation of B cells by serum proteins (Borrebaeck, 1984).

In the present study, comparison was made on the performance of different sera in the human *in vitro* immunization system using immobilized antigens. Both

FCS and human AB serum could evoke a significantly higher AFC response as compared with horse serum and rabbit serum. The optimal serum concentration for obtaining maximal AFC response was found to be 10 % as reported earlier (Volkman *et al.*,1982; Yarchoan *et al.*, 1984).

Leucyl-Leucine methyl ester is a lysosomotropic agent and its effect on cytotoxic cells, mainly monocytes and natural killer cells has been delineated (Thiele *et al,* 1983; Thiele and Lipsky, 1985; 1986). The dipeptide exhibits an irreversible cytotoxicity to large granular lymphocytes, alloantigen-specific cytotoxic T cells and a subset $CD8^+$ T cells (Thiele and Lipsky, 1986). Leu-Leu-OMe has no demonstrable adverse effects on B cells, helper ($CD4^+$) T cells, endothelial cells and fibroblasts (Thiele and Lipsky, 1986). The large granular lymphocytes have been reported to contain killer and natural suppressor cells (Maier *et al.,* 1986) and both subsets have been implicated in the suppression of B cell functions (Arai *et al.,* 1983; Targan *et al.,* 1985; Layton *et al.,* 1983).

When the PBL population was treated with Leu-Leu-OMe were stimulated with soluble and sepharose-linked antigens, a significant increase in the AFC response was obtained in the cultures treated with soluble antigen as compared with the Leu-Leu-OMe untreated controls. Whereas, there was no enhancement in the AFC response following Leu-Leu-OMe treatment in the cultures stimulated with immobilized antigens. Hence, the AFC response of immobilized antigens is not influenced by the presence of cytolytic/suppressor T cells, unlike the response with soluble antigens. The B cell activation by immobilized antigens is therefore predominantly a T-independent response.

CHAPTER -VI

SUMMARY AND CONCLUSIONS

TT and OVA were immobilized on CNBr-activated sepharose-4B and immunomagnetic beads (Dynabeads). The immobilized antigens were characterized and were used in the *in vitro* immunization system to study their efficacy over the antigen in the soluble form.

The amount of TT immobilized on sepharose beads was quantified using ^{125}I labeled TT. About 80% of the TT used for coupling was bound to the sepharose beads. The autoradiogram of sepharose-^{125}I-TT showed an uniform distribution of TT on the surface of the bead. Stability of the immobilized antigens during storage and in cell culture was studied by monitoring the ^{125}I-TT released from the sepharose beads. At 4 °C only (0.625%) a negligible amount of TT was released from beads over a period of one month. In cell culture for 5 days, almost 98 % of the TT immobilized on the sepharose was found to be with the beads.

The distribution of TT on various sizes of sepharose beads was studied using sepharose-TT FITC conjugated anti-TT antibody interaction. Bigger beads (>50|i showed lot of fluorescence as compared to the smaller ones. The antibody binding capacity of sepharose-TT was studied by reacting sepharose-TT with anti-TT antibody, followed by the addition of anti-rabbit IgG-HRPO conjugate. The antigen-antibody reaction was visualized by the addition of 4-chloro-l-naphthol as substrate.

The amount of antigen immobilized on the surface of Dynabeads was quantified by solid phase ELISA. The stability of Dynabead-TT was studied by monitoring the release of ^{125}I-TT over a period of one month, and it was found that the Dynabead-TT was very stable when stored at 4 °C. The stability of Dynabeads-TT was monitored by solid phase ELISA. It was observed that 95 % of TT was in the immobilized state during a 5 day culture period.

After characterizing the immobilized antigens they were used in the murine and human *in vitro* immunization systems. Sepharose-TT was found to be a potent immunogen when administered *in vivo* to mice. At all concentrations of antigen used in *in vitro* immunization system the immobilized antigens showed a higher AFC response as compared to their soluble counter parts. The kinetics for the optimal antigen stimulation period was studied and was found that a 5 day antigen stimulation yielded maximal AFC response. FCS at 10 % (v/v) concentration was found to be optimal for *in vitro* AFC response. Splenic lymphocytes were treated with Leu-Leu-OMe for depletion of lysosome-rich cytolytic cells. When the Leu-Leu-OMe treated cells were cultured with soluble and sepharose-TT, an enhancement in the AFC response was observed in the soluble TT treated cultures as compared with the Leu-Leu-OMe untreated cultures. Whereas, in sepharose-TT cultures the AFC response was unaltered.

Certain physical characteristics of the immobilized antigens which influence the AFC response like, the density of antigen on the sepharose bead, size of the bead and the geometry of the culture vessel were studied. The use of sepharose-TT at 5mg antigen/ml beads, and immobilized on beads of size >50 u was found to be optimal for use in the *in vitro* immunization system. The AFC response was not influenced by the type of the culture vessel used for *in vitro* immunization.

The effect of lymphokines on the *in vitro* immunization system was studied by using PMA stimulated EL-4.IL-2 supernatant, and soluble and sepharose-Con A stimulated rat splenic lymphocyte supernatant. An assay system was developed to estimate the levels of EL-2 in these supernatants using sepharose-Con A blasts. The IL-2 levels were determined in terms of the proliferative response of the sepharose-Con A blasts upon supplementation of the supernatant.

The EL-4.IL-2 supernatant showed a maximal activity at 10% (v/v) concentration. Soluble Con A supernatants showed a maximal activity at 2.5%

followed by a steep decline in the activity at higher concentrations. Whereas with sepharose-Con A supernatant this decline in activity was not observed.

The soluble Con A and Sepharose-Con A supernatants are quantitatively similar in terms of IL-2 activity. But, they are different qualitatively in terms of T cell proliferation and also in their effect on AFC response when supplemented in the *in vitro* immunization cultures.

Upon supplementing the *in vitro* immunization system with the EL-4.IL-2 and soluble Con A supernatants, the soluble TT stimulated cultures showed an enhancement in the AFC response, whereas, the AFC response was unaltered in the sepharose-TT treated cultures. The AFC response was enhanced in both soluble and sepharose-TT cultures upon supplementation of sepharose-Con A supernatants.

When the unprimed murine splenic lymphocytes were stimulated with Dynabeads-TT and Dynabeads-OVA in a concentration range of 160 - 640 ng a significant AFC response was observed. Dynabeads could easily be separated from the cultures and were reused for *in vitro* immunization to obtain a similar AFC response as obtained with the antigen used for the first time. Upon supplementation of Dynabead-TT cultures with EL-4.IL-2 supernatant, the AFC response was enhanced significantly.

TT and OVA were used both in soluble and in sepharose-linked forms in the *in vitro* immunization system of human PBL. The immobilized antigens evoked significantly higher AFC response as compared to their soluble counterparts. The AFC response was maximal on day 6 in soluble TT and OVA cultures where as the response was maximal on day 5 in sepharose-TT and sepharose-OVA cultures. The AFC response was maximal when FCS and human AB serum were used at 10% concentration. The Leu-Leu-OMe treated cultures showed an enhancement in the AFC response in soluble antigen cultures whereas the AFC was unaltered in the cultures treated with sepharose linked antigens.

CONCLUSIONS :

1. TT immobilized on sepharose beads is very stable at 4 °C and at 37 °C in culture conditions.
2. Distribution of TT is uniform on the surface of the sepharose bead.
3. Immobilized TT is able to interact with anti-TT antibody indicating that the epitopes are well exposed.
4. Both soluble and immobilized forms of TT can interact efficiently with B lymphocytes as they evoke a significant proliferative response of TT primed lymphocytes derived from TT immunized mice.
5. Sepharose-TT acts as a potent immunogen when administered *in vivo* as compared with soluble TT.
6. Sepharose linked antigens are potent stimulators of murine splenic lymphocytes and human PBL in *in vitro* immunization, compared with soluble antigens.
7. A 5 day antigen stimulation period is required to evoke a maximal AFC response in murine *in vitro* immunization system with both soluble and sepharose-TT.
8. Sepharose linked antigens can evoke a maximal AFC response at an earlier time point (5 days) as compared to their soluble counterparts (6 days) in human *in vitro* immunization system.
9. The stimulation of B lymphocytes by immobilized antigens is not influenced by the presence of suppressor T cells, macrophages and other lysosome rich cytolytic cells.
10. An assay was developed to estimate the levels of IL-2 in the PMA stimulated EL-4.IL-2 supernatant and soluble and sepharose-Con A stimulated rat splenic lymphocyte supernatant using sepharose-Con A blasts.
11. The stimulation of B cells by sepharose antigens involves a T-independent mechanism is confirmed since the addition of helper factors from culture supernatants did not enhance the AFC response.

12. A sensitive and specific solid phase ELISA is developed to quantify the TT immobilized on Dynabeads.
13. The Dynabeads-TT can evoke a significant AFC response when used at nanogram concentration range in an *in vitro* immunization system.
14. Dynabeads-TT can be separated very easily from the culture and can be reused in the *in vitro* immunization system.
15. FCS and human AB serum support the *in vitro* immunization of human PBL better than the rabbit serum and horse serum.
16. The *in vitro* immunization protocol developed using antigens immobilized on sepharose 4B does not require exogenous supplementation of lymphokines.

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In vitro immunization of murine lymphocytes using immobilized immunogens

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Murine splenic lymphocytes were immunized *in vitro* using immobilized antigens. Immobilization was achieved by covalently linking the antigens to Sepharose beads. Tetanus toxoid (TT) was used as test antigen in soluble and immobilized forms. The outcome of *in vitro* immunization was assayed in terms of the number of antigen-specific antibody-forming cells (AFC) enumerated by filter immuno plaque assay. The AFC specific to TT were significantly higher in cultures stimulated with immobilized antigen as compared with soluble antigen. The effect of various concentrations of antigen, time kinetics, effect of serum, and leucyl-leucine *O*-methyl ester treatment on the *in* vitro-immunization system has been studied. The results indicate that immobilized antigens are more potent than their soluble counterparts *in vitro* and hence are useful in *in vitro*-immunization protocols.

Introduction

In vitro immunization has been gaining importance in recent years as a novel approach in immunotechnology to prepare murine and human monoclonal antibodies [1]. Induction of specific antibody response has been demonstrated following *in vitro* immunization of murine and human lymphocytes with a variety of antigens [1,2]. In all cases the in vitro sensitization was dependent upon the exogenous addition of lymphokines derived from mixed lymphocyte culture or stimulated cell line supernatants to the cell culture [3]. The antigen used for *in vitro* immunization was always presented to the lymphocytes in the soluble form [1–3].

Earlier, we and others have demonstrated that Sepharose-coupled anti-immunoglobulin (Sepharose-anti-Ig) acts as a potent stimulator of B lymphocytes as compared with soluble anti-Ig [4,5]. In the presence of Sepharose–anti-Ig, the B lymphocytes could be driven to differentiation (antibody secretion), whereas only proliferative response was induced by soluble anti-Ig [5]. As anti-Ig-induced triggering of B cells is supposed to mimic antigenic stimulation via the interaction with the surface Ig of B lymphocytes, we have examined whether immobilized antigens could serve as better stimulants of B lymphocytes compared with the same antigen in soluble form. The efficacy of immobilized and soluble forms of the antigen to stimulate B lymphocytes *in vitro* was determined in terms of induction of antigen-specific antibody-forming cell (AFC^2) response. The results obtained indicate that AFC response induced by immobilized antigens was significantly higher than that obtained with soluble antigen under the same conditions.

Materials and methods

Immunogens

Tetanus toxoid (TT) was generously provided by Vaccines Division, Biologicals Evans, Hyderabad, India. The homogeneity of TT was checked by SDS/PAGE, performed as described by Laemmli [6], and the protein was silver-stained. There was no major contaminant, and 99% of the protein consisted of TT.

Animals

Female Balb/c mice of age 8–12 weeks obtained from National Institute of Nutrition, Hyderabad, India, were used in all the experiments.

In vivo immunization

The mice were primed with 10 μg of TT emulsified in Freund's complete adjuvant (FCA). Control mice were injected with saline/FCA emulsion.

Cell preparation

Single-cell suspensions of mouse splenic lymphocytes were prepared as described previously [7]. The lymphocytes were cultured in RPMI-1640 containing 10% FCS and antibiotics (penicillin, 100 units/ml; streptomycin. 100 μg/ml).

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· Abbreviations used: AFC, antibody-forming cell, TT, tetanus toxoid; FIPA, filter immuno plaque assay. Leu-Leu-OMe, leucyl-leucine *O*-methyl ewer: anti-Ig, anti-immunoglobulin. FCS. fetal-calf serum: FCA, Freund's complete adjuvant; sIg, surface immunoglobulin.

Preparation of Sepharose-coupled TT

TT was coupled to CNBr-activated Sepharose 4B as described previously [4]. The amount of TT bound to the gel was calculated as the percentage of the initial quantity of protein used for coupling. The percentage of protein coupled to the gel was 80%. Concentration of the Sepharose-bound TT was calculated based on the amount of protein and volume of gel taken and expressed as /ig/ml of gel.

[^{3}H]Thymidine incorportaion

Cultures for proliferation assay were set up with 2×10^5 primed mouse splenic lymphocytes in 0.2 ml of medium containing 5% foetal-calf serum (FCS) in 96-well microtitre plates in a humidified atmosphere of 5% CO_2 at 37 °C for 3 days. The cultures were pulsed for the last 16–18 h of the culture period with 0.5 μCi of [^{3}H]thymidine (specific radioactivity 5-6 Ci/mmol), and the cultures were processed using a Skatron cell harvester and the results expressed as c.p.m./10^6 cells.

Treatment of splenic lymphocytes with leucyl-leucine O-methyl ester (Leu-Leu-OMe)

Splenic lymphocytes were suspended (10×10^6 cells/ml) in serum-free RPMI 1640 containing freshly prepared 250 mM Leu-Leu-OMe. After 15 min incubation at room temperature the cells were washed three times in RPMI 1640 containing 2% FCS [8].

In vitro immunization

Unprimed splenic lymphocytes were immunized *in vitro* at a concentration of 2×10^6 cells/ml of medium in 12 mm x 75 mm screw-capped tubes. The cells were cultured with the indicated concentrations of TT for 5 days in a humidified incubator with 5% CO_2 at 37 °C.

Filter immuno plaque assay (FIPA)

Antibody-secreting cells were enumerated using FIPA as described previously [9]. Briefly, nitrocellulose membranes (15 mm diameter) were coated with 0.15 mg/ml solution of TT in 24-well microtitre plates for 3 h. Then the filters were treated with 5% lactalbumin peptide solution to block the unoccupied sites by a Hot-blott technique [10]. *In vitro*-immunized lymphocytes suspended in RPMI 1640 containing 1% FCS were put on the nitrocellulose membranes in wells at a concentration of 0.5×10^6 cells/well. Incubation was carried out at 37 °C for 3 h in a humidified incubator. Then the filters were washed with PBS and incubated with peroxidase-labelled goat anti-mouse IgM (1:800 dilution) for 1 h. After washing the membranes with PBS, plaques were developed by adding 4-chloro-1-naphthol solution (3 mg of 4-chloro-1-naphthol in 1 ml of methanol was added to 5 ml of PBS containing 0.003% H_2O_2). The filters were rinsed, dried, and the blue plaques were counted microscopically under low magnification.

Results

In order to ascertain that the antigen TT can effectively interact with lymphocytes in a Sepharose-linked form, a proliferation assay using TT-primed murine splenic lymphocytes was performed. The effect of soluble and Sepharose-TT on the proliferative response and induction of antigen-specific B cells was studied. It was observed that primed murine splenic lymphocytes when stimulated *in vitro* with Sepharose-TT showed a significant proliferative response and an antigen-specific AFC response (Tables 1 and 2). Sepharose beads alone have no effect on the proliferative response. Also soluble TT added along with Sepharose beads (not covalently linked) was not as effective as the antigen immobilized covalently on Sepharose.

Table 1 Proliferative response **of primed murine splenic lymphocytes** to **soluble and Sepharose-TT**

Values presented are means +S.E.M. of triplicate cultures. Immunization and cell culture was carried out as described in the text. Results are from a representative experiment out of three performed.

	[^{3}H]Thymidine incorporation into DNA	
Addition to culture	(c.p.m./IO' cells)	(A c.p.m)
	12275±370	
Sepharose beads (25 µl)	11930±280	—
+ 0.05 µg/ml soluble TT	12995±255	720
+ 0.5 µg/ml soluble TT	14865+1375	2590
Soluble TT (/ig/ml)		
0.05	13325+105	1050
0.10	14385±950	2110
0.05	14685±1120	2410
1.00	17685±1315	5410
Sepharose-TT (µg/ml)		
0.05	12005±550	—
0,'0	14430±425	2155
0.50	15730±575	3455
1 00	21555+425	**9280**

Table 2 AFC response of primed **murine** splenic lymphocytes to soluble and Sepharose-TT

Primed murine splenic lymphocytes were stimulated in vitro with different concentrations of soluble and Sepharose-TT. AFC response was determined on day 5 by FIPA. Values represented are means+S.E.M. for three experiments. *P < 002.

	IgG plaques/10^6 cells	
[TT] (µg/ml)	Soluble TT	Sepharose-TT
0	15	2
5	47±2	96
10	140±2	161±5*
15	156±2	194+5*

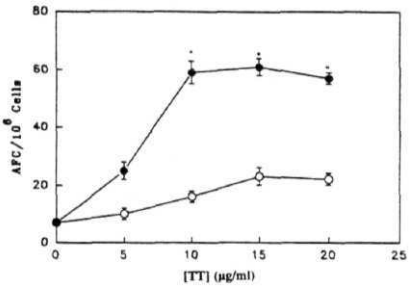


Figure 1

AFC response of murine splenic lymphocytes immunized *in vitro* with soluble (o) and Sepharose–TT (•). Results presented are means+S.E.M. for five experiments. *$P < 0.001$.

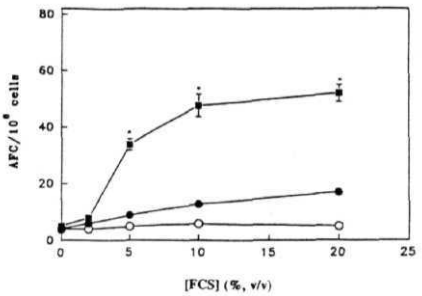


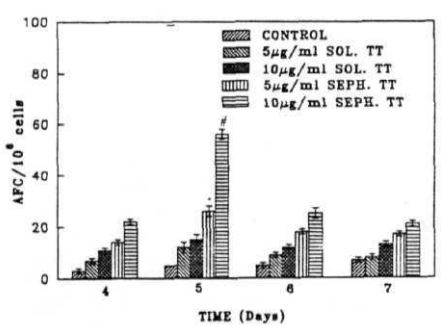
Figure 3

Effect of serum concentration on the AFC response of *in vitro*-immunized murine splenic lymphocytes. Values presented are means+SEM. for four experiments. *$P < 0.001$. 0. Control. ●. 0.5 μg/ml soluble TT. ▪ 0.5 μg/ml Sepharose–TT.

In vitro immunization

The AFC response using different concentrations of soluble and Sepharose-TT ranging from 5-20 μgs/ml was determined during a 5-day immunization period in *vitro*. The results showed a dose-dependent increase in AFC response determined by FIPA, with an optimal concentration of 10 μg/ml of antigen. At all the concentrations tested, the AFC response with Sepharose-TT was found to be significantly higher as compared with those immunized with soluble TT (Figure 1).

The kinetics of antigen-specific AFC response of *in vitro*-immunized murine splenic lymphocytes was determined on days 4, 5, 6 and 7. Day 5 of the antigen-stimulation period revealed a more-than-twofold increase in the number of AFC as compared with day 4 (Figure 2).



Figure

Time course of AFC response of *in vitro*-immunized murine splenic lymphocytes. Results presented are mean+S.E.M. for five experiments *$P < 0.01$; #$P < 0.001$. SOL. soluble. SEPH. Sepharose.

The data indicates that a 5-day *in vitro*-immunization period is optimal for antigen-specific antibody production.

The effect of serum concentration on *in vitro* immunization of murine splenic lymphocytes was also investigated. It was observed that a 10% (v/v) serum concentration in the culture gave an optimal AFC response with both soluble and Sepharose-TT. At concentrations above 10%, no further increase in AFC response was seen (Figure 3).

Treatment with lysosomotropic agents causes depletion of lysosome-rich cell populations, namely monocytes, large granular lymphocytes, cytotoxic T cells and a subset of $CD8^+$ T suppressor cells from the splenic lymphocyte preparation [11,12]. When Leu-Leu-OMe-treated cells were immunized *in vitro* with soluble and Sepharose—11, the AFC response increased almost twice with soluble antigens as compared with controls not treated with Leu-Leu-OMe. However, the cultures stimulated with Sepharose-linked TT did not show any significant increase in the number of AFC (Figure 4).

Discussion

Immobilization of immunogens on the surface of an insoluble matrix results in the formation of array of immunogen molecules at a fixed density. Upon interaction with the surface immunoglobin (sIg) receptor on B cells, the antigen–sIg complex gets internalized or gets redistributed, resulting in cap formation or shedding into the medium [4]. It has been shown previously that, in the case of Sepharose-linked anti-Ig, the sIg receptors on B cells do not undergo internalization and are present on the cell surface throughout the stimulation period of culture [13,14]. Since internalization of the antigen does not occur in the case of

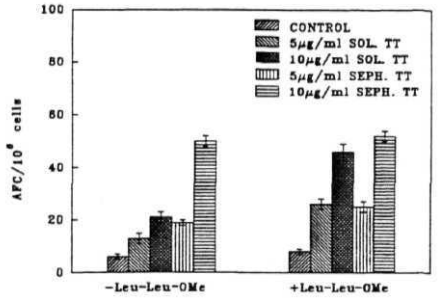


Figure 4

Effect of Leu-Leu-OMe on the AFC response of *in vitro*-immunized of murine splenic lymphocytes. Results presented are mean +SEM for five experiments SEPH. Sepharose: SOL. soluble.

Sepharose-linked antigens, there is a constant interaction of the antigen with the cell-surface receptor. It is possible that, in the case of Sepharose-linked antigens, the interaction and delivery of signal to the B cell at the sIg receptor level could be stronger and sustained as compared with soluble antigens. The activation of B cell by Sepharose-linked antigen can be thought to be analogous to the presentation of a polymeric antigen, thereby achieving greater degree of receptor cross-linking to the sIg receptor than it is possible with soluble antigens. Further, it is likely that the crucial requirement of lymphokines for growth and differentiation of B cells is circumvented by the use of immobilized antigens. In this context, there is evidence to show that multiple lymphokines are produced by a B cell line A20.1, within 3-4 h after surface Ig cross-linking by immobilized anti-Ig [14]. Treatment of lymphoid cell population with Leu-Leu-OMe removes monocytes/macro- ¬ ‐phages and other lysosomotropic cells with no adverse effects on B cells and T helper cells. In the present study, stimulation with Sepharose-TT showed similar AFC response in both Leu-Leu-OMe-treated and untreated lymphocyte cultures, indicating that the enchancement of AFC response with Sepharose-TT is probably not due to stimulation of monocytes/macrophages. However, most of the previous reports on *in vitro* immunization using a variety of cell types and antigens clearly indicated a requirement of lymphokines in the culture medium [3]. The interesting and important aspect of the present study is that the AFC response could be induced in an *in vitro* system without the addition of any exogenous lymphokines. In conclusion, the present study indicates the potential use of antigens in immobilized form for effective *in vitro* immunization.

Acknowledgments

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