INTESTINAL MUCOSAL IMMUNITY IN VITAMIN A-DEFICIENT RATS

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This is to certify that I, G. Santhi, had carried out the work embodied in this thesis for the full period prescribed under the Ph.D. ordinances of University of Hyderabad.

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

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TO MY MOTHER

CONTENTS

LIST OF ABBREVIATIONS

Con A	Concanavalin A
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme linked immunosorbent assay
E/T	Effector : Target ratio
FCA	Freund's complete adjuvant
FCS	Fetal calf serum
GALT	Gut associated lymphoid tissue
HBSS	Hank's balanced salt solution
HEPES	N-2-Hydroxyethyl piperazine-N-2-ethane sulphonic acid
IEL	Intraepithelial lymphocytes
IFN	Interferon
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interluekin
LPL	Lamina proprial lymphocytes
LPS	Lipopolysaccharide
MLR	Mixed lymphocyte response
NK	Natural killer
PBS	Phosphate buffered saline
PHA	Phytohaemagglutinin
PMSF	Phenyl methyl solphonyl fluoride
POPOP	1-4-Bis[2-(5-phenyloxazolyl) benzene]
PP	Peyer's patches
PPO	2,5-Diphenyloxazole
SDS-PAGE	SDS-Polyacrylamide gel electrophoresls
Seph. anti-IgG	Sepharose coupled anti-rat IgG
sIgA	Secretory IgA
TCR	T cell receptor
Vit A	Vitamin A
Vit A^+	Vitamin A-sufficient (Pair-fed control)
Vit A"	Vitamin A-deficient

CHAPTER I

GENERAL INTRODUCTION

Lymphocytes play an important role in conferring immunity against pathogens. The two types of lymphocytes are, a) B lymphocytes (Bursa/ Bone marrow-derived) **and** b) T lymphocytes (Thymus-derived) which carry **out** humoral and cell-mediated immune responses respectively. The mature T and B lymphocytes circulate via the blood and the secondary lymphoid organs, which are the sites of lymphocyte-antigen interactions. The secondary lymphoid organs include spleen, lymph nodes and the lymphoid tissues of gastrointestinal, respiratory and urogenital tracts. Apart from lymphocytes, these organs also contain macrophages and dendritic cells that trap and process antigens which are presented to the antigen specific T and B cells.

Lymphoid tissues associated with gastrointestinal, respiratory and urogenital tracts are always exposed to the external environment. They participate in the defense against various viral, bacterial and parasitic antigens that enter the body via the mucosal route. Besredka (1919) proposed the existence of mucosal protective local immune systems which function fairly independent of systemic immunity. A common mucosal immune system has been proposed to be associated with **the** above mentioned tissues (Bienenstock *et al,* 1978; Mestecky and McGhee, 1987). When an antigen is administered orally, intestinal lymphoid tissue is stimulated and the sensitized lymphocytes leave **the** intestine, circulate in the blood stream and colonize the mucosal surfaces throughout the body.

Gut associated lymphoid tissue (GALT)

The gut associated lymphoid system has been shown to exhibit humoral or cell-mediated responses **or both.** The immune response of

this system differs from the responses of regional lymph nodes or systemic lymphoid compartment (Bienenstock and Befus, 1980). GALT is unresponsive to the vast array of complex harmless antigens present in the gastrointestinal lumen, but it somehow recognizes and removes the potentially harmful infectious organisms. Thus, it plays an important role in host defense against harmful antigens gaining entry along with the ingested food on one hand, and in oral tolerance against the harmless food antigens on the other.

GALT is one of the largest lymphoid compartments in the body, and it comprises more than one half of the total lymphocyte population (Walcsman and Ozer, 1976). GALT consists of lymphoid cells organized in discrete structures such as Peyer's patches, and those present in epithelial and lamina proprial layers. The lymphocytes present in between the epithelial cells are called Intraepithelial lymphocytes (IEL), while those disseminated diffusely in the lamina propria are called Lamina proprial lymphocytes (LPL). The subset composition and functions of IEL and LPL differ considerably from each other.

Peyer's Patches

Small deposits of lymphoid tissue in the from of nodules located in the submucosal layer of intestine are called Peyer's patches (PP). In PP, lymphoid cells are arranged in a fashion analogous to lymph nodes, with B cell germinal centers surrounded by T cells, interspersed macrophages and dendritic cells (Bloom and Fawcett, 1972). An important characteristic of PP is the presence of a unique epithelium covering the dome region consisting of cuboidal epithelial cells, and specialized antigen sampling cells called mlcrofold cells or M cells

(Owen and Jones, 1974). The antigens that are transported from gut lumen by the M cells are processed and presented to the lymphocytes by macrophages and dendritic cells of PP (Castro, 1982). PP are the major sites at which the activation and programming of IgA-producing B cells and mucosally-directed T cells are initiated. T cells with both helper and suppressor function are present in PP. Approximately 60% of PP T cells are T helper cells including those which support IgA responses (Hanson and Brandtzaeg, 1989). T cells capable of suppressing IgA production have also been described in PP (Elson *et al,* 1979). The antigenically primed T and B lymphocytes migrate from PP through the mesenteric lymph nodes and blood circulation and finally home onto the lamina propria and intraepithelial spaces of different mucosal surfaces (Husband and Gowans, 1978; Ottaway, 1990).

Intraepithelial lymphocytes (IEL)

Intraepithelial lymphocytes are among the largest populations of T lymphocytes in the body (Mowat, 1984). They are located in very close proximity to the intestinal lumen. IEL in the gut are intriguing cells with poorly understood functions. IEL contain a large proportion of granulated lymphocytes (Guy-Grand et *al,* 1978; Mayrhofer, 1980; Rudzik and Bienenstock, 1974; Ferguson, 1977; Davles and Parrott, 1981; Cerf-Bensussan et *al,* 1983). Most of the T cells of IEL have a CD8 phenotype (cytotoxic/suppressor) (Selby *et al,* 1981; Lyscom and Breuton, 1982; Ernst et *al,* 1985; Van der Heijden, 1986; Vaage et *al,* 1990). A small subpopulation of T helper cells (CD4) is also present among IEL. Spontaneous cytotoxic activities like natural killer cytotoxicity and antibody dependent antibacterial activity are associated with large granular T lymphocytes (Herberman, 1981; Timonen et *al,*

1981; Tagliabue *et al,* 1982). Almost 50% of T cells of mouse IEL which are cytotoxic are thymus-independent (Klein, 1986). Recently it has been reported that a majority of murine intestinal cytotoxic T lymphocytes express the T cell receptor γ/δ (Bonneville *et al,* 1988; Viney *et al,* 1990). In contrast, majority of rat and human IEL bear T cell receptor α/β rather than γ/δ (Vaage *et al,* 1990; Brandtzaeg, 1989).

As majority of the IEL are cytotoxic T cells, it is implied that they are involved in the protection of mucosa by participating in local cell-mediated immunity; they also participate in epithelial renewal (Cerf-Bensussan *et al,* 1984; Mowat and Ferguson, 1982).

The intestinal epithelial cells express Class II antigens of the major histocompatibility complex (Wiman *et al,* 1978; Barlay and Mason, 1982; Mayrhofer *et al,* 1983; Bland, 1988), which are characteristic surface antigens of antigen-presenting cells.

Lamina Proprial lymphocytes (LPL)

Lamina propria of the intestine contains a large number of lymphocytes and plasma cells, along with a few eosinophils and macrophages. In response to the antigens of gut lumen, IgA committed B cells and T cells which regulate B cell responses, migrate selectively to lamina propria (Fiocchi, 1989).

LPL populations are remarkably different from IEL populations in their composition. LPL consist of heterogeneous populations of cells including B cells, helper T cells ($CD4^+$) and suppressor/cytotoxic T cells ($CD8^+$) (Parrott *et al,* 1983), while IEL appear to consist

predominantly of T cells with CD8 phenotype. Unlike IEL, very small numbers of large granulated lymphocytes are present in the lamina propria of mice (Tagliabue *et al,* 1982). The T lymphocytes of LPL have the capacity to provide help for Ig synthesis by B cells as well as suppressor cell activity similar to the lymphocytes of peripheral blood (James *et al,* 1985; Elson *et al,* 1985). The predominant class of immunoglobulin secreted by the B cells of lamina propria is IgA (Tseng, 1982; Guy-Grand et *al,* 1974).

The macrophages of lamina propria play an important role in phagocytosis of bacteria and in antigen processing and presentation (Bull and Bookman, 1977).

Secretory IgA

IgA is the major immunoglobulin of intestinal secretions and participates in antibody-mediated defense at mucosal surfaces (Tomasi *et al,* 1963; Brandtzaeg, 1974; Mestecky, 1987; Nagura and Sumi, 1988). The mucosal secretory IgA consists mainly of dimeric molecules complexed with the J chain and possesses an additional glycoprotein called secretory component (SO (Tomasi *et al,* 1965). IgA is secreted by the plasma cells of lamina propria and is transported into the lumen through intestinal epithelial cells where the SC secreted by epithelial cells binds to dimeric IgA to form a complex molecule called secretory IgA (sIgA) (Brandtzaeg, 1974). The secretory component protects IgA from the proteolysis by gastrointestinal proteases.

The surface of the intestinal epithelial layer is protected by IgA in conjunction with mucus. sIgA, effectively blocks both bacterial

adherence and antigen association with the mucosa, thereby reducing or eliminating the mucosal penetration by microbes (McNabb and Tomasi, 1981). sIgA protects the mucosal surfaces from bacterial and viral infections by neutralizing bacterial exotoxins and viruses. Receptors for Fc portion of IgA on lymphocytes have been reported (Strober *et al,* 1978) and sIgA also participates in antibody-dependent cell-mediated bacteriolytic activity carried out by CD8 subset of T cells (Nencioni et al, 1983). Thus, sIgA in the gut has three major roles: antigen exclusion, antibacterial function and participation in cell mediated immunity.

Lymphokines in GALT

Lymphokines are a group of molecules produced by activated T cells and have multiple effects on the growth and function of the cells of immune system and also on other cell types. Immunoregulatory T cell-help is required for IgA responses and also for the function of cytotoxic lymphocytes (Elson and Heck, 1979; Kawanishi *et al*, 1983; McGhee and Mestecky, 1989). These T cells operate through lymphokines secreted by them. Activation of antigen specific CD4 T cells leads to the secretion of IL-2, IL-4, IL-5 and IL-6 needed for B cell responses and Ig synthesis. The IgM expressing B cells of PP switch over to IgA expressing B cells in presence of IL-4, while IL-5 and IL-6 are needed for the differentiation of IgA expressing B cells in the lamina propria into IgA secreting plasma cells (Kawanishi *et al,* 1982; 1983). In addition, antigen-activated CD4 T cells modulate the function of cytotoxic lymphocytes with the help of lymphokines such as IL-2, IFN-γ and lymphotoxin (tumor necrosis factor-β). Both CD4 and CD8 T cells require the T cell growth factor, IL-2, for proliferation. IL-2 is

also involved in the induction of cytotoxic activity (Liebson *et al,* 1992). IFN-γ modulates spontaneous cytotoxic activities of CD8 lymphocytes (Chen *et al,* 1986; Weigent *et al,* 1983; Simon et *al,* 1986) and lymphotoxin causes hemorrhagic necrosis of tumors (Beutler and Cerami, 1987). IFN-γ also inhibits the activity **of** IL-4 **(Rabin** *et al,* 1986).

Lymphocytes from all the compartments of gut associated lymphoid tissue contain higher numbers of IL-5 and IFN-γ secreting cells when compared with spleen (Taguchi *et al,* 1990). **LPL** which is a major IgA effector site, contains large number of IL-5 producing CD4 T cells and a few IFN-γ secreting cells. In contrast, **PP** which is a major IgA inductive site contains a few IL-5 producing CD4 T cells (Taguchi *et al,* 1990). IL-5 and IFN-γ producing cells are also present in IEL (Dillon *et al,* 1986; Taguchi *et* **ai,** 1990). IL-2 is secreted only by LPL cells but not by IEL. The major role of IL-2 in the gastro-intestinal immune system is probably to expand cell populations through proliferation. IL-2, also enhances the lytic function of natural killer cells and is necessary for the differentiation of cytolytic T cells (Simon *et al,* 1986; Chen *et al,* 1986).

Oral tolerance

Suppression of immune responses against harmless non-replicating luminal antigens is called "Oral tolerance". Protection against harmful systemic types of immune reactions elicited by IgG, IgE and T cell mediated delayed type hypersensitivity is also afforded by the same suppressive mechanism (Mowat, 1987). This phenomenon of hypo-responsiveness involves multiple immunoregulatory events (Nicklin and

Miller, 1983). Oral tolerance seen in GALT is mediated by suppressor cells. Selective presentation of processed antigens by gut epithelium leads to the generation of specific and non-specific suppressor T cells (Meyer and Shlien, 1987). Though suppression is a general phenomenon induced by soluble non-replicating agents, The responses of IgA against harmful, non-replicating soluble agents are regulated by the action of contra-suppressor T cells which are of CD8 phenotype (Green *et al,* 1988; Brines and Lehner, 1988).

Oral vaccination

Vaccination offers the best prophylactic approach to build up immunity to a variety of viral and bacterial diseases, especially amongst children. Now-a-days oral vaccination is gaining importance over systemic immunization to protect an individual against enteric infections such as cholera, typhoid fever, Schigellosis, etc. (Holmgen et *al,* 1982). The stimulation of local gut mucosal immune system is better achieved by oral route rather than through the systemic route (Mestecky, 1987). Based on the concept of a common mucosal immune system associated with gastrointestinal, respiratory and urogenital tracts, oral vaccines against respiratory and urogenital tract infection have also been developed (Holmgren et *al,* 1992). The functional status of the gut immune system determines the outcome of the oral vaccines.

Mucosal immunity in intestinal diseases

In certain gastrointestinal diseases, striking changes in mucosal lymphocytes and secretory IgA production which results in mucosal damage have been observed (Nicklin and Miller, 1983; Nagura, 1992). In

chronic gastritis, coeliac disease and inflammatory bowel disease overproduction of IgG has been reported (Brandtzaeg et *al,* 1985). In these diseases abnormalities in mucosal T and B cells were also observed (Brandtzaeg *et al,* 1987). A striking feature of coeliac disease is the increased number of T cells (CD4) in jejunal epithelium (Freedman et *al,* 1988). The number of γ/δ T cells is also increased and this may contribute to the villous atrophy and destruction of enterocytes (Halstensen *et al,* 1989).

Several aspects of immunity are impaired in diseased conditions resulting from nutritional deficiencies, viz., protein-energy malnutrition, vitamin and mineral deficiencies which are common in developing countries. Increased frequency and severity of infections, especially enteric infections have been observed in protein-energy malnutrition and Vitamin A (Vit A) deficiency states (Scrimshaw *et al,* 1968; Sommer et *al,* 1984).

Vitamins and immune response

The relationship between Vitamins and immunity is well recognized in recent years. Both humoral and cellular responses are shown to be altered in Vit A (Bendich, 1991), Vit B (Bendich and Cohen, 1988), Vit C (Long, 1950; Zweiman et *al,* 1966), Vit D (Rook, 1988) and Vit E (Tengerdy and Brown, 1977; Corwin and Shloss, 1980) deficiencies. Among all the Vitamins, Vit A is known as anti-infection Vitamin (Perla and Marmorston, 1941) because of its effect on both epithelial cells and immune system.

Vitamin A

Animals and humans are not capable of synthesizing Vit A. They depend on natural plant sources which contain significant amounts of Vit A precursors such as β-carotene. Vit A plays an important role in metabolism, in addition to its role in photoreceptor mechanism of visual cycle (Moore, 1957). Further, it is essential for the growth, differentiation and replication of the epithelium and mesenchymal tissue (Sklan, 1987). It plays a role in maintaining the structural integrity of cellular and subcellular membranes. It is suggested to be involved in the transport of metabolites across the cell membrane and in bone formation (Mellanby, 1941). Vit A is known to be necessary to maintain the integrity of barriers such as skin, mucous membranes and cilia or tears which comprise the first line of defense against penetration by pathogens. Functional defects of epithelium during Vitamin A deficiency are suggested to result in high frequency of infections.

Vitamin A deficiency

Vit A deficiency as a cause of Xerophthalmia and blindness is very well established. Vit A deficiency results in keratinizing metaplasia (xerosis) of mucus-secreting epithelial tissues (Wolbach, 1937). The epithelium of intestinal mucosa does not normally keratinize, but shows a decline in goblet cells (De Luca *et al,* 1969; Rojanapo *et al,* 1980). Reduction in epithelial cell turnover in the small intestine of Vit A-deficient rats was reported (Zile *et al,* 1977). Loss of protective mucoid secretions compromises the surface integrity of epithelium resulting in enhanced susceptibility to microbial invasion. Various specific and non-specific components of

immune response were shown to be impaired in Vit A deficiency (Nauss, 1986; Vyas and Chandra, 1984).

Vitamin A deficiency and susceptibility to infection

Studies in Humans :

Vit A deficiency has been recognized as a major cause of childhood morbidity and mortality in the developing countries. It is well established that Vit A deficiency enhances susceptibility of humans to various types of infections (West *et al,* 1989). Respiratory tract infections and diarrhea are very frequent in children associated with low levels of serum Vit A (Arroyave and Calcano, 1979). A depressed serum Vit A level has been reported to be associated with pneumonia, rheumatic fever, measles and chicken pox (Shank *et al,* 1944; Jacobs *et al,* 1954; Sommer, 1982; Arroyave and Calcano, 1979). Studies on Indonesian and Indian children showed that mild Vit A deficiency is associated with increased mortality due to increased respiratory and diarrheal infections (Sommer *et al,* 1984; Milton *et al,* 1987). From these reports, it is apparent that Vit A deficiency increases the susceptibility to infection.

Few attempts were made to study the basis of immunological defects associated with the increased incidence of infections in Vit A deficiency (Bhaskaram and Reddy, 1975; Srisinha *et al,* 1975; Semba *et al,* 1991; Semba *et al,* 1993). As Vit A deficiency in humans is usually associated with protein calorie malnutrition, the interaction of Vitamin A with immune system is better understood using experimental animals.

Studies in experimental animals :

Increased susceptibility of Vit A-deficient animals to various infections like Mycobacterium tuberculosis, Salmonella typhimurium, Pseudomonas aureginosa, etc., has been reported (Scrimshaw *et al*, 1968). Atrophy of thymus and decreased cellularity of spleen was observed in Vit A-depleted rats (Wolbach and Howe, 1925). Depressed cell-mediated and humoral immune responses were observed in experimental Vit A-deficient animals (Vyas and Chandra, 1984). Phagocytosis, mitogen induced lymphoproliferation, delayed type hypersensitivity and specific antibody responses were suppressed in Vit A-deficient animals.

The majority of studies in Vit A-deficient humans and animals mentioned above deal with the immune function of splenic and peripheral blood lymphocytes. Information on the immune system associated with mucosal surfaces is scanty.

AIM AND SCOPE OF THE PRESENT WORK

It is very well established that Vitamin A modulates immune responses in experimental animals as well as in humans. The dietary deficiency of Vitamin A exists to a significant extent in developing countries like India. Vit A deficiency per se, may influence the host-pathogen interaction and impair the outcome of prophylactic vaccination programmes. Oral vaccination has been gaining importance in recent years to combat a variety of bacterial and viral infections. In order to achieve effective oral immunization, the humoral and cellular responses of orally administered vaccines at the gastrointestinal level has to be understood. This will require the phenotypic and functional characterization of intestinal B and T cells as well as their response to antigens. As pointed out above, Vitamin A deficiency could seriously impair the outcome of oral a vaccine in a deficient individual by affecting the function of intestinal lymphocytes.

The main objective of the present study has been to assess the immune response of the intestinal lymphocytes in experimental Vitamin A deficiency using a rat model in terms of

a) the function of intestinal intraepithelial lymphocytes as determined by

i. total number of T cells and subsets,

ii. polyclonal mitogenic and antigen specific activation of T cells,

iii. natural killer cell activity and

iv. sIgA-mediated anti-bacterial activity,

b) the humoral response of intestinal lamina proprial lymphocytes as determined by

i. total number of IgA positive B cells,

ii. mitogenic and antigen specific activation of B cells and

iii. induction of antigen specific IgA secreting plasma cells by bacterial antigens.

CHAPTER II

ISOLATION AND CHARACTERIZATION OF IEL AND LPL FROM RAT SMALL INTESTINE

INTRODUCTION

The lymphocytes of epithelial and lamina proprial compartments of intestine are phenotypically and functionally distinct. To study their functions, it is necessary to isolate pure populations of IEL and LPL. In this chapter the isolation and characterization of IEL and LPL from rat small intestine is described.

Intraepithelial lymphocytes :

Various techniques have been described so far for the isolation of IEL from small intestine of mice (Davies et *al,* 1981; Laventon et *al,* 1983; Dillon *et al*, 1984; Mosley and Klein, 1992), rats (Lyscom et *al,* 1982; Vaage *et al,* 1990) and humans (Greenwood et *al,* 1983; Lundqvist *et al,* 1992). IEL fractions have been isolated by gentle mechanical manipulation (Lyscom et *al,* 1982; Lundqvist *et al,* 1992), by EDTA treatment (Davies et *al,* 1981; Dillon et *al*, 1984; Nauss et *al,* 1984; Mosley and Klein, 1992) and by enzymatic treatment (Laventon et *al,* 1983).

Characteristics of IEL :

IEL population is quite heterogeneous and differs in composition from species to species. Studies on human intestinal IEL have consistently shown that majority of them (80%) are T lymphocytes (Janossy et *al,* 1980; Selby et *al,* 1981; Cerf-Bensussan et *al,* 1983; Greenwood et *al,* 1983). A few reports claimed the proportion of T cells in IEL of rats and mice to be more than 80% (Guy-Grand et *al,* 1978; Van der Heijden, 1986; Vaage et *al,* 1990) while others consider it to be around 50% (Lyscom et *al,* 1982; Tagliabue et *al,* 1982; Parrott et *al,* 1983). Majority of T cells bear surface antigens that characterize the

cytotoxic/ suppressor subset. Approximately half of the murine IEL are Thy 1^- (Parrott *et al,* 1983) and mature extrathymically (Lefrancois, 1991). These thymic-independent murine IEL express TCR $\gamma\delta$ instead of conventional TCR $\alpha\beta$ (Bonneville *et al,* 1988). On the contrary, human IEL and rat IEL contain very few TCR $\gamma\delta$ cells and majority of them are TCR $\alpha\beta^+$ cells (Brandtzaeg *et al,* 1989; Vaage *et al,* 1990).

Marsh (1975) reported that transformation of lymphocytes occurs within the interepithelial cell spaces of the small intestinal mucosa, suggesting that epithelial lymphocytes are immunocompetent cells responsive to local antigenic stimulation. However, reports on *in vitro* proliferative response of IEL to T cell mitogens have been varied. Nauss *et al* (1984) showed that IEL from rat large intestine were unresponsive to Con A. A small stimulatory effect was noted when splenic adherent cells and 2-mercaptoethanol were added. Mowat *et al* (1986) have reported that murine IEL by themselves are unresponsive to Con A and the addition of adherent accessory spleen cells and MLR supernatants enabled IEL respond to Con A. Contrary to the above findings, Dillon and MacDonald (1984) demonstrated that murine IEL proliferate in response to Con A, PHA and LPS, and this response could be enhanced by the addition of Con A-stimulated supernatants. The proliferative response of IEL to mitogens was shown to vary from species to species. Human and rabbit IEL were unresponsive to T cell mitogens (Greenwood *et al,* 1983; Ramsay and Holmes, 1990), while IEL isolated from pigs showed strong blastogenic response to T cell mitogens, PHA and Con A (Wilson *et al,* 1986). Murine IEL displayed minimal proliferative response towards antl-CD3, anti-TCR $\alpha\beta$ and anti-TCR $\gamma\delta$ (Mosley and Klein, 1991).

Significant natural killer (NK) cell activity and antibody -dependent cell-mediated cytotoxicity (ADCC) has been shown to be mediated by IEL obtained from different species (Tagliabue *et al,* 1982; Nauss *et al,* 1984). The NK cell activity has been reported to be associated with large granular IEL as well as nongranular IEL (Flexman *et al,* 1983).

Lamina proprial lymphocytes :

Many procedures have been developed for the isolation of LPL (Bull and Bookman, 1979; Davies and Parrott, 1981; Lyscom *et al,* 1982; Nauss *et al,* 1984; Van der Heijden and Stok, 1987; Woolverton *et al,* 1992). Most of these procedures make use of enzymatic digestion of the intestinal pieces obtained after the removal of epithelial layer by EDTA treatment. Proteases like collagenase (Bull and Bookman, 1979; Davies and Parrott, 1981; Van der Heijden and Stok, 1987) and dispase (Woolverton *et al,* 1992) are used for the isolation of LPL.

Characteristics of LPL :

LPL subset composition is remarkably different from that of IEL population. LPL were shown to contain a heterogeneous population of cells, including B cells, helper T cells and suppressor/cytotoxic T cells (Bull and Bookman, 1979; Lyscom *et al,* 1982; Kanof *et al,* 1988). In contrast to IEL, lymphocytes from lamina propria showed vigorous proliferative response to both T and B cell mitogens (Greenwood *et al,* 1983; Nauss *et al,* 1984; Ramsay and Holmes, 1990). LPL is also known to possess natural killer activity (Shanaham *et al,* 1987; Tagliabue *et al,* 1982). Majority of B cells in lamina propria are IgA (Craig and Cebra, 1971). Antigen specific IgA responses can be induced by administering the antigen orally. Induction of antigen specific IgA

responses at mucosal surfaces depends on the antigen nature, route and dose (Pierce, 1984; Dunkley and Husband, 1990).

MATERIALS AND METHODS

ANIMALS :

Female Wistar, Sprague-Dawley, Holtzman and Fischer rats, and Balb/c mice, of age 8-12 weeks, bred in the animal facility of National Institute of Nutrition, Hyderabad, were used in the present experiments.

1. ISOLATION OF IEL :

Chemicals :

RPMI 1640 with 2mM glutamine and 25mM HEPES, Foetal calf serum (FCS) and penicillin G were obtained from Sigma Chem. Co., USA. Dithiothreitol (DTT) and HEPES were obtained from SRL Chemicals, India. Ethylene diamine tetra acetic acid (EDTA) was from Glaxo, India and Phenyl methyl sulfonyl fluoride (PMSF) from Merck, Germany. Nylon wool was obtained from Fenwal laboratories, USA and membrane filters (0.22μ and 0.45μ) from Millipore, USA. Percoll was supplied by Pharmacia, Sweden. Streptomycin was from Sarabhai Chemicals, India. All other chemicals used were of analytical grade.

Reagents :

i. RPMI 1640 : The powdered medium was dissolved in distilled water and penicillin G (100U/ml) and streptomycin (100μg/ml) were added. pH was adjusted to 7.2 and the solution was made up to 1 litre with distilled water. The medium was sterile filtered through 0.45μ membrane filter using millipore filtration unit and stored at 4°C.

ii. Complete medium : RPMI 1640 supplemented with 5% FCS.

iii. Hank's balanced salt solution (HBSS) : KCl, 400mg; KH_2PO_4, 60mg; Na HPO , 48mg; glucose, 1g; NaCl, 8g; phenol red, 17mg; HEPES, 4.76g, were dissolved in distilled water. Penicillin G (100U/ml) and streptomycin (100µg/ml) were added and pH of the solution was adjusted to 7.2. Volume was made upto 1 litre with distilled water, and the solution was sterile filtered as above and stored at 4 C.

iv. Isolation medium : HBSS supplemented with 2% FCS and 100µM PMSF.

v. Phosphate buffered saline (PBS) : 0.01M sodium phosphate buffer, pH 7.4, containing 0.15M NaCl.

Procedure :

Rats were killed under mild ether anesthesia and small intestine from the first part of duodenum to the end of ileum was collected immediately and was flushed with large volumes of cold PBS. Peyer's patches, fat and mesentery were removed. Intestine was opened longitudinally and cut into l-2cm pieces. To remove mucus, intestinal pieces were incubated in isolation medium with 1mM DTT for 5mln at 37 C with gentle shaking. Supernatant was discarded and the pieces were incubated in isolation medium with 0.25mM EDTA at 37 C for 10min with shaking (120rev/min). Incubation with EDTA was repeated atleast 5-6 times until the supernatant became clear. All the supernatants collected from EDTA incubations were passed through a loosely packed nylon wool column to remove clumps and debris. Cells were sedimented by centrifugation at 400 xg for 10min and resuspended in the complete medium. Cells were washed twice with complete medium and finally resuspended in the same.

To obtain enriched live population of IEL a discontinuous

percoll density gradient (60%/40%/30%; density= 1.08/1.06/1.04 respectively) was used. Cells were suspended in 30% percoll and layered over the gradient. The gradient was spun at 600 xg for 20min at 4 C. Cells at 60/40% interface were collected and washed thrice with the complete medium. Viability of the cells was checked at each step using Trypan blue dye exclusion.

For comparative studies, IEL were also isolated in the absence of PMSF in isolation medium. IEL from mouse small intestine were isolated by following the same procedure described above.

2. HISTOLOGICAL EXAMINATION OF SMALL INTESTINAL TISSUE :

Intestinal pieces were collected at different stages of the isolation method and they were preserved in buffered formalin. Tissues were processed for histological examination using standard protocols. Wax embedded tissues were sectioned and stained with hematoxylin and eosin.

3. ESTIMATION OF AMINO ACID CONTENT :

Amino acid contents in EDTA-HBSS supernatants were estimated by Ninhydrin method as described earlier (Stanford Moore *et al,* 1954).

4. **ISOLATION** OF LPL :

LPL were isolated according to the method of Van der Heijden *et al* (1987).

Intestinal pieces after the isolation of IEL were incubated in complete medium for 10min and washed with the same to remove residual EDTA. They were incubated for 90min in 25ml complete medium with 75 U/ml collagenase type XI (Sigma Chem. Co, USA) at 37°C in a shaking waterbath. Supernatant was collected and the pieces of intestine

remaining were gently squeezed through a stainless steel wire mesh. Both the supernatant and the suspension obtained after squeezing the tissue were passed through a loosely packed nylon wool column. Cells were spun down at 400 xg for 10min and resuspended in the complete medium.

LPL population was further purified by using discontinuous percoll gradient as described above for IEL isolation. The 60/40% interface was collected and washed thrice with complete medium. Viability was checked using trypan blue dye exclusion.

5. DETERMINATION OF VIABILITY BY TRYPAN BLUE DYE EXCLUSION :

Principle :

Viable cells exclude the dye, while nonviable cells take up the dye thereby fostering a visual distinction between unstained viable cells and blue-stained nonviable 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:

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

6. LYMPHOCYTE COUNTING :

Principle :

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

Procedure :

A small volume of cell suspension was diluted with Turk's solution (0.01°/. gentian violet, w/v, in 3% v/v, acetic acid) and mixed thoroughly. 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 :

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

7. **COUPLING OF ANTI-RAT IgG TO CNBr-ACTIVATED SEPHAR0SE-4B** :

Principle :

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

Chemicals and reagents :

CNBr-activated sepharose-4B and affinity purified anti-rat IgG were purchased from Sigma Chem. Co., USA.

i. Coupling buffer : 0.1M bicarbonate buffer, pH 8.4, in 0.5M NaCl.

ii. Ethanolamine, 1M, pH 8,0.

iii. Acetate buffer : 0.1M acetate uffer, pH 4.0, in 1M NaCl.

iv. Borate buffer : 0.1M borate buffer, pH 8.0, in 1M NaCl.

v. RPMI 1640.

Procedure :

All operations of coupling were carried out under sterile conditions. 1g of CNBr-activated Sepharose-4B was washed with 100ml of 1mM

HC1 followed by large volumes of double distilled water. The swollen gel was taken into 5ml of coupling buffer containing 2mg of goat anti-rat IgG and 2mg of goat IgG (purified on a protein-A Sepharose column from goat serum). The mixture was rotated end-over-end for overnight at 4 C. The coupled gel was gently centrifuged at 100rpm for 2min and the supernatant was saved. The gel was washed with 2.5ml coupling buffer to remove unbound material. Supernatant at every step was saved to calculate finally the amount of protein that has been coupled to the gel.

In the next step, gel was treated with ethanolamine at room temperature for 1h by end over rotation. This is to block the remaining reactive groups. Three washing cycles were used to remove non-covalently adsorbed protein to the gel, each cycle consisting of one acid wash with acetate buffer and one alkaline wash with borate buffer. Finally the gel was suspended in RPMI 1640 and stored at 4 C.

Absorption at 280nm was taken for all the supernatants against their corresponding buffer blanks. From this the amount of protein bound to the gel was calculated.

8. LYMPHOCYTE PROLIFERATION ASSAY USING MITOGENS :

Principle :

Mitogens activate lymphocytes to proliferation *in vitro* when all the growth requirements are given. The proliferative response is measured by H-thymidine incorporation into DNA.

Chemicals and reagents :

Concanavalin A (Con A) and 2-mercaptoethanol were obtained from Sigma Chem. Co., USA. Fungizone (amphoterecin B) was supplied by Sarabhai Pharmaceuticals, India. Phytohaemagglutinin (PHA) was purchased

from Gibco Labs, USA and lipopolysaccharide (LPS) from *E. coli* was supplied by Sigma Chem. Co., USA. ^{3}H-thymidine was supplied by BRIT, India.

i. Culture medium : RPMI 1640 supplemented with 10% FCS, 50μM 2-mercaptoethanol and 2.5μg/ml fungizone.

ii. Sepharose coupled anti-rat IgG (Seph. anti-IgG).

iii. The mitogens were dissolved in RPMI 1640, sterile filtered and stored at 4°C.

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

Procedure:

Cell cultures containing 2x10 IEL, LPL or splenic lymphocytes in 0.2ml of culture medium were kept in 96-well flat bottomed sterile microtitre plates (Laxbro, India). T cell mitogen, Con A was added to IEL cultures at the indicated concentrations. Con A and Seph. anti-IgG were used to stimulate LPL, and Con A, PHA, LPS and Seph. anti-IgG were used to stimulate splenic lymphocytes.

Cultures with and without mitogens were taken in triplicates and incubated at 37°C in a humidified incubator with 95% air and 5% CO atmosphere. The cultures were pulsed with 0.5μCi of H-thymidine/well for the last 18h culture period and processed using Skatron cell harvester. Dried filters were transfered into the scintillation cocktail and the radioactivity was measured using Beckman Liquid Scintillation Counter. Viability of the cells in culture was checked at regular intervals using trypan blue dye exclusion method.

a. Effect of splenic adherent cells on proliferation of IEL :

Preparation of splenic adherent cells :

Single cell suspension of spleen cells from syngenic animals was obtained by teasing the spleen in complete medium on a stainless steel wire mesh. Cells were washed thrice with the complete medium and the cell suspension was adjusted to a concentration of 10x10 cells/ml. 5ml of this cell suspension was taken into a sterile plastic petri dish (90mm dia.) and incubated for 3h at 37°C in an incubator with 5% CO atmosphere. Nonadherent cells were discarded and the plate was gently washed with complete medium to remove the remaining non-adherent cells. Adherent cells were removed gently using a sterile rubber policeman. Cells were washed twice with complete medium and counted using Turk's solution.

Proliferation assay :

Adherent cells were added to the lymphocyte cultures (IEL) along with the mitogens and the proliferation assay was carried out.

b. Effect of ConA-stimulated splenic lymphocyte supernatants on proliferation of **IEL**

Preparation of Con A-stimulated rat splenic lymphocyte supernatants :

Syngenic splenic lymphocytes were cultured in bulk with Con A at a concentration of 3μgs/ml. Cell concentration was adjusted to 4x10 cells/ml of complete medium with 50μM 2-mercaptoethanol and 7ml of this cell suspension was taken into a siliconized glass tissue culture bottle. Cultures were kept at 37°C in a CO incubator. After 24h the supernatant was collected, filter sterilized through a low protein binding 0.45μ membrane filter and stored at -70 C until use.

Proliferation assay :

Con A-stimulated splenic lymphocyte culture supernatants were added to the IEL cultures at various concentrations (10%, 20'/. or 40%). Mitogens were added to these cultures and proliferation assay was carried out. Control cultures were maintained without mitogens in presence of Con A-stimulated splenic lymphocyte supernatant.

9. Mixed Lymphocyte Response :

Principle :

The mixing of two populations of allogenic lymphoid cells results in T cell proliferation, which is termed as mixed lymphocyte response (MLR). Cell surface antigens encoded by the genes of the major histocompatibility complex or the M locus or both, are the major stimuli of this response. One population of allogenic cells serves as the antigenic source (the stimulator cells) and is inhibited from proliferating by procedures that arrest cell division. Then, the proliferation by the second cell population (the responder cells) is measured.

Procedure :

The two different strains of rats used were Sprague-Dawley and Wistar. Splenocytes from Sprague-Dawley rats were used as stimulator cells. IEL and splenocytes isolated from Wistar rats were used as responders.

The stimulator cells were treated with mitomycin C (Biochem Pharmaceuticals, India), a DNA cross linker, to inhibit proliferation. To 1.0 ml of cell population containing 3x10 cells, 25µg of mitomycin C was added and incubated at 37°C for 20mln. Cells were washed thrice with excess of complete medium and used as stimulator cells.

The untreated responder cells and the treated stimulator cells were suspended in complete medium with 50μM 2-mercaptoethanol and the cell concentration was adjusted to 2x10 cells/ml. 2x10 responder cells in 100μl were cultured with 2x10 stimulator cells in 100μl. For control cultures 100μl of complete medium was added to the stimulator cells. Cultures were incubated at 37°C in a CO incubator for 96 or 120h and were pulsed with o.5μCi ^{3}H-thymidine for the last 18h. Cells were harvested and the radioactivity was counted as described above.

10. NATURAL KILLER CELL ASSAY :

Principle :

The quantitative assay of natural killer cell activity makes use of Cr labeled tumor cells as targets. The effector cells (IEL) were incubated with the targets at different effector to target cell ratios. Cr released into the supernatant due to cell lysis was measured.

Maintanance of target cells **(YAC-1)** :

YAC-1 is a Moloney leukemia virus induced murine T cell lymphoma. YAC-1 cells were used as tumour targets for IEL as they were found to be suitable targets (Tagliabue *et al*, 1982).

Freezing and storage of the cells :

YAC-1 were obtained from NFATCC, Pune, India. 2-3x10 cells/ml of RPMI 1640 containing 15% FCS and 15% dimethyl sulfoxide (DMSO) **were** taken in sterile plastic vials and were frozen at -70 C overnight and then transfered to liquid nitrogen for future use.

Revival and subculture :

Cells were taken out from liquid nitrogen and thawed quickly using a 37 C water bath. They were washed with warm complete medium for at least three times to remove DMSO. Cultures were maintained in 24 well plates (Laxbro, India) at a concentration of 2x10 cells/ well in one ml of complete medium. Cells were subcultured every 48h.

NK cell activity :

Loading YAC-1 with ^{51}Cr :

Cr was obtained as Sodium chromate ($Na\ CrO_4$) in saline from BRIT, India. YAC-1 were loaded with ^{51}Cr by incubating $8\text{-}10x10^6$ cells /ml complete medium with 300μCi of Cr at 37°C for 1h. Cells were washed at least three times with large volumes of RPMI to remove free Cr, and finally suspended in complete medium.

Micro-well assay :

Reagents :

i. Bray's mixture : 60g of naphthalein, 4g PPO, 200mg POPOP were dissolved in 1,4-Dioxane. 100ml of methanol and 25ml of ethylene glycol were added and the final volume was made upto 1 litre with dioxane.

Procedure :

YAC-1 target cells loaded with Cr were incubated with effector cells (either IEL or spleen cells) in a 'v' bottomed microtitre plate (Laxbro, India) at effector to target (E/T) ratios ranging from 6.25:1 to 100:1. Concentration of the target cells was maintained constant as 10 cells/well and the effector cell concentration was changed accordingly. After 6h of incubation at 37°C in 5% CO_2 atmosphere, the plate

was spun down at 500 xg for lOrain. An aliquot of the supernatant was taken from each well into the scintillation cocktail (Bray's mixture) and the radioactivity was measured using a Beckman liquid Scintillation counter provided with a Cr-progrmmer (model number LS-1701) or in a Gamma counter (model no. 1275, LKB-Wallac, USA).

To know the total Cr incorporated, the target cells were treated with 1% Nonidet-P40 and for the spontaneous release 10 loaded target cells were plated without the effector cells. Percent specific cytolysis was calculated by using the formula :

$$\% \text{ lysis} = \frac{\text{Cr released from the cells during incubation} - \text{spontaneous release}}{\text{total release} - \text{spontaneous release}} \times 100$$

11. IMMUNOFLUORESCENCE :

Total T cell number and its subsets and the total B cell number, in IEL, LPL and splenic lymphocyte populations were enumerated by indirect immunofluorescence.

Chemicals and reagents :

i. The monoclonal antibodies W3/13, 0X8 and W3/25 which identify a Pan T cell, cytotoxic/suppressor and helper/inducer markers respectively, were purchased from Sera-Lab, UK.

ii. RGL-2, a monoclonal actibody specific for rat intestinal T lymphocytes was a generous gift from Dr. Cerf-Bensussan, France.

iii. Goat anti-rat IgG (for total B cells) and goat anti-rat IgA (for IgA B cells) were purchased from Sigma Chem. Co., USA.

iv. FITC conjugated goat anti-mouse IgG was from Sigma Chem. Co., USA and FITC conjugated sheep anti-goat IgG was prepared by conjugating FITC (Sigma Chem. Co., USA) to sheep anti-goat IgG (Cappel labs, USA) as described below.

a. Preparation of FITC conjugates :

Reagents :

i. Bicarbonate buffered saline (BBS), pH 9.2 ; 0.05M sodium bicarbonate buffer, pH 9.2, in 0.15 M Nacl.

ii. BBS, pH 8.5 : 0.05M Sodium bicarbonate buffer, pH 8.5, in 0.15M Sodium chloride.

Procedure :

The IgG sample was first dialysed at 4°C against 0.15M sodium chloride and then against BBS, pH 8.5, for 4-5h, following with BBS, pH 9.2, for 2h. The IgG sample was next dialysed against a solution of 100µg of FITC/ml BBS, pH 9.2, for 14-16h. The reaction was stopped by changing the dialysis buffer to PBS, pH 7.0. Sample was extensively dialysed against this buffer to remove uncoupled free FITC. Fluorescein/protein ratio was determined according to Wells *et* ai (1966).

b. Fluorescent labeling and visualization :

Procedure :

IEL, LPL or splenic lymphocytes, 3-5x10 cells in 50µl of complete medium containing 0.05% azide, were first incubated with respective antibody for 1h on ice. Cells were washed three times and incubated with FITC conjugated second antibody under the same conditions. Cells were washed free of excess FITC conjugate and were observed using Leitz fluorescence microscope with appropriate filter optics. A minimum of 300 cells were counted in each sample.

12. ANTIGEN SPECIFIC IMMUNE RESPONSE :

The test bacterium selected was *Salmonella typhimurium* (strain LT-2).

Growth of S. *typhimurium* in suspension culture :

S. *typhimurium* suspension cultures were obtained by incubating 100µl of frozen culture in 10ml of Luria broth (LB medium, supplied by Himedia, India) containing 0.5"/ glucose at 37°C with shaking for 12h.

Freezing and storage :

0.85 ml (10^9 cells) of overnight grown culture was added to 0.15 ml of sterile glycerol and frozen in liquid nitrogen. The frozen cultures were stored at -70 C till use.

Preparation of outer membrane antigens :

Outer membrane antigens were isolated using the method of Senda *et al* (1989). The bacterial cells from overnight grown cultures were resuspended in cold PBS. Amount of protein in the cell suspension was determined by the method of Lowry *et al* (1951). Cell pellet was collected by centrifugation of the suspension at 12,000 xg for 30min. The pellet (containing approximately 20mg protein) was suspended in 5ml of 0. 02M Sodium phosphate buffer containing 2% N-lauroyl sarcosine (Sigma Chem. Co., USA) and 1. 5mM EDTA. After incubating for 1h at 37°C, suspension was centrifuged at 100,000 xg for lh. Pellet was washed twice with phosphate buffer and incubated at 37 C for 2h with 5ml of the same buffer containing 20µg deoxyribonuclease, 20µg ribonuclease (Sigma chem. Co., USA) and 10mM $MgCl_2$. The pellet was collected by centrifugation at 100,000 xg for 1hr and washed twice with PBS. Finally the outer membrane antigens were suspended in PBS and the amount of protein was determined.

13. RAISING ANTISERA AGAINST S. *typhimurium* **:**

a. **Antiserum in** rabbits :

Membrane antigen preparation (1mg/ml) in PBS was thoroughly mixed with 1ml of Fruend's complete adjuvant (FCA, Sigma chem. Co., USA) and the emulsion was injected intramuscularly on the dorsal surface of a rabbit at 5-6 sites. First booster dose (0.5 mg protein) in adjuvant was given after one month. A second booster dose of same concentration was given fifteen days later. The rabbit was bled 15 days after the second booster and the serum was separated, heat inactivated and stored at -20°C in aliquots.

b. Antiserum in **rats** :

Seven weeks-old rats were injected intraperitoneally with 50μg of S. *typhimurium* membrane protein in FCA. A booster dose was given after 15 days intraperitoneally with same amount of protein in adjuvant. On the fifth day after the booster injection, rats were killed and blood was collected by heart puncture. Serum was heat inactivated and stored at -20 C until use.

The presence of antigen specific antibody (IgG) in rat **and** rabbit sera was confirmed by western blot analysis.

c. **Rat intestinal fluid** :

Rats were primed by injecting intraperitoneally 50μg of S. *typhimurium* membrane proteins in FCA. After 15 days, the rats were boostered orally with 10 live bacteria in LB medium. The oral dose was given after the administration of 0.2M bicarbonate orally to minimize the lysis of bacteria by gastric acids. Three oral doses were given with an interval of 5 days between doses. On the fifth day after the last booster dose rats were killed and the intestinal fluids **were**

collected. Same protocol was followed to obtain *in vivo* sensitized IEL, LPL and splenocytes. But the rats were killed on the third day after the last oral booster to obtain antigen sensitized lymphocytes.

Collection of intestinal fluid :

The intestine was flushed and washed with minimal amounts (5ml) of PBS containing 0.1mg/ml Soybean trypsin inhibitor (Sigma Chem. Co., USA). PMSF was added to a final concentration of 1mM and the washings were vortexed for 1min. To obtain a clear solution the suspension was centrifuged at 10,000 xg for 30min, the supernatant was collected and stored at -20 C. Presence of antigen specific IgA in intestinal fluid was confirmed by ELISA (Senda *et al*, 1989).

14. ELECTROPHORETIC AND WESTERN BLOT ANALYSIS OF S. *typhimurium* MEMBRANE ANTIGENS :

Reagents :

i. Electroblotting buffer : 48mM Tris, 39mM glycine with 20% methanol.

ii. Blocking buffer : 5% w/v, Bovine serum albumin in PBS.

iii. Incubation buffer : 37. w/v, Bovine serum albumin in PBS.

iv. Tris-buffered saline (TBS) : 50mM Tris buffer, pH 8.0, containing 0.15M Nacl.

v. Substrate solution : 3μg of 1-chloro, 4-naphthol (Fluka, Switzerland) in 1ml of methanol was added to 5ml TBS containing 0.17. H_2O_2.

Procedure :

Polyacrylamide gel electrophoresis in presence of SDS (SDS-PAGE) of the S. *typhimurium* membrane antigens was run according to the

method of Laemmli *et al* (1970) using a 10% gel.

The proteins separated on SDS-PAGE were transfered on to a nitrocellulose membrane filter (Scheicher and Schuell, USA) electrophoretically using a blotting apparatus (Hoefer Scientific Instruments, USA) at a constant voltage (14v) at 4°C overnight in electroblotting buffer. The membrane filter was washed with PBS and incubated for 1h at room temperature with blocking buffer. Membrane was washed with PBS and incubated with rat antiserum or rabbit antiserum (1:4 dil in the incubation buffer). The incubation was carried out at room temperature with constant agitation for lh. After washing away the non-specifically bound primary antibody with excess amounts of PBS, the membrane was then incubated in appropriately diluted secondary antibody for lh at room temperature with constant shaking. The secondary antibodies used were Horse radish peroxidase (HRPO) conjugated goat anti-rabbit IgG (Lupin Laboratories, India) or HRPO-goat anti-rat IgG (Sigma Chem. Co., USA). Membrane was washed with PBS at least four times and finally with TBS. Membrane was developed with the substrate solution and the reaction was stopped after 2-3min by rinsing the membrane with distilled water.

15. ANTIBODY-DEPENDENT CELL-MEDIATED ANTIBACTERIAL ACTIVITY :

Principle :

The targets used in this assay are *S. typhimurium.* The effector cells (IEL or splenocytes) were incubated with bacteria in the presence of antibodies to bactierial antigens and the bacteriolysis was monitored.

Procedure :

The assay was carried out by following the method of Tagliabue

et al (1983).

IEL and splenocytes were isolated from *in vivo* sensitized rats (one ip followed by three oral boosters as described in protocol 13). S. *typhimurium* (10^4) in complete medium without antibiotics were taken in conical tubes together with either appropriately diluted antibodies, rat antiserum (IgG source) or intestinal fluid (sIgA source), or medium and were centrifuged at 1300 xg for 10min at 4°C. The lymphoid cell suspension was then added to these tubes at different effector to target (E/T) ratios. Tubes were again centrifuged at 500 xg for 5min at 4 C. Experimental and control tubes were incubated at 37 C in a 5% CO- atmosphere for 2h. Two controls were maintained, one with bacteria but no lymphocytes and antibody, and the other with bacteria and antibody but no lymphocytes.

After incubation, pellets were thoroughly suspended in 1ml of PBS. Appropriately diluted aliquots were plated on petri dishes containing agar-tryptose using top-agar method and incubated overnight at 37 C. Colony forming units (CFU) were counted and the percentage of antibacterial activity expressed as antibacterial index was calculated as follows:

'/. Antibacterial index =

$$100 \left(100 \times \frac{\text{No. of CFU of experimental plates}}{\text{No. of CFU of control plates}}\right)$$

16. ANTIGEN INDUCED LYMPHOCYTE PROLIFERATION ASSAY :

Proliferative response of *in vivo* sensitized LPL and splenocytes to S. *typhimurium* antigens *in vitro* was assessed. Various concentrations of S. *typhimurium* antigens were added to LPL cultures and the lymphocyte proliferation assay was carried out by measuring ^{3}H-thymidine incorporation as described in protocol 8.

17. GEL ANALYSIS OF DNA FROM CULTURED RAT AND MOUSE IEL :

Principle :

The programmed cell death (apoptosis) of IEL is monitored by the ladder-like pattern of DNA banding following electrophoresis in agarose gels.

Reagents :

i. NTE buffer : 100mM Nacl in 10mM Tris buffer, pH 8.0, containing 1mM EDTA.

ii. TBE buffer : 89mM Tris, pH 8.4, containing 89mM boric acid and 2mM EDTA.

iii. Loading buffer : TBE buffer containing 15%. Ficoll 400, 0.5% SDS, 50mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol.

Procedure :

Rat and mouse IEL were incubated at 4 C and 37 C for 24h and the DNA was extracted from these cells as described earlier (Viney *et al*, 1990). Briefly, cells were washed with cold RPMI 1640 and disrupted by incubating them in NTE buffer containing 1% SDS and 0.2mg/ml proteinase K (Sigma Chem. Co., USA) for 24h at 37°C. DNA was extracted with phenol, chloroform, isoamyl alcohol mixture (25:24:1, v/v/v) and precipitated with ethanol. DNA was air dried and dissolved in 20µl of NTE buffer. Samples were then digested with 1mg/ml ribonuclease (Sigma Chem. Co. , USA) for 1h at 37°C. 10µl of loading buffer was added to each digested sample and the mixture was run on a 1% agarose gel with TBE buffer. Gel was stained with 0.5µg/ml ethidium bromide solution and analysed using UV-light in a transilluminator (Fotodyne, USA).

18. Statistical analysis :

The data obtained in the present study was subjected to statistical analysis and the significance of the difference between two values was calculated according to Student's t-test.

RESULTS

ISOLATION AND CHARACTERIZATION OF IEL :

Cell yield and **viability** :

Various procedures described earlier were tried for the isolation of viable IEL from rat small intestine (Davies et *al,* 1981; Lyscom *et al,* 1982; Nauss et *al*, 1984), but none of them gave a good yield of viable lymphocytes. Hence, a modified procedure for the isolation of intraepithelial lymphocytes was developed. This procedure makes use of a protease inhibitor- PMSF, during the incubation for the release of IEL. Yield and viability of IEL was higher in presence of PMSF as compared to those isolated in the absence of PMSF (Table 1). Almost 80% of cells **were** contributed by epithelial cells in the cell population obtained after EDTA treatment. When PMSF was included in the isolation medium, viability after EDTA treatment was about 85%. After percoll gradient centrifugation, most of the epithelial cells, dead cells and debris remained in 30% fraction, while the 30-40% interface had rest of the epithelial cells with few IEL. **A pure** population of IEL with 98% viability was collected from 40-60% interface. The yield and viability of IEL was significantly higher in presence of PMSF ($p < 0.01$, Table 1).

TABLE 1

CELL YIELD AND VIABILITY OF IEL ISOLATED IN PRESENCE OF PMSF

Treatment	cell yield		viability	
	-PMSF	+PMSF	-PMSF	+PMSF
	($\times 10^6$ cells)		%	
(i) After EDTA incubations				
Total cells	72 + 5	84 + 6	75 + 5	85 + 4
IEL	13.7 + 0.9	17.2 + 1.3	—	—
(ii) After percoll gradient				
IEL (40-60% Percoll interface)	6.8 + 1.5	10.2 + 0.9#	87 + 3	98 + 0.2

Values presented are mean + SEM of ten experiments.

Viability was determined as described in the methods section.

• $p < 0.01$, # $p < 0.05$ as compared to -PMSF

Histological observation of the intestinal tissue :

Histological observations showed that EDTA treatment removes only the epithelial layer from the intestinal wall without disrupting lamina propria (Figure **1).**

Amino acid concentrations of EDTA-supernatants :

Isolation of IEL was carried out in presence and absence of PMSF, and the amino acid content of the supernatants obtained at each step of incubation was determined.

Figure 2 shows that the amino acids released into the medium in presence of PMSF was comparatively lower than the supernatants obtained in absence of PMSF showing that the protease activity of the tissue was inhibited to a significant extent.

Proliferative response of IEL and splenic lymphocytes to mitogens :

Proliferative response of IEL and splenic lymphocytes was tested using T cell mitogen, Con A. IEL were found to be unresponsive to Con A (Table 2). However, same concentrations of Con A (2μg/ml, 4μg/ml) induced a strong proliferative response of autologous splenic lymphocytes maintained under the same conditions. Different concentrations of Con A (from 2 to 200μg/ml) were added to the IEL cultures and it was found that IEL were unresponsive to Con A even at higher concentrations. Even at different cell densities IEL showed no response towards Con A *in vitro.*

To rule out the possibility that Wistar IEL alone might be unresponsive to mitogens, IEL from different strains, Sprague-Dawley, Holtzman and Fischer rats, were isolated and the lymphocyte transform-

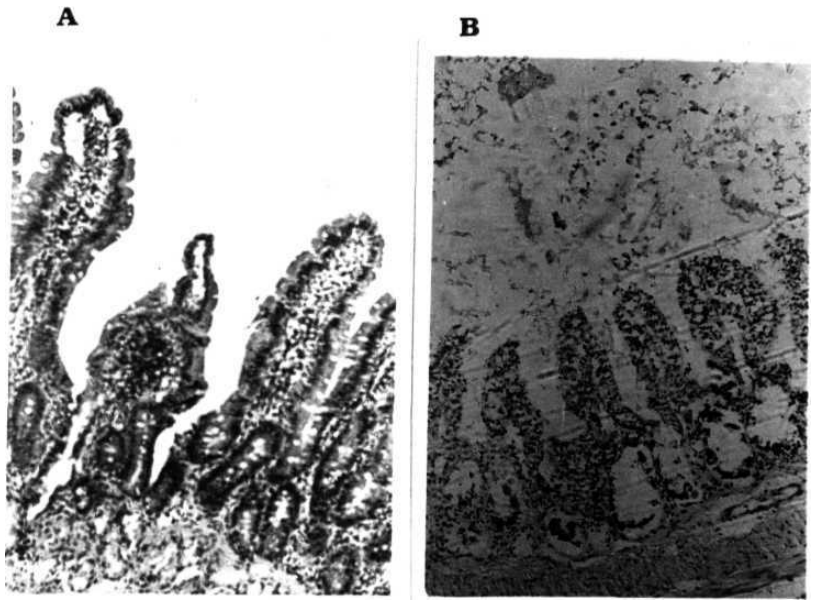


FIGURE 1:

Histologicai appearance of a segment of small intestine A) before and B) after four incubations in presence of EDTA. Magnification, 120X.

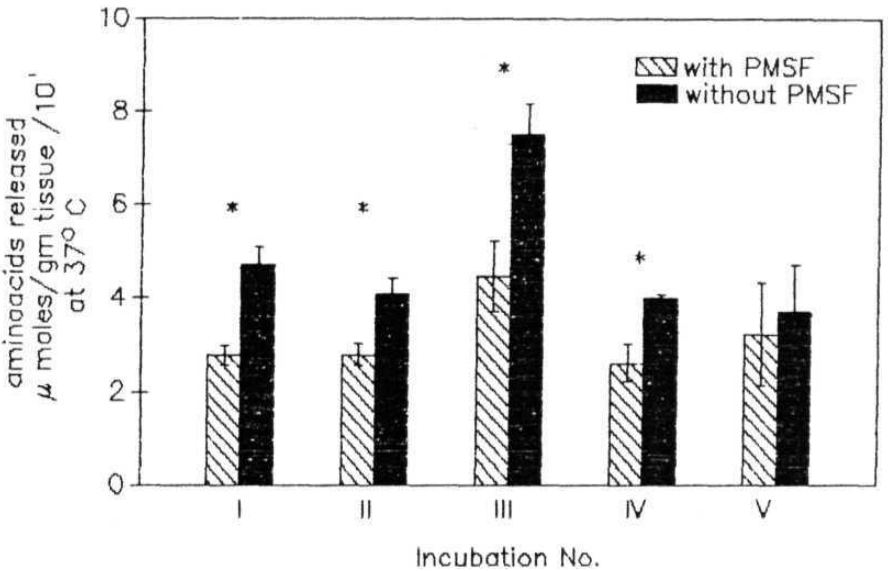


FIGURE 2: Amino acid content of the supernatants collected during the isolation of IEL in presence and absence of PMSF.

Values are expressed as mean + SEM of five observations. Incubation I was with 1mM DTT and incubations II, III, IV and V were with 0.25mM EDTA. • $p < 0.01$

TABLE 2

PROLIFERATIVE RESPONSE OF IEL TO DIFFERENT CONCENTRATIONS OF CON A

Cell population	H-thymidine incorporation into DNA			
	None	Con A		
		2μg/ml	4μg/ml	10μg/ml
		cpm/10 cells		
EXPT 1				
IEL	397 + 32	**428 + 48**	421 + 74	432 + 82
splenocytes	810 + 30	45750 + 330	56520 + 350	ND
EXPT 2				
IEL	757 + 59	427 + 47	**845** + 45	780 + 89
splenocytes	2923 + 382	44078 + 1490	69935 + 597	ND
EXPT 3				
IEL	1120 + 37	1250 + 17	735 + 15	ND
splenocytes	5302 + 232	72208 + 1087	98349 + 2807	ND
EXPT 4				
IEL	582 + 35	**556 +83**	611+72	ND
splenocytes	5914 + 453	67860 + 1741	69572 + 897	ND
EXPT 5				
IEL	**834 + 44**	757 + **55**	723 + 87	**ND**

H-thymidine incorporation was measured between 72-96h for **IEL** and between 24-48h for splenocytes. Values presented are mean + SEM of triplicate cultures from representative experiments.

ND= Not done.

ation assay was carried out. IEL from all strains were found to be unresponsive to Con A *in vitro* (Table 3).

In order to see whether the unresponsiveness of IEL was not due to the method used to isolate IEL, which makes use of PMSF, EDTA, etc., the proliferative response of splenocytes isolated in presence and absence of PMSF-EDTA was checked. There was no significant difference in the proliferative response of splenocytes isolated in presence of PMSF- EDTA to that of the cells isolated in absence of the same (Table 4).

Viability of rat and mouse IEL in culture :

In order to check whether the lack of response to Con A is due to cell death during culture, rat IEL were maintained at 37 C and the viability was checked at regular intervals. For comparative purposes IEL from Balb/c mice were also cultured along with rat IEL. Table 5 shows that upto 75-80% of rat IEL were viable even after 48h of culture period. However, in case of mouse **IEL only 50%** of cells were viable after 24h of culture period as reported earlier (Viney *et al*, 1990).

Gel electrophoretic analysis of DNA extracted from rat and mouse IEL cultured at 37°C :

DNA was extracted from IEL after 24h culture period and examined by gel electrophoresis to determine whether the cell death was accompanied by DNA fragmentation, a process indicative of apoptosis. Figure 3 shows the gel pattern of DNA from rat and mouse **IEL** maintained at 37°C for 24h. The ladder like pattern indicative of apoptosis was observed only with mouse IEL.

TABLE 3

PROLIFERATIVE RESPONSE OF IEL ISOLATED FROM DIFFERENT STRAINS OF RATS TO CON A

Strain	^{3}H-thymidine incorporation into DNA		
	None	Con A	
		2μg/ml	4μg/ml
	cpm/ 10 cells		
EXPT 1			
Sprague-Dawley	438 + 30	471 +32	511+41
Holtzman	375 + 50	330 + 17	421 + 28
Fischer	362 + 35	377 +56	353+15
EXPT 2			
Sprague-Dawley	388 + 45	412 + 36	373 + 54
Holtzman	419 + 51	406 + 28	397 + 58
Fischer	368 + 62	351 + 19	402 + 47

H-thymidine incorporation was measured between 72-96h. Values are expressed as mean + SEM of triplicate cultures.

TABLE 4

PROLIFERATIVE RESPONSE OF SPLENOCYTES ISOLATED IN MEDIUM WITH EDTA AND PMSF TO CON A

Con A μg/ml	^{3}H-thymidine incorporation into DNA	
	cells isolated in complete medium	cells isolated in medium with EDTA and PMSF
	cpm/ 10^5 cells	
None	13576 + 126	19051 + 153
2.0	1080047 + 4910	1144756 + 3078
4.0	1083985 + 15467	1198105 + 4126
6.0	837912 + 13742	891960 ± 6049

Values are expressed as mean + SEM of triplicate cultures. Data shown are from a representative experiment.

TABLE 5

VIABILITY OF RAT AND MOUSE IEL DURING CULTURE

Cell population	viability		
	0 h	24 h	48 h
		%	
EXPT 1			
Rat IEL	98.0	92.0 +1.0	80.0+3.0
Mouse IEL	97.0	58.0+3.0	61.0+4.0
EXPT 2			
Rat IEL	99.0	84.0 +1.0	74.0+4.0
Mouse IEL	98.0	57.0 +4.0	45.0+6.0
EXPT 3			
Rat IEL	99.0	87.0+1.0	78.0 +3.0
Mouse IEL	98.0	55.0+4.0	51.0+5.0

The viability of cells was checked at different time periods using trypan blue dye exclusion. Values expressed are mean ± SEM of triplicate cultures from three representative experiments.

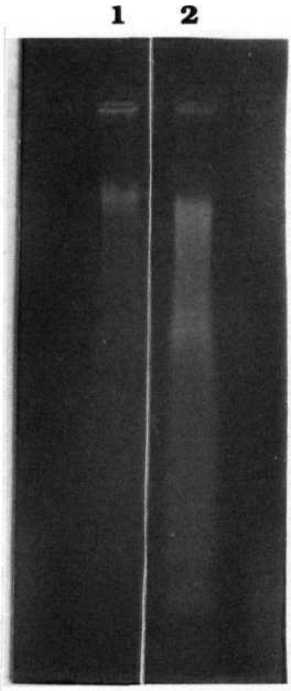


FIGURE 3: Electrophoretic analysis of DNA from rat and mouse IEL on agarose gels.

Lane 1 : rat IEL cultured at 37°C for 24 h,

Lane 2 : mouse IEL cultured at 37°C for 24 h.

Effect of splenic adherent cells on proliferative response of IEL :

The unresponsiveness of IEL to mitogens in vitro could be due to the absence of accessory cells that are required for the transformation response. Hence, to test the role of accessory cells in IEL transformation response, autologous splenic adherent cells were added to IEL cultures at different concentrations ranging from $2x10^4$ to $8x10^4$ cells/ culture. IEL were unresponsive to Con A even in presence of splenic adherent cells (Table 6).

Effect of Con A-stimulated splenic lymphocyte culture supernatants on proliferative response of IEL :

The addition of Con A-stimulated autologous splenic lymphocyte culture supernatants to Con A-activated IEL cultures resulted in a significant proliferative response (Table 6). Optimal response was obtained when the supernatant was added at a concentration of 40%.

Mixed lymphocyte response of IEL :

The MLR response of IEL was assessed by culturing Wistar IEL with mitomycin C treated Sprague-Dawley spleen cells as stimulator cells. IEL failed to show any response to allogenic stimulator cells (Table 7). However, a vigorous proliferative response of Wistar spleen cells to Sprague-Dawley stimulators was seen.

Enumeration of T cell subsets of IEL :

T cell subset composition of IEL was determined using monoclonal antibodies specific for helper/inducer (W3/25) and cytotoxic/ suppressor (0X8) subsets. A monoclonal antibody which recognizes pan T cell marker (W3/13) was used to enumerate total number of T cells in

TABLE 6

EFFECT OF SPLENIC ADHERENT CELLS AND CON A-STIMULATED SPLENIC LYMPHOCYTE CULTURE SUPERNATANTS ON THE PROLIFERATIVE RESPONSE OF IEL

cell population	^{3}H-thymidine incorporation into DNA			
	None	Con A		
		2μg/ml	4μg/ml	10μg/ml
	cpm/ 10 cells			
EXPT 1				
IEL	403 + 52	386 + 33	394 + 18	358 + 75
IEL + SAC	332 + 12	380 + 49	426 + 13	423 + 71
IEL + SCS**	3080 + 108	11725 + 2284	8755 + 293	1735 + 69
EXPT 2				
IEL	864 + 71	780 + 43	753 + 56	ND
IEL + SAC	1530 + 40	865 + 61	960 + 60	ND
IEL + SCS	2513 + 137	13282 + 275	13075 + 388	ND
EXPT 3				
IEL	831 +64	811+53	774 + 80	ND
IEL + SAC	1811 + 52	1711 + 70	1813 + 67	ND
IEL + SCS	7485 + 125	30500 + 1290	31590 + 894	ND
EXPT 4				
IEL	565 + 43	593 + 64	543 + 17	ND
IEL + SAC	617 + 47	598 + 54	634 + 23	ND
IEL + SCS	5989 + 134	10405 + 978	11965 + 1286	ND
EXPT 5				
IEL	703 + 62	686 + 58	693 + 38	ND
IEL + SAC	826 + 65	852 + 43	806 + 51	ND
IEL + SCS	4087 + 243	9635 + 545	9539 + 760	ND

^{3}H-thymidine incorporation was measured between 72-96h. Values presented are mean + SEM of triplicate cultures. ND= Not done.

• SAC = splenic adherent cells were added at a final concentration of $4x10^4$ cells. ** SCS = Con A-stimulated splenic lymphocyte culture supernatant was added to a final concentration of 40%.

TABLE 7

MIXED LYMPHOCYTE REACTION OF IEL

Responder	Stimulator	H-thymidine incorporation into DNA		
		Expt 1	Expt 2	Expt 3
		cpm/10 cells		
—	Sprague-Dawley splenocytes	540 + 10	525 + 65	825 + 12
Wistar IEL	—	505 + 35	530 + 40	820 + 10
Wistar IEL	Sprague-Dawley splenocytes	480 + 20	620 + 90	750 + 15
Wistar splenocytes	—	8175 + 160	28335 + 360	7340 + 10
Wistar splenocytes	Sprague-Dawley splenocytes	179475 + 1290	112745 + 2280	11740 + 245

IEL and spleen cells were cultured with mitomycin C treated stimulator cells for 120h. The results are expressed as mean + SEM of triplicate cultures from representative experiments.

the IEL population. About 90% of IEL were positive for pan T cell marker, of which 70% were cytotoxic/suppressor type and 30% were of helper/inducer subset (Table 8).

Using a monoclonal antibody, RGL-2, which has been shown to be specific for rat intestinal T cells, it was found that almost 90% of IEL were positive to RGL-2. Under the same conditions only 4% of syngenic splenic lymphocytes labeled positive with RGL-2 (Table 8).

Natural killer cell activity :

Rat intestinal lymphocytes showed strong cytotoxic activity against the NK-sensitive targets 'YAC-1' lymphoma cells (Figure 4). At 25:1, 50:1 and 100:1 effector:target (E/T) ratios significant NK activity was observed with no detectable lysis at 12.5:1 E/T ratio.

Humoral and cell mediated immune response **towards** S. *typhimurium* :

Antibodies against S. *typhimurium* membrane proteins in serum and intestinal secretions :

SDS-PAGE analysis of S. *typhimurium* membrane proteins showed that more than 20 polypeptides were present in the extract (Figure 5A). Western blot analysis using rabbit antiserum and rat antiserum raised against S. *typhimurium* protein antigens showed antibodies specific for some of the major proteins (Figure 5B).

Rat antiserum was used as a source of antigen specific IgG and the intestinal fluid as a source of IgA in the antibody-dependent cell-mediated antibacterial activity assay of IEL. The presence of S. *typhimurium* specific IgA in the intestinal fluid was confirmed by ELISA.

TABLE 8

T CELL SUBSETS IN IEL BY INDIRECT IMMUNOFLUORESCENCE ANALYSIS

Monoclonal Antibody	% positive
1. W3/13 (Pan T cell marker)	89.0 +5.0
2. OX 8 (cytotoxic/ supressor)	70.0 +6.0
3. W3/25 (helper/ inducer)	32.0 +4.0
4. RGL-2	88.0
5. RGL-2	4.0

Three hundred cells were counted per sample and results were given as mean + SEM of five experiments.

• Spleen cells were used instead of IEL. In case of RGL-2 mean of two experiments is presented.

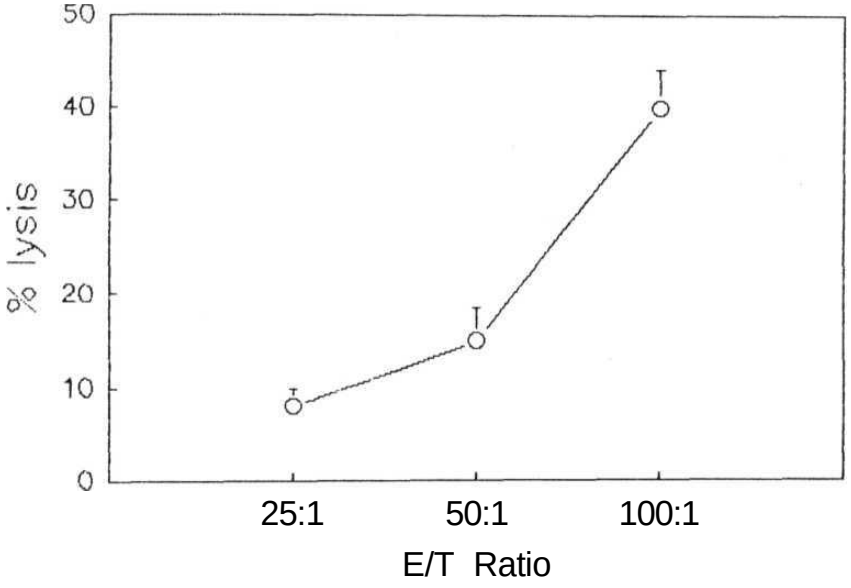


FIGURE 4: Natural killer cytotoxic activity of IEL. Values are expressed as mean + SEM of six experiments.

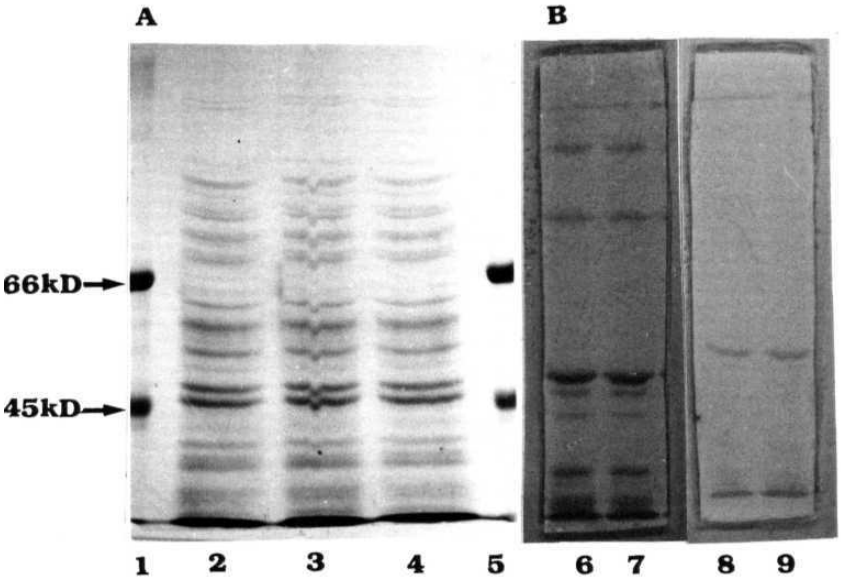


FIGURE 5:

A, SDS-polyacrylamide gel (10%) electrophoresis of S. *typhimurium* membrane antigen extract.

B, Western blot analysis for the detection of S. *typhimurium* specific IgG.

Lanes 1,5 : Molecular weight markers
Lanes 2-4 : S. *typhimurium* membrane antigen extract, stained with commassie blue.
Lanes 6,7 : Blotted with rabbit antiserum to *S. typhimurium* followed by peroxidase staining.
Lanes 8,9 : Blotted with rat antiserum to S. *typhimurium* followed by peroxidase staining.

Antibody-dependent cell-mediated antibacterial activity :

IEL were isolated from the rats sensitized *in vivo* with S. *typhimurium* and the antibacterial activity was assessed in presence of rat antiserum containing IgG and rat intestinal fluid containing sIgA against S. *typhimurium* membrane antigens.

Heat inactivated serum was used at a dilution of 1/100 whereas the intestinal fluid was used at a dilution of 1/3. Table 9 shows the antibacterial activity of IEL against *S. typhimirium.* Around 50% lysis was observed in presence of rat antiserum and intestinal fluid. Higher activity was observed at 100:1 effector:target (E/T) ratio than at 50:1. No lysis was observed when bacteria were incubated with antiserum or intestinal fluid alone.

ISOLATION AND CHARACTERIZATION or LPL :

Yeild and viability of LPL :

The intestinal pieces used for the isolation of IEL in presence of EDTA and PMSF were subsequently used for the isolation of lamina proprial lymphocytes according to the method of Van der Heijden *et ai* (1989). The procedure involves lesser incubation time (90min) as compared to other procedures (Davies et *al*, 1981; Lyscom *et ai,* 1982; Tagliabue et *al*, 1982; Nauss et *al*, 1984), which enables the recovery of a good number of viable lymphocytes. After gradient purification, an average of 10^7 lymphocytes per rat were obtained and the viability was always around 98%.

TABLE 9

ANTIBODY-DEPENDENT CELL-MEDIATED ANTIBACTERIAL ACTIVITY OF IEL

E : T ratio	* lysis	
	IgG	sIgA
50: 1	40 + 6	31 + 4
100: 1	52 + 3	50 + 3

Rat antiserum containing IgG was used at 1/100 dilution and the intestinal fluid (collected by washing the intestine in 10ml of PBS) containing sIgA was used at 1/3 dilution. Values are exressed mean + SEM of four experiments.

Lymphocyte transformation response :

Rat LPL showed good proliferative response against the T cell mitogen, Con A (Table 10). The addition of Con A-stimulated splenic lymphocyte culture supernatant was not necessary as in the case of IEL. Con A concentration of 2μg/ml was found to be optimal. LPL were also responsive to the B cell mitogen, Sepharose coupled anti-rat IgG (heavy & light chain specific). However, the proliferative response of LPL to these mitogens was lesser than that of splenocytes. Unlike splenocytes, LPL showed optimal stimulation on day 4 (96h).

Enumeration of T cell subsets and IgA B cells in LPL :

Unlike IEL, only 45% of LPL were found to be positive for pan T cell marker (W3/13) (Table 11). The cytotoxic/ suppressor (OX 8) subtype constituted about 37% of LPL and the helper/inducer subset were about 16%. The number of IgA B cells in LPL was around 17%.

Antigen specific proliferative response of LPL :

Proliferative response of LPL isolated from *in vivo* sensitized rat was tested *in vitro* against the same antigen (S. *typhimurium* membrane proteins). A significant response was observed towards the antigen as detected by H-thymidine incorporation (Figure 6).

DISCUSSION

In the first part of the present investigation the functional characteristics of IEL and LPL isolated from small intestine of Wistar rats were studied. This was necessitated due to the paucity of information about the function of rat small intestinal IEL and LPL.

TABLE 10

PROLIFERATIVE RESPONSE OF LPL TO T AND B CELL MITOGENS

Cell population	^{3}H-thymidine incorporation into DNA		
	None	Con A (2μg/ml)	Seph. anti-IgG (10μg/ml)
		cpm/ 10 cells	
LPL	1238 + 226	8889 + 364	2969 + 141
Splenocytes	9071 + 88	405915 + 2804	28465 + 504

Values are expressed as mean + SEM of three experiments.

TABLE 11

Enumeration of LPL for T cell subsets and B cells

Antibody	% positive cells
1. W3/13 (Pan T cell marker)	45 + 3
2. OX 8 (cytotoxic/ suppressor)	37 + 2
3. W3/25 (helper/ inducer)	16 + 2
4. Anti-IgA	17 + 2

A minimum of three hundred cells were counted per sample.

Values expressed are mean + SEM of four observations.

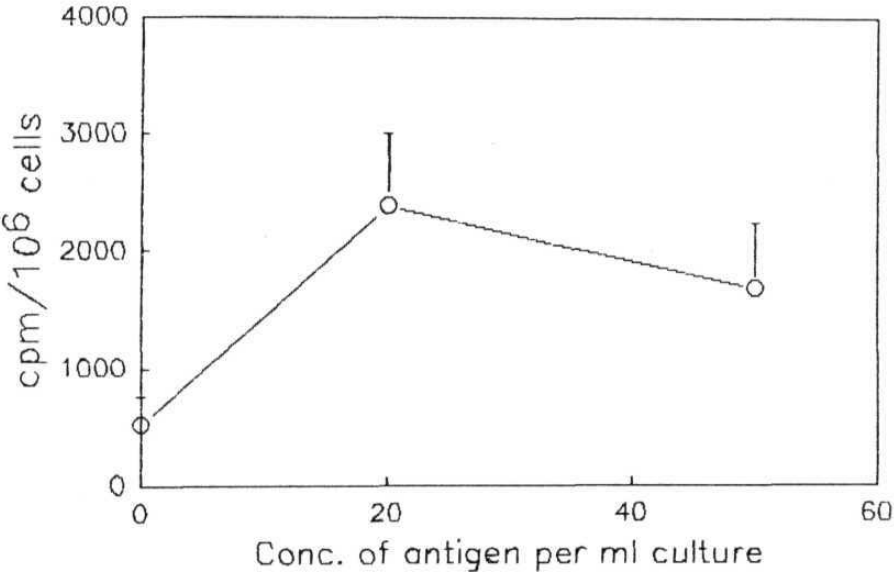


FIGURE 6: Antigen specific proliferative response of LPL. LPL were isolated from the rats immunized with S. *typhimurium* as described in methods. *In vitro* proliferative response of these cells to S. *typhimurium* membrane antigens were tested at above mentioned concentrations and the values are expressed as mean + SEM of three experiments.

Following the methods published earlier (Davies *et al,* 1981; Lyscom *et al,* 1982; Tagliabue *et al,* 1982; Mayrhofer and Whately, 1983) it was not possible to obtain a good yield of viable lymphocytes from rat small intestine. Hence, a modified method was developed for the isolation of IEL from rat small intestine, which makes use of a protease inhibitor, PMSF, in the isolation medium along with EDTA during incubation for the release of IEL. IEL population isolated in presence of PMSF showed 99% viability and the yield was also higher when compared to that obtained in the absence of PMSF. Addition of PMSF during EDTA treatment to release IEL probably protects the cells from being acted upon by putative 'protease' activity associated with the small intestine. The effective inhibition of proteases by PMSF is revealed by the fact that the release of amino acids is significantly lower in presence of PMSF at different steps of incubation. Hence, inhibition of the 'protease' activity associated with small intestine might have resulted in good yield and high viability of IEL.

Histological examination of intestinal tissue after EDTA treatment showed that during EDTA-PMSF incubations, only the epithelial layer was detached from the intestinal wall leaving lamina propria intact. Using RGL-2, a monoclonal antibody specific for rat intestinal T cells, almost 90% of IEL were positive to RGL-2 by immunofluorescence staining, as reported earlier (Cerf-Bensussan et *al,* 1986). It was also shown that only 50% of LPL and 2% of PP lymphocytes stain positive to RGL-2. Hence, this data along with histological analysis shows that the IEL populations were predominantly derived from epithelium.

IEL were analyzed for total T cells and its subsets by indirect

immunofluorescence method using monoclonal antibodies. The percentage of cells positive to W3/13, a pan T cell marker, was 90V.. Van der Heijden (1986) reported that 83% of rat IEL were W3/13 positive by immunocytochemical analysis in tissue sections. Vaage *et al* (1990) reported that 84% of isolated IEL were $CD3^+$. However, Lyscom et ai (1982) showed that only 30% of rat IEL were W3/13 positive. The percentages of helper/inducer and cytotoxic/suppressor cells of IEL were similar to those reported earlier (Lyscom *et al,* 1982; Van der Heijden, 1986; Vaage, *et al,* 1990).

IEL failed to show any proliferation on stimulation with Con A. Addition of autologous splenic adherent cells also did not induce any proliferative response in Con A-stimulated IEL. Reports on the proliferative response of IEL from different species to polyclonal mitogens have been highly contradictory (Greenwood et *al,* 1983; Nauss et ai, 1984; Dillon and MacDonald, 1984; Mowat et *al,* 1986; Wilson et *al,* 1986; Ramsay and Holmes, 1990). Some have reported that IEL respond to mitogens (Dillon and MacDonald, 1984) whereas others showed that IEL were unresponsive (Greenwood et ai, 1983; Nauss et ai, 1984; Mowat et *al,* 1986; Ramsay and Holmes, 1990). Wilson et *al* (1986) reported that mouse IEL were unresponsive to T cell mitogens, whereas IEL Isolated from pig showed strong blastogenic response.

In the present study, the failure of IEL populations to respond to Con A as a result of a). the isolation procedure employed, or b) due to poor viability in culture has been ruled out based on the following observations. Firstly, splenocytes isolated in presence of PMSF showed identical proliferative response to those isolated in the absence of PMSF. Hence, use of PMSF during IEL isolation probably does

not effect their proliferative capacity. Secondly, when the viability of IEL was checked in culture from Oh to 48h, it is observed that almost 80% of cells were still viable at the end of 48h. This clearly indicates that under the culture conditions employed in the present study viability of IEL was not a factor responsible for the lack of mitogenic response. Unlike rat IEL, only 50% of mouse IEL were viable after 24h culture period confirming the observations of Ernst *et al* (1985a). It has been reported recently that γ/δ murine IEL rapidly die in tissue culture due to apoptosis (Viney *et al,* 1990). It is interesting to note here that unlike mouse IEL majority of rat and human IEL bear T cell receptor α/β rather than γ/δ (Vaage *et al,* 1990; Brandtzaeg *et al,* 1989) and hence the behavior of these cells in culture is also probably different compared to murine IEL. Further, the gel electrophoretic analysis of DNA extracted from rat and mouse IEL which were cultured at 37 C for 24h shows that mouse IEL undergo apoptosis showing a ladder like pattern indicative of DNA fragmentation. No such programmed cell death was seen in rat IEL when cultured at 37°C.

Addition of Con A-stimulated splenic lymphocyte culture supernatants which were supposed to be rich in lymphokines made IEL responsive to Con A. The results obtained are in accordance with those of Nauss *et al* (1984) and Mowat *et al* (1986). The unresponsiveness of IEL to Con A alone could be due to the lack of growth factors like IL-2, which are required for proliferation. The analysis of IEL for T cell subsets showed that only a small percentage of T cells are of T helper phenotype. Hence, it is probable that the appropriate T helper cell which produces IL-2 needed for proliferation is present at a low frequency. The inability of mouse IEL to produce significant amounts

of IL-2 has been reported (Dillon *et al,* 1986). IL-2 secreted by LPL may be helping IEL proliferation *in vivo.* However, *in vitro,* IEL need the addition of culture supernatants containing IL-2 for proliferation. IEL showed no proliferative response in one way mixed lymphocyte reaction in response to splenic alloantigens. This observation is similar to those with mouse IEL (Mowat *et al,* 1986). The unresponsiveness of IEL to mitogens and alloantigens might be due to the low frequency of mitogen or antigen reactive precursors.

Further, rat IEL showed significant natural killer cell activity against YAC-1 target cells. NK cell activity of intestinal IEL has been studied in a variety of mammalian species. Significant NK cell activity of IEL was observed in humans (Timonen *et al,* 1981), mice (Tagliabue *et al,* 1982), rats (Nauss *et al,* 1984; Flexman *et al,* 1983) and guinea pigs (Arnaud-Battandier *et al,* 1978). Rat IEL isolated in our study in presence of PMSF and EDTA showed significant natural killer activity. The NK cell activity in humans (Timonen *et al,* 1981), as well as in rats has been shown to be mediated by large granular lymphocytes. While, Flexman *et al* (1982) reported that both granular and nongranular IEL exhibit natural killer cell activity towards NK susceptible tumor cells. Tagliabue *et al* (1982) suggested that mouse intestinal IEL effectors of NK activity has a phenotype distinct from that of splenic NK cells.

IEL showed significant antibacterial activity in presence of both IgG and IgA. This shows that IEL has the capacity to mediate IgG -dependent and IgA-dependent bacteriolytic activity (Nencioni *et al,* 1983).

LPL were isolated from rat small intestine by the procedure of Van der Heijden and Stok (1987). This procedure involves shorter incubation time period (90 min) when compared to other procedures (Nauss *et* ai, 1984; Davies *et* ai, 1981; Lyscom et ai, 1982; Tagliabue *et al,* 1982) which enables the recovery of good number of viable lymphocytes. Phenotypic analysis of LPL populations showed that only 45% of LPL were T cells and almost 20% of LPL are IgA^+ B cells. The results obtained are in accordance with previous reports (Lyscom et ai, 1982; Woolverton *et al,* 1992; Bartnik et ai, 1980). In contrast to IEL, LPL showed good proliferative response to both T and B cell mitogens. No addition of Con A-stimulated splenic lymphocyte supernatants was needed for LPL proliferation. The proliferative capacity of LPL to mitogens was not as good as that of splenic lymphocytes (Woolverton et *al,* 1992). Antigen specific proliferative response was also observed with LPL.

These results show that IEL and LPL differ considerably from each other with different subset compositions and varied responses towards mitogens. The IEL and LPL populations obtained are functionally active and are amenable for further experimentation *in vitro.*

CHAPTER III

INTESTINAL IMMUNE RESPONSE IN VITAMIN A-DEFICIENT RATS

INTRODUCTION

Studies carried out on Vitamin A-deficient children and experimental animals have established that Vitamin A is essential for the maintenance of optimal immune response.

Epidemeological studies

Children with low levels of serum Vit A were found to be susceptible to respiratory tract infections and diarrhea (Arroyave and Calcano, 1979). Incidence of chronic diarrhea, parasitic infections and measles virus infection was shown to be higher in Vit A -deficient children (Patwardhan, 1969; Sommer *et al,* 1984; Bhaskaram *et al,* 1984). Increased mortality in children with mild xerophthalmia has been reported (Sommer *et al,* 1983). Supplementation with retinol significantly decreased childhood morbidity and mortality rates (Sommer *et al,* 1986). Low levels of serum Vit A has been reported to be associated with numerous infectious diseases like pneumonia, rheumatic fever (Jacobs *et al,* 1954) and urinary tract infections (Brown *et al,* 1979).

Changes in **lymphoid** tissue

Wolbach and Howe (1925) characterized the histopathological changes in lymphoid organs of Vit A-depleted rats, and reported thymic atrophy and decreased lymphoid cells in spleen. Krishnan *et al* (1974) described thymic and splenic atrophy in Vit A-deficient rats with markedly depleted T cell areas. However, no changes in lymphoid tissues were observed in mild Vit A deficiency (Nauss *et al,* 1979). Chicken fed Vlt A-deficient diet had reduced number of lymphocytes in

bursal and nasal lymphoepithelial tissues with a moderate cellular depletion in thymus (Bang *et al,* 1973). Vit A deficiency associated with infection of New castle disease virus led to a rapid and complete loss of lymphocytes from respiratory lymphoid tissues, thymus and bursa in chicken (Bang *et al,* 1975). Decreased number of circulating lymphocytes was observed in Vit A-deficient rats (Nauss *et al,* 1979).

Cell-mediated immunity

Cell-mediated immunity has been shown to be impaired in Vit A-deficient children as well as in experimental animals. Bhaskaram *et al* (1975) showed depressed cell-mediated immune response of peripheral blood lymphocytes in children with low serum Vit A levels. Splenic lymphocytes from Vit A-deficient rats showed one-third of the transformation response to mitogens, Con A, PHA and LPS, as compared to the lymphocytes from pair-fed control rats (Nauss *et al,* 1979; Friedman and Sklan, 1989). Repletion of deficient animals with retinyl acetate for three days restored the transformation responses to normal (Nauss *et al,* 1979). Chandra and Au (1981) reported depressed mitogenic transformation response even in mild Vit A deficiency. Decreased natural killer cell activity and interferon production of splenocytes in Vit A-deficient rats has been reported (Nauss *et al* 1985a; Bowman *et al,* 1990).

Humoral immunity

Humoral immune response in Vit A-deficient mice has been shown to be impaired (Smith *et al,* 1987; Carman *et al,* 1992) with decreased antibody responses to protein antigens. IgG1 antibody response is diminished in Vit A-deficient mice owing to decreased frequency of

IgG1 secreting B lymphocytes (Smith *et al,* 1987). Helper T lymphocytes from Vit A-deficient mice failed to provide B cell stimulus for antigen specific IgGl responses (Carman *et al,* 1989). Vit A-deficient rats produced very low concentrations of tetanus toxoid specific IgM and IgG antibodies in both primary and secondary antibody responses (Kinoshita *et al,* 1991). Repletion with retinol two days before booster immunization resulted in normal IgM and IgG responses towards tetanus toxoid.

Local immune response

The functional integrity of local immune system is compromised at number of levels in Vit A-deficient animals. Mc Dermott et *al* (1982) reported that the localization of mesenteric lymph node lymphocytes into the intestine was altered in Vit A deficiency. Takagi and Nakano (1983) reported defective trapping of lymphocytes by the draining lymph nodes in mild Vit A deficiency. Regarding the status of mucosal surfaces in Vit A deficiency, Zile *et al* (1977) have reported decreased turnover of epithelial cells due to impaired DNA synthesis in the small intestine of Vit A-deficient rats. Number of Peyer's patches in ileum of Vit A-deficient rat were significantly reduced (Majumder *et al,* 1987). No changes were observed in the histology of intestinal Peyer's patches of Vit A-deficient animals (Van Bennekum *et al,* 1991). Srisinha et *al* (1980) showed that Vit A -deficient rats had low levels of secretory component on immunofluorescence staining. Lowered levels of secretory IgA in nasal washings of Vit A-deficient children has been reported (Srisinha et *al,* 1975). Chandra (1988) reported increased bacterial adhesion to respiratory epithelium, in children with Vit A deficiency.

There are no detailed studies on the functional status of mucosal lymphocytes in Vit A deficiency. Hence, in the present study the status of intestinal mucosal immune system with respect to intestinal lymphocyte function has been studied in Vit A-deficient rats.

MATERIALS AND METHODS

1. ANIMALS AND EXPERIMENTAL DESIGN :

The experimental design followed was according to Nauss *et al* (1985). Female Wistar rats of 21 days age were purchased from National Institute of Nutrition, Hyderabad, India. Animals were housed individually in wire mesh bottomed cages. They were divided into two groups, designated as Vitamin A-deficient (Vit A) and pair-fed controls (Vit A). Both the groups were weaned onto a Vit A-free diet when they were 25 days old. The composition of the Vit A-free diet is given in Table 12. Vit A rats were supplemented orally with 300IU of retinyl palmitate (Vital Pharmacal Pvt. Ltd., India) once for every three days in 0.1 ml of refined groundnut oil. Each Vit A rat was initially weight matched to a rat in Vit A group. Thereafter, each Vit A rat received the same amount of food that its Vit A pair-mate had eaten the previous day. Food consumption was measured every day and the weight gain was recorded twice weekly. As each deficient rat ceased to gain weight it was killed along with its pair-fed control and tissues were collected for further experiments.

2. SERUM AND LIVER RETINOL CONCENTRATIONS :

Serum and liver retinol concentrations were determined micro-fluoremetrically by the method of Selvaraj *et al* (1970).

TABLE 12

COMPOSITION OF THE DIET

Ingredient	**g/kg diet**
Casein (Vitamin-free)	200
Sucrose	100
Refined groundnut oil	50
Maize starch	600
Vitamin mix (-A)	10
Mineral mix	40

• Vitamin mix constitutes (mg/g Vit mix prepared in starch, unless otherwise stated): menadione, 0.5; tocopherol acetate, 10 IU; thiamine, 0.5; riboflavin, 1; pyridoxine, 0.5; pantothenic acid, 4; niacin, 4; inositol, 10; para amino benzoic acid, 10; cyanocobalamine, 0.003; biotin, 0.02; folic acid, 0.2; chole-calciferol, 200 IU; choline chloride, 200.

•• Mineral mix constitutes the following (g/100g mineral mix): KH_2PO_4, 38.9; $CaCO_3$, 38.1; NaCl, 14; $MgSO_4$, 5.73; $FeSO_4 \cdot 7H_2O$, 2.7; $MnSO_4 \cdot H_2O$, 0.401; KI, 0.078; $ZnSO_4 \cdot 7H_2O$, 0.0548; $CuSO_4 \cdot 5H_2O$, 0.0477; $CoCl_2 \cdot 6H_2O$, 0.0023.

Briefly, 50µl of the sample (serum or 10% liver homogenate) was taken into a glass micro-centrifuge tube and it was saponified with 50µl of 1N alcoholic potassium hydroxide. Tubes were kept in a hot water bath maintained at 60 C for 20 min, to extract and hydrolyze Vit A esters to retinol. Tubes were cooled and retlnol was extracted with 0.3ml of xylene by vigorously mixing the two phases on a vortex mixer for 20 sec. The upper xylene layer was separated by centrifugation and transfered to microcuvettes. Fluorescence was measured in Hitachi spectrofluoremeter at 490nm with excitation at 350nm. Samples were then irradiated for 10min under UV light and fluorescence was measured again. The fluorescence destroyed by UV irradiation was calculated.

A series of standards of retinyl acetate (Sigma Chem. Co., USA), containing 0.01µg-0.05µg, were treated in a similar way as the unknown sample. A standard curve was prepared from which the Vit A concentration in the unknown samples was calculated.

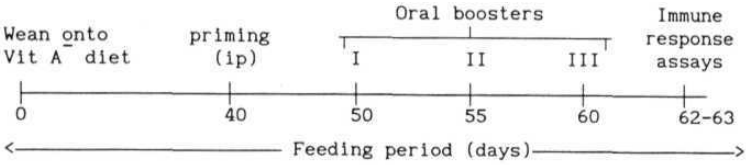
3. IMMUNE RESPONSE ASSAYS :

Lymphocyte transformation assays of IEL, LPL and splenocytes isolated from Vit A^{-} rats and Vit A rats were carried out as described in Chapter II. Proliferative response of IEL to Con A was studied in presence of Con A-stimulated splenic lymphocyte culture supernatant.

Natural killer cell assay with IEL and splenocytes, enumeration of total T and B cells and T cell subsets from IEL, LPL and splenocytes by immunofluorescence technique were performed as described in Chapter II.

4. ANTIGEN SPECIFIC IMMUNE RESPONSES :

To study antigen specific immune responses in Vit A deficiency, Vit A and Vit A rats were sensitized *in vivo* as described in the methods section of Chapter II (protocol 13). Immunization schedule was as follows :



IEL, LPL and splenocytes were isolated from *in vivo* sensitized Vit A and Vit A rats and the *iv vitro* proliferative response of LPL to the *S. typhimurium* antigen, and the antibody-dependent anti-bacterial activity of IEL and splenocytes were studied as described in Chapter II.

Antigen specific antibody response was studied by measuring antibody secreting cells in the lamina propria using immunocyto-chemical technique.

Immunocytochemistry :

Chemicals and reagents :

Poly-L-lysine, goat anti-rat IgA and diaminobenzidine were supplied by Sigma Chem. Co., USA. HRPO conjugated donkey anti-goat IgG and goat anti-rabbit IgG were purchased from Lupin laboratories, India. Antiserum to *S. typhimurium* antigenic extract was raised in rabbits as described in the methods section of chapter II.

i. Phosphate buffered saline (PBS), pH 7.4.

ii. 0.05M Tris buffer, pH 7.6 containing 0.15M NaCl (TBS).

iii. Developing solution : 0.5µg Diaminobenzidine /ml of TBS containing 0.01% H_2O_2.

Procedure :

Small intestinal pieces from Vit A and Vit A~ rats were washed with cold PBS and snap frozen in liquid nitrogen. The pieces were stored at -80 C until use. Cryosections of thickness 6µ were made using a cryostat maintained at -20°C. Sections were collected on to poly-L-lysine coated glass slides.

The sections were air dried for 3h and stained for IgA and antigen specific antibody secreting B cells in lamina propria. Endogenous peroxidase was inhibited by the method of Steefkerk (1972). For this the sections were fixed with methanol for 10min followed by a treatment with 0.006% H_2O for 20min. They were washed with PBS at least three times (3min each) after every incubation. Non-specific binding was minimized by incubating the sections with 5% milk powder peptide solution in PBS for 30min.

To enumerate total IgA secreting B cells, sections were first incubated with 1:100 diluted goat anti-rat IgA (α-chain specific) for 60min at room temperature. They were washed with PBS and incubated with HRPO conjugated anti-goat IgG (1-100 dil) for 30min at room temperature. After washing with PBS they were incubated with developing solution for 5 minutes.

Antigen specific antibody secreting B cells were stained by

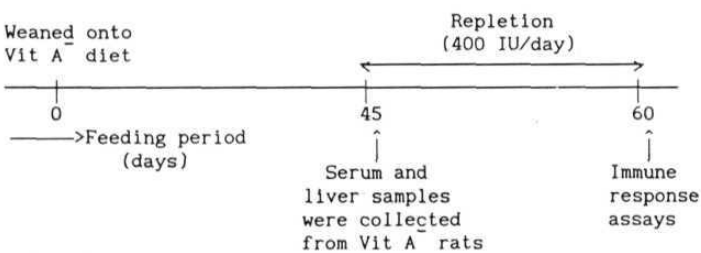
incubating the sections first with 100µg/ml solution of *S. typhimurium* antigenic extract overnight at 4°C. Sections were washed with PBS and incubated with 1:50 diluted rabbit antiserum to S. *typhimurium* antigens for 60min at room temperature. Finally they were incubated with HRPO conjugated goat anti-rabbit IgG (1:100 dil) for 30min at room temperature. Sections were incubated for 5min with developing solution.

All the sections were counter stained with hematoxylin, dehydrated and mounted with DPX.

REPLETION STUDIES :

ANIMALS :

Vitamin A deficiency was induced in Wistar female rats as described above. When the Vit A rats reached the weight plateau, ie., after 6-7 weeks of feeding period, they were repleted with 3mg of retinyl palmitate given orally for 15 days (400IU/day). Repletion schedule is schematically represented below :



After the repletion period, rats were killed and phenotypic analysis and mitogen induced proliferative response of IEL, LPL and splenocytes were studied.

Vit A levels in serum and liver samples collected from Vit A-deficient, Vit A-repleted and pair-fed control rats were estimated microfluoremetrically.

RESULTS

Induction of Vitamin A deficiency :

Rats which were fed Vit A-free diet ceased to gain body weight and entered the weight plateau after 6 to 7 weeks of feeding period. Figure 7 shows the growth curve of Vit A and Vit A rats. Two stages of Vit A deficiency were observed. Early Vit A-deficient state (Stage 1), when the Vit A rats reached weight plateau, and late Vit A deficiency (Stage 2), when they showed a loss of body weight. Experiments to study the immune status were carried out using the rats which were beyond Stage 1 of deficiency to stage 2. The deficient state was confirmed by low serum and hepatic concentrations of Vit A.

Serum and Liver Vit A concentrations :

Serum Vit A concentration of Vit A~ rats was 1.3μg/dl, while that of Vit A rats was around 40μg/dl. Hepatic Vlt A values were less than 3.6μg/g wet tissue weight in Vit A~ rats in comparison to 20μg/g wet tissue wt. in Vit A^+ rats ($p < 0.01$, Vit A~ vs Vit A^+, Table 13).

Serum Proteins :

Serum total protein concentration in Vit A rats was not significantly different from that of Vit A^+ rats (Table 13). Also, there was no change in the serum albumin concentration in Vit A rats

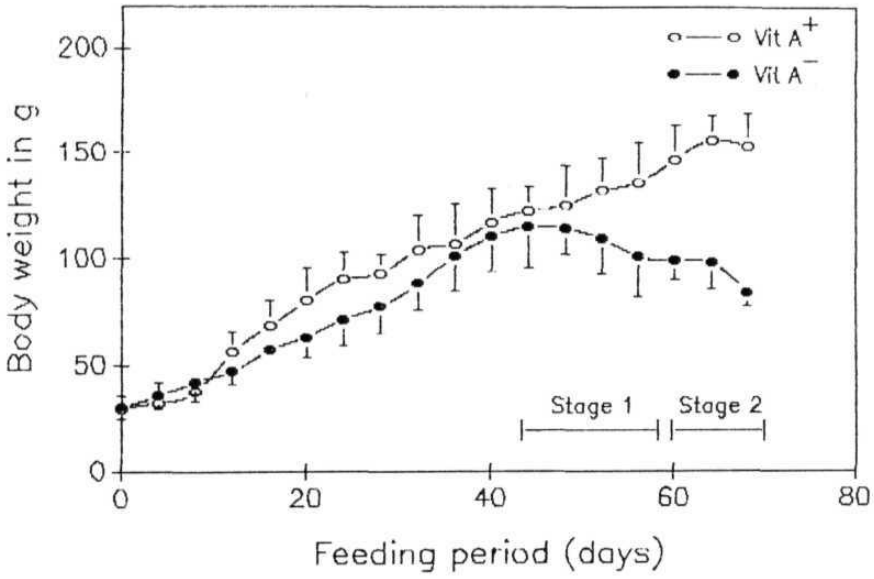


FIGURE 7: Growth curve of Vit A and Vit A rats. Body weights were recorded twice weekly. Each point is a mean + SD of 10 animals.

TABLE 13

CONCENTRATIONS OF SERUM AND LIVER VIT A AND SERUM PROTEINS IN VIT A^+ AND VIT A~ RATS

Parameter	Vit A^+	Vit A^-
Serum Vit A (μg/dl)	42 + 4	1.3+0.3
Liver Vit A (μg/g wet tissue)	20 + 1	3.6 + 0.6»
Serum protein (g/dl)	5.5+0.2	5.3+0.2

Values presented are mean + SEM of ten rats per group.

• p< 0.01, Vit A^+ vs Vit A".

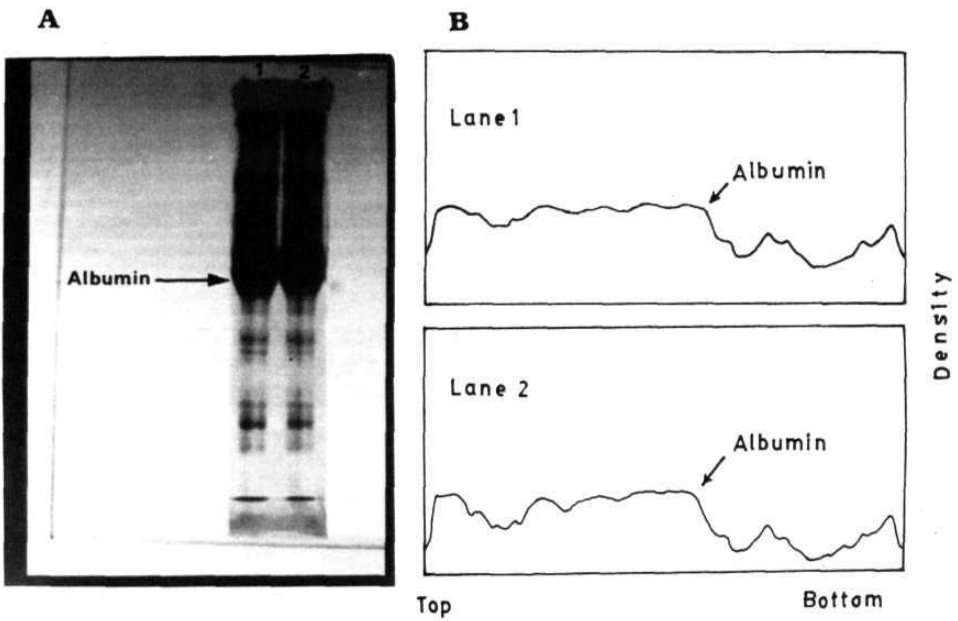


FIGURE 8:

A, SDS-polyacrylamide gel (10%) electrophoresis of serum samples obtained from Vit A (lane 1) and Vit A (lane 2) rats. Gel was stained with Commassie blue.

B, Densitometric analysis of protein patterns separated on SDS gels as in Figure 2A.

compared to Vit A rats as determined by electrophoretlc separation followed by densitometric analysis (Figure 8A & B).

Changes in the weights of lymphoid organs :

Weights of spleen and thymus from Vit A rats were significantly lower as compared to Vit A rats ($p < 0.01$, Table 14). However, the decrease in organ weight was not significant when expressed as percent body weight. The yeild of splenic lymphocytes per gram weight of spleen from Vit A rat was not significantly different from Vit A rat. The number of Peyer's patches were significantly reduced in Vit A rats ($p < 0.01$) as compared to Vit A rats. Fluid accumulation was observed in the ileal lumen of Vit A rats.

Morphology of intestine and spleen :

Small intestinal pieces obtained from Vit A and Vit A rats were sectioned and stained with haematoxylin-eosin. Histological observations showed that epithelial integrity was altered in Vit A deficiency (Figure 9). Decreased cellularity of lamina propria was also observed. No gross changes were observed in the histological sections of spleen from Vit A rats (Figure 10). The cellularity in Peyer's patches was less in Vit A rats compared to Vit A rats (Figure 11).

Yield of IEL and LPL :

The yield of IEL from Vit A^- rats was significantly lower than Vit A^+ rats ($p < 0.01$, Table 14). Number of LPL obtained from Vit A rats was also low when compared to Vit A rats ($p < 0.05$).

TABLE 14

SPLEEN, THYMUS WEIGHTS AND YEILD OF IEL, LPL AND SPLENIC LYMPHOCYTES FROM VIT A^+ AND VIT A~ RATS

Parameter	Vit A^+	Vit A~
Wt. of spleen in g	0.46 + 0.02	0.29 + 0.05*
Wt. of spleen as % **body** weight (g)	0.30 + 0.02	0.25 + 0.03
Yield of splenic lymphocytes (10^6 cells/g spleen)	687 + 26	617 + 25
Wt. of thymus in g	0. 14 + 0.02	0.09 + 0.016*
Wt of thymus as % **body** weight (g)	0.097 + 0.01	0.09 + 0.01
Number of Peyer's patches	9 + 0 . 7	2.7+0.5*
Yield of IEL/g wet tissue (x 10^6 cells)	2 + 0.1	0.7 + 0.08
Yield of LPL/g wet **tissue** (x 10^6 cells)	1.5 +0.1	1.1 + 0.1#

Values presented are as mean + SEM of six rats per group.

• $p < 0.01$; t $p < 0.05$, Vit A^+ vs Vit A~.

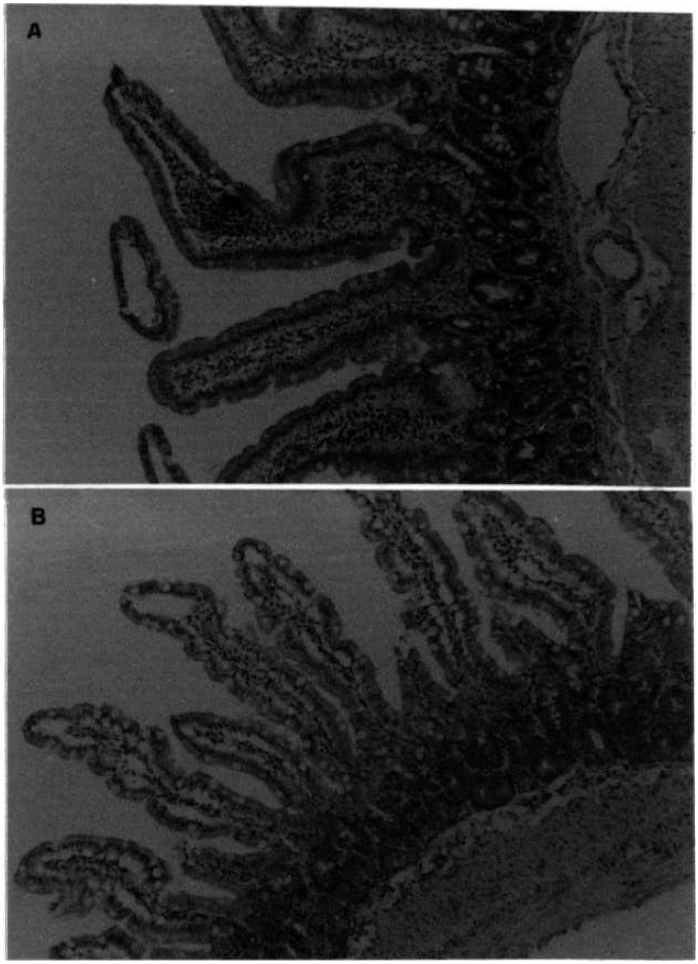


FIGURE 9:

Histology of small intestine in Vit A deficiency.

A, Vit A^{+}; B, Vit A".

Magnification, 120X.

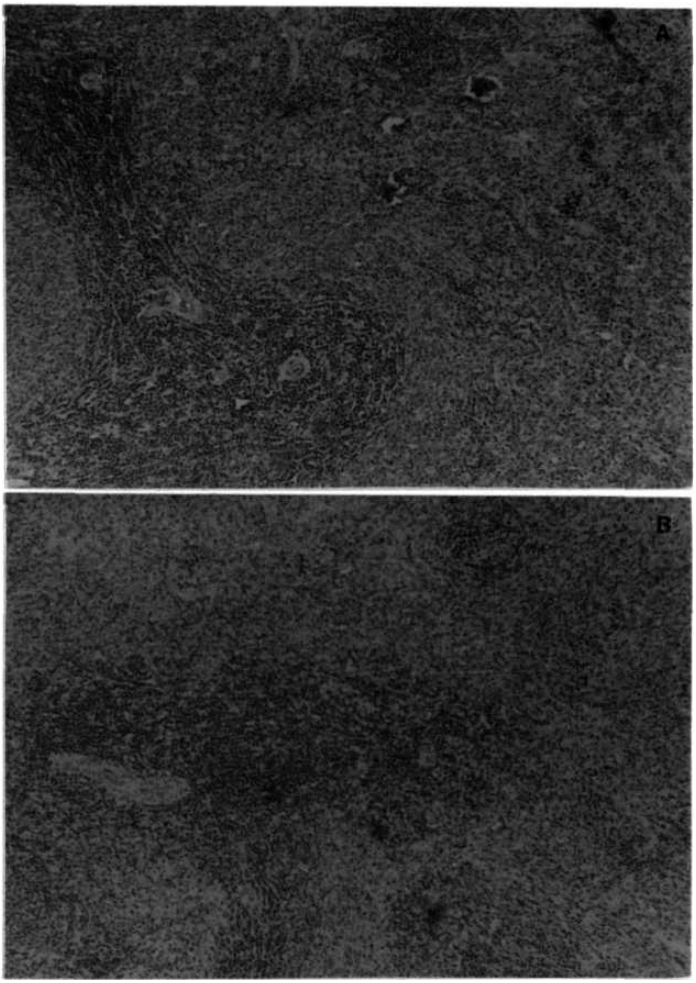


FIGURE 10:

Histology of spleen in Vit A deficiency.

A, Vit A^{+}; B, Vit A".

Magnification, 120X.

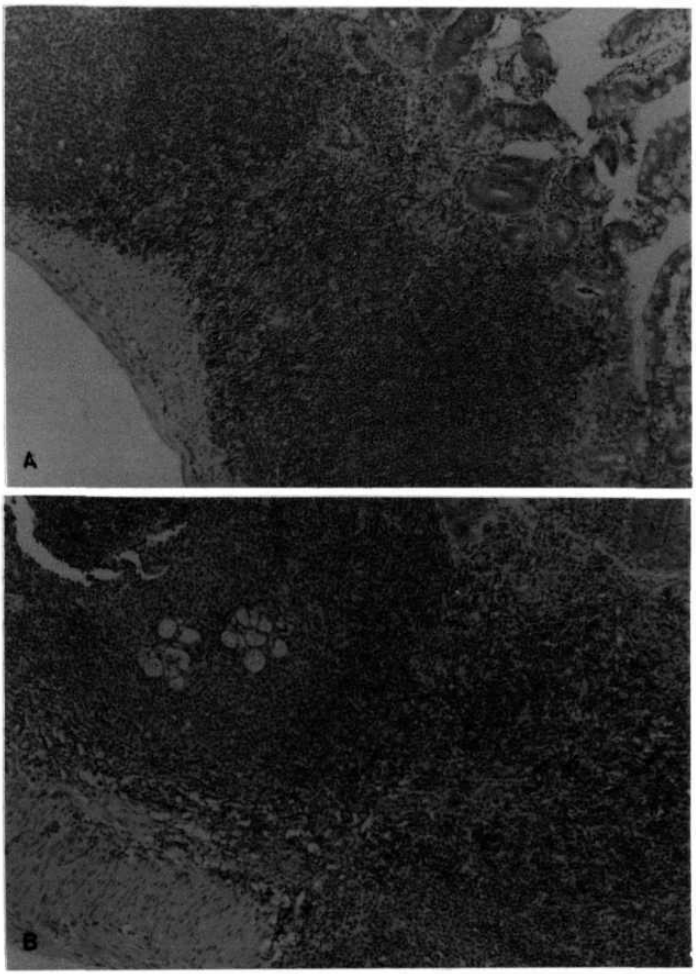


FIGURE 11:

Histology of Peyer's patches in Vit A deficiency.

A, Vit A^{+}; B, Vit A .

Magnification, 120X.

IEL, LPL and Splenic lymphocyte transformation response to mitogens :

Lymphocyte transformation assay of IEL towards Con A was carried out in presence of Con A-stimulated splenic lymphocyte culture supernatants. IEL from Vit A and Vit A rats showed optimal response at a Con A concentration of 2μg/ml and the response of IEL from Vit A~ rats was significantly reduced as compared to that of Vit A^+ rats (Table 15).

The proliferative response of LPL isolated from Vit A rats to T cell mitogens, Con A and B cell mitogen, Seph. anti-IgG was significantly reduced as compared to pair-fed controls (Table 16).

Studies were also carried out with splenic lymphocytes obtained from Vit A^+ and Vit A^- rats. Table 17 & 18 show the proliferative response of splenic lymphocytes obtained from Vit A and Vit A rats towards the T cell and B cell mitogens. The proliferative response of splenic lymphocytes from Vit A rats was significantly lower towards all the mitogens used as compared to Vit A rats.

Enumeration of T lymphocytes and its subsets in IEL, LPL and splenic lymphocytes :

Total number of T cells, as identified by the monoclonal antibody which recognizes a Pan T cell marker, were found to be significantly decreased in the IEL population of Vit A rats as compared to Vit A rats ($p< 0.01$, Figure 12). The percentage of helper/inducer subset of IEL was also found to be decreased significantly in Vit A rats. However, suppressor/cytotoxic subset number of IEL was not significantly altered.

TABLE 15

MITOGENIC RESPONSE OF IEL FROM VIT A^+ AND VIT A~ RATS

Expt No.	Mitogen	H-thymidine incorporation into DNA			
		Vit A^+		Vit A"	
		cpm/ 10 cells			
			Δcpm		Δcpm
1	-	648 + 35		891 + 52	
	+	6713 + 222	6065	3208 + 200	2317
2	-	5850 + 187		735 + 63	
	+	29991 + 384	24141	935 + 54	200
3	-	7845 + 209		3528 + 44	
	+	30500 + 1290	22655	12840 + 208	9312
4	-	3463 + 68		1905 + 48	
	+	6665 + 171	3202	3040 + 140	1135

H-thymidine incorporation was studied on day 4 of culture. Con A was used as the mitogen at a concentration of 2μg/ml. Values are expressed as mean + SEM of triplicate cultures. Data shown are from four representative experiments.

TABLE 16

MITOGENIC RESPONSE OF LPL ISOLATED FROM VIT A AND VIT A RATS

Expt. No.	Mitogen	^{3}H-thymidine incorporation into DNA			
		Vit A$^+$		Vit A$^-$	
		cpm/ 10 cells			
			A cpm		A cpm
1.	None	1230 + 110		1095 + 113	
	Con A (2μg/ml)	11342 + 594	10112	4278 + 138	3183
	Seph. anti-IgG (10μg/ml)	3025 + 280	1795	736 + 91	
2.	None	1198 + 132		950 + 45	
	Con A (2μg/ml)	7893 + 182	6695	3358 + 176	2408
	Seph. anti-IgG (10μg/ml)	2935 + 93	1737	636 + 48	
3.	None	940 + 83		794 + 42	
	Con A (2μg/ml)	8973 + 251	8033	4911 + 132	4117
	Seph. anti-IgG (10μg/ml)	3126 + 182	2186	933 + 51	139
4.	None	1050 + 242		910 + 102	
	Con A (2μg/ml)	7349 + 210	6299	5112 + 122	4202
	Seph. anti-IgG (10μg/ml)	2793 + 87	1743	1267 + 120	357

Values are expressed as mean + SEM of triplicate cultures obtained from four experiments.

TABLE 17

PROLIFERATIVE RESPONSE OF SPLENIC LYMPHOCYTES FROM VIT A^{+} AND VIT A~ RATS TO T CELL MITOGENS

Mitogen	^{3}H-thymidine incorporation into DNA			
	Vit A^{+}		Vit A	
	cpm/ 10 cells			
EXPT 1		Δcpm		Δ cpm
None	37268 + 586		7368 + 296	
Con A (2 μg/ml)	1266250 + 2934	1228982	152596 + 2807	145288
PHA 1-10 dil	578150 + 7571	540882	98656 + 1243	91288
EXPT 2				
None	10996 + 85		3798 + 11	
Con A (2 fig/ml)	406143 + 2934	395147	65543 + 1084	61745
PHA 1-10 dil	170501 + 4674	159505	72866 + 446	69068
EXPT 3				
None	42141 + 115		2143 + 49	
Con A (2μg/ml)	937316 + 11219	895175	59477 + 2987	57334
PHA 1-10 dil	93793 + 113	51652	3636 + 152	1493
EXPT 4				
None	2683 + 53		**4963** + 74	
Con A (2μg/ml)	293100 + 3086	290417	98428 + 2626	**93465**
PHA 1-10 dil	93452 + 1056	90769	48892 + **45**	43929

Values expressed are mean + SEM of triplicate cultures of four representative experiments.

TABLE 18

PROLIFERATIVE RESPONSE OF SPLENIC LYMPHOCYTES FROM VIT A^{+} and VIT A~ RATS TO B CELL MITOGENS

Mitogen	^{3}H-thymidine incorporation into DNA				
	Vit A^{+}		Vit A"		
	cpm/ 10 cells				
EXPT 1	A cpm				Δ cpm
None	37268 + 586		7368 + 296		
LPS (25μg/ml)	82871 + 1071	45603	10998 + 188		3630
Seph. anti-IgG (10 μg/ml)	660766 + 1171	623498	195885 + 596		188517
EXPT 2					
None	10996 + 85		3798 + 11		
LPS (25μg/ml)	40000 + 730	29004	3521 + 29		-277
Seph. anti-IgG (10μg/ml)	198485 + 1346	187489	42013 + 73		38215
Expt 3					
None	42141 + 115		2143 + 49		
Seph. anti-IgG (10μg/ml)	107315 + 2043	65174	12076 + 291		9933
Expt 4					
None	2683 + 53		4963 + 74		
LPS (25μg/ml)	9598 + 89	6915	5112 + 45		149
Seph. anti-IgG (10μg/ml)	312775 + 3150	310092	153715 + 1043		148752

Values expressed are mean + SEX of triplicate cultures of four representative experiments.

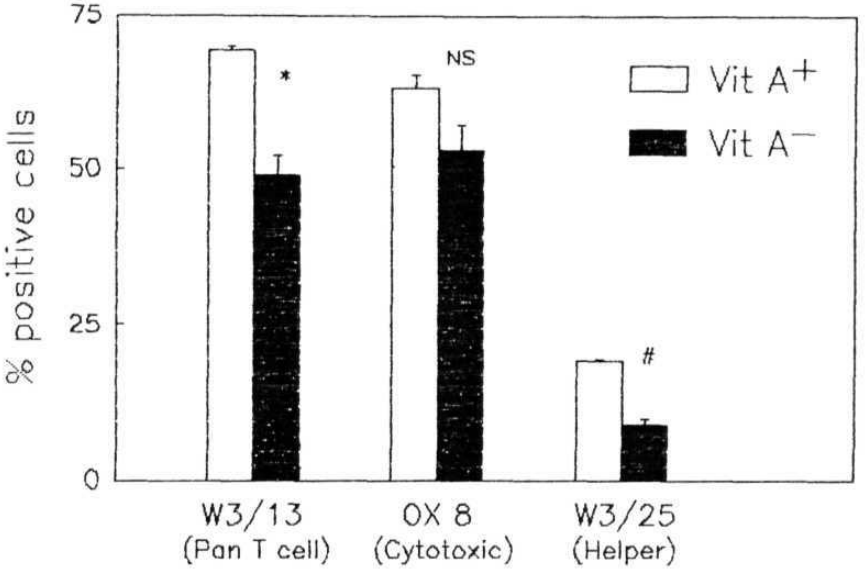


FIGURE 12: Total T cells and its subsets in IEL of Vit A and Vit A rats. Data presented are mean + SEM of six experiments. • p< 0.01; # p< 0.05; NS= not significant, Vit A^+ vs Vit A .

LPL populations obtained from Vit A rats showed a significant decrease in the percentage of total T cells as well as in cytotoxic T cells (Figure 13). Though a decrease in helper/inducer subset was observed in Vit A rats, it was not statistically significant ($p < 0.1$). IgA B cell number of LPL isolated from Vit A~ rats was very low as compared to that of LPL obtained from Vit A^+ rats.

Enumeration splenic lymphocytes of Vit A rats for total T cells, its subsets and total B cells was carried out and were compared with Vit A rats. There was significant decrease in total T cells, its subsets and also in Ig B cells in Vit A rats as compared to that of Vit A^+ rats (Figure 14).

Natural Killer cell activity :

The natural killer cell activity of IEL was tested using Cr-labelled YAC-1 cells as targets, at different effector: target ratios. The NK cell activity of IEL in Vit A~ rats was found to be significantly increased at 50:1 & 100:1 E:T ratios as compared to Vit A rats (Figure 15). The percent specific lysis of IEL from Vit A rats at 100:1 E:T ratio was around 27, while that of Vit A rats was around 40 ($p < 0.05$).

Splenic lymphocytes isolated from the same Vit A rats showed significant decrease in NK cell activity as compared to their pair-fed controls at all E:T ratios tested ($p < 0.01$, Figure 16).

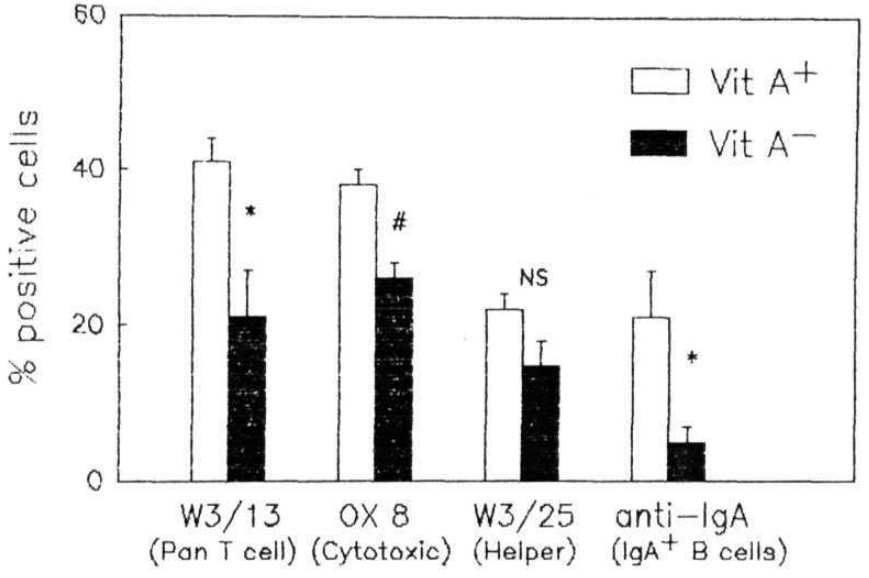


FIGURE 13: Enumeration of LPL from Vit A and Vit A rats, for total T cells and its subsets, and IgA B cells. Data presented are mean + SEM of six observations • p< 0.01; # p< 0.05; NS= not significant.

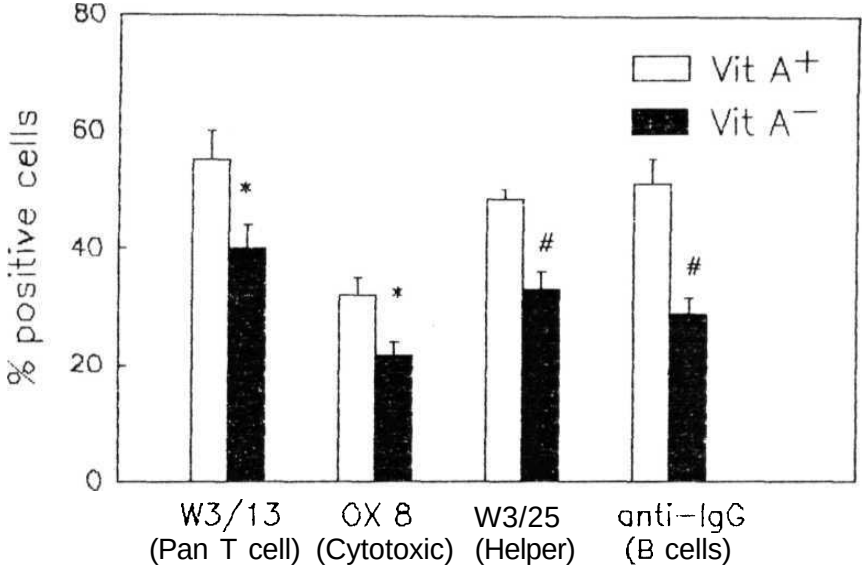


FIGURE 14: Enumeration of splenic lymphocytes from Vit A and Vit A rats for total T cells and its subsets, and Ig B cells. Data presented are mean + SEM of six observations. * $p < 0.01$; # $p < 0.05$.

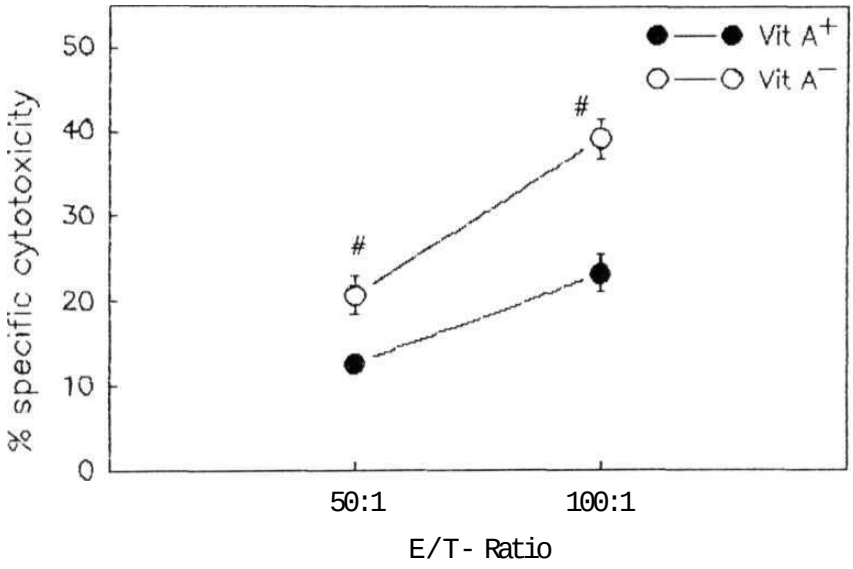


FIGURE 15: NK cell activity of IEL from Vit A^+ and Vit A^- rats. Values presented are mean + SEM of six experiments. # $p < 0.05$, Vit A^+ vs Vit A .

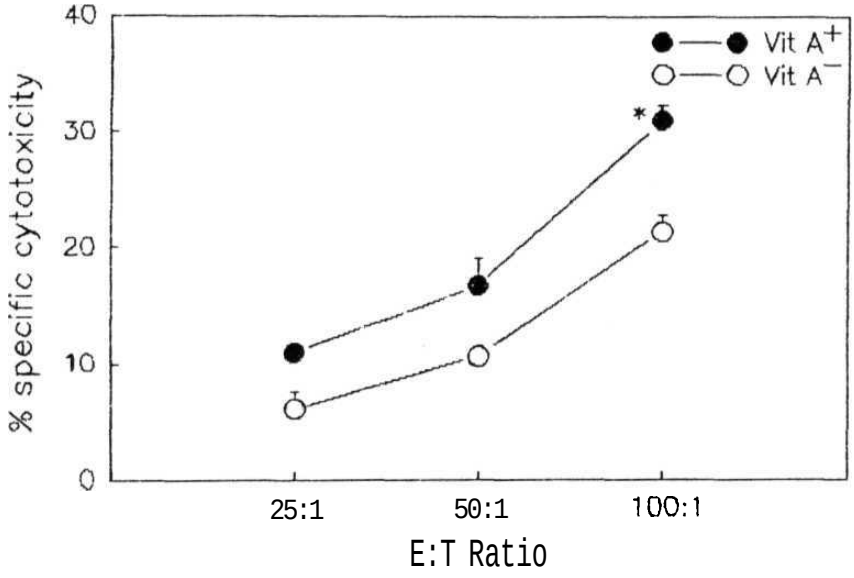


FIGURE 16: NK cell activity of splenic lymphocytes from Vit A and Vit A rats. Values presented are mean + SEM six experiments. • p< 0.01, Vit A^{+} vs Vit A~.

HUMORAL AND CELL-MEDIATED IMMUNE RESPONSE TOWARDS *S. typhimurium* :

Antibody-dependent cell-mediated antibacterial activity :

Antibody-dependent antibacterial activity of IEL was assayed using *S. typhimurium* as test organism. Antiserum raised against S. *typhimurium* antigen extract in rats was used as IgG source and the rat intestinal fluid from S. *typhimurium* immunized rats was used as IgA source. The assay was done in presence of rat antiserum and rat intestinal fluid. Both IgG and IgA-dependent antibacterial activity of IEL were found to be reduced in Vit A rats (Table 19). Rat antiserum was used at a final dilution of 1/300 and the intestinal fluid at a dilution of 1/3.

Antibody-dependent antibacterial assay of splenic lymphocytes was carried out only in presence of rat antiserum. IgG-dependent antibacterial activity of splenic lymphocytes against *S. typhimurium* was also decreased in Vit A^- rats (Table 20).

Antigen specific proliferative response of LPL :

LPL isolated from S. *typhimurium* primed Vit A and Vit A rats were cultured *in vitro* with S. *typhimurium* membrane antigens and the proliferative response was measured by H-thymidine incorporation. THe proliferative response of LPL was found to be decreased in Vit A rats (Figure 17).

Immunohistology :

Cryosections of small intestine obtained from Vit A and Vit A rats were stained for IgA and antigen-specific antibody secreting B

TABLE 19

ANTIBODY-DEPENDENT ANTIBACTERIAL ACTIVITY OF IEL AGAINST S. *typhimurium* IN VIT A$^+$ AND VIT A~ RATS

Antibody	E:T ratio	Vit A$^+$	Vit A~
IgG	100:1	63 + 4	38 + 2 *
	50:1	38 + 2	27 + 2 #
IgA	100:1	57 + 3	41 + 3 #
	50:1	47 + 4	27 + 3 *

Values are expressed as mean + SEM of four experiments.

* p< 0.01; # p< 0.05, Vit A$^+$ vs Vit A~.

TABLE 20

IgG-DEPENDENT ANTIBACTERIAL ACTIVITY OF SPLENIC LYMPHCYTES AGAINST S. typhimurium IN VIT A$^+$ AND VIT A~ RATS

E:T ratio	Vit A$^+$	Vit A
100: 1	68 + 4	37 + 7*
50: 1	47 + 5	34 + 2*

Values are expressed as mean + SEM of four experiments.

* $p < 0.05$, Vit A$^+$ vs Vit A~.

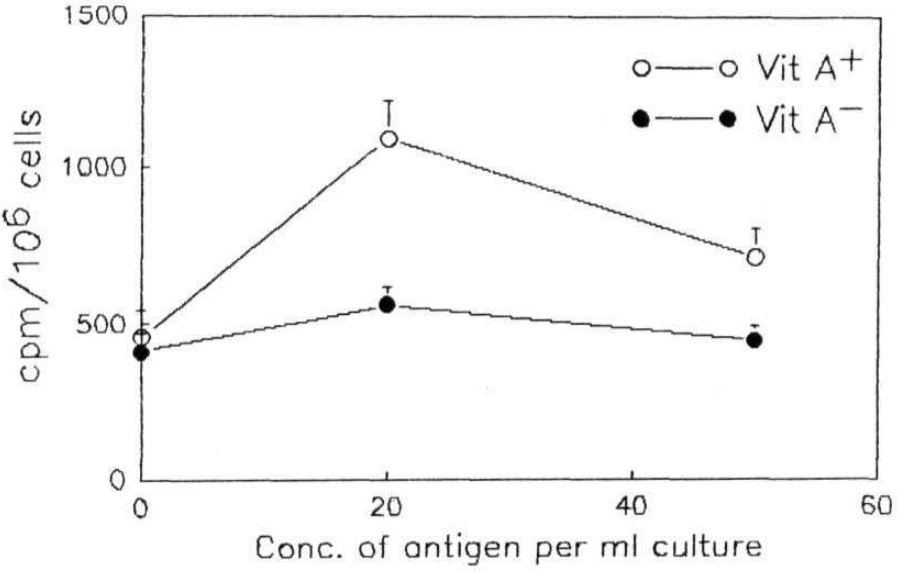


FIGURE 17: Antigen specific proliferative response of LPL from Vit A and Vit A rats. *S. typhimurium* antigenic extract was used as the antigen. Values expressed are mean + SEM of three experiments.

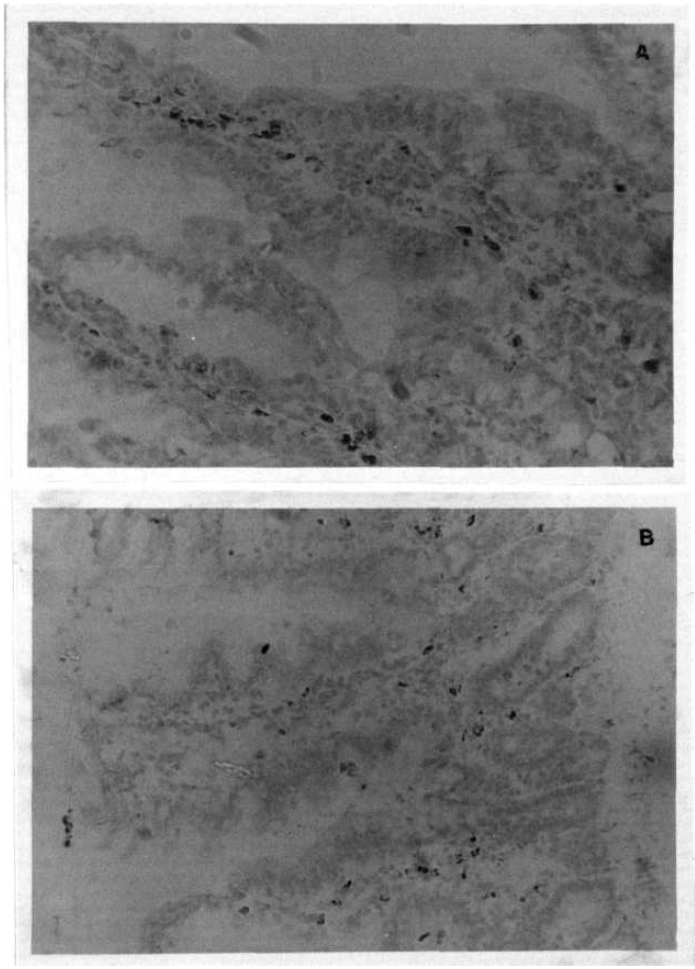


FIGURE 18: Total IgA^+ B cells in the lamina propria of A) Vit A^+ and B) Vit A~ rats.

Magnification, 200X.

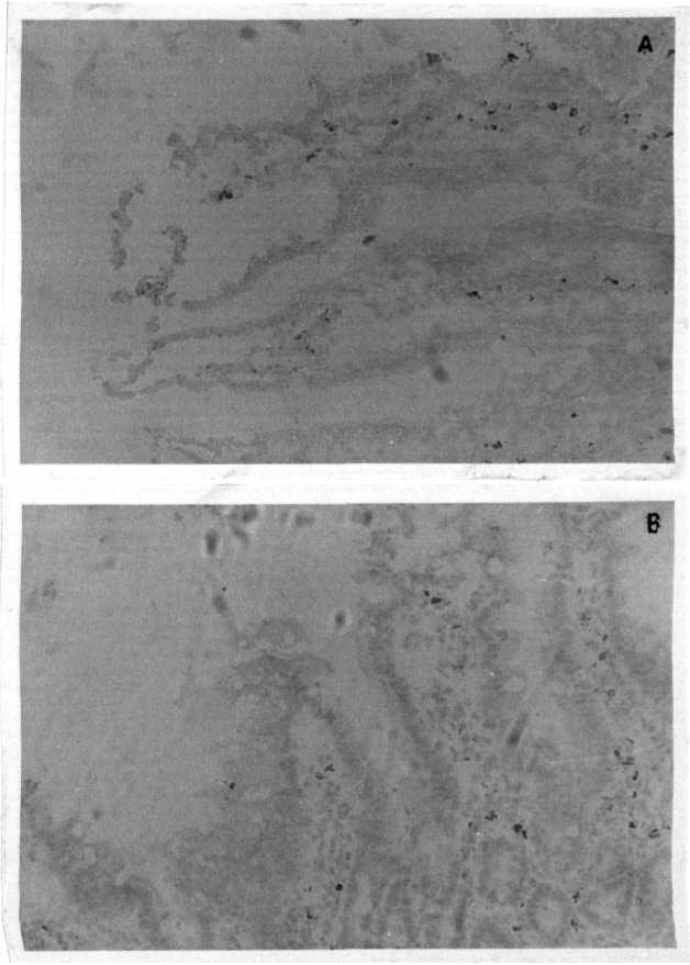


FIGURE 19: Antigen specific antibody secreting B cells in the lamina propria of A) Vit A and B) Vit A rats immunized with S. *typhimurium*. Magnification, 200X.

TABLE 21

IgA$^+$ AND ANTIGEN SPECIFIC ANTIBODY SECRETING B CELLS IN THE LAMINA PROPRIA OF VIT A$^+$ AND VIT A~ RATS

	Positive cells per 1 cm of intestine	
	IgA	anti-S. *typhimurium* antibody secreting B cells
Vit A$^+$	2560 + 190	1580 + 75
Vit A~	995 + 60*	750 + 70*

Values are expressed as mean + SEM of four obsevations.

• p< 0.01, Vit A$^+$ vs Vit A~.

cells. As shown in Figure 18 and Table 21, the number of IgA-containing cells in lamina propria were markedly decreased in Vit A rats. S. *typhimurium* specific antibody secreting cells were also decreased in Vit A deficiency (Figure 19, Table 21).

REPLETION STUDIES :

Animals :

Rats during stage 1 of Vit A deficiency were repleted with 3mg of retinyl palmitate, given orally in a duration of 15 days. Repleted rats showed increase in their body weights which was similar to that of the pair-fed control rats.

Serum and liver Vit A concentrations of Vit A-repleted animals :

Serum Vit A concentration of repleted animals was around 35μg/dl, while that of the control rats was 40μg/dl. Before repletion, the Vit A-deficient rats showed a serum Vit A concentration of 3μg/dl (Table 22).

Liver Vit A levels of repleted rats was around 18μg/g wet wt. and the control rats showed a concentration of 20μg/g wet wt. of the tissue. Liver Vit A levels of Vit A-deficient rats, before repletion, was around 4μg/g wet wt. (Table 22).

Transformation response of IEL, LPL and splenocytes towards mitogens was studied in the repleted animals. IEL, LPL and splenocytes of Vit A-repleted animals were also enumerated for the total T cells and its subset and B cell numbers.

Table 22

SERUM AND LIVER VIT A CONAENTRATIONS OF VIT A-REPLETED ANIMALS AND THEIR PAIR-FED CONTROLS

Animals	Serum Vit A µg/dl	Liver Vit A µg/g wet tissue
Vit A deficient	**2.8+1.7**	**4.5+1**
Pair-fed control	41.2+6	19.8 + 3.2
Vit A-repleted	35.5 + 5[NS]	17.2 + 29[NS]

Values were expressed as mean + SEM of five observations.

• p< 0.01; NS = Not significant, pair-fed controls vs Vit A -depleted or Vit A-repleted rats.

Proliferative response of IEL, LPL and splenocytes :

IEL and LPL isolated from Vit A repleterd rats showed similar proliferative responses towards mitogens as compared to the proliferative responses of lymphocytes obtained from pair-fed control rats (Figure 20 & 21). The proliferative response of splenocytes to T cell and B cell mitogens from repleted rats was also similar to that of controls (Figure 22). Slight decrease in B cell response of splenocytes was still observed even after repletion.

Enumeration of **IEL,** LPL and splenocytes :

Figure 23 shows the percentages of total T cell and its subsets of IEL isolated from Vit A-repleted and pair-fed control rats. Figures 24 and 25 show the T cell subset composition and IgA B cell number of LPL and splenocytes respectively, which were isolated from Vit A-repleted and pair-fed control rats. The percentages of total T cells, cytotoxic/suppressor cells and helper/inducer cells in all the cell populations from Vit A-repleted rats were similar to pair-fed controls.

DISCUSSION

In this chapter the results obtained on the functional status of intestinal and splenic lymphocytes in experimental Vit A deficiency are discussed.

Effects of Vit A deficiency on immune function have been examined in human subjects and experimental animals. As Vit A deficiency is usually associated with protein-energy malnutrition in humans, it is

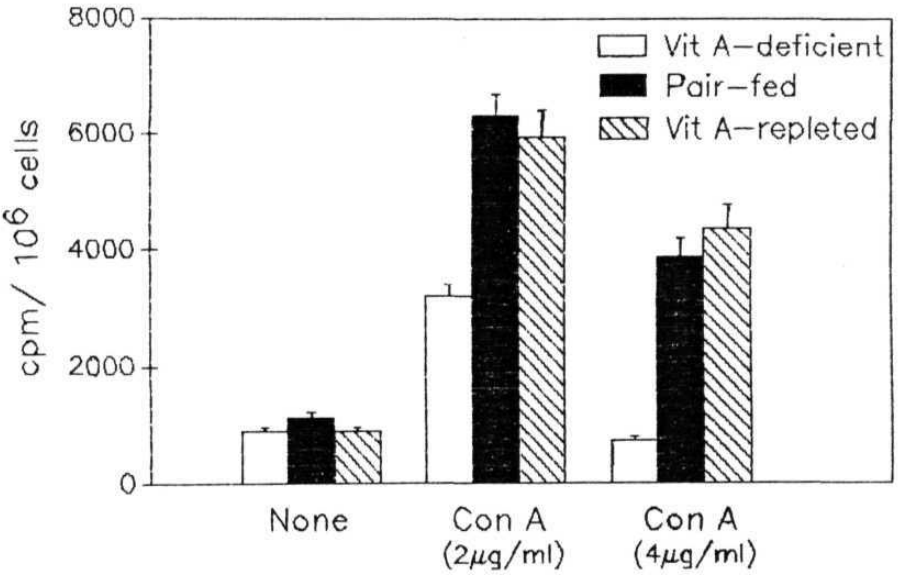


FIGURE 20: Proliferative response of IEL isolated from Vit A-repleted rats. Values presented are mean + SEM of three experiments.

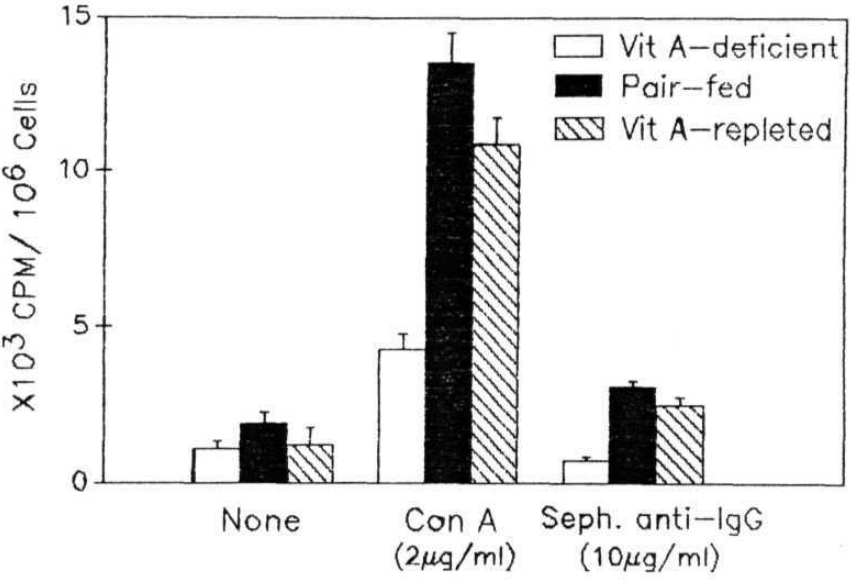


FIGURE 21: Proliferative response of LPL isolated from Vit A-repleted rats. Values are expressed as mean + SEM of three experiments.

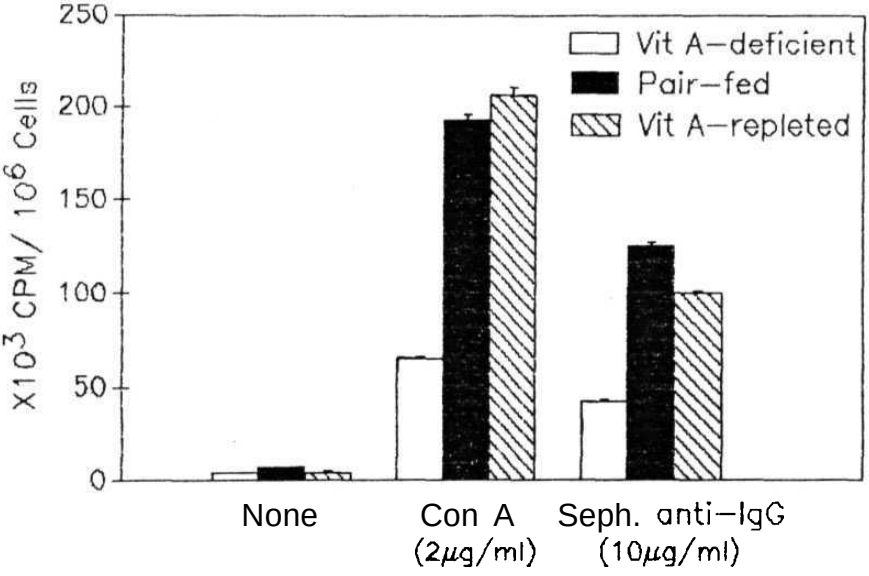


FIGURE 22: Proliferative response of splenic lymphocytes from Vit A-repleted rats. Values are expressed as mean + SEM of three experiments.

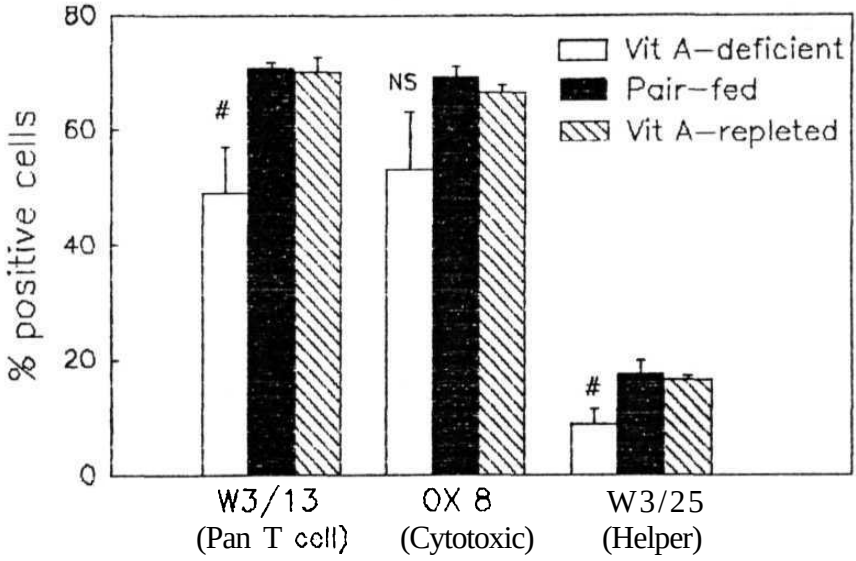


FIGURE 23: Enumeration of IEL from Vit A-repleted rats. # $p < 0.05$; NS= not significant, pair-fed controls vs Vit A-depleted or Vit A-repleted rats. Values are expressed as mean + SEM of four observations.

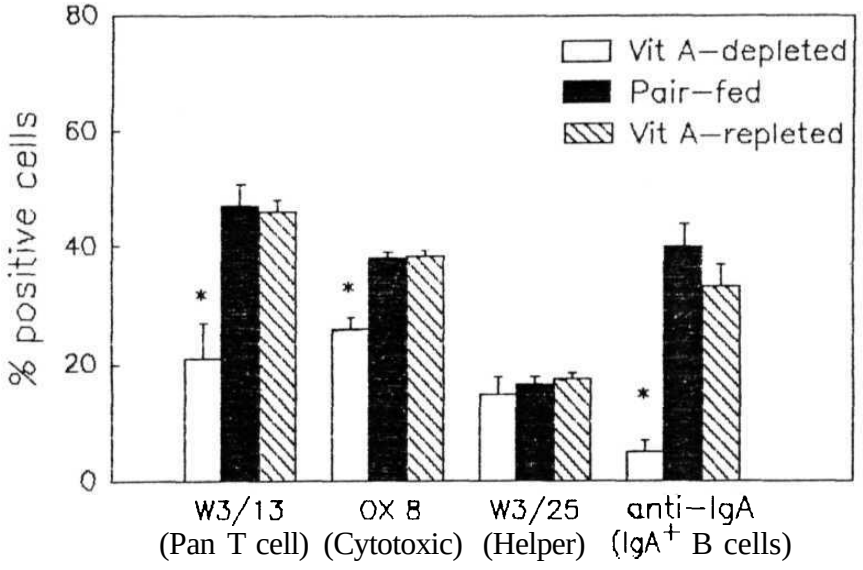


FIGURE 24: Enumeration of LPL from Vit A-repleted rats. • p< 0.01, pair-fed controls vs Vit A-depleted or Vit A-repleted rats. Values are expressed as mean + SEM of four observations.

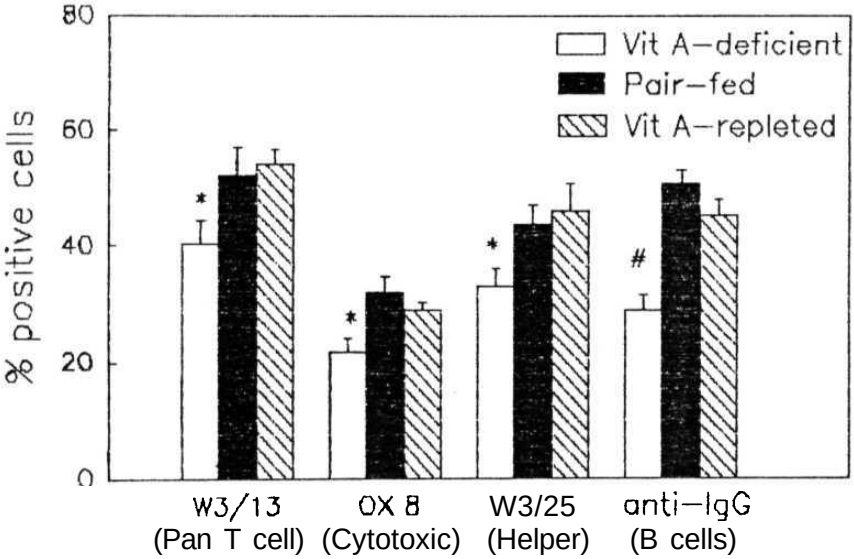


FIGURE 25: Enumeration of splenic lymphocytes from Vit A-repleted rats. • $p < 0.01$; # $p < 0.05$, pair-fed controls vs Vit A-depleted or Vit A-repleted rats. Values are expressed as mean + SEM of three observations.

difficult to interpret the observations obtained from Vit A-deficient human subjects. Studies using animal models are preferred, where the effect of single nutrient deficiency can be examined in isolation. The animal models also allow the usage of proper controls. Although mouse is used extensively for immunological studies, it has not been used frequently for Vit A deficiency studies as it requires initiation of Vit A-deficient diet during fetal development. Rat has been used extensively for Vitamin A deficiency studies as it satisfies certain criteria like, a) feasibility to induce deficiency in post-weaning stage and b) decrease in weight gain as an early sign of deficiency state. Another important reason for the usage of rat model in our studies was that the T cell subset composition and the T cell receptors expressed by rat IEL are similar to that of human, while mouse IEL markedly differ from human IEL (Bonneville *et al,* 1988; Vaage *et al,* 1990; Brandtzaeg *et al,* 1989). Hence, it can be suggested that the results obtained by using the rat model can be extrapolated to the human situation.

Vit A deficiency was induced in Wistar rats by maintaining them on a Vit A-free diet. Pair-fed controls were used to compensate for the effects of anorexia and associated decrease in weight gain observed in Vit A rats. Maximum possible attention was given to housing and sanitation. After 6-7 weeks of feeding period the Vit A-deficient rats reached a weight plateau. Low serum and liver Vit A levels of Vit A rats after 7 weeks of feeding period showed that by this time Vit A deficiency was induced to a significant level. However, serum total protein and serum albumin concentrations of Vit A~ rats was unaltered as compared to pair-fed controls, suggesting

that the Vit A deficiency state was not complicated with protein -energy malnutrition.

It has been reported that Vit A deficiency is associated with atrophy and decreased cellularity of thymus, bursa and other lymphoid tissues (Wolbach and Howe; Bang *et al,* 1973). In our studies, we observed a decrease in absolute weights of thymus and spleen in **Vit A rats,** but the relative weights (percentage of body weight) were not significantly different. The gross cellularity of spleen was not altered, but a decrease in the cellularity of Peyer's patches was observed. The number of PP were significantly low in Vit A rats and similar observation was reported in Vit A guinea pigs (Majumder *et al,* 1987). The morphology of small intestinal mucosa has been reported not to be significantly affected in Vit A deficiency (De luca *et al,* 1969), unlike other mucus-secreting epithelial tissues. However, in the present studies, decreased cellularity of lamina propria, and changes in epithelial structure were observed in histological sections. The decreased yield of IEL and LPL isolated from the small intestine of Vit A rat are consistent with the histological changes. Fluid accumulation seen in the ileum of Vit A rats might be due to the enhanced secretory activity of Vit A-deficient small intestine as reported by Nazegwu and Levin (1991).

Phenotypic analysis of IEL, LPL and splenocytes of Vit **A and Vit A^- rats** showed that the percentage of B cells in LPL and splenocytes decreased significantly in Vit A deficiency. The percentage of total T cells was also decreased in IEL, LPL and splenocytes of Vit A rats. However, the T cell subset composition and B cell number of spleno-

cytes were reported to be unaltered in Vit A deficiency (Nauss *et al,* 1985). The altered T cell subset composition and B cell number of splenocytes in our study could be due to the severe deficiency state of the animals. Nauss *et al* (1985) suggested that the effect of Vit A deficiency on cell-mediated immune response is complex and depends on the state of deficiency as well as lymphoid organ under examination. It is interesting to note that the suppressor/cytotoxic T cell number of IEL in Vit A rats was unaltered and this could be due to the enhanced migration of these T cells to the epithelium of the intestine from other lymphoid organs. It was suggested earlier (Ernst *et al,* 1985) that under diseased conditions cytotoxic lymphocytes which cause destruction of diseased enterocytes escape from normal regulation and migrate to the intestinal epithelium. Recently it is reported that children with Xerophthalmia had lower number of CD4 T cells and higher proportions of CD8 T cells in peripheral blood (Semba *et al,* 1993). The reduction in the percentage of helper T cells of IEL in Vit A rats is reflected in the reduced transformation response of IEL towards Con A in Vit A rats as compared to their pair-fed controls. However, the percentage of T helper cells of LPL in Vit A rats was not altered and a significant reduction in the percentage of cytotoxic/ suppressor T cells was observed. Hence, it is clear that in Vit A deficiency the distribution pattern of T helper and T cytotoxic lymphocytes in intestinal epithelium and lamina propria is altered significantly. Migration of cytotoxic/suppressor T cells into the intestinal epithelium might have increased in Vit A deficiency leaving fewer $CD8^+$ T cells in lamina propria. The percentage of helper T cells of LPL was not significantly decreased, while a decrease in transformation response of LPL to Con A was observed.

The decreased transformations of IEL, LPL and splenic lymphocytes to both T and B cell mitogens in Vit A deficiency could be due to the defect at the receptor level. Majority of cell surface receptors for several biological effectors including mitogens are glycoprotein in nature (Gesner and Ginsberg, 1964; Woodruff and Gesner, 1968). A glycoprotein coat has been found on both T and B lymphocytes (Saunter *et al,* 1973). As, Vit A has been reported to be essential for glycoprotein biosynthesis, where it acts as a carrier for glycosyl moieties (De Luca, 1977), Vit A deficiency probably results in a reduced synthesis of important membrane glycoproteins which are needed for antigen recognition and mitogen interaction. Vit A was shown to increase *in vitro* blastogenesis of thymocytes by modulating receptors for IL-2 (Sidell and Ramsdell, 1988). Alterations in IL-2 receptor expression in Vit A deficiency might be responsible for the decreased proliferative response towards Con A.

With respect to the B cell responses, Vit A has been shown to have direct effect on the differentiation of B cell hybridomas (Sherr *et al,* 1988), and it is thought to be involved in the differentiation of less mature B cells through its effect on gene expression. Hence, lack of Vitamin A may impair B cell differentiation resulting in the reduction of mitogen-reactive mature B cells.

The NK cell activity of splenic lymphocytes was significantly decreased in Vit A deficiency. This is in accordance with the earlier reports. (Nauss and Newberne, 1985a; Bowman *et al,* 1990). However, increased NK cell activity of IEL was observed in Vit A rats. This

could be due to the increased homing of cytotoxic cells with natural killer activity into the epithelial layer of intestine in Vit A~ rats. Enhanced NK activity of IEL in contrast to the reduced NK activity of splenic lymphocytes observed in Vit A rats indicates the apparent independent behavior of NK cells from mucosal sites and from secondary lymphoid organs. Carman et al (1992) reported that in Vit A deficiency mesenteric lymph node cells secreted more IFN-γ and less IL-2, IL-4 and IL-5. The increased IFN-γ secretion in intestinal immune system might result in increased cytolytic function of IEL. It has also been suggested that IFN-γ overproduction might enhance the gut inflammatory response (Carman *et al,* 1992).

The antibacterial activity of IEL and splenocytes in presence of antibody was found to be decreased in Vit A rats. This may be due to the reduction in the number of cytotoxic T cells which participate in the antibacterial activity. The reduced antibacterial activity indicates an altered host-pathogen interaction in the intestine resulting in enhanced susceptibility to infections.

The antigen specific proliferative response of LPL was also depressed in Vit A rats and this might be due to the defective induction of antigen specific effector cells in the intestine in Vit A deficiency. Antigen specific T lymphocyte proliferative responses have been reported to be decreased in Vit A-deficient rats and chicks (Friedman and Sklan, 1989). This was suggested to be either due to a direct effect of Vit A on T lymphocyte IL-2 production, which was shown to be increased by Vit A supplementation (Colizzi and Malkovsky, 1985), or due to an indirect effect resulting from a macrophage

mediated decrease in antigen presentation, Ia expression and IL-1 production (Goldman, 1984, 1985; Wirth and Kierszenbaum, 1986).

The number of IgA B cells and antigen specific antibody secreting B cells were decreased in the lamina propria of Vit A rats. It has been shown that helper T lymphocytes from Vit A mice fall to provide the stimulus to B cells for antigen specific Ig responses (Carman et *al,* 1989). The molecular basis of this failure may be due to the elevated IFN-γ production by T cells of Vit A rats (Carman *et al,* 1991). Excess IFN-γ could inhibit IL-4 and also IL-4 stimulated B cell class switching. Decrease in the production of IL-2, IL-4 and IL-5 has been reported with mesenteric lymph node cells (Carman *et al,* 1992). Reduced interluekin secretion by LPL in response to an antigen may result in altered B cell proliferation and differentiation. Reduction of IgA B cells and antigen specific antibody secreting B cells in the lamina propria of Vit A rats indicates the impairment of humoral response which may influence the course of a bacterial infection.

In order to see whether the observed changes in immune function of IEL and LPL are of reversible nature, the Vit A rats were rehabilitated with Vit A and the proliferative response and subset composition were determined in repleted animals. Repletion studies showed that the subset composition of IEL, LPL and splenocytes and also the proliferative responses of these lymphocytes to mitogens were restored to normal levels. Nauss et *al* (1979) and Friedman and Sklan (1989) reported that the impaired transformation responses of splenic lymphocytes in Vit A rats were recovered rapidly on supplementation

with Vit A. Repletion with retinol has been shown to effectively restore the antibody responses in Vit A rats (Sri Kantha *et al,* 1992).

Finally, considering the possible role of Vit A in membrane glycoprotein biosynthesis and differentiation of many cell types, it can be postulated that lymphocytes could have an altered distribution pattern in Vit A animals. The depressed immune response in Vit A deficiency may not be due to the malfunction of any one cell type, a generalized dysfunction in regulatory metabolic reactions could be expected in Vit A deficiency. This is supported by the rapid recovery of immune function following the supplementation of depleted rats with Vit A (Nauss *et al,* 1979).

CHAPTER IV

SUMMARY AND CONCLUSIONS

IEL and LPL were isolated from rat small intestine and the functional characteristics of them were studied in detail.

A modified method was developed to isolate IEL from rat small intestine. A protease inhibitor, Phenyl Methyl Sulphonyl Fluoride (PMSF) was included in the isolation medium along with EDTA for the isolation of IEL. The inclusion of PMSF resulted in good yield with 98% viability. This was shown to be due to the inhibition of 'protease' activity associated with the intestine by PMSF.

Indirect immunofluorescence analysis showed that majority of IEL were T lymphocytes with almost 70% of cytotoxic/suppressor subtype. About 30% of cells were found to be helper/inducer subtype.

IEL were unresponsive to the T cell mitogen, Con A as measured by the proliferation assay (H-thymidine incorporation into DNA). Addition of adherent accessory cells obtained form autologous spleen cells also failed to improve the proliferative capacity of IEL in presence of Con A. However, the addition of supernatants from Con A -stimulated autologous splenocyte culture (a source of lymphokines) to Con A-activated IEL cultures resulted in a significant proliferative response. IEL failed to recognize alloantigens in a one way mixed lymphocyte reaction. However, IEL showed significant natural killer activity against YAC-1 target cells.

The LPL populations were found to contain both T and B lymphocytes, majority of B lymphocytes being IgA positive. LPL responded well to T cell (Con A) and B cell (Seph. anti-IgG) mitogens.

Unlike IEL, no addition of lymphokine rich Con A stimulated splenic supernatants was required for the proliferation of LPL.

The antigen specific responses of IEL and LPL were studied using *Salmonella typhimurium* as test organism. After *in vivo* sensitization of IEL and LPL, proliferative response of *in vivo* sensitized cells was determined *in vitro.* The antigen-sensitized IEL were also used to detect secretory IgA and IgG-dependent antibacterial activity against *S.typhimurium.* IEL failed to show a proliferative response to *S. typhymurium* antigens *in vitro.* However, a significant proliferative response to *S.typhymurium* antigens was observed with LPL. IEL showed both IgA and IgG dependent bacteriolytic activity.

After characterizing IEL and LPL, the functional status of intestinal immune system was studied in Vitamin A-deficient (Vit A) and pair-fed control (Vit A) rats.

Vit A deficiency was induced in Wistar female rats. Rats were weaned onto a Vit A-free diet and were continued on the same for 6-9 weeks, at which time the deficient rats showed growth retardation followed by loss of body weight. There was a significant reduction in serum and liver retinol concentrations in Vit A rats as compared to Vit A^{+} rats. The functional status of intestinal immune system was studied with respect of IEL and LPL. Along with the studies on IEL and LPL, effect of Vit A deficiency on the immunological status of lymphocytes obtained from spleen was also studied.

Functional characterization of IEL, LPL and splenic lymphocytes from Vit A^+ and Vit A^- rats showed that,

1. the percentage of T cells and helper/inducer subtype of IEL were significantly reduced in Vit A rats as compared to Vit A rats, however, the percentage of T cells bearing cytotoxic/suppressor phenotype was altered in Vit A deficiency,

2. in LPL, the percentage of T and B cells was significantly reduced in Vit A rats, significant reduction was observed in cytotoxic/suppressor subtype of LPL, while the decrease in helper inducer subtype was not significant,

3. the percentage of T cells, its subsets and B cells of splenic lymphocytes isolated from Vit A rats was significantly less,

4. the proliferative responses of IEL, LPL and splenic lymphocytes to mitogens were significantly reduced in Vit A rats,

5. the natural killer cell activity of IEL against YAC-1 lymphoma cells was increased in Vit A rats, splenic lymphocytes isolated from the same deficient rats showed decreased NK cell activity,

6. antigen specific proliferative response of LPL towards *S. typhimurium* antigens was also reduced in Vit A rats,

7. both IgG and IgA-dependent bacteriolytic activities of IEL were depressed in Vit A rats, IgG-dependent bacteriolytic activity of splenic lymphocytes was also decreased in Vit A deficiency,

Further, histological studies showed that the intestinal epithelial integrity was disturbed in Vit A deficiency and that the cellularity of lamina propria was also decreased. Immunohistological studies, carried out to enumerate total IgA B cells and antigen specific antibody secreting B cells in lamina propria of Vit A rats,

showed that the number of IgA and antigen specific antibody secreting B cells was decreased in Vit A rats.

After being on Vitamin A deficiency for eight weeks, those rats were repleted with Vit A and subset composition and proliferative response of IEL, LPL and splenic lymphocytes were studied to see whether the altered responses could be reversed. The total T cell and subset compositions of IEL, LPL and splenic lymphocytes were restored to normal after repletion. The proliferative responses of IEL, LPL and splenocytes isolated from Vit A-repleted rats were similar to those of the pair-fed controls.

These results suggest a depressed immune function of mucosal associated lymphocytes of small intestine in Vit A deficiency. Several epidemiological and experimental studies have demonstrated a significant increase in the incidence of respiratory and diarrheal infections in Vit A-deficient states. Altered intestinal immune function as observed in this study seem to significantly contribute to the altered host-pathogen interactions at the mucosal surfaces and enhanced susceptibility to infection.

The role of Vitamin A in cellular metabolism in general and immune function, in particular have been discussed.

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