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 (Lefrançois, 1991). These thymic-independent murine IEL express TCR γδ instead of conventional TCR αβ (Bonneville et al., 1988). On the contrary, human IEL and rat IEL contain very few TCR γδ cells and majority of them are TCR αβ⁺ 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 anti-CD3, anti-TCR αβ and anti-TCR γδ (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:

1. 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$_2$PO$_4$, 60mg; NaHPO$_4$, 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 up to 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 1-2cm pieces. To remove mucus, intestinal pieces were incubated in isolation medium with 1mM DTT for 5min 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 at least 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 10 min 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.
7. COUPLING OF ANTI-RAT IgG TO CNBr-ACTIVATED SEPHAROSE-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 buffer, 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 (amphotericin 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. 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 2x10^6 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\(_2\) 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 transferred 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 10x10^6 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% CO₂ 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 3μg/ml. Cell concentration was adjusted to 4x10^6 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 CO₂ incubator. After 24h the supernatant was collected, filter sterilized through a low protein binding 0.45μm 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 3x10^6 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 2x10⁶ cells/ml. 2x10 responder cells in 100μl were cultured with 2x10⁶ 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 CO₂ incubator for 96 or 120h and were pulsed with 0.5μCi ³H-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.

Maintenance of target cells (YAC-1):

YAC-1 is a Moloney leukemia virus induced murine T cell lymphoma. YAC-1 cells were used as tumor 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. 2-3x10⁶ 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 transferred 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 2x10^6 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 CrO₄) in saline from BRIT, India. YAC-1 were loaded with ^51^Cr by incubating 8-10x10^6 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 up to 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% CO₂ atmosphere, the plate
was spun down at 500 xg for 10 min. 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-programmer (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:

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

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 antibody 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 0.15M 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 al (1966).

b. Fluorescent labeling and visualization:

Procedure:

IEL, LPL or splenic lymphocytes, 3-5x10⁶ 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 (10⁹ 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 1h. 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 MgCl₂. 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 Freund’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 boosterized 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 H₂O₂.

Procedure:

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

The proteins separated on SDS-PAGE were transferred onto 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 1h. 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 1h 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 bacterial antigens and the bacteriolysis was monitored.

Procedure:

The assay was carried out by following the method of Tagliabue.
IEL and splenocytes were isolated from in vivo sensitized rats (one ip followed by three oral boosters as described in protocol 13). 

*S. typhimurium* (10⁴) 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% CO₂ 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:

\[
\text{Antibacterial index} = \frac{\text{No. of CFU of experimental plates}}{\text{No. of CFU of control plates}} \times 100
\]

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 °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**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>cell yield $(x \times 10^6$ cells)</th>
<th>viability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-PMSF</td>
<td>+PMSF</td>
</tr>
<tr>
<td>(i) After EDTA incubations</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cells</td>
<td>72 + 5</td>
<td>84 + 6</td>
</tr>
<tr>
<td>IEL</td>
<td>13.7 + 0.9</td>
<td>17.2 + 1.3</td>
</tr>
<tr>
<td>(ii) After percoll gradient</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IEL (40-60% Percoll interface)</td>
<td>6.8 + 1.5</td>
<td>10.2 + 0.9</td>
</tr>
</tbody>
</table>

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 transfor-
FIGURE 1:

Histological 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

<table>
<thead>
<tr>
<th>Cell population</th>
<th>H-thymidine incorporation into DNA</th>
<th>2µg/ml</th>
<th>4µg/ml</th>
<th>10µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>IEL</td>
<td>397 + 32</td>
<td>428 + 48</td>
<td>421 + 74</td>
</tr>
<tr>
<td></td>
<td>splenocytes</td>
<td>810 + 30</td>
<td>45750 + 330</td>
<td>56520 + 350</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>IEL</td>
<td>757 + 59</td>
<td>427 + 47</td>
<td>845 + 45</td>
</tr>
<tr>
<td></td>
<td>splenocytes</td>
<td>2923 + 382</td>
<td>44078 + 1490</td>
<td>69935 + 597</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>IEL</td>
<td>1120 + 37</td>
<td>1250 + 17</td>
<td>735 + 15</td>
</tr>
<tr>
<td></td>
<td>splenocytes</td>
<td>5302 + 232</td>
<td>72208 + 1087</td>
<td>98349 + 2807</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>IEL</td>
<td>582 + 35</td>
<td>556 + 83</td>
<td>611 + 72</td>
</tr>
<tr>
<td></td>
<td>splenocytes</td>
<td>5914 + 453</td>
<td>67860 + 1741</td>
<td>69572 + 897</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>IEL</td>
<td>834 + 44</td>
<td>757 + 55</td>
<td>723 + 87</td>
</tr>
</tbody>
</table>

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

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

³H-thymidine incorporation was measured between 72-96h. Values are expressed as mean ± SEM of triplicate cultures.
<table>
<thead>
<tr>
<th>Con A (µg/ml)</th>
<th>H-thymidine incorporation into DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cells isolated in complete medium</td>
</tr>
<tr>
<td>None</td>
<td>13576 ± 126</td>
</tr>
<tr>
<td>2.0</td>
<td>1080047 ± 4910</td>
</tr>
<tr>
<td>4.0</td>
<td>1083985 ± 15467</td>
</tr>
<tr>
<td>6.0</td>
<td>837912 ± 13742</td>
</tr>
</tbody>
</table>

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

### TABLE 5

**VIABILITY OF RAT AND MOUSE IEL DURING CULTURE**

<table>
<thead>
<tr>
<th>Cell population</th>
<th>viability (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 h</td>
</tr>
<tr>
<td><strong>EXPT 1</strong></td>
<td></td>
</tr>
<tr>
<td>Rat IEL</td>
<td>98.0</td>
</tr>
<tr>
<td>Mouse IEL</td>
<td>97.0</td>
</tr>
<tr>
<td><strong>EXPT 2</strong></td>
<td></td>
</tr>
<tr>
<td>Rat IEL</td>
<td>99.0</td>
</tr>
<tr>
<td>Mouse IEL</td>
<td>98.0</td>
</tr>
<tr>
<td><strong>EXPT 3</strong></td>
<td></td>
</tr>
<tr>
<td>Rat IEL</td>
<td>99.0</td>
</tr>
<tr>
<td>Mouse IEL</td>
<td>98.0</td>
</tr>
</tbody>
</table>
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 $2 \times 10^4$ to $8 \times 10^4$ 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

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

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

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

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**

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

*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. typhi-murium 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. typhimurium*. 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** of 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 al (1989). The procedure involves lesser incubation time (90min) as compared to other procedures (Davies et al, 1981; Lyscom et al, 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**

<table>
<thead>
<tr>
<th>E : T ratio</th>
<th>IgG lysis</th>
<th>slgA lysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>50:1</td>
<td>40 + 6</td>
<td>31 + 4</td>
</tr>
<tr>
<td>100:1</td>
<td>52 + 3</td>
<td>50 + 3</td>
</tr>
</tbody>
</table>

Rat antiserum containing IgG was used at 1/100 dilution and the intestinal fluid (collected by washing the intestine in 10ml of PBS) containing slgA was used at 1/3 dilution. Values are expressed 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>
<thead>
<tr>
<th>Cell</th>
<th>Population</th>
<th>$^{3}\text{H}$-thymidine incorporation into DNA</th>
<th>cpmp/ 10 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
<td>Con A (2µg/ml) Seph. anti-IgG (10µg/ml)</td>
<td></td>
</tr>
<tr>
<td>LPL</td>
<td>1238 ± 226</td>
<td>8889 ± 364</td>
<td>2969 ± 141</td>
</tr>
<tr>
<td>Splenocytes</td>
<td>9071 ± 88</td>
<td>405915 ± 2804</td>
<td>28465 ± 504</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM of three experiments.
**Enumeration of LPL for T cell subsets and B cells**

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

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 90%. 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 al. (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 al., 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 al., 1983; Nauss et al., 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 0h 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 al, 1984; Davies et al, 1981; Lyscom et al, 1982; Tagliaabue 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 al, 1982; Woolverton et al, 1992; Bartnik et al, 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.