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INTRAEPITHELIAL INTESTINAL LYMPHOCYTES AND ENTEROCYTES IN INTESTINAL IMMUNE RESPONSE

A Thesis

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ABSTRACT

The study was undertaken to investigate some functional aspects of the intraepithelial intestinal lymphocytes (IEL). The emphasis was placed on their possible role in an immune response. Putative cooperation between IEL and the epithelial cells in an intestinal immune response was investigated. The populations of IEL, spleen cells, peritoneal macrophages, IEL with peritoneal macrophages, and IEL with epithelial cells were stimulated with Concanavalin A (ConA) and the supernatants were then examined for the presence of interleukin-2 (IL-2) and interferon (IFN). Elisa and bioassay were applied to detect presence of IFN. Microtitration assay with vesicular stomatitis virus was used for IFN detection.

IEL were found to produce both IL-2 and IFN. IEL co-cultured with the absorptive intestinal epithelium responded by a significantly augmented IL-2 production, and this was interpreted as a cooperation of the two cell types in an immunological sense. The widely accepted bioassay for IL-2 was found to be non-specific. This conclusion was derived from the discrepancies between the results of the bioassay and the Elisa, and also from the failure of the monoclonal antibodies specific to IL-2 to inhibit the IL-2 bioassay.

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1.0 GENERAL INTRODUCTION

The small intestinal mucosa represents an enormous area of interface between the external and internal milieus, with intense antigenic stimulation leading to both positive and negative immune responses. The inner surface of the small intestine is, with the exception of the area overlying Peyer's Patches (PP), lined with columnar absorptive epithelium (enterocytes). Currently, PP is thought to be the place of the afferent arm of an immune response, and that implies that only antigens entering through the specialized M epithelium overlying PP stimulate local immune response. However, normal absorptive epithelium (enterocytes) are also known to play a role in antigen uptake. Once an antigen is transported through the epithelium and released into the lamina propria, it would be processed by accessory cells of macrophage or dendritic cell lineage, and as in other situations, antigens are probably presented to lymphocytes in association with major histocompatibility complex (MHC) antigens of the Class II (or Ia antigens) in a MHC-restricted fashion.

Recent observations of Ia on the absorptive epithelium of the small intestine (1, 2, 3, 4) raise the possibility that epithelium could play a more active part in antigen presentation, possibly by directly interacting with cells of the immune system in the same fashion as accessory cells.

If the Ia antigens on the absorptive epithelium have an immunological function, the most likely one seems to be presentation of antigens to receptive lymphocytes. Intraepithelial lymphocytes (IEL) of the small intestine are dispersed in high numbers among enterocytes (5, 6). Thus the two cell types are well located for putative interaction. Since class II antigen expression in intestinal tissue is primarily seen in enterocytes close to the location of IEL (2), and Ia expression has been shown to be induced by interferon (IFN) (7), the ability of IEL to synthesize IFN was investigated.

Enterocytes displaying Ia antigens may cooperate with T helper (T_h) cells, and promote production of interleukin-2 (IL-2) by T cells, which is necessary for T cell clonal expansion. Accessory cells produce IL-1 (8), and it might be this lymphokine that induces production of IL-2 in T helper cells. Therefore, the capacity of IEL to synthesize IL-2 was studied. Two assays were used for IL-2 detection, the bioassay and the monoclonal antibody based enzyme linked immunoassay (Elisa). The reason for this was the concern that the bioassay might react to lymphokines other than IL-2, and the fact that monoclonal antibody specific to IL-2 recently became available. Both pure IEL and IEL co-cultivated with epithelial cells were examined with respect to IL-2 production, following polyclonal stimulation with Concanavalin A (Con A). A possible helper effect of

enterocytes on IL-2 production by IEL, would indicate immunological co-operation of the two cell types.

2.0 INTRODUCTION

2.1 Intraepithelial Lymphocytes (IEL)

2.1.1 Morphology of IEL

A significant percentage of murine IEL contains cytoplasmic granules that stain with May-Grunwald-Giemsa or toluene blue stain (9, 10). Rat IEL stain with alcian blue (pH 2) and toluidine blue (pH 4) (11) and also incorporate sulphate, suggesting content of sulphated mucopolysaccharides (5). Granulated cells account for 19% of the rat IEL on the first day of life (12), while in adult human they reach 31% of total IEL (13). The granules are seen mainly in medium size human and rat IEL (13, 14) and they are clustered in the concavity of the nucleus. Small IEL, similar in size to resting splenic or lymph node lymphocytes, constitute 44% of IEL from normal mice and 38% of IEL from nude mice. Large IEL, two to four times the size of small lymphocytes, constitute 56% of the population in normal mice and 62% in nude mice (15). Blast cells and cells in mitosis are rarely seen amongst IEL. Electron microscopy reveals that cytoplasm of rat IEL contains complexes of Golgi and centrioles (14). The nuclei are of a kidney shape containing marginated metachromatin and nucleoli. Some granules consist of electron-opaque core and electron-lucent rim, and some contain homogeneously electron-opaque granules. Granules of cells isolated from immune rats were

reported to contain the enzymes glycosamine and serine esterase (14). Although histamine, which is specific for mucosal mast cells, was detected in murine IEL (16) and mast cells were also observed in IEL of bovine origin (10), the majority of IEL are not mast cells. In one report eosinophils accounted for 5.6% of rat intraepithelial intestinal leucocytes (5).

2.1.2 Number of IEL

The number of IEL can be estimated by a differential count in relation to the epithelial cells. Results have been expressed as IEL/100 epithelial cells, or as a number of IEL lying in a fixed area of mucosal surface. In humans, the number of IEL per 100 epithelial cells is between 30 and 40 (average 34.1/100). In cattle $2x10^6$ viable cells were isolated per each cm^2 of the intestinal mucosa (10). In rats, the reported average yield was $2.20x10^6$ per one gram of gut (5), and in mice between 1.6 to $4.0x10^7$ cells per gram of gut (17).

2.1.3 IEL age dependence

In rats IEL are low in numbers at birth (3-4/100 epithelial cells). The number increases significantly during the first week of life, then decreases the next 2 weeks, and rises again to reach mature values about 6 weeks after birth (12). Within the IEL cell population, T suppressor cells appear to peak 1 week after delivery, then they decline and slowly increase to peak again at 12 weeks of life. T helper cells

are first detected 2 weeks after birth. The number of the cells with T helper and pan T markers is low in neonates and it reaches maturity by 6 weeks of age.

2.1.4 Surface phenotype

Most of the mature IEL isolated from humans are of the T lineage (83%) and express the phenotype associated with cytotoxic-suppressor T cells (68-83%) (18). In human IEL, the mean percentage of erythrocyte rosette (E+) forming cells was reported to be 68%, and that of T helper cells 10% (13, 18). A freshly isolated population of mouse IEL contained 57% Lyt2+ (suppressor) cells, 17% Lyt1+ (helper) cells, 30% Thy 1.2+ (T cell) cells, 3% Mac-1+ (macrophage) cells and 6% sIg+ (B cell) cells (19). In cattle 60% of IEL were E+ T cells, while 10% were B cells, (10). In rats, cells bearing the T-suppressor marker predominated in IEL (73%), but were present in lower concentration in lamina propria (26%) and Peyer's Patches (6%). IEL were unusual in containing a population of cells which were negative for the pan T-cell marker, but positive for the T-suppressor marker (5). Similar results were found in mice, where nearly 70% of IEL bore Thy1-, Lyt2+ phenotype (pan T negative, suppressor positive)(15). Two other surface markers have been also found on IEL; the marker present on activated cytotoxic cells CT-1 and the marker present on B cells and on immature T cells J11d (15). Two different types of CTL cells, which bear Thy1, Lyt2, and assialo GM antigens on the

surface, were detected in IEL from primed mice. One is strictly antigen specific and functionally similar to splenic derived CTL clones. The second ..e is antigen dependent for proliferation but requires the presence of lymphokines for lytic activation (20). All these results indicate that IEL is a unique population of cells, resembling neither population of spleen cells nor PBL.

2.1.5 Origin of IEL

Two fundamental characteristics distinguish the IEL from cells of other peripheral immune compartments: their phenotype and their variation in natural effector activities. The origin and the function of IEL are controversial. In both thymus-bearing and athymic nude unprimed mice a high proportion of IEL expresses an antigen associated with activated cytotoxic cells (CT-1) and a lymphocyte marker present on immature T cells (J11d) (15). This is evidence that some cytotoxic IEL may originate by a thymus independent lineage (15). Alternatively Thy1-, Lyt2+ IEL may be derived from peripheral CTL that have lost Thy1 antigen expression. Presence of CT1 antigen on unprimed cells indicates that those cells are highly activated and lytic. CT-1 antigen can be associated with the population of Thy1+ and Thy1 cells. These findings suggest that some IEL originate as non-thymus-derived cells that acquire lytic activity within the epithelium itself (15).

2.1.6 Kinetics of IEL

Decreased numbers of IEL are found in athymic (thymectomized, thymectomized and irradiated, neonatally thymectomized and nude), and in germ-free mice. In nude mice, the majority of IEL are replaced within a week (21). IEL with a lifespan of several weeks were also detected in the epithelium. Long-lived IEL in normal mice form a more morphologically uniform population than long-lived lymphocytes in nude mice (21). The rate of appearance of newly formed IEL in epithelium was the same in thymectomized, nude, and normal mice (21). The rate of disappearance of ^{3}H thymidine labelled cells from the intestine was the same in germ-free and normal mice. A relatively high percentage of cells was seen the day after injection of tritiated thymidine and thereafter a linear decrease was observed. A few labelled IEL were still detected 6-9 weeks after the injection (21). IEL have excellent locomotor activity *in vitro* and migrate into collagen gels rapidly (22). *In vivo*, radiolabelled IEL were found 2h after injection mainly in the liver and lungs. By 18h fewer IEL appeared in the lungs, some were detected in the blood and none were found in the lymph nodes. There were numerous IEL in the kidneys after 24h, however, still no accumulation was found in the gut (22). IEL were also found to have a very low mitotic rate, with only 0.4-0.8% labelled after 1h of incubation (21).

2.1.7 Responsiveness of IEL to mitogens

Mouse IEL can be stimulated to proliferate by T cell mitogens (Concanavalin A, Con A, pokeweed, PWM, phytohemaglutinin, PHA) as well as the B cell mitogen lipopolysaccharide (LPS) (23, 24). Others were not able to show any response of murine IEL to optimal mitogen concentrations even in extended culture at different cell densities (23). A strong proliferative response occurred when IEL were cocultured with adherent spleen cells at the ratio 15-20% IEL (25). The yield of IL-2 was reported to be minimal in stimulated murine IEL, and that of IFN γ was intermediate in comparison to spleen cells (23). Supernatant from Con A stimulated murine IEL, tested in bioassay, contained low levels of IL-2 (10U/ml), compared to 70U/ml produced by spleen cells (16). Murine IEL produced slightly higher levels of IL-3 than IL-2 (23). The level of IL-2 and IL-3 was higher when IEL were costimulated with antigen and mitogen, or were cocultured with mitogen and recombinant IL-2 (23). A weak blastogenic response of mouse IEL compared to pig IEL has been reported (24).

2.1.8 Function of IEL

Intestinal epithelium contains a population of potent natural killer (NK) cells which may represent a type of NK cell different from that found in other tissues (25). IEL are effector cells in spontaneous cell-mediated cytotoxicity (SCMC) (27, 28) and antibody dependent cell mediated

cytotoxicity (ADCC) (29, 30, 28). However, there is a controversy concerning NK activity of IEL in the small intestine. In the mouse, it was demonstrated that IEL possess very strong NK activity, even higher than that mediated by spleen cells or Peyer's Patch lymphocytes (30, 31). In other studies, NK activity of murine IEL was found to be minimal compared to the activity of spleen lymphocytes (26). Lytic activity of murine IEL could be enhanced by extension of incubation time with lectins to 18h (26). In rats, IEL exhibited cytotoxic activity similar to spleen cells (29). In humans, NK activity of IEL was reported to be minimal compared to the activity of PBL (27), or even nonexistent (13). In one study, mouse IEL were found to suppress the NK activity of spleen cells, and this suppressor activity was not mediated by macrophages or T cells (26). With IEL the cytotoxicity was first detected at the ratio 100:1, while with PBL it was first detected at the ratio 5:1. It is suspected that the functional state of intestine may influence the activity of effector cells isolated from the intestine (27). Lack of NK activity in IEL of nude mice would indicate that IEL and spleen NK cells belong to two different populations, since spleen cells of nude mice contain high levels of NK cells (26). Strain differences in NK activities of IEL were observed among C57B1, C3H, NIH, BALB/c strains of mice. NIH strain had the highest cytotoxic activity. Nude mice have more NK activity

than other strains (26). Culturing of IEL in different conditioned media also changes NK activity. Cells either lose their cytotoxic activity, or this activity is enhanced (9). The cytotoxic activity of IEL can not be boosted by interferon treatment (13, 29) which is at variance with the report in which IFN ß treatment of IEL potentiated their lytic activity (30). Poor response to IFN treatment might be explained by the relatively small pool of pre-NK cells (29). Unlike spleen cells, IEL do not induce systemic graft versus host reaction (GvHR). IEL were unable to induce systemic GvHR even in the presence of accessory cells or lymphokines. In the mouse, IL-2, which is known to enhance a GvHR, did not have any influence on the systemic reaction (22, 25). In an *in vivo* graft versus host assay, popliteal lymph node from mice treated with IEL showed increased weight, (19) which demonstrates that IEL are able to recognize and respond to antigen. *In vitro*, mitomycin treated IEL provided the stimulus for moderate to strong mixed lymphocyte reaction (MLR) response by spleen cells. Proliferative response was strong in allogenic MLR (19, 25), indicating presence of MHC on IEL.

2.1.9 Antibody production

Isolated murine IEL have been examined for surface IgG, IgA, IgM by direct immunofluorescence. The IEL preparations contained an average of 29.1% Ig positive cells (17). Bovine IEL contained 10% IgG positive cells while lamina

propria lymphocytes (LPL) had 28% (10). Freshly isolated murine IEL contained only 0-0.3% IgG+ cells, however after 4 days in culture the percentage of cytoplasmic Ig+ cells increased to 26% (16). In rats, B cells were absent in IEL but were detected in lamina propria lymphocytes (LPL) (28%) and peripheral blood lymphocytes (PBL) (55%) (5). In humans, IEL were found to be IgG negative (18) while others detected <1% cells containing IgG (13). The fact that more recent studies (in the mouse) report no B cells among IEL, and that their appearance was induced by in vitro cultivation (16) suggests that the intestinal epithelium might indeed act as Bursa equivalent in mammals, as previously suggested (32).

2.2 Role of intestinal cells as accessory cells

Peyer's Patches (PP) are currently thought to be the site of the afferent arm of the immune response i.e. antigenic stimulation. Induction of an immune response at the mucosal level requires interaction between lymphocytes and accessory cells. The macrophages have long been recognized to fulfil the function of an accessory cell (33). The major pathway of entry for antigens has been thought to be through M cells overlaying PP. Recently, it was found that mouse and rat absorptive intestinal epithelium express Ia antigens (1, 2, 11) belonging to the Major Histocompatibility Complex (MHC) class II antigens similar to those described on macrophages.

Mouse H-2 or human HLA are membrane glycoproteins that act as restriction elements, regulating the presentation of antigen to immunomodulatory cells. The role of the absorptive intestinal epithelium in the induction and regulation of mucosal immune responsiveness has not been established (1, 2). Ia antigens in the rat intestinal epithelium (11) were detected on the epithelial cells of villi and crypts, with stronger staining in the brush border. Presence of Ia on intestinal absorptive epithelial cells (EC) suggests that they may act as antigen presenting cells. Their putative function is to provide accessory signals to T cells directly at the mucosal portal of entry (2, 3, 11). Presence of HLA (class II) molecule was also reported in the epithelia adjacent to lymphoid tissue in the stomach and in the ileum of healthy humans (34).

2.2.1 Presence and location of Ia antigen

The presence of Ia on intestinal epithelial cells of mice was documented by immunoferritin labelling (35). Staining was distributed on the lateral and basal cell membrane. An immunofluorescence study with monoclonal antibodies (mAb) against Ia revealed both cytoplasmic and membrane staining in the small intestinal epithelium of rats (1). The cytoplasmic staining appeared to be granular and concentrated in the supranuclear region. Membrane staining was both linear and granular, and restricted to the basolateral membrane with occasional Ia expression on

microvilli. Adult rats showed staining for Ia antigens in both villi and crypts, with accentuation in the brush border of the epithelial cells (11). Immunoperoxidase staining of human gut revealed Ia antigens in the enterocytes of villous apexes but not in the cytoplasm of crypt enterocytes (4). In humans, epithelium close to the lymphoid cells was shown to express HLA class II. Expression was independent of cell type. It appears both on the normal and inflamed tissues (34). In humans and in rats, Ia expression on the absorptive epithelium seems to be related to the presence of IEL (34, 11). In humans, Ia was demonstrated on the normal epithelium of terminal ileum, appendix and colon. Appearance of Ia was correlated with the presence of inflammatory reaction (34).

2.2.2 Expression of Ia by epithelial cells of small intestine.

Sixty to seventy percent of epithelial cells (EC) immediately after the isolation expressed cytoplasmic and plasma membrane Ia antigens, as shown by indirect immunofluorescence with monoclonal antibodies (1). Epithelial cells cultured in normal medium did not express Ia antigen (11). In the same study epithelial cells cultured in the medium containing supernatant from Con A stimulated IEL or spleen cells contained an increased number of Ia bearing cells (27-99%), indicating that stimulated lymphocytes were releasing an Ia inducing factor. The

characteristics and the kinetics of the Ia-inducing factor were similar to that of interferon-like activity, but not interleukin 2 (11). Lymphocyte proliferation was not essential for the expression of the Ia on cells, as reported in the study performed in rats (11). Supernatants from lymphocytes (spleen or IEL) treated with protein synthesis blocking agent mitomycin C were able to induce appearance of Ia. Persistence of Ia molecule expression by intestinal epithelial cells requires the lasting presence of Ia inducing factor in the medium (11).

2.2.3 Kinetics of <u>in vitro</u> Ia expression on enterocytes

Ia expression by EC was detectable after 12h of incubation with Con A induced supernatants. Maximum expression was shown when incubation time was extended to 24h (11). The same study also indicated that extension of incubation beyond 24h did not enhance Ia appearance, rather, the activity was decreased.

2.2.4 Synthesis of Ia antigen

Ia expression requires endogenous protein synthesis (11), as shown with the use of agents blocking protein synthesis: cycloheximide or puromycin. The inhibition of Ia expression by the inhibitors of protein synthesis was dose dependent.

2.2.5 Age-dependence of Ia expression

In new born and young rats, and also in fetuses acquired from 10 to 14 days of gestation, mucosal epithelial cells

were found to be Ia negative (11).

2.2.6 Antigen presentation by epithelial cells

Human small intestinal absorptive epithelial cells can present antigen in a way comparable to conventional accessory cells (3). EC pulsed with TT (tetanus toxoid) were able to process and present antigen to tetanus primed T cells in a way similar to control monocytes/dendritic cells. It was also demonstrated that EC were effective stimulators in both autologous and allogeneic MLR. EC treated with mAb directed against Ia were not able to stimulate effector cells in MLR (3) or to induce suppressor cells (2). It was further shown that binding of antigen by rat EC reached a maximum level within 30 min. and was inhibited by lowering the temperature to 4^0C (1). The same study indicated that binding was not receptor specific.

2.2.7 Putative accessory function of EC

Rat T cells co-cultured with EC showed no proliferative response (1, 2). T cells incubated with antigen followed by co-culture with EC responded with proliferation which reached a maximum level at 96h (1). The same study demonstrated that the addition of IL-2 amplified T cell proliferation in MLR (1). A similar study in humans showed that epithelial cells together with T cells caused proliferation of PBL T suppressor cells in MLR (3). It was also shown that culture of T cells with EC and antigen induced antigen-dependent suppressor cells as well as the

expression of suppressor phenotype on the surface of T cells (2). T suppressor cells induced by EC and antigen, caused diminished proliferative response of cocultured lymph node T cells (2). A study in humans revealed that T suppressor generated by EC stimulation, mediated antigen nonspecific suppression in MLR. These cells were able to cause inhibition of T cell proliferation and B cell differentiation (3).

2.3 Interleukin-2

Interleukin-2 (IL-2), formerly known as a T Cell Growth Factor (TCGF), is one of the lymphokines produced by T cells. It is a glycoprotein, and its biological activity is not inactivated by treatment with neuraminidase and glycosidase (37). Biological activity of IL-2 is associated with a globular protein, with a m.w. at 15-20 Kd (36). IL-2 stimulates T cells to undergo cell cycle progression via a specific membrane receptor (38, 39, 36). IL-2 has the ability to promote the long-term culture of normal T lymphocytes from peripheral blood or bone marrow, and of established cell lines of T cytotoxic cells (40,41).

2.3.1 Kinetics of IL-2 production

Minimal IL-2 activity was found in cultures of murine spleen cells stimulated with Con A for less than 12h (less than 0.15 U) and maximal yield occurred between 12 and 48h. Extension of culture beyond 48h resulted in decreased

activity, and 98h of stimulation revealed no detectable activity of IL-2 (41). It was proposed that the loss of IL-2 activity after prolonged Con A stimulation was due to the active removal of the lymphokine by the proliferating cells (41). Pretreatment of murine spleen cells with anti-Thy1 serum and complement (C) resulted in 70% inhibition of IL-2 production in response to Con A stimulation (41). It was further shown that LPS stimulation (B cell mitogen) did not result in IL-2 production, indicating that IL-2 is produced by T cells.

2.3.2 Species crossreactivity

Cross-activity of IL-2 was described in different species (41, 42). It was found that both rat and mouse IL-2, prepared from spleen cells, were able to stimulate murine cytotoxic cells (41). The same study also demonstrated that human IL-2 prepared from PHA stimulated PBL caused proliferation of murine cells. Later it was shown that porcine and bovine IL-2 produced by PBL responded optimally in the homologous systems, whereas bovine IL-2 gave better response in the ovine system than the ovine IL-2 itself (42). That study also showed that IL-2 produced by gibbon cell line MLA 144 reacted well with porcine, ovine and bovine T cell blasts. Porcine IL-2 could be measured on murine blast cells but these cells were 50% less sensitive than the pig system (42). All these results indicate that IL-2 is a highly conserved structure among various species

of mammals.

2.3.3 Mechanism of action of interleukin-2

Interleukin-2 binds to a specific receptor on a cell. Binding of IL-2 to murine cytotoxic T cell line (CTLL) cells is largely reversible, and is characterized by rapid association and slow dissociation (35, 43). These authors also examined the influence of temperature on association and dissociation of IL-2 to receptors. At 37^0C maximum association of radiolabeled IL-2 was completed within 15 min. The rate of dissociation was slower, with the half-time of 60-70 min. Despite degradation, the level of radiolabeled IL-2 associated with the cell surface was stable for at least 60 min. at 37^0C. The number of receptors was also examined. It was found that the number of sites per cell reached 15000 for the CTLL (Cytotoxic T Lymphocyte Line) and 13000 for the HTLL (Helper T Lymphocyte Line). The number of receptors on unstimulated murine splenocytes and thymocytes was below the detectable level, and 200 receptors per cell were detected on human PBL. Upon stimulation with lectins or alloantigens, the level of binding sites dramatically increased (36). Others showed increased level of receptor mRNA after such stimulation (44).

2.3.4 IL-2 producing cells

IL-2 is produced by spleen cells or established T cell lines after stimulation with lectins or antigens. Stimulation of

murine spleen cells with Con A results in IL-2 production (45, 46). Stimulation of spleen cells with LPS does not produce any IL-2 in supernatants (41). T cell clones D1.1, D1.5, D1.6 generated from lymph nodes of Balb/c mice immunized with rabbit IgG, and subjected to multiple cycles of restimulation and rest, also produced IL-2 (47). An adequate source of human IL-2 is provided by PHA stimulated T leukemia cell line (JURKAT) (43). Results of the study in mice indicate that IL-2 secretion and production is limited to T helper cells (46, 47, 45). It was proposed that there were two subsets of murine helper/inducer T lymphocytes. One is Thy1, producing IL-2 and IFN γ, the other is Thy2, which produced IL-4 (47, 45). Stimulation by mitogen causes expression of several lymphokine genes (44). The synthesis follows the pattern of protein synthesis. The induction of IL-2 can be blocked by cyclosporin A (CsA) which blocks transcription (44).

2.3.5 IL-2 production by IEL

Murine IEL stimulated in vitro with Con A produced very low levels of IL-2 as assessed by bioassay and the activity did not increase after a prolonged incubation with Con A (23, 19, 16). The same study revealed that IEL from mice previously infected with Trichinella spiralis produced higher levels of IL-2 (10U/ml).

2.4 Interferon

In 1957 a new factor that interfered with viral growth was discovered (48). This factor was released following the incubation of a heat inactivated virus with a chorio-allantoic (CA) membrane. The substance was called interferon (IFN). The authors reported that the time intervals that elapsed between the application of interfering and challenge viruses had no influence on the degree of interference. The factor was unstable at 37^0C. The membrane extracts had an inhibitory effect on the interfering activity of heated virus. The interfering virus was rapidly taken up by the cell but interference in the cell took some time to establish. There was a difference in time between the onset of the interfering activity in the membrane and the release of the factor into the medium, and the amount of interferon depended upon the amount of the inducing virus used. At that time it was not known whether interferon was part of the virus liberated by CA membrane, or whether it was a soluble cellular product (48). Presently it is known that IFN is a cell coded polypeptide (49). The National Institute of Allergy and Infectious Diseases (U.S.A.) and The World Health Organization define interferon as a protein which exerts virus non-specific antiviral activity in at least homologous cells, through cellular metabolism involving synthesis of RNA and proteins (50, 51).

2.4.1 Types of interferons

Interferons are glycoproteins which are synthesised in cells following viral infection or induction with specific inducers. Interferons are excreted by the cell and interact with replicative events of a broad range of viruses by acting on neighbouring cells and making them resistant to viral infection. In murine and human systems, there are three types of IFN; α, ß and τ. In earlier nomenclature IFN α and ß were described as type I, and IFN τ was described as type II (51). IFN α is produced by leucocytes and IFN ß by fibroblasts (51). Type I IFN is acid (pH 2) and heat stable (51). The molecular weight of human IFN α is about 20000 dalton as determined by gel electrophoresis (52). IFN τ is labile at pH 2 and sensitive to high temperature (53). IFN τ is inducted by a polyclonal mitogen or an antigen stimulation of T cells (51). A significant level of IFN production occurs within one hour of stimulation by polyI-polyC. It reaches peak shortly thereafter and declines by four to six hours (54).

2.4.2 Inducers other than viruses

The interferons can be induced in vivo and in vitro by double-stranded (ds) RNA of viral and bacterial origin (55, 54, 56) or by synthetic inducers like polyI-polyC (55).

2.4.3 Genetic differences in IFN production

Interferon production was compared in four strains of inbred

mice. Differences were noted among C57BL/6, BALB/c, DDD and C3H strains, and they related to IFN inducers used. Newcastle disease virus generated the highest level in C67BL/6 and DDD strain. PolyI-polyC and LPS induced significant amounts in C56BL/6 and C3H/He mice. Sensitization of mice with BCG followed by injection with PPD caused the highest IFN production in C57BL/6 mice (57).

2.4.4 Cross-species activity

Bovine, porcine and human origin cells were protected in vitro by human IFN (58). The effect depended on the virus or inducer used and on the type of IFN. Human leukocyte IFN had greater protective effect than human embryonic IFN (produced by human embryonic fibroblasts stimulated with NDV). Bovine embryonic skin IFN (produced by bovine embryonic skin stimulated by NDV) exhibited slight antiviral effect, and porcine kidney IFN did not exhibit detectable activity on human fibroblasts. Mouse and rabbit cell cultures were not protected by human IFN (58). Human IFN did not produce significant protection of murine cells (55).

2.4.5 Antiviral effect of interferon

Interferons do not interfere with the early events of viral replication, absorption, penetration and uncoating (59). IFN binds to a cell through specific receptor. About 5000 binding sites per cell was reported for Daudi cells (52). All types of human IFN α and ß bind to the same receptor while IFN τ binds to a different receptor (52).

The internalization of murine IFN-receptor complex is temperature sensitive (60). Low temperature (4^0C) prevents internalization. Exposure of a cell to IFN causes an increase in the amount of activated synthetase (61) and kinase (62). After induction with inducers (dsRNA), there is degradation of viral mRNA by synthetase which involves two phases. The first one is activation of cellular mRNA and ATP. The second is endonuclease action which degrades viral mRNA (63). DsRNA promotes phosphorylation of two proteins P1 (m.w.64000) and P2 (m.w.37000). The effect of a kinase is higher if cells are pretreated with IFN for at least 24h (55). Interferon τ may induce a portion of its antiviral activity through the induction of another interferon (64). The authors demonstrated the presence of IFN α in the supernatant fluid of IFN τ treated mouse cells and showed that under certain conditions antiviral activity of IFN τ in mouse and human cells can be reduced by antibodies to IFN α or IFN ß. They also proved that maximal antiviral activity develops as a consequence of the induction of a second interferon (64).

2.4.6 Effect of IFN on cells

The effects depend on the type of the cell and the type of IFN. Bovine IFN causes decreased migration of PMN cells, bacterial phagocytosis, generation of H_2O_2, activity of ADCC and virus-plaque inhibition (65). IFN treatment of bovine colonic mucosal lymphoid cells increased their cytotoxicity

to target cells in ^{51}Cr released assay. In a single cell cytotoxic assay, IFN treatment of gut mucosal NK cells increased efficiency of lysis of target cells as well as fresh epithelial cells (66). The cytotoxic activity of murine IEL in vitro could be increased by treatment with homogeneous IFN ß (30). Expression and shedding of Ia antigens were examined in human cells treated with IFN α and ß. A dose-dependent increased accumulation and shedding of HLA-A,B,C, ß-microglobulin and Ia antigens were observed after treatment with IFN ß. IFN α did not influence Ia expression (7). Pretreatment of adult porcine PBL with IFN in vitro augmented SCMC for PK-15 and PK-15 infected with TGEV (28). Pretreatment of PK-15-TGE cell line with IFN protected PK-15 cells against lysis (28). Mature and inflammatory murine macrophages respond to IFN ß treatment with increased cytotoxicity (67).

2.4.7 Interferon and viral infection

The interferon response during rotavirus infection of calves and transmissible gastroenteritis virus infection (TGEV) was studied in swine (68, 69). In rotavirus infected calves, IFN synthesis was transient and was detected in the intestine and in the blood at the time of the diarrhoeic symptoms. During TGEV infection of the newborn piglet, IFN was found at very high titers in the intestine and other organs, and in the blood and urine. Intestinal synthesis of IFN started a few hours after the onset of diarrhoea and its

duration was short, while high level of serum IFN lasted longer (69). These results suggest an extra-intestinal origin of IFN during TGE infection. The same authors concluded that IFN in calves was synthesized in the intestine, almost exclusively in the jejunum and ileum. In rotavirus infection IFN was characterized as type I. The cell type responsible for interferon production in the intestine was not determined. IFN was found in association with isolated enterocytes, which were obtained by enzymatic digestion of mucosa, but its production by villous epithelial cells was not proven (68). The IFN could protect the host either via a direct antiviral effect, or by indirect effect on the early immune defense mechanisms (NK activity)(69). Correlation between rotavirus excretion in the faeces and IFN production was also investigated (70). IFN production correlated well with periods of virus excretion and the level of IFN production was dependent on the dose and the pathogenicity of virus. With a massive rotavirus inoculum, IFN was produced immediately and no clinical symptoms were observed (70). In another study interferon treatment did not completely inhibit virus replication and secretion, but it reduced the level of rotavirus excretion in stools. Seroconversion was also observed. Interferon was completely cleared from calves within 24h after intra muscular injection. There were two peaks of antiviral activity due to endogenous interferon

production (71). Exogenous recombinant human IFN α (produced by bacteria) reportedly suppressed clinical symptoms in rotavirus infected, colostrum deprived calves (71).

3.0 METHODS

3.1 Animals

Six week old Balb/c male mice (IEL isolation) and retired breeders (hybridoma production), and 200g female Spraque-Dawley rats (Rat Growth Factor production), were purchased from Charles River Laboratory. Animals were given Rat Chow (Purina) and water ad libitum, and taken care according to "Guide to care and use of experimental animals", volume I ans II, available from Canadian Council on Animal Care.

3.2 Media

Roswell Park Memorial Institute (RPMI) 1640 medium, cat.no.430-1800, and Minimal Essential Medium Eagle (MEME), cat.no.410-1600 were purchased from Gibco. Heat inactivated (56^0C, 30 min.) Fetal Bovine Serum (FBS) (Gibco, cat.no. 310-4080) was aliquoted and stored at -90^0C. Bovine testicular hyaluronidase (Sigma Chemical Co., St.Louis, cat.no. H-3884) was dissolved in Ca^{+2} and Mg^{+2} free Dulbecco's Phosphate Buffer Saline (D-PBS) (Gibco, cat.no. 310-4200), dispensed in 1 ml amounts and stored at -90^0C. Glass wool (Fisher, cat.no.11-390) was prewashed in distilled water at 100^0C for 20 min. with three changes of water. The glass wool was then tightly packed up to 10 ml in 50 ml syringes, sterilized and stored at room temperature. Concanavalin A (Con A)(Sigma Chemicals Co, cat.no.C5275) was dissolved in Ca^{+2} Mg^{+2} free PBS (5mg/ml)

and stored at -90^{0}C.

3.3 Cell lines

An Interleukin 2 (IL-2) dependent cell line (CTLL-2) was purchased from American Type Culture Collection (ATCC, cat.no.TIB 214). It was subcultured in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin (Gibco, cat.no.600-5140), 15mM Hepes (Gibco, cat.no.845-1344), 2mM L-Glutamine (Gibco, cat.no. 320-5030), 2mM Sodium pyruvate (Gibco, cat.no. 890-1840), in a humidified atmosphere with 5% CO_2 at 37^{0}C. The medium is referred to as RPMI complete medium (RPMI-C). The medium was supplemented with 40% Rat Growth Factor (RGF) (see section 2.4). Cells were grown at very low densities, and they were passed every time the concentration reached 1x10^5/ml. They were centrifuged (600xg for 10 min.) and resuspended to 1-2x10^4/ml. The interferon sensitive murine cell line L929 (kindly provided by Dr.S.H.S.Lee from Dalhousie University, Halifax) was grown in MEME supplemented with 10% FBS and penicillin-streptomycin (MEME-CM). Cells were passed at the split ratio 1:2 each time they reached confluence. Hybridoma DMS1, producing monoclonal antibodies to IL-2, was kindly supplied by Dr.Kendall Smith from Dartmouth Medical School, Hanover in New Hampshire, USA. The hybridoma cells were grown in RPMI supplemented with 15% FBS, 2mM Sodium pyruvate, penicillin-streptomycin, 2.5g/l sucrose (Sigma Chemical Co, cat.no. S

1888), 2mM glutamine and it was grown in the presence of macrophages or medium in which peritoneal macrophages were grown (see section 2.6).

3.4 Rat growth factor

RGF was prepared according to ATCC instruction. Spleens were removed aseptically from female Spraque-Dawley rats after euthanasia with halothane overdose. They were homogenized by forcing tissue fragments through a No.60 mesh sieve (Belco Biotechnology, cat.no. 1985-00060) using a syringe plunger. The cells were washed three times with RPMI 1640 and resuspended at the density of $1.0-1.5x10^6$ of viable cells /ml in $150cm^2$ flasks containing 150-200 ml of RPMI. Culture medium consisted of RPMI 1640, 1% heat inactivated FBS, 0.05mM ß mercaptoethanol (Sigma Chemical Co, cat.no.M-6250), 15 mM Hepes, 100U/ml penicillin, 100µg/ml streptomycin and 1.0 µg/ml Con A. The yield of viable cells was $39.8x10^6$/ml. The cells were incubated at 37^0C in a 5% CO_2 humidified atmosphere for 48h. The supernatant was checked before collection for bacterial contamination by inoculation on blood agar and into broth. Supernatant was harvested by centrifugation at 16,000xg/10 min. at 4^0C, filter sterilized through a 0.22 micron membrane, aliquoted, and stored in -90^0C.

3.5 Preparation and purification of monoclonal antibodies

The DMS-1 hybridoma producing antibodies to IL-2 was grown in RPMI. Balb/c female mice (retired breeders), were given

0.5ml/mouse of Pristine (Sigma Chemical Company, cat.no.P-1403) intraperitoneally. Ten to fourteen days later $3x10^6$ hybridoma cells in 1ml of PBS were given intraperitoneally per mouse. Ascites developed within the next 3 weeks, and the fluid was collected by peritoneal lavage with 3x7 ml of RPMI with antibiotics. Purification by affinity chromatography with Protein A was performed (72). Briefly, ascites fluid was filtered through the Whatman No.1 filter paper. The sample was mixed 1:1 with the binding buffer containing 1.5 M glycine and 3 M NaCl (pH 8.9). Purification was performed on a Protein A-Sepharose (Pharmacia) CL-4B column. The column was washed with 50 ml of deionized water, then primed with 10ml of a starting buffer (0.1M Phosphate, pH 7.0). One ml of Protein A-Sepharose CL-4B was added to the column. The flow rate was set at 15-20 ml/h. When sedimentation was completed the column was washed with 10 bed volumes of binding buffer. The sample was added when the buffer from above the gel was drained. Once the sample had been loaded on the gel, binding buffer was added and a column was run until the spectrophotometrical reading returned to the baseline. Elution of immmunoglobin was performed by addition of 0.1 M citric acid buffer, pH 6. The IgG1 fraction was collected on fraction collector (LKB Bromma). The protein content was assessed spectrophotometrically (LKB Biochrom Ultrospec II). The fraction with the highest protein content was desalted

on 5 ml Sephadex G-25 column (Pharmacia). The column was washed with 25 ml of PBS and then 2.5 ml of sample was added. After the sample was desalted the column was washed with 3.5 ml of PBS, pH 7.27. The fractions containing antibodies with lower protein content was dialysed against 1000 volumes of PBS overnight. Final concentration of antibodies was 0.596 mg/ml for the sample dialysed against PBS, and 0.769 mg/ml for the sample passed through the Sephadex column.

3.6 Macrophage isolation

Mice were killed by cervical dislocation. The skin of the abdominal wall was washed with 70% alcohol, cut longitudinally and dissected. The peritoneal cavity was lavaged with RPMI 1640 supplemented with penicillin and streptomycin using an 18 gauge needle attached to a 10 ml syringe. The abdomen was flushed 4 times, and the cells were removed by centrifugation at 600xg for 10 min. The supernatant was discarded and the pellet resuspended in 2 ml of RPMI CM. Macrophages were cultured in a 96 well plate (Corning, cat.no. 25860) at a density of $2x10^4$/well/200 μl and incubated at 37^0C for 2h prior to the addition to IEL.

3.7 IEL isolation

The method of intraluminal digestion was adopted (17). Six week old male Balb/c mice were given Apo-metronidazole (lot no. 74610) in drinking water (2g/l) 48h before they were sacrificed (31), in order to eliminate protozoal parasites.

Three mice were used for collection of intraepithelial lymphocytes (IEL) at each time. The small intestines were dissected, an 18 gauge needle with tubing was inserted into the lumina and the contents were flushed with 20 ml of Ca^{+2} and Mg^{+2} free PBS. Three millilitres of hyaluronidase (4700U/ml) were instilled into each intestine. Both ends of intestines were clamped with haemostats, and the intestines were kept on ice until digestion, which took place in a water bath at $37^{0}C$ for 1h. The intestines were then flushed with 20 ml RPMI-CM [RPMI, 10% FBS, 50 μl/ml gentamycin (Gibco, cat.no. 600-5710)]. The contents were divided in two equal parts. One part, which was not processed further, is referred to as intraepithelial lymphocytes with epithelial cells (IEL+ep). The second part was vortexed with glass beads for one minute to break the cell clumps and to release free lymphocytes. The cells were then filtered through prewashed glass wool. The cells recovered by this procedure are referred to as pure intraepithelial lymphocytes (pIEL). The suspension was centrifuged at 70xg for 5 min., the supernatant was discarded and the pellet resuspended in 1 ml of RPMI-CM. The cell viability was determined by Trypan Blue exclusion. The cells were counted in a hemocytometer, resuspended to $2x10^{6}$ cells/ml and 100 μl was placed in wells of flat bottom microtiter plates. The pIEL were cultured either alone (pIEL) or with macrophages ($2x10^{4}$/well)(M+IEL). The cells were stimulated with Con A

(1μg/100μl/ well) for 48h at 37^{0}C in 5% CO_2 humidified atmosphere. The culture was then collected and centrifuged at 600xg for 10 min. The supernatant was stored at -90^{0}C until tested.

3.8 Spleen lymphocytes

Spleens were minced through a wire mesh screen with a syringe plunger and passed through a 23 gauge needle to break the cell clumps. The cells were centrifuged at 600xg for 10 min. The red blood cells were lysed by adding 0.2 ml of sterile distilled water to the pelleted cells. The cells were vortexed for 20 sec and the same volume of double strength RPMI was added. The lymphocytes were then washed three times. The viable cells (Spl) were resuspended at the density of 1x10^6/ml and 200 μl placed into wells of 96 well flat bottom microtiter plate.

3.9 Bioassay

The method of Palladino et. al., (73) was modified and adopted. The CTLL-2 cell line was grown in RPMI-C supplemented with 40% of RGF. The cells were washed 3 times in RPMI before use, to eliminate lymphokines obtained in RGF. Viability was determined by the trypan blue exclusion test. The cells were seeded in flat bottom 96 well plates (Corning, cat.no. 25860) at the density of 1x10^4/well/ 100μl. The supernatants from Spl, M, M+IEL, pIEL, IEL+ep cells were added to each well (100μl/well) in three replicates. The plates were incubated for 24h at 37^{0}C in 5%

CO_2 humidified atmosphere. The following day the cell cultures were pulsed with 1μCi/μl/well of H^3 Thymidine (ICN Chemicals, specific activity 20-40 Ci/mM), and incubated for 6h. The cells were harvested using an automated cell harvester (Skatron, A.S. Lier, type 7020) onto glass fibre filter disks (Skatron Lies, cat.no. SK-7031). After collection, the filter disks were dried in the oven. Each disk was put into a separate scintillation vial (Fisher, cat.no. 03-337-1A), to which 4 ml of scintillation cocktail (1.25gm of POPOP and 100gm of PPO in 1L of toluene) (ICN Chemicals, cat.no. 882427) was added. The cells (CTLL-2) plus complete medium (RPMI-CM) were used as a control. The samples were counted in a Beckman Scintillation Counter (model LS1701).

3.10 IL-2 neutralization assay

The rationale behind performance of this assay was to determine whether the stimulation of the indicator CTLL cells by the supernatants of the spleen cells was indeed due to IL-2. The CTLL cell line is widely accepted as a IL-2 dependent cell line. Serial two-fold dilutions of supernatants from Con A stimulated spleen cells were made, and 50 μl of each was dispensed to 96 well plates in triplicates. Each dilution was mixed with an equal volume of 100μg/ml of mAb to IL-2 and incubated for 1h at 37^0C. After the incubation, 100 μl of $1x10^4$ CTLL were added to each well, and further incubation took place for 24h. The

cells were pulsed for 6 h with 1μCi/well of H^3 thymidine. The cells were harvested on a filter paper, and after the paper was dried it was placed in 4 ml of scintillation cocktail for counting.

3.11 Elisa for IL-2 detection

The modified method of Smith (74, 75) was used. Immunolon 2 removawells (Dynatech Lab.Inc., cat.no. 011-010-6302) were used for the antigen coating in an indirect test. One hundred μl/well of supernatant from Con A stimulated Spl cells, M, M+IEL, pIEL, IEL+ep were dispensed in triplicates wells. RPMI-CM was used as a control. The wells were incubated for 4h at 37^0C. The supernatants were discarded, and wells were treated with a blocking buffer [(3% BSA Sigma, cat.no.A-7030) in D-PBS with Ca^{+2}, Mg^{+2} (Gibco)], and incubated for 1h at 37^0C. The wells were then washed 5 times with a washing buffer containing D-PBS supplemented with $CaCl_2$ (Gibco) and 0.05% Tween 20 (J.T.Baker Chemicals Inc., cat.no. 9005-64-5). The mAb against IL-2 were suspended in D-PBS with Ca^{+2}, Mg^{+2} (Gibco) at concentration of 10μg/ml, and 100 μl of the working dilution was dispensed into each well. The plate was incubated overnight at 37^0C. On the following day, the wells were washed 5 times with washing buffer. A working dilution (1:5000) of goat-anti mouse IgG conjugated to alkaline phosphate (Boehringer-Mannheim, cat.no.605260) was added to each well (100μl/well), and incubated for 1h at 37^0C. The wells were

washed again 5 times, and 100 μl of substrate prepared according to the manufacturer's instructions were added (4-nitrophenyl phosphate cat.no. 726923 in substrate buffer cat.no. 7266915, both Boehringer-Mannheim). After 30 min. of incubation at 37^0C the readings were taken at wavelength of 405 nm using microplate Elisa reader (Dynatech MR600).

3.12 Virus

The Indiana strain of Vesicular Stomatitis Virus (VSV) was obtained from Dr.J.B. Derbyshire, University of Guelph. The virus was adapted to growth in L929 cells. The cytopathic effect (CPE) was seen after 16h of incubation at 37^0C. The flask was subjected to one freeze-thaw cycle (-90^0C) to disrupt cell membranes and to release the virus. The culture medium was then centrifuged at 200xg for 10 min. to remove the cell debris, and the supernatant was stored in -90^0C.

3.13 Tissue culture infective dose 50 ($TCID_{50}$)

Cells L929 were seeded in a 96 well plate at the density of $3x10^4$ cells/well. Twenty four hours later, 25 μl of each of the serial 10 fold dilutions of virus made in serum free medium, were added to 6 wells. The plate was incubated for 48h at 37^0C in a CO_2 humidified atmosphere. The CPE was monitored under an inverted microscope. Two hundred $TCID_{50}$ were used for the interferon assay.

3.14 Interferon assay

The method described by Steward (50) was used. Serial

dilutions of supernatants from Spl, M, M+IEL, pIEL, IEL+ep cells were prepared in a 96 well plate in MEME-C using titertek octapet (Flow, cat.no. 77-942-00). Fifty μl of medium were introduced into each well, and 25 μl of the sample was pipetted into the first row. The solution was mixed and 25 μl were transferred to the next row. The L929 cells were seeded at the density of $3x10^4$ cells/well in 100 μl of the MEME-CM. Cultures were incubated for 24h at 37^0C. Twenty four hours later 50 μl of 200 $TCID_{50}$ of VSV were dispensed into each well and left for incubation for further 26h. The controls contained cells without virus and cells with virus, but no IFN. At 26h the test was read under an inverted microscope. The highest sample dilution that inhibited CPE in 50% of wells represented the titre of interferon.

3.15 Statistical analysis

The analysis of the bioassay was done on the Minitab program using Student's T test. Statistical value of the bioassay neutralization test was measured by analyzing differences between points of lines by regression analysis and T test. Results of the Elisa were transformed into logarithmic form (Log_{10}) for stabilization and SAS General Linear Models Procedure was applied. Means of groups were compared by multiple comparison with application of the the Student-Newman-Keuls test. The P value for all tests applied was set at 0.05 level.

4.0 RESULTS

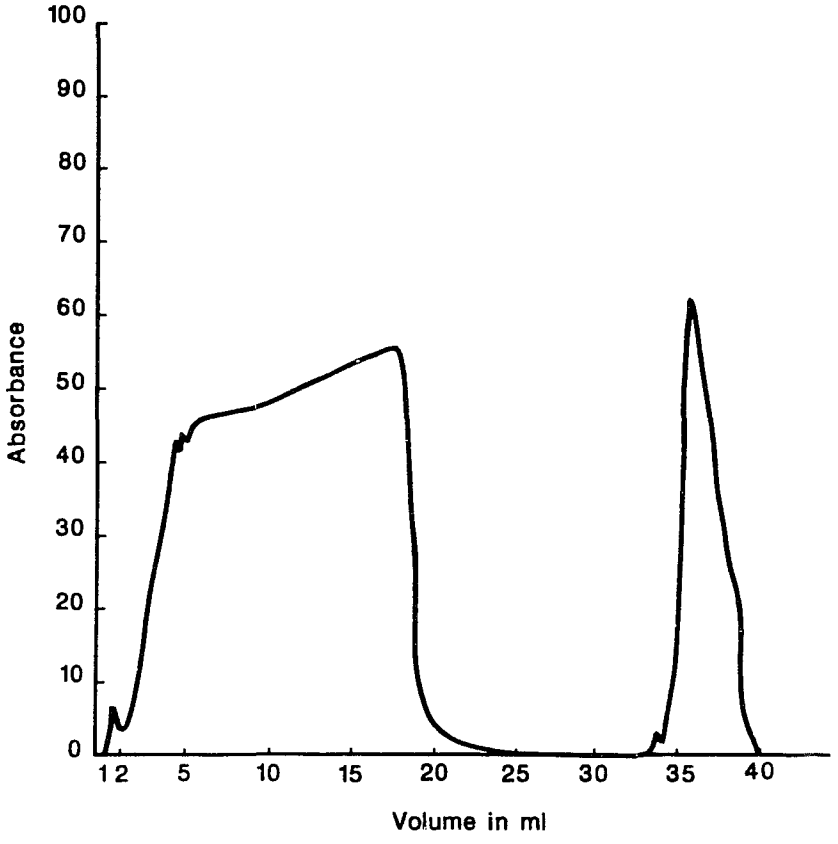
4.1 Purification of monoclonal antibodies (mAb) from ascites fluid

MAb specific to IL-2 were purified on protein A-Sepharose. The specificity of this mAbs for IL-2 was established by Smith (1986). One ml fractions were collected and the protein content was assessed spectrophotometrically at the wavelength of 280 nm (Figure 1). The amount of protein in mg/ml was calculated by dividing the spectrophotometric reading by 1.4. Fractions contained from 0.596 to 0.769 mg protein/ml of protein per ml. Fractions with the highest protein content were desalted on Sephadex G-25 column, while the fractions with lower readings were desalted by dialysis against 10 volumes of PBS for 24h.

4.2 Isolation of IEL and collection of macrophages

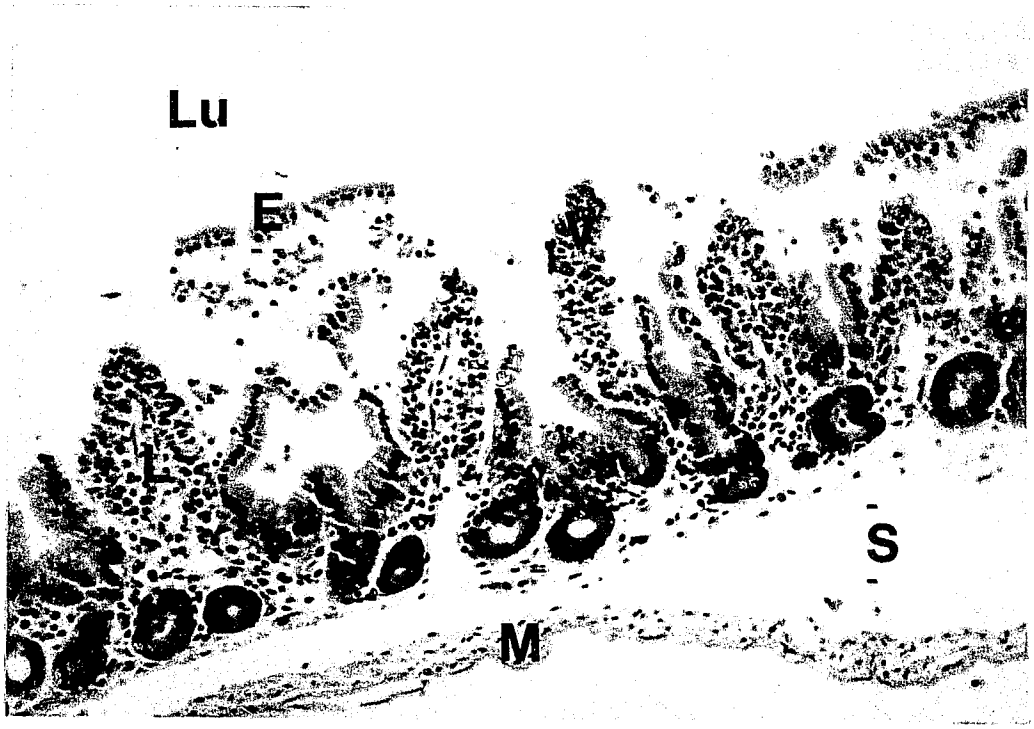
The effect of hyaluronidase on detachment of the epithelium was evaluated histologically. Incubation for 1h at 37^0C caused detachment of the epithelial layer from the lamina propria. Lifting of the epithelium was most pronounced in the area of the tips of the villi and sometimes was observed on the entire villi (Figure 2). Histological examination confirmed that the LP was not digested. The yield of pIEL from three mice varied from $2.35x10^6$/ml to $4.53x10^6$/ml (Table I). The cells were checked for viability by trypan blue exclusion (Table I), and stained smears (Wright Giemsa)

Figure 1: Diagram of antibody purification from mouse ascities



Purification of monoclonal antibodies (IgG-1) to interleukin-2 (IL-2) from mouse ascities by Protein A-Sepharose CL-4B chromatography.

Figure 2: Photomicrograph of mouse small intestine after enzymatic digestion



Enzymatic digestion of intestine with hyaluronidase was examined on histological sections stained with Harris Hematoxilin (magnification 100x times). Digested epithelium (E) was detached form villi (V) and remains of it are seen in the lumen (Lu). Lamina propria (L), crypts (C), submucosa (S) and muscularis (M) were intact.

Table I: Isolation of IEL and macrophages

Cell Source	Experiment number						
	1	2	3	4	5	6	7
IEL [a]	1.39(82%)	2.53(88%)	3.5(96%)	3.52(93%)	3.98(93%)	4.53(97%)	2.35(93%)
Macrophages [b]	0.43	0.43	0.72	2.21	4.82	3.44	3.35

a total cell count in millions, obtained from three mice by method of enzymatic digestion. Numbers in brackets indicate percentage of viable cells.
b total count in millions obtained from one mouse.

were prepared for assessment of morphology. The macrophage yield obtained from one mouse was from $0.215x10^6$/ml to $3.6x10^6$/ml.

3.3 Detection of IL-2 by Bioassay

A significant stimulation of CTLL cells occurred with supernatants of spleen cells. No stimulation was observed with the supernatants from pure IEL, IEL with epithelial cells and IEL with macrophages (Table II).

3.4 Detection of IL-2 by Elisa

The means of IL-2 levels detected in all samples were significantly different from controls (Table III). The IL-2 level in IEL+ep cells supernatant was significantly different from that in pIEL. Readings from supernatants from pIEL and spleen cells fell into the same group and were not significantly different from each other. The mean values of IL-2 detected in the supernatants of M+IEL were not significantly different from either pIEL, spleen cells or macrophages.

3.5 Neutralization assay of IL-2 activity

Incubation of the supernatant from spleen lymphocytes with mAb to IL-2 resulted in a significant decrease of stimulation of CTLL cells (Figure 3).

3.6 Comparison of bioassay and Elisa

Since Elisa and bioassay gave contradictory results, identical dilutions of spleen and IEL+ep cell supernatants were analyzed by both assays. Bioassay (Figure 4a) showed a

Table II: Detection of IL-2 by bioassay in supernatants of ConA stimulated cells

Cell source	Experiment number													
	1		2		3		4		5		6		7	
contr[a]	162[f]	(1)[g]	162	(1)	372	(1)	372	(1)	734	(1)	734	(1)	734	(1)
spleen*	5548	(34.03)	12875	(78.98)	151717	(407.84)	55440	(149.03)	32931	(66.39)	75821	(154.88)	41054	(82.77)
Macro[b]	108	(0.66)	108	(0.66)	647	(1.74)	582	(1.56)	744	(1.51)	832	(1.68)	592	(0.89)
M+IEL[c]	130	(0.80)	122	(0.75)	218	(0.58)	386	(1.04)	476	(0.96)	577	(1.16)	452	(0.91)
pIEL[d]	131	(0.81)	128	(0.79)	198	(0.53)	329	(0.88)	423	(0.8?)	1761	(3.55)	233	(0.47)
IEL+ep[e]	168	(1.04)	162	(0.94)	401	(1.08)	193	(0.52)	554	(1.12)	238	(0.48)	233	(0.47)

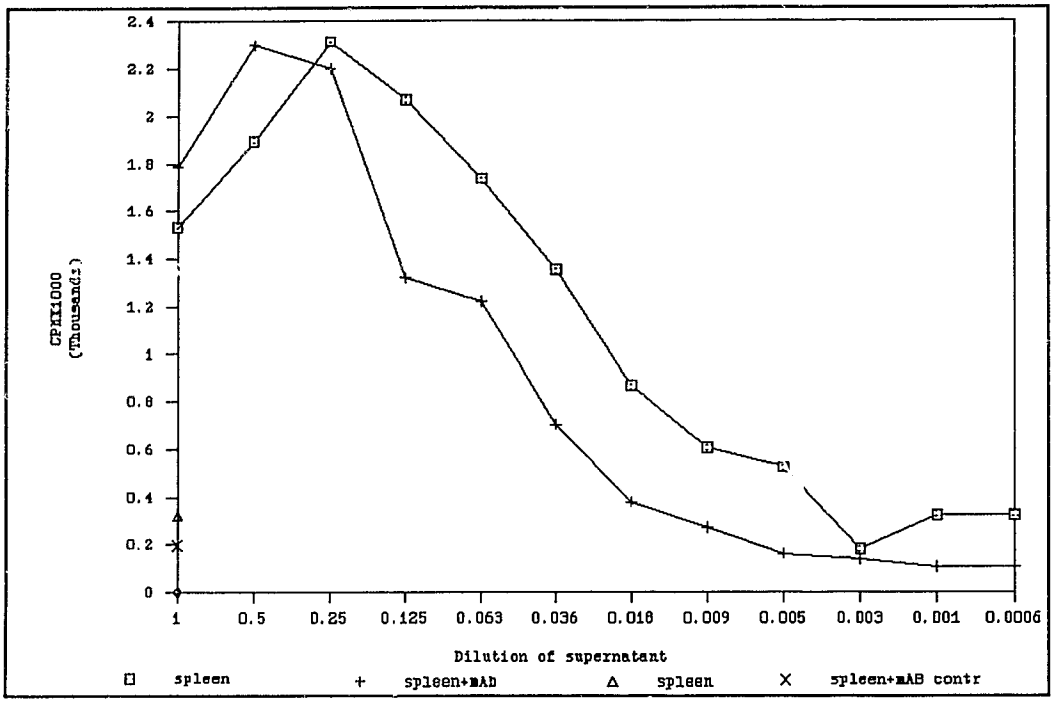
a - CTLL-2 cells cultured with controled medium alone (RPMI-C)
b - peritoneal macrophages
c - peritoneal macrophages and intraepithelial lymphocytes
d - pure intraepithelial lymphocytes
e - intraepithelial lymphocytes and epithelial cells
f - count per minute (CPM), mean of three replicates
g - stimulation index (Mean reading of sample/meadn reading of control for given experiment)
* - results statistically significantly different from controls (P=0.05)

Table III: Detection of IL-2 by Elisa in supernatants of ConA stimulated cells

Cell source	Experiment number							Mean *
	1	2	3	4	5	6	7	
contr[a]	0.162[f]	0.162	0.176	0.176	0.140	0.140	0.140	**0.157 (D)**
spleen	0.255	0.249	0.202	0.166	0.194	0.332	0.315	**0.249 (B)**
macro[b]	0.202	0.239	0.175	0.197	0.189	0.269	0.124	**0.199 (C)**
M+IEL[c]	0.203	0.211	0.265	0.244	0.157	0.283	0.120[g]	**0.234 (B,C)**
pIEL[d]	0.275	0.239	0.277	0.233	0.268	0.310	0.130[g]	**0.259 (B)**
IEL+ep[e]	0.623	0.576	0.932	0.688	0.942	0.634	0.895	**0.756 (A)**

a - medium alone (RPMI-CM)
b - peritoneal macrophages
c - peritoneal macrophages and intraepithelial lymphocytes
d - pure intraepithelial lymphocytes
e - intraepithelial lymphocytes and epithelial cells
f - optical density (OD), mean of three replicates
g - only one replicate
* - mean of 21 data within group (contr, spleen, macro, M+IEL, pIEL, IEL+ep). Means labeled with different letters are significantly different from each other (P=0.05). Means labeled with the same letter are not significantly different from each other(P=0.05). Mean of cell population M+IEL falls into groups B and C which indicates that it is not significantly different from either of them.

Figure 3: Diagram of IL-2 neutralization by mAb



The effect of mAb to IL-2 on the bioassay activity of the supernatant from spleen cells.

Figure 4: Comparison of Elisa and bioassay for IL-2 detection

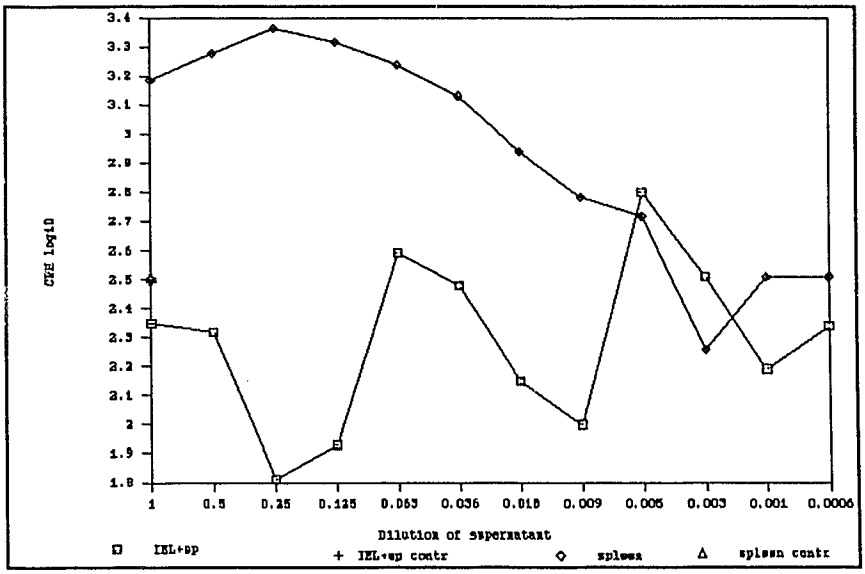


Figure 4a: Determination of IL-2 in spleen and IEL+ep cells supernatants by bioassay

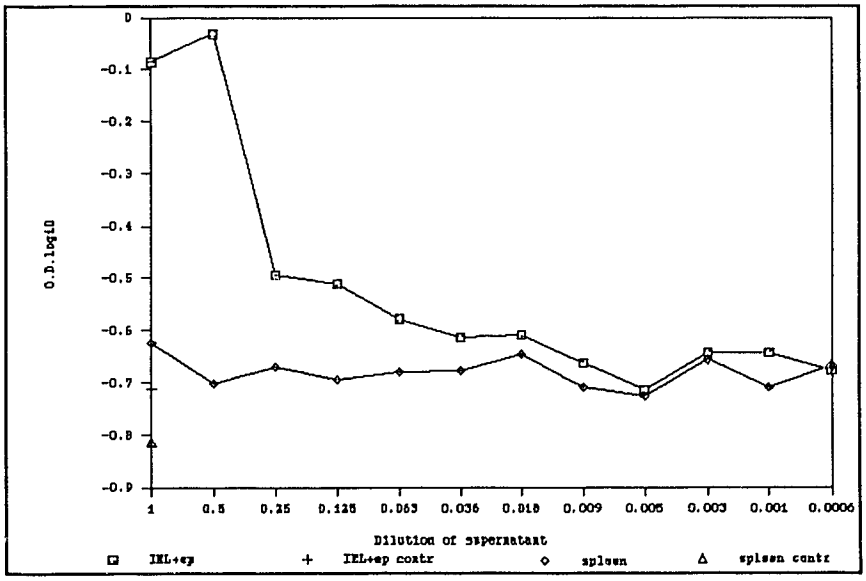


Figure 4b: Determination of IL-2 in spleen and IEL+ep cells supernatants by Elisa

dose dependent stimulation with spleen supernatant but no significant reading with IEL+ep cells population. Elisa (Figure 4b) gave a dose dependent reading with the IEL+ep supernatant but no response was observed with the spleen cell supernatant.

3.7 Interferon assay

Results are summarized in Table IV. Supernatants from Spl, M, M+IEL and IEL+ep cells were tested. Due to insufficient sample, pIEL supernatants were tested only in exp 4, 5, 6. In experiments 1, 2, 3, and 5 the level of IFN produced by IEL+ep cells was higher than the level of IFN in spleen cells supernatant. In experiments 6, 7, 8, on the other hand, spleen cells produced higher amounts of IFN then IEL+ep cells. The variation observed could have been caused by the differences in physiological activities of the intestines at the time of IEL isolation. Interferon was detected in the supernatants of macrophages in experiments 2, 4 and 6.

Table IV: Detection of IFN in supernatants of ConA stimulated cells

	Exp 1					Exp 2				
Cell source	Spl[a]	M[b]	M+ IEL[c]	IEL[d]	IEL +ep[e]	Spl	M	M+ IEL	IEL	IEL +ep
Dil										
1:3	+	-	ND	ND	+	+	+	ND	ND	+
1:9	+	-	ND	ND	+	+	-	ND	ND	+
1:27	+	-	ND	ND	+	+	-	ND	ND	+
1:71	-	-	ND	ND	+	-	-	ND	ND	+
1:213	-	-	ND	ND	-	-	-	ND	ND	-
1:639	-	-	ND	ND	-	-	-	ND	ND	-
1:1917	-	-	ND	ND	-	-	-	ND	ND	-
1:5751	-	-	ND	ND	-	-	-	ND	ND	-

	Exp 3					Exp 4				
cell source	Spl	M	M+ IEL	IEL	IEL +ep	Spl	M	M+ IEL	IEL	IEL +ep
Dil										
1:3	+	-	ND	ND	+	+	+	+	-	ND
1:9	+	-	ND	ND	+	+	+	+	-	ND
1:27	-	-	ND	ND	+	+	+	-	-	ND
1:71	-	-	ND	ND	+	+	+	-	-	ND
1:213	-	-	ND	ND	+	-	-	-	-	ND
1:639	-	-	ND	ND	-	-	-	-	-	ND
1:1917	-	-	ND	ND	-	-	-	-	-	ND
1:5751	-	-	ND	ND	-	-	-	-	-	ND

Table IV continued p.50

Table IV continued

	Exp 5					Exp 6				
Cell source	Spl	M	M+ IEL	IEL	IEL +ep	Spl	M	M+ IEL	IEL	IEL +ep
Dil										
1:3	+	-	+	+	+	+	+	+	-	-
1:9	+	-	+	+	+	+	-	+	-	-
1:27	+	-	-	+	+	+	-	+	-	-
1:71	+	-	-	+	+	+	-	-	-	-
1:213	-	-	-	+	+	+	-	-	-	-
1:639	-	-	-	+	+	+	-	-	-	-
1:1917	-	-	-	-	+	-	-	-	-	-
1:5751	-	-	-	-	-	-	-	-	-	-

	Exp 7					Exp 8				
Cell source	Spl	M	M+ IEL	IEL	IEL +ep	Spl	M	M+ IEL	IEL	IEL +ep
Dil										
1:3	+	-	-	+	+	+	-	ND	ND	+
1:9	+	-	-	+	+	+	-	ND	ND	+
1:27	+	-	-	+	+	+	-	ND	ND	+
1:71	+	-	-	+	+	+	-	ND	ND	+
1:213	+	-	-	+	-	+	-	ND	ND	-
1:639	+	-	-	+	-	+	-	ND	ND	-
1:1917	-	-	-	+	-	+	-	ND	ND	-
1:5751	-	-	-	+	-	-	-	ND	ND	-

\+ IFN present (no VSV CPE)
\- IFN absent (VSV CPE)
a - spleen cells
b - peritoneal macrophages
c - peritoneal macrophages and intraepithelial lymphocytes
d - pure intraepithelial lymphocytes
e - intraepithelial lymphocytes and epithelial cells

5.0 DISCUSSION

5.1 The isolation of IEL

The method of intraluminal enzymatic digestion was adopted for liberation of IEL (17). The digestion method with hyaluronidase was employed in order to release IEL from the epithelial layer without disturbing the integrity of LP. The technique allowed contact of the entire mucosal membrane with the enzyme. Only very mild massage of intestines with digested epithelium, resulted in release of IEL and preservation of intact lamina propria (Figure 2, p.41).

5.2 Bioassay and Elisa results

Significant stimulation ($P< 0.05$) of CTLL-2 cells cultured with the supernatant of spleen cells was observed in the bioassay. No stimulation was obtained with the supernatants from pure IEL, IEL with epithelial cells, macrophages and IEL with macrophages (Table II, p.44). This is in disagreement with the results of Elisa where supernatants of IEL+ep, pIEL, M+IEL, spleen and macrophages gave statistically significant results from controls (Table III, p.45). Production of IL-2 by macrophages can be explained by contamination of this cell population with lymphocytes. Although the Elisa readings in the supernatants of spleen cells were low, these levels of IL-2 were detectable by the bioassay. On the other hand, the Elisa readings were high in the supernatants of IEL+ep but no IL-2 was detected by

the bioassay. These contradictory results support the view that the bioassay was negatively influenced by unrelated substances, and the following discussion represents an attempt to reconcile these findings.

5.2.1 CTLL indicator cells and the specificity of their response.

Structural similarities and functional cross-reactivity among individual lymphokines (38) have been observed. Although the CTLL-2 cell line is widely accepted to be dependent on IL-2 for growth, significant stimulation of this line with IL-4 was reported (46). Other lymphokines like granulocyte macrophage-colony stimulating factor (GM-CSF), colony stimulating factor (CSF-1), interleukin-1 (IL-1), interleukin-3 (IL-3) and INF gamma did not induce proliferation of CTLL-2 cells (46). Thus the bioassay may have detected IL-4 in the spleen cell supernatants, in addition to or rather than IL-2, and this is supported by the results of the IL-2 neutralization test (Figure 3, p.46). Only approximately one third of the bioassay response was neutralizable by mAb against IL-2, and thus was due to IL-2. The rest of the activity was due to an unrelated substance, probably IL-4 (46).

The levels of IL-2 detected by the bioassay in the supernatants other than from spleen cells, may have been masked by the negative effect of other lymphokines (e.g. IFN) on the indicator cells. High levels of interferon were

detected in the supernatants of IEL (Table IV, p.47); others also found that spleen cells yielded low titers of IL-2 when measured by a bioassay (76).

It was also reported that certain concentrations of phorbol myristate acetate (PMA) can mimic IL-2 activity giving high background readings, indicating that low levels of IL-2 can not be distinguished from PMA effects (46). It is, therefore, possible that Con A might have had the same effect on CTLL cells. However, lack of CTLL-2 stimulation by supernatants of IEL, IEL with epithelial cells, and IEL with macrophages indicated that Con A was not responsible for the stimulation of the indicator CTLL cells.

Low yields of IL-2 detected in the supernatants of IEL by Elisa may have been caused by the low frequency of the IL-2 producing cells. It was reported that mouse PBL of helper phenotype (L3T4+) had a high frequency of IL-2 producing cells (1:1, 1:2), higher than spleen (1:7-1:17), and PBL of Lyt2+ phenotype (suppressor cells) (1:11-1:28) (46). Elimination of L3T4+ cells by monoclonal antibodies and complement caused 99% decrease in IL-2 production. Others reported that cells producing IL-2 belong to a subpopulation of T helper cells called Th1 (45, 47). Most of the cells isolated from small intestine epithelium, however, are of T cytotoxic-suppressor phenotype. The reported percentage of T suppressor phenotype in the intestinal epithelium is 68% and that of T helper is 10% (5,

17, 18, 12, 15). The small percentage of T helper cells is probably responsible for the low IL-2 level detected in the supernatants of Con A stimulated IEL. A recently published report also indicates low production of IL-2 by murine IEL when tested by the bioassay (19). Interleukin 2 binds to activated T cells by means of specific membrane receptors. The rate of T cell proliferation is dependent on the concentrations of free IL-2 available to the cells, cellular IL-2 receptor concentration, and the affinity of IL-2 for the receptor (38). Since CTLL cells were cultured in the conditioned medium containing lymphokines and were subcultured not later than two days before performing the bioassay, it is presumed that the cells maintained a high level of the receptor expression, and that T cell activation was dependent only on IL-2 availability.

5.2.2 Proliferation inhibiting factors

Low bioassay readings with supernatants from IEL+ep cells (Table II, p.44) were in disagreement with the high reading of the same supernatants in Elisa test (Table III, p.45). Non-dialysable factors produced by epithelial cells and capable of inhibiting proliferation of T cells (7, 32), may have been responsible for inhibition of proliferation of CTLL-2 despite the presence of IL-2. Others speculated that contamination of IEL with epithelial cells as well as contamination with the intestinal content may result in altered responsiveness of IEL (18). The fact that the

epithelial cells when cultured with IEL stimulated IL-2 production, as measured by Elisa, argues against the negative influences of the epithelial cells on IL-2 production by IEL in our procedure.

A possible explanation for the low IL-2 reading in bioassay may be the presence of IFN τ in the supernatants of stimulated IEL and epithelial cells (Table IV, p.49, 50). IFN was detected in the supernatants (Table IV, p.49, 50) and it may have inhibited proliferation of CTLL cells. Inhibition of CTLL proliferation beyond the level of the controls supports this thesis. Bovine IFN α and τ was reported to reduce the proliferative response of PBL to mitogens for 24h after treatment, and effect of unresponsiveness was overcome by addition of IL-2 (65). In the mouse system IFN ß was shown to activate mouse spleen suppressor cells which in turn produce a factor suppressing immune response (77). It is also possible that stimulated IEL suppressor cells consumed IL-2 produced by T helper, consequently leading to a low bioassay reading, however this mechanism would have inhibited IL-2 levels detected by both assays. The response of bovine PBL of T cells lineage to IL-2 was inhibited by INF α and τ in a dose dependent manner (65). The high Elisa reading observed in supernatants of IEL with epithelial cells (Table III, p.45) is interpreted as a provision of an accessory signal by the epithelial cells to IEL. Enhanced polyclonal stimulation of IEL in the

presence of spleen cells or macrophages was demonstrated previously (23, 25). The fact that these supernatants were not active in a bioassay would argue for the presence of a T cell proliferation inhibition substance e.g. IFN, causing the bioassay to be negative. IFN presence was detected in intestines of calves inoculated with rotavirus (68, 69, 71). This IFN was described as type I according to physico-chemical properties but its origin was not defined. IFN was found in intestinal fractions containing isolated enterocytes but it was not determined whether it was produced by villous epithelial cells. It was further observed that although intestinal IFN appeared at the same time as systemic IFN, the titre of intestinal IFN was higher. We demonstrated IFN in the supernatants of Con A stimulated spleen cells and ILE+ep cells (Table IV, p.49, 50). Thus the sensitivity of the bioassay might have been decreased by IFN. IL-2 production in the supernatants of polyclonally stimulated spleen cells and IEL, as assessed by bioassay and Elisa, is difficult to compare because of the possible positive influence of IL-4, and negative influence of IFN on the bioassay. CTLL-2 stimulation would, therefore, not be reflective only of IL-2 content but would also reflect presence of IL-4 and IFN. Elisa on the other hand should have detected IL-2 with high specificity (43), and it should not have been influenced by the presence of other factors in the supernatants.

5.3 Interaction between IEL and epithelial cells

The presence of Ia molecules on intestinal epithelial cells and their ability to process and present antigen to T lymphocytes by those cells was recently documented in mice (1, 2) and humans (3). It was shown that Ia+ EC are located in close proximity to lymphoid tissue (34). Moreover, in both humans and mice, epithelial cells presenting antigens to lymphocytes were capable of activation of T suppressor cells (2, 3). The correlation was even more significant when T cells were previously sensitized (1, 2). Also, as shown by the same authors, blocking of the Ia molecule on EC with specific antibody completely diminished the ability of epithelial cells to deliver antigen and stimulate T suppressor cells. The ability of IEL to modulate expression of the Ia on epithelial cells was previously suggested (11). We have shown production of IL-2 by IEL, and this is in agreement with the recent report of others (16, 19, 23). In addition, we have demonstrated that IEL responded by increased production of IL-2 when co-cultured in the presence of enterocytes. Others reported that the blastogenic response of IEL to Con A was stronger when accessory cells were added to culture (25). These results taken together support the view that normal enterocytes act as accessory cells in immune response, and that they cooperate with IEL. The failure of peritoneal macrophages to augment IL-2 production (Table III, p.45) is difficult

to explain but we speculate that IEL may have a specialized requirement for the accessory cell, that is not met by the peritoneal macrophage but is satisfied by the absorptive intestinal epithelium.

Human IFN ß causes enhanced expression as well as shedding of class II MHC molecules (7) and also induces activation of human T suppressor cells (77). In the cattle a dose-dependent correlation between IFN τ and its effect on T suppressor cells from PBL was documented (65). Low doses of IFN τ were stimulative while high doses were suppressive. Suppression was overcome by addition of IL-2 (65). In our study IFN was detected in supernatants from IEL and IEL+ep cells (Table IV, p.49, 50).

IEL and absorptive intestinal epithelial cells may interact in the following fashion: IFN α and ß present during a primary antigenic stimulation, and IFN τ synthesized during an anamnestic response influence Ia expression on epithelial cells. Expression of Ia by these cells enables them to activate "helper" IEL which in turn release IL-2. IL-2 stimulates suppressor cells previously activated by IFN and antigen presented together with Ia molecule of enterocytes.

6.0 CONCLUSIONS

The digestion method with hyaluronidase was employed in order to release IEL from the intestinal epithelial layer without disturbing the integrity of the lamina propria (LP). As shown on histological sections of the digested intestine (Figure 2, p.41), the LP was not damaged, thus allowing isolation of pure IEL uncontaminated with LP lymphocytes. This was important, since the population of LP lymphocytes phenotypically differs from the population of IEL. IEL are mainly T lymphocytes with the suppressor-cytotoxic surface marker, while LP cell population contains B cells and T cells of the helper lineage (16, 19, 23).

We utilized two assay systems for IL-2 detection; the conventional bioassay and the newly developed Elisa. While the Elisa was undoubtedly specific for IL-2 because of the mAb used for IL-2 detection, the bioassay yielded results that were in disagreement (Figure 4a, 4b, p.47). The presence of IFN and IL-4 in the samples analyzed by the bioassay was probably responsible for the false negative and the false positive results, respectively. IFN was detected in the supernatants of spleen cells, pIEL, IEL+ep and macrophages. Although IL-4 was not monitored in our samples, its presence can be assumed, at least, in the supernatants of the spleen cells. Only marginal suppression of the bioassay reading of spleen cell supernatant by the

monoclonal antibodies specific for IL-2 supports the conclusion that the majority of the IL-2 activity detected by the bioassay in spleen cell supernatants was caused by a factor other than IL-2, perhaps IL-4. This conclusion is also supported by the recent report of others (46).

The observation of augmented IL-2 production by IEL co-cultured with the epithelial cells strongly suggests that the absorptive epithelial cells act as accessory cells, and that they cooperate with IEL in the afferent arm of an immune response. The fact that peritoneal macrophages were not capable of cooperating with IEL suggests that the the mechanism of the accessory cell-like activity of the absorptive epithelium differs from that of the macrophage.

Synthesis of IFN by IEL has already been mentioned in relation to its possible effect on the IL-2 bioassay. More importantly, however, IFN secreted by IEL (Table IV, p.49, 50) may provide the stimulus for Ia expression (7), and activation of T suppressor cells (77). Although the T cell activation by IFN did not occur with high doses of IFN, this dose related suppression was overcome by the addition of IL-2 (65). Thus IL-2 synthesized by IEL, may in addition to its well defined direct action on T cell lymphocyte modulate the effect of IFN on T suppressor cells. The detection of IFN and IL-2 in the supernatants of Con A stimulated IEL, and the "helping" effect of enterocytes on IL-2 production by IEL, are interpreted as highly suggestive evidence that

normal enterocytes act as accessory cells in the immune response. Upon antigenic stimulation IFN influences Ia expression on epithelial cells. Expression of Ia by these cells enables them to activate IEL which in turn release IL-2. IL-2 stimulates cells previously activated by IFN and by antigen presented together with Ia molecule of enterocytes.

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