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Physiological inflammation of the small intestine during weaning in the rat

A thesis submitted to the University of Adelaide as the requirement for the Degree of Doctor of Philosophy

by

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In

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Summary

This thesis explored the hypothesis that physiological inflammation in the small intestine and the mesenteric lymph nodes (MLN) is upregulated during the weaning period. Weaning is associated with exposure of the intestinal mucosa to food, bacteria and environmental antigens. The aim of this research was to determine changes in the number, phenotype, and activation status (using interleukin-2 receptor expression, IL-2R) of intraepithelial lymphocytes (IEL), lamina propria lymphocytes (LPL), mucosal mast cells (MMC) and MLN cells from pre-weaning to post-weaning in rats. This work was supplemented with the phenotypic analysis or activation status of cells in lymphocyte-filled villi (LFV), Peyer's patches and crypto-patches. Most of this work was performed from cryostat histological sections using a variety of monoclonal antibodies, using either the immunoperoxidase or alkaline phosphatase staining detection method. IEL, LP and MMC cells were counted as density per mm of muscularis mucosae.

$CD45^+$ cells expanded significantly both in the epithelial and LP compartments during the weaning period. Approximately 10-50% of expanding $CD45^+$ cells had the $\alpha/\beta TCR^+$ phenotype, but the remaining cells were eosinophils, NK cells and presumably early T-cell precursors, as there were very low numbers of $\gamma/\delta TCR^+$ IEL, B-cells, and macrophages. However, after weaning and in adult rats at 10 weeks of life, nearly 65% of $CD45^+$ cells were T cells with the α/βTCR phenotype. Eosinophils (peroxidase positive) were abundant (72%) in the epithelial layer in weanling rats, particularly at day 17. Proliferation ($Ki\text{-}67^+$) remained low both for IEL and LP cells. In the middle of weaning (day 21 of life), 7% of T-cells were positive for IL-2R in the MLN, as compared with 2% at post-weaning (day 42). $IL\text{-}2R^+$ activated cells peaked in the LP and epithelial layers at days 21 and 23, respectively, but lower

expression of this marker was present after day 35 in the epithelial layer. There was a significant expansion of MMC from mid-weaning (day 21) to post-weaning (day 35). This co-incided with a broad peak of T-cell activation in the LP in the second week of weaning.

LFV were recognisable in suckling rats prior to weaning. Total number of LFV cells expanded approximately 2-fold during the weaning phase. Despite this increase, no detectable Ki-67 staining was evident over this time. Longitudinal studies showed that the initial population of lymphocytes within LFV was negative for most T-cell and B-cell markers, but lymphocytes started to express α/βTCR and CD45RA (specific for B-cells) at days 19 and 25 of life, respectively. Moreover, IL-2R$^+$ cells and NK cells were first observed at days 7 and 17 of life, respectively. An unexpected finding in LFV was an early increase of NK cells (at day 19) that remained greater than the proportion of α/βTCR$^+$ T-cells. LFV cells also contained a much higher proportion of IL-2R$^+$ cells in suckling rats compared to cells in the epithelial layer and LP compartments, and their proportion remained almost constant after mid-weaning.

Structures like mouse crypto-patches were first seen at the end of weaning (day 28). Preliminary work revealed that the phenotype and activation of rat crypto-patch cells were almost identical to LFV cells, as most crypto-patch cells expressed IL-2R (e.g. at day 45), and CD4$^+$ cells predominated over CD8$^+$ cells (e.g. at day 70). Therefore, the phenotype and activation of crypto-patch were similar to that of LFV cells.

The results presented in this thesis suggest that weaning is associated with a peak of physiological intestinal inflammation and expansion of α/βTCR$^+$ cells in the gut-associated lymphoid tissues (GALT). The expansion of α/β-T-cells in the epithelial layer, LP and LFV probably occurred under the influence of intestinal food and bacterial antigens. This expansion presumably relates to recruitment of cells from other sites, as Ki-67 antigen was rarely detected. LFV were phenotypically different from populations in IEL and LP. They contained lymphocytes with similarities in phenotype to immature T-cells. The phenotypic similarity

between LFV compartment and the thymus suggests that LFV could be a site of extrathymic differentiation and maturation with NK cells originating there and circulating to the LP and epithelial compartments. Collectively, the findings of this work are consistent with the hypothesis that physiological inflammation in the small intestine and MLN complex is upregulated in infancy, particularly during weaning.

Declaration

This work contains no material which has been accepted for the award of any degree or diploma in any University or other tertiary institution and, to the best of my knowledge and belief, no material previously published or written by another person, except where due reference is made in the text.

I give my consent to this copy of my thesis, when deposited in the University library, being made available for loan and photocopying.

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Publications arising from this thesis

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Masjedi M, Cummins AG, and Tivey DR. (1996). Expansion of intestinal intraepithelial lymphocytes in rats during weaning. Proceedings of North Western Adelaide Health Service (NWAHS); The Queen Elizabeth Hospital's Research Day. S19.

Masjedi M, Cummins AG, and Tivey DR. (1997). Expansion and activation of rat mucosal lymphocytes of the small intestine during weaning. Proceedings of Annual Scientific Meeting, ASMR (SA). O15.

Masjedi M, Cummins AG, and Tivey DR. (1997). Expansion and activation of rat small bowel mucosal lymphocytes during weaning. Proceedings of NWAHS, The Queen Elizabeth Hospital's Research Day, S27.

Masjedi M, Cummins AG and Tivey DR. (1997). Expansion of intestinal intraepithelial lymphocytes in DAxPVG/c rats during weaning. Ninth International Congress of Mucosal Immunology. *Immunol Cell Biol*; **75** (supplement): W 4.5.8.

Masjedi M and Cummins AG. (1995). Expansion of intestinal intraepithelial lymphocytes (IEL) in DAxPVG/c rats during weaning. Proceedings of Annual Scientific Meeting, Medical Research Week, ASMR (SA).

Masjedi M, Tivey DR, and Cummins AG (1996). Expansion of intestinal intraepithelial lymphocytes in DAxPVG/c rats during weaning. Proceedings of the First Congress of the Federation of Immunological Societies of Asia-Oceania (FIMSA), p 180.

Masjedi M, Tivey DR, and Cummins AG (1997). Expansion and activation of rat small bowel mucosal lymphocytes during weaning. Proceedings of the Thirty-Sixth National Scientific Conference of ASMR, Adelaide, SA, O74.

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Abbreviations used in the thesis

APC	Antigen-presenting cells
DTH	Delayed-type hypersensitivity
γ-IFN	gamma-interferon
GALT	Gut-associated lymphoid tissue
GIEL	Granulated intraepithelial lymphocytes
GvHR	Graft-versus-host reaction
hsp	Heat-shock protein
IBD	Inflammatory bowel disease
IEL	Intraepithelial lymphocyte
IL-3	Interleukin-3
LP	Lamina propria
MLN	Mesenteric lymph node
MMC	Mucosal mast cell
NGIEL	Non-granulated intraepithelial lymphocytes
PBS	Phosphate-buffered saline
Phytohaemmaglutinin	PHA
RMCPII	Rat mast cell protease II
SCID	Severe combined immunodeficiency
TBS	Tris-buffered saline
TCR	T-cell receptor antigen
TGF-β	Transforming growth factor-beta
TSA-2	Thymic shared antigen-2

Chapter 1

Introduction and Literature Review

Introduction to T-cells

1.1. What are T-cells?

T-cells are lymphocytes which were originally characterized as being thymus-derived (hence 'T' for thymus), and that were found to have a certain anatomical distribution in lymphoid organs. They are now recognized by the presence of certain surface markers (CD2, CD3, and CD7) and may not always be thymus-derived (Roitt *et al.*, 1996).

The success of the specific immune system depends on the substantial ability of T lymphocytes to identify, differentiate and respond to a large variety of foreign antigens. Antigen recognition activates T-cells to fulfil their effector functions leading, for instance, to the production of lymphokines that augment proliferation, maturation, and immunologic function of other cell types including B-cells (Abbas *et al.*, 1994; Roitt *et al.*, 1996).

1.2. Phenotype of peripheral T-lymphocytes

Mature functional T lymphocytes express a number of characteristic surface proteins in addition to T cell receptors (TCRs) for antigens (**Table 1.1.**). Many of these proteins are designated by a cluster of differentiation (CD) number. In fact, CD antigens are a group of antigens that differentiate leucocyte populations (Klein, 1991; Imboden and Stites, 1994).

Almost all mature T lymphocytes in blood and peripheral lymphoid organs express $CD2,^+$ $CD3^+$ and $CD7^+$ surface markers. There are distinct subpopulations that have various immunologic roles and express their own distinctive cell surface markers (**Table 1.1**). The two most important T-cell subsets are identified by CD4 and CD8 surface proteins. Mature, functional T lymphocytes express one of these two proteins, and this is associated with important differences in cell function (Abbas *et al.*, 1994; Imboden and Stites, 1994; Roitt *et al.*, 1996).

Table 1.1. Some important surface molecules of T lymphocytes.

Marker	Major known or proposed function or significance
TCR	Antigen binding
CD2	Lineage-specific marker, ligand for CD58 (LFA-3)¶; forms an adhesion pair, e.g. between T cell and antigen-presenting cell
CD3 complex	Signal transduction as a result of antigen recognition by T-cells; lineage-specific marker
CD4	Subset-specific marker (mainly on helper cells); interaction with class II MHC proteins.
CD5	Lineage-specific marker, ligand for the B-cell specific molecule CD72 §; signal transduction associated with TCR
CD7	Lineage-specific marker (earlist T-cell marker); function unknown
CD8	Subset-specific marker (mainly on cytotoxic cells); interaction with class I MHC proteins.
CD45RA	Primary 'naive' T-cells; a tyrosine phosphatase required for TCR signaling
CD45RO	Secondary 'memory' T-cells; general function as above
IL-2 receptor (CD25)	Activation-specific marker; the α chain of IL-2R
Class II MHC proteins	Activation-specific antigens; responsible for (antigen presenting molecules) rapid graft rejection between allogeneic individuals, and regulate immune responses to all antigens
Fc receptors	Immunoglobulin binding

¶ LFA-3, lymphocyte function-associated antigen;
§ Although CD5 can bind to CD72, it is debated whether this is the physiological ligand.
(MacDonald and Spencer 1990; Stamenkovic *et al.*, 1991; Imboden and Stites, 1994; Kumar and Clark, 1994; Abbas *et al.*, 1994; Roitt *et al.*, 1996).

Table 1.2. Major T-cell subsets found in blood and secondary lymphoid tissues

Surface phenotype	Major function	Proportion of total blood T-lymphocytes	TCR type
$CD4^+ CD8^-$	Helper	70%	α/β
$CD4^- CD8^+$	Cytotoxic	25%	α/β, seldom γ/δ
$CD4^- CD8^-$	Cytotoxic	4%	γ/δ
$CD4^+ CD8^+$	Unknown (transient activation state)	1%	α/β

(Modified from Imboden and Stites, 1994).

Approximately 70% of T cells in human blood or in peripheral tissues express $CD4^+$ $CD8^-$ (helper/inducer T-cells), while about 25% express $CD4^-$ $CD8^+$ (cytotoxic T-cells). These phenotypes are often referred to as single-positive lymphocytes, and are the cells usually involved in immune responses. Nearly 4% of T cells outside the thymus express neither CD4 or CD8 molecules (double negative lymphocytes). They express the γ/δTCR. The remaining 1% of extrathymic T lymphocytes express both CD4 and CD8 accessory molecules (double-positive cells), whose function remains elusive (Abbas, *et al.*, 1994; Imboden and Stites, 1994; Roitt *et al.*, 1996).

1.3. The TCRs

1.3.1. Structure of the α/βTCR

The T cell receptors form a heterogeneous class of membrane proteins, which are comprised of a pair of transmembrane polypeptides, either αβ chains (TCR2) or γδ (TCR1) (**Fig. 1.1**). TCRs are closely related to immunoglobulins in ontogeny and share several structural and functional properties, including the ability to detect specific small molecular ligands (antigens). The amino-terminal regions of the α and β chains are remarkably polymorphic, so that within the whole T cell population there are a large number of various TCR α/β dimers. Each has the ability to recognize a specific combination of peptide fragments and MHC. The TCRs on single T cells contain only an individual type of α/β dimer; hence, individual T-cells respond only to a particular combination of antigen and MHC non-self antigen and self-MHC molecules (Abbas *et al.*, 1994; Stites and Imbodden, 1994; Roitt *et al.*, 1996).

The α/β dimer is strongly linked to a cluster of five polypeptide chains, referred to as the CD3 molecule complex (**Fig. 1.1**). In contrast to the α/βTCR, the CD3 chains are not polymorphic and range in size from 16-28 KDa. They are responsible for signal transduction and thus allow the invariant chains (constant region) of TCR to convert the recognition of antigen bound to MHC antigens into intracellular signals for T-cell activation. In contrast to

α/β TCR chains, the CD3 chains do not have an extracellular large domain. In fact, the intracellular region for the α/β dimer is several amino acids in length, while CD3 chains have large cytoplasmic domains ranging from 45-55 amino acids for CD3 ε, δ, and γ (**Fig. 1.1**) to 113 amino acids for CD3 ζ (Imboden and Stites 1994; Cotran, 1994).

1.3.2. Functions of α/β T-cells

The functions of most α/β T-cells in blood and peripheral lymphoid organs are well-characterized. Broadly speaking, the functions of α/β bearing T-cells are as follows:

(**1**) Helper function for B-cells via lymphokine secretion,

(**2**) Cytotoxicity,

(**3**) Mediation of delayed-type hypersensitivity (DTH),

(**4**) Allograft rejection,

(**5**) Graft versus host reaction (GvHR).

This group of lymphocytes exert their functions via the recognition of foreign antigens complexed with class I or II MHC molecules by both cytotoxic T-cells ($CD4^{-}$ $CD8^{+}$) and by helper/inducer T-cells ($CD4^{+}$ $CD8^{-}$), respectively (Janeway *et al.*, 1988; Cheng *et al.* 1991; Haas *et al.*, 1993). Certain α/β T-cells are activated by heat shock proteins (hsp), an effect which is independent of antigen recognition (O'Brien and Born, 1991). The function of α/βTCR^{+} intraepithelial lymphocytes (IEL) might be to induce and maintain oral tolerance to food antigens (Mowat, 1987)

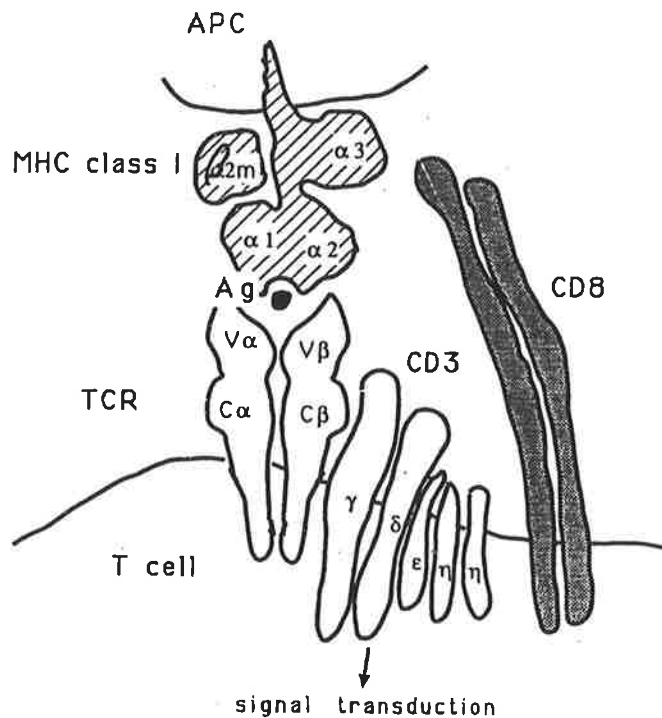


Fig. 1.1. Schematic illustration of the α/βTCR CD3 complex and its function in antigen-presentation to a $CD8^{+}$ T cell. Antigen recognition gives rise to T-cell activation through signals transmitted by the CD3 molecule that is strongly associated to the TCR in the T-cell membrane. The CD8 molecule is able to boost the interaction of T cells with class I major histocompatibility complex (MHC) antigens, while CD4 regulates interaction of T cells with MHC class II antigen (Cerf-Bensussan *et al.*, 1991).

APC; antigen-presenting cell

1.3.3. Structure of γ/δ T-cells

The second type of receptor (TCR1) consists of a pair of transmembrane polypeptides known as the γ and δ chains. The γ/δ heterodimer is similar to the α/β heterodimer in structure and in genomic organization. These two subclasses of T-cells share some common features such as the association with CD3 molecules, the expression of certain cell surface markers, and demonstration of similar T-cell functional activities (cytotoxicity and release of certain lymphokines).

It has been postulated that the functions of mucosal $\gamma/\delta TCR^+$ T-cells are as follows:

(1) Regulation of systemic immune response to foods via γ interferon (γ–IFN)

(2) Production of epitheliotropic cytokines

(3) Stimulation of epithelial renewal during infection/enteropathy in mice

(4) Help for IgA antibody production

(Boismenu and Havran, 1994; Komano *et al.*, 1995; Mowat and Viney, 1997)

Concerning the homeostatic regulation of intestinal epithelia by these cells, it has been shown that the absence of γ/δ T-cells is associated with a reduction in epithelial cell turnover and downregulation of the expression of major histocompatibility complex class II molecules. No such effects are observed in α/β T-cell deficient mice. These findings indicate that intraepithelial γ/δ T-cells, at least in mice which have high numbers of γ/δ T-cell mucosally, modulate growth and differentiation of enterocytes (Komano *et al.*, 1995).

1.4. T-cell ontogeny

1.4.1. Stages of thymocyte development

T-cells arise from bone marrow-derived precursor cells that undergo maturation in the thymus (**Fig. 1.2.**). The thymus provides an environment for the development of early T-cells into mature antigen specific T-lymphocytes. At the earliest stages of development, thymocytes express several T-cell surface molecules (such as CD2 and CD7) that are characteristic of the T-cell lineage, but they do not express many others, including CD4 and CD8 'co-receptors',

and therefore are designated as double-negative thymocytes ($CD4^- CD8^-$). Rearrangement of the TCR genes begins at the double-negative stage. Thymocytes that are destined to become α/β^+ T cells, rearrange first the βTCR gene and subsequently the αTCR gene. At this stage of development, the cells begin to express both CD4 and CD8α/β and thus become double-positive thymocytes ($CD4^+ CD8^+$). Both positive and negative selection occur in the cortex or at the corticomedullary junction when thymocytes are at the $CD4^+CD8^+$ double positive stage. When thymocytes become more mature, they begin to lose the expression of either CD4 or CD8 and to increase α/β TCR expression, becoming single positive thymocytes in the medulla (**Fig. 1.2.**). At this point, thymocytes have the characteristics of functional, mature peripheral T-cells and leave the thymus to populate the blood and peripheral lymphoid tissues (Abbas, *et al.*, 1994; Imboden and Stites, 1994; Roitt *et al.*, 1996).

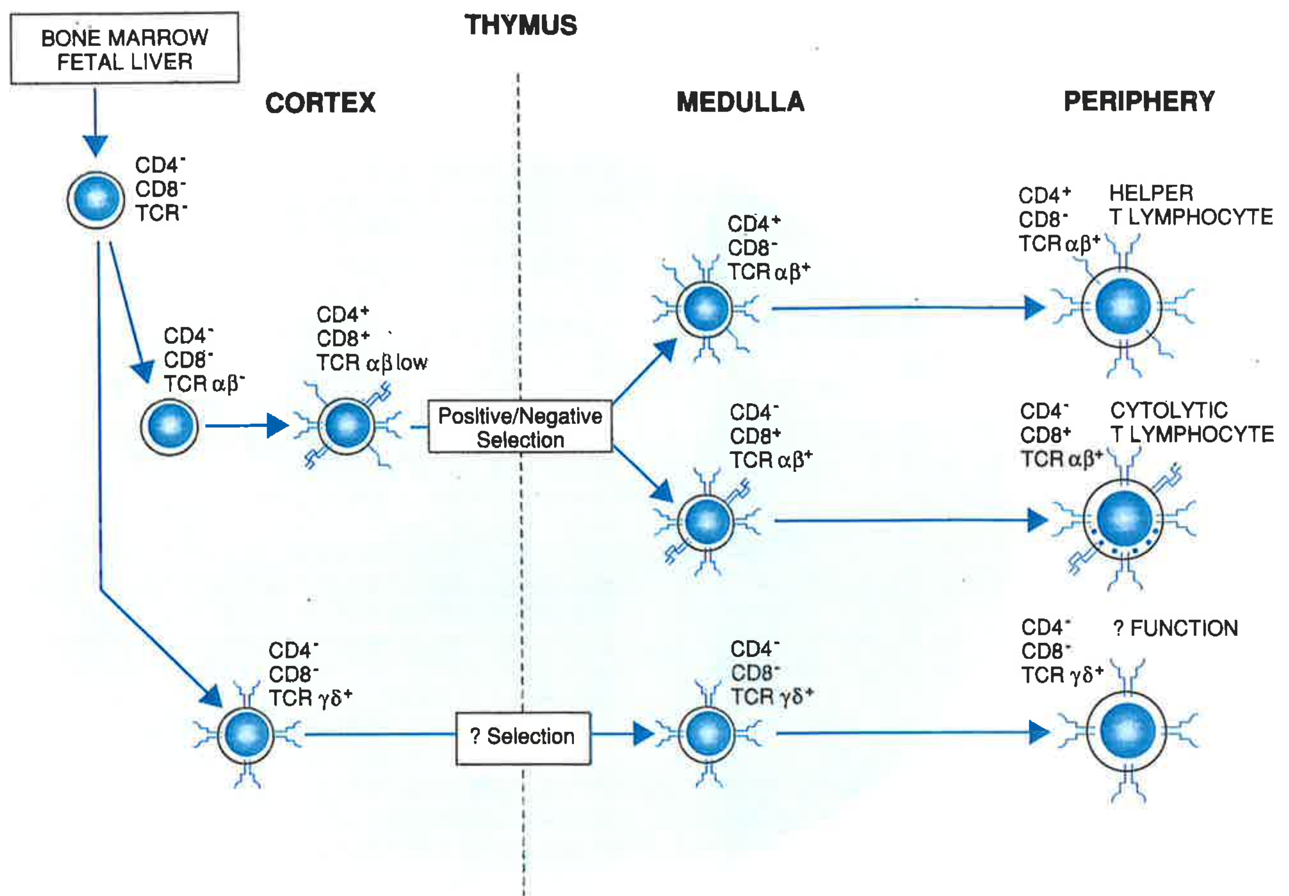


Fig. 1.2. Ontogeny and lineage relationships of maturing T-cells. γ/δTCR⁻ and α/βTCR⁻ expressing cells are separate lineages that develop from a common progenitor, most of thymocytes express both CD4 and CD8. TCR expression begins in this double-positive stage, starting with low numbers of receptors on each cell and increasing as maturation proceeds. Single-positive, *i.e.*, CD4+ or CD8+ α/βTCR⁻ expressing mature cells are selected from this population. Some γ/δ cells express CD4 or CD8 (Abbas *et al*., 1994).

Introduction to natural killer (NK) cells

1.5. NK cells

NK cells or null cells are non-T, non-B lymphocytes, and constitute part of the innate, non-antigen dependent immune system, and perhaps represent a more primitive immune system. NK cells kill other target cells within 4 h and thus are different from cytotoxic T-cells that typically need 4 days of antigen priming *in vivo* and *in vitro* (Britten *et al.*, 1986; Canesa *et al.*, 1988; Klein, 1991).

Approximately 10-15% of large peripheral blood lymphocytes and 3-4% splenic lymphocytes do not express TCR-CD3 complex or surface immunoglobulins, and contain cytoplasmic azurophilic granules. These lymphocytes are endowed with inherent ability to lyse a variety of tumor cells, virally infected cells, and some normal cells. Substantial numbers of NK cells are found in the lung interstitum, in the intestinal mucosa, and in the liver. In contrast to T cells, they are seldom found in the thymus or lymph nodes and are not commonly found in the thoracic duct lymph (Trinchieri, 1989, Klein, 1991).

1.5.1. Functional properties of NK cells

NK cells are part of the 'inherent' (as opposed to 'adaptive') immune system that appear to be involved in host defense against many obligate and facultative intracellular pathogens and, probably, against neoplastic cells. NK cells can be also stimulated to synthesize and to secrete numerous cytokines which may have complex immunoregulatory functions. However, the precise physiologic functions of NK cells remain unknown (Britten *et al.*, 1986; Canessa *et al.*, 1988; Klein, 1991; Cotran *et al.*, 1994; Imboden and Stites, 1994).

1.5.2. Development of NK cells

Like T and B lymphocytes, NK cells arise from stem cells in the bone marrow, but little is known concerning their ontogeny. The requirements for development of the NK cell lineage are discrete from those of T cells and B cells. For instance, children with severe combined immunodeficiency (SCID) may be devoid of T and B cells but have normal or increased NK cells. This shows a different cell lineage for NK cells than for T and B lymphocytes. By contrast, a few patients have no NK cells, but have normal B and T cells (Imboden and Stites, 1994).

The features of NK cells in addition to their spontaneous killing of target cells have been reviewed by Klein (1991), and are as follows:

(1) late maturation (in the mouse, NK activity is absent in embryos and neonates, appears at 3-4 weeks of age, is maximal at 5-8 weeks, and subsequently decreases to low levels in mice aged 6-12 months);

(2) radioresistance (NK cells sustain activity even when irradiated with a dose of over 1000 rads, which is lethal for many other cells);

(3) thymus independence;

(4) bone marrow dependence;

(5) unique cell-surface marker profile.

1.5.3. Distinction between NK and T-cells

In contrast to T-cells, NK cells do not rearrange TCR genes, and do not express a cell surface TCR-CD3 complex. In addition, most NK cells express two cell-surface molecules, CD16 (a low affinity Fc receptor that binds IgG in immune complexes) and CD56 (pan NK marker, adhesion molecule) (**Fig. 1.3**). These cell-surface molecules are not found on T cells. Hence, analysis of the expression of CD3, CD16, and CD56 surface markers discriminates NK cells (which are always $CD3^-$ and commonly $CD16^+$ $CD56^+$) from mature T cells (which are

always CD3+ and usually $CD16^{-}CD56^{-}$) (Abbas *et al.*, 1994; Cotran *et al.*, 1994; Imboden and Stites, 1994; Roitt *et al.*, 1996).

1.6. NK1.1^{+} T-cells: a new member of T-cells

NK T cells are a subset of mature α/β T-cells which co-express the NK marker (NK1.1) and have NK cell function. They were first identified in the mouse thymus by Fowlkes and Budd (1978). These NK T-cells express α/βTCR, and consist of $CD4^{-}$ $CD8^{-}$ double-negative cells (40-60%) or $CD4^{+}$ cells. NK1.1^{+} T-cells express a restricted TCR repertoire consisted of invariant TCRα chain, Vα14-J α 281, associated with polyclonal Vβ8, Vβ7, and Vβ2 TCRβ chains. These cells recognize the products of the conserved family of MHC class I-like CD1 genes and a single invariant α chain is mostly involved in this process. (Bendelac, 1995; Vicari and Zlotnik, 1996; Bendela *et al*., 1997).

1.6.1. Tissue distribution of NK1.1^{+} T-cells

They constitute 10-20% of the mature thymocytes (i.e., 0.5% of the total thymocytes in the adult thymus). NK T-cells are also found in the bone marrow and liver (15-50% of the T-cells), spleen (1% of T-cells), and peripheral lymph nodes (0.3% of T-cells) (**Table 1.3**; Bendelac, 1995; Vicari and Zlotnik, 1996). These cells are virtually absent from the intestinal epithelium (IEL), but their frequency in the lamina propria (LP) and Peyer's patches is unknown (Ohteki and MacDonald, 1994).

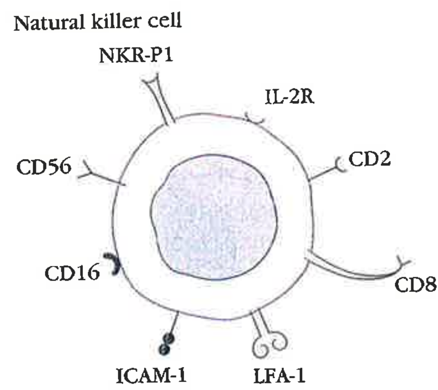


Fig. 1.3. Schematic representation of some common membrane molecules present on NK cells. CD16 (FcγRIII) is receptor for Fc region of IgG (Kuby, 1994)

Table 1.3. Tissue distribution of NK1.1$^+$ T-cells

Tissue	Mature T-cells (%)
Thymus	25
Spleen	2
Lymph nodes	1
Bone marrow	40
Liver	30
Intestinal lamina propria	+§

§ The authors detected positive cells, which were not enumerated (Bendelac, 1995; Vicari and Zlotnik, 1996).

1.6.2. Phenotype of NK1.1$^+$ T-cells

The phenotype of thymic and splenic NK1.1$^+\alpha/\beta$TCR cells looks like mature, activated or memory T-cells, because they express high levels of CD44 and low levels of CD24, CD26 ligand, CD5, 3G11 and thymic shared antigen 2 (TSA-2) (Hayakawa *et al.*, 1992; Bendelac *et al.*, 1992; Arase *et al.*, 1993; Bendelac *et al.*, 1994; Vicari *et al.*, 1994). However, unlike memory T-cells, they express high levels of CD45RB and many express Ly-6C$^+$ (Takahama, *et al.*, 1991; Vicari *et al.*, 1994; Vicari *et al.*, 1996). Upon activation *in vitro*, most CD4$^-$CD8$^-$ α/βTCR$^+$ thymocytes express CD38, a predominant B-cell marker, while peripheral NK1.1$^+$ T-cells occasionally express B220, another B-cell marker (Koyasu, 1994). The most impressive phenotypic characteristics of NK1.1$^+$ T-cells is the expression of a variety of NK-cell markers, including CD16, Ly-49A, Ly-49C and the IL-2R β chain (CD122) (Sykes, 1990; Arase, *et al.*, 1992; Arase *et al.*, 1993; Lantz, 1994). They have NK function, but their morphology with electron microscopy and their granule contents are intermediate between T-cells and NK cells (Watanabe, *et al.*, 1995).

1.6.3. Functions of NK1.1$^+$ T-cells

The most important function of NK1.1$^+$ T-cells is production and release of cytokines, especially large amounts of IL-4, both *in vivo* and *in vitro*. The cytokine release is particularly striking, because it occurs *in vivo* within an hour of TCR engagement. The early secretion of IL-4 provides early help for T-and B-effector cells (e.g., helper T-cells, cytotoxic T-cells) and regulates the Th_1 or Th_2 differentiation of some immune responses, suggesting that these cells are immunoregulatory (Bendelac, 1995; Vicari and Zlotnik, 1996; Bendelac et al., 1997).

1.6.4. Origin and development of NK1.1$^+$ T-cells

Controversy surrounds the source of NK1.1$^+$ T-cells. It has been shown that they can arise from fetal thymic cultures (Bendelac *et al*., 1994), suggesting that they mainly differentiate within the thymus. However, there is also evidence that they could arise extrathymically-namely, from the periphery of nude mice, in which the thymus is rudimentary (Kikly and Dennert, 1992; Ohteki *et al*., 1992). Moreover, NK1.1$^+$ T-cells selectively develop in the liver of thymectomized mice reconstituted with syngeneic bone marrow cells (Sato *et al*., 1995).

Nevertheless, NK1.1$^+$ T-cells appear to originate from a subset of thymocyte progenitors that randomly express CD1-specific Vα14-Jα281/Vβ8 TCRs that recognize CD1-expressing cortical thymocytes. The recognition of CD1 probably occurs at the double-positive stage, and for reasons relevant to the affinity for the CD1 ligand, there is a positive selection for CD4$^+$ and double-negative cells on the one hand, and negative selection applied to CD8$^+$ cells through DNA fragmentation followed by nuclear fragmentation (apoptosis) and cell death on the other hand (**Fig. 1.4.**). The expression of such TCRs is likely to be random rather than directed by self-antigens. This suggests that the unique phenotype of these cells, including the expression of NK receptors, activation markers, and IL-4 cytokine release

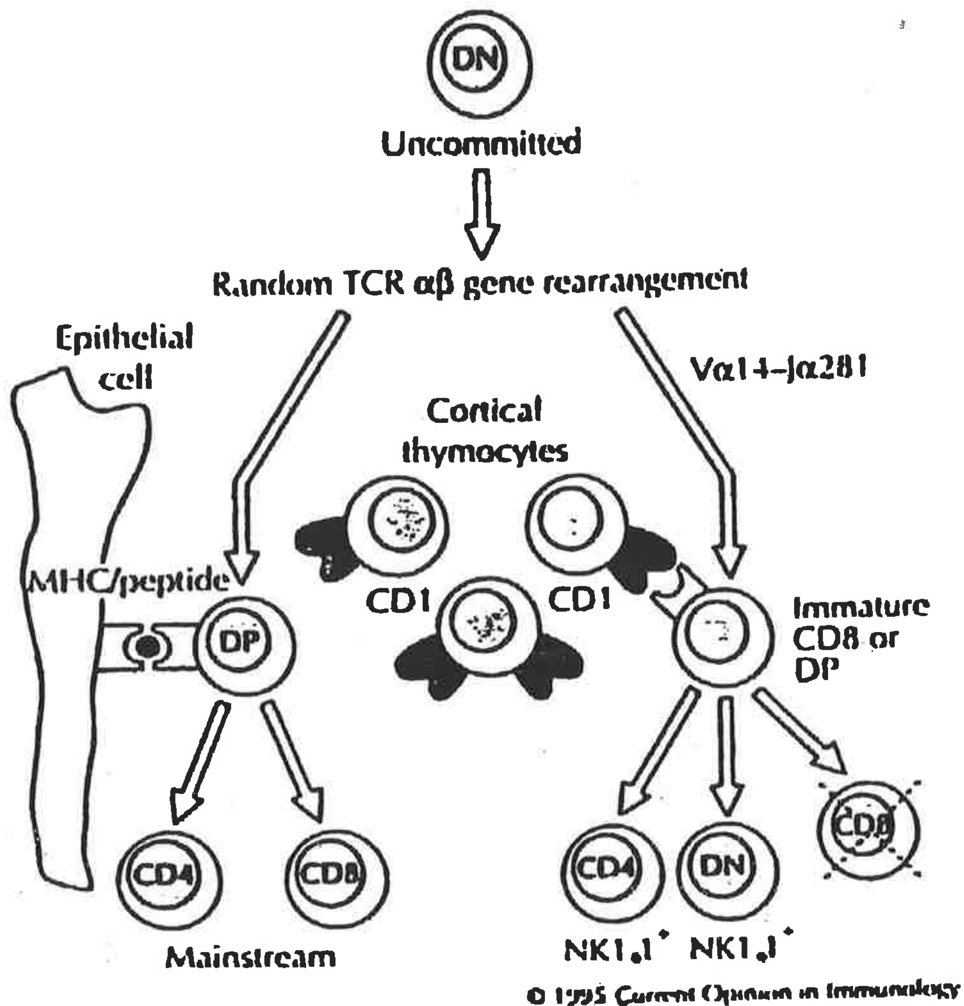


Fig. 1.4. Thymic selection pathways of mainstream and NK1.1+ T-cells. A cortical thymocyte randomly expressing a Vα14-Jα281/Vβ8 TCR will recognize the CD1 ligand (CD8) expressed by cortical thymocytes, probably in the absence of peptide. Mainstream T-cells recognize classical MHC ligands that form complexes with peptides on epithelial cells. These differences, rather than a genetic precommitment, are likely to be responsible for the distinct phenotype of NK1.1+ T-cells (Bendelac, 1995).

following primary activation, are the outcome of positive and negative selection, rather than being pre-programmed (Abbas, *et al*., 1994; Bendelac, 1995).

Introduction to mucosal immunity, mucosal mast cells and the small intestine

1.7. The mucosal immune system

Mucosal surfaces are the major sites in the body in which antigens are confronted. Throughout life, they are constantly exposed to antigens, such as food particles, microbes, chemical products, parasites, or allergens (Klein, 1991). The mucosal immune system is made up of lymphoid tissues that are related to the mucosal surfaces of the gastrointestinal, respiratory, and urogenital tracts. The development of the mucosal immune system is influenced by the complex and distinctive antigenic compounds present at various mucosal surfaces (Strober and James, 1994). It is now established that the mucosal immune system is a discrete immunologic entity and is regulated in a different manner to that of peripheral lymphoid tissues (McGhee and Kiyono, 1994).

1.7.1. Distinction between the mucosal and systemic immune systems

The mucosal immune system may be distinguished from the systemic immune system by several characteristics. These consist of:

(1) A mucosal-related immunoglobulin, mucosal IgA;

(2) Regulatory T lymphocytes with mucosal-specific properties; and

(3) specific mucosal recirculation and homing for cells (Th cells, $CD8^+$ cytotoxic T cells, and B cells). These are initially induced in the mucosal follicles (Peyer's patches) and migrate to the diffuse mucosal lymphoid tissues (mucosal effector sites) of the LP (**Fig. 1.5**). In fact, the cells of the mucosal immune system are not constant, but are highly dynamic, and are

characterized by a higher proportion of activated cells [eg, interleukin-2 receptors positive ($IL-2R^+$) T-cells] and a high proportion of IgA plasma cells that have differentiated from B-cells. Accordingly, the mucosal immune system should be regarded as a separate immunological unit (Strober and James, 1994; McGhee and kiyono, 1994; Roitt *et al.*, 1996).

1.7.2. Function of the mucosal immune system

The primary action of the mucosal immune system is to provide a host defense against the antigenic environment at mucosal areas (Klein, 1991; Strober and James, 1994; Roitt *et al.*, 1996). In this function, it is assisted by non-immunological protective factors, including acid and pepsin secretion by the stomach, the presence of bile salts in the small intestinal lumen, and clearance of antigen by peristaltic contractions. A mucosal barrier is provided by secreted mucus overlying the epithelium. Finally, substances such as lactoferrin, lactoperoxidase, and lysozyme exert inhibitory effects on one or other specific microorganisms (Van Garderen *et al.*, 1991; Strober and James, 1994).

Another equally important function of the mucosal immune system is to prohibit the entry of mucosal antigens across the mucosal barrier and hence protect the systemic immune system from unsuitable antigenic exposure. In fact, the second function of the mucosal immune system is defence against antigens by forming an extrinsic barrier. In addition, the mucosal immune system responds to antigens which penetrate across the mucosal barrier (Kumar and Clark, 1994; Strober and James, 1994).

1.8. The immune system of the gut

The gut immune system constitutes the largest proportion of immunological tissue in the body, comprising approximately 40% of the total lymphoid tissue of adults (MacDonald, 1991; Marsh and Cummins, 1993). At the interface between the surface of the mucosa and the

gut lumen, there is a single layer of epithelium which separates the lymphoid tissue from a large number of luminal antigens comprised of bacteria, food and the many potential pathogens which colonize the gut (MacDonald, 1992; Strober and James, 1994; Kumar and Clark, 1994).

1.8.1. Function of the gut immune system

The immune system of the gut has two major functions:

(1) protection of the host from enteric infections by pathogens (viruses, bacteria, and parasites) (James, 1993);

(2) regulatory function: tolerance to intraluminal antigens as a consequence of suppression elements (probably IEL) (Strober and James, 1994).

This effect minimizes the induction of immune responses to the immense and varied load of dietary proteins (James, 1993) Thus, the immune system of the gut has the double function of defence against penetration of potential pathogens and of down-regulation of immune responses that prevent excessive stimulation of the peripheral immune system (Cerf-Bensussan *et al.*, 1991).

In addition, the gut epithelium may play a significant immunological role since isolated rat enterocytes process and degrade complex antigens to simple peptides in lysosomes, and express class II MHC products (Bland and Warren, 1986; MacDonald and Spencer, 1990), suggesting that epithelial cells in the gut may be involved in the presentation of soluble antigens such as dietary proteins to $CD4^+$ IEL. This suggestion is corroborated by *in vitro* experimental studies demonstrating that isolated rat or human enterocytes are able to present soluble protein such as ovalbumin (nominal antigen) to antigen-primed peripheral blood T lymphocytes (Bland and Warren, 1986). Hence, it presently appears more likely that enterocytes act as antigen processing cells rather than as antigen presenting cells (APCs), and

that the precise *in vivo* role of enterocytes in the presentation of soluble proteins to IEL remains unanswered.

1.8.2 Anatomy of the immune system of gut

The human gastrointestinal tract has as much lymphoid tissue as the spleen. The immune system of the gut can be morphologically and functionally separated into two fundamental parts:

(**1**) Mucosal lymphoid aggregates, consisting of Peyer's patches and mesenteric lymph nodes (MLN) and;

(**2**) Diffuse mucosal lymphoid tissues consisting of cell populations present in two separate compartments; IEL and the LP (Strober and James 1994; Roitt *et al.*, 1996).

1.8.3 Traffic and homing of mucosal gut lymphocytes

A characteristic feature of the mucosal immune system is the homing capability of cells developing in mucosal follicles. In fact, different lymphocytes have different homing receptors that allow them to reside in a particular organ or tissue. Lymphocytes are also like migratory birds: they settle down only temporarily and then move on further, but keep coming back to the same place. This feature shows that they are not stationary, but are highly dynamic (Klein, 1991; James, 1991; Strober and Stephen, 1994).

The organized tissues are 'afferent' lymphoid areas, where intraluminal antigens enter the system (**Fig. 1.5**). In fact, a specialized epithelial cell above the Peyer's patches called the 'M' or 'membrane' cell allows luminal antigens to pass through the follicle-associated epithelium (MacDonad and Spencer, 1990; Strober and Stephen, 1994). The lymphocytes (both T and B cells) lying below the epithelial layer are stimulated by these antigens and undergo expansion in the Peyer's patches. These antigen-stimulated cells (blast cells) pass to the MLN and

subsequently via the thoracic duct, to the circulation (**Fig. 1.5**). Studies of mucosal cell circulation show that MLN-derived B lymphoblasts selectively migrate into mucosal areas (**Fig. 1.5**), and that the majority (70-90%) of the resident B cells are IgA B cells (Strober and James, 1994). MLN and thoracic duct-derived T lymphoblasts also colonize mucosal sites, both in the LP and in the IEL compartments; however, in comparison with B-cells, a smaller proportion of mucosal follicle-derived T-cells have this characteristic. In conclusion, how T-cells travel to the intraepithelial compartment and are subsequently retained at this site remain elusive. The circulation of lymphocytes also indicates that T-cells which colonize the LP and IEL regions of the intestine appear to derive, at least in part, from the Peyer's patch (Guy-Grand, 1978).

Mucosal homing of cells in the gut mucosa via the Peyer's patch is antigen independent, because experimental studies have shown that mucosal lymphoblasts travel to antigen-free intestinal grafts in extraintestinal sites (under the kidney capsule). However, subsequent proliferation and maturation into IgA plasma cells (plasma cell producing IgA immunoglobulin) and induction of cytotoxic reaction by T-cells is antigen dependent. Both the organized (Peyer's patch and MLN) and the diffuse mucosal lymphoid regions (LP) are highly antigen-dependent. This has been well-demonstrated in:

(**1**) Germ-free animals in which their numbers dramatically decline and;

(**2**) Conventionally reared animals (conventional condition of housing and feeding) in which their numbers expand under conditions of increased antigenic stimulation (Cerf-Bensussan *et al.*, 1985; Monk *et al.*, 1988; Lefrancois, 1990; Jarry *et al.*, 1990; Umeski *et al.*, 1993; Guy-Grand and Vassalli, 1993; Strober and James, 1994; Okuyama *et al.*, 1995).

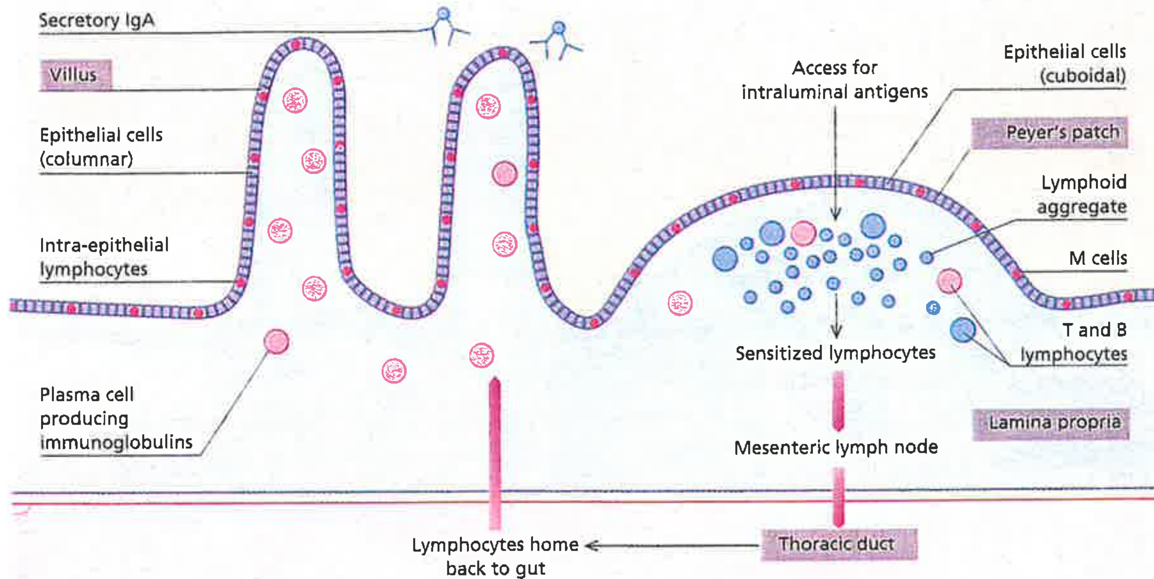


Fig. 1.5. Small intestinal mucosa showing the GALT. This diagrammatic illustration also shows migration between the systemic and mucosal immune systems, so that lymphoid cells which are stimulated with antigen in Peyer's patches migrate via mesenteric lymph nodes (MLN) and thoracic duct into the LP of the gut (modified from Kumar and Clark, 1994).

1.9. Peyer's patches

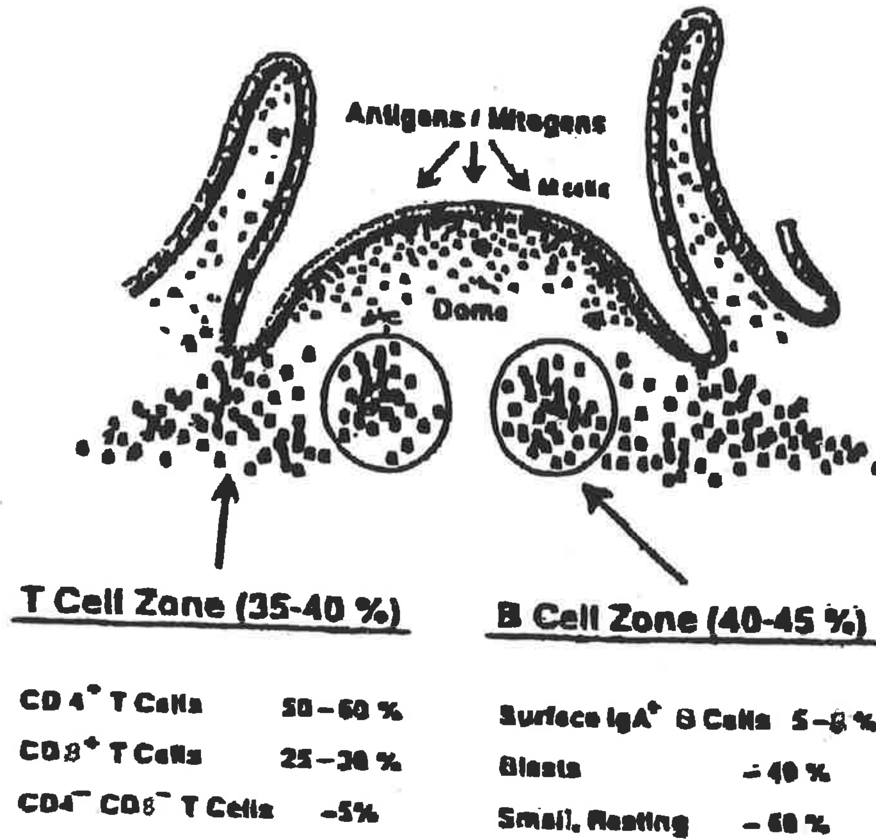
Animal studies have demonstrated the influence of the luminal environment on the development of Peyer's patches. Within days of birth, Peyer's patches contain the greatest density of proliferating lymphoid cells within the body, probably reflecting the initial exposure to exogenous antigens (Reynolds and Morris, 1983). Moreover, in germ-free mice, Peyer's patches are small but enlarge when animals are exposed to a conventional environment (Crabbe *et al.*, 1970; Shanahan, 1994). Peyer's patches are well-developed at birth in mice (Joel *et al.* 1971, 1972); they are first populated by T-cells from the thymus and only later develop B-cell regions. Peyer's patch lymphocytes respond to gut antigens. They produce specific antibody-producing cells in the lamina propria (Craig and Cebra, 1971) and systemic suppressor cells (Mattingley and Waksman, 1978; Nagan and Kind, 1978; Elson, Heck and Strober, 1979). Little information is available on the activation status of Peyer's patch cells during weaning. The important features contained by Peyer's patches are as follows:

(1) A specialised epithelium which consist of cuboidal epithelial cells and no goblet cells; the epithelium contains little mucus to prevent antigen uptake. Therefore, it absorbs enteric antigens (soluble, particulate, bacteria, viruses).

(2) Epithelial M cells (membranous cells) which are specialized for pinocytotic uptake and transport of antigen into Peyer's patches.

(3) A subepithelial dome region which is rich in MHC class II positive dendritic cells.

(4) Follicles with germinal centres (B-cell areas) and interfollicular regions containing T-cells and interdigitating cells. They do not have appreciable numbers of plasma cells (**Fig. 1.6**, McGhee *et al*., 1993).



Antigen Presenting Cells
Dendritic and B Cells and MØ

Fig. 1.6 Schematic illustration of a human Peyer's patch lymphoid nodule with distinct T and B-cell zones. Note that dome area is rich in antigen-presenting cells (modified from McGhee *et al.*, 1993; Strober *et al.*, 1994).

Introduction to the mesenteric lymph nodes (MLN) complex

1.10.1. Histology of the MLN complex

The MLN is a major organ in the intestinal immune system. It is not one single lymph node, but a series of lymph nodes that are more accurately called the MLN complex (Carter and Collins, 1974). The lymph node consists of a B-cell area (cortex), a T-cell area (paracortex), and a central medulla, which has cellular cords containing T cells, B-cell, numerous plasma cells and abundant macrophages (Stites *et al.*, 1994; Roitt *et al.*, 1996) (**Fig. 1.7**). The paracortex contains many APCs (dendritic cells or interdigitating cells) which express high conentrations of MHC class II surface antigens. Each lymph node contains its own arterial and venous supply. Lymphocytes leave the circulation and enter the node through specialized high endothelial venules in the paracortex area (Stites *et al.*, 1994; Abbas *et al.*, 1994; Roitt *et al.*, 1996). The cortex contains a few aggregates of mainly B-cells (primary follicles), most of which are stimulated (secondary follicles) and have a site of active proliferation, or germinal centre (Stites *et al.*, 1994; Roitt *et al.*, 1996).

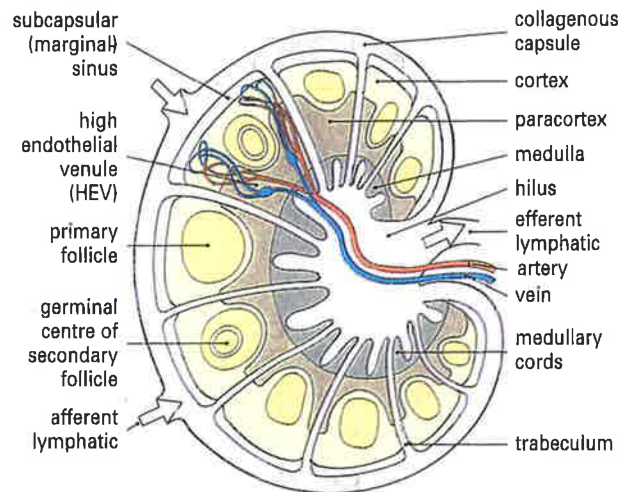


FIg. 1.7. The major structural features of a typical lymph node showing the distinct cortex containing aggregate of B-cells (primary follicles) most of which are stimulated (secondary follicles) and possess a site of active proliferation or germinal centre. The parafollicular region contains predominantly T-cells, many of which are associated with the interdigitating cells (antigen-presenting cells). The medulla with lymphatic cords and vessels, contains both T and B cells, as well as most of the lymph node plasma cells organized into cords (Roitt *et al.*, 1996).

1.10.2. Phenotype of the MLN complex

There are no differences in the phenotype of T-cells isolated from the different nodal segments of the MLN complex (Gautreaux *et al.*, 1994). MLN cells consist of approximately 65-80% T-cells (nearly all $\alpha/\beta TCR^+$) in mice, rats and humans (Gautreaux *et al.*, 1994; Thompson *et al.*, 1996; Brandtzaeg *et al.*, 1997). The remaining 20-30% in mice and humans are $CD19^+$ B-lymphocytes with a small residual population of macrophages, eosinophils and dendritic cells (Gautreaux *et al.*, 1994; Brandtzaec *et al.*, 1997). T-cells ($CD3^+$) consist predominantly of $CD4^+$ (~70%) cells as shown by flow cytometry of rat and human intestinal lymph cells (Thompson *et al.* 1996; Brandtzaeg *et al.* 1997). Brandtzaeg *et al.* found that less than 0.5% of T-cells express both CD4 and CD8 in humans.

1.10. 3. Activation and proliferation status of the MLN T-cells

Previous studies have indicated that weaning in the rat is associated with increased immune activity in the GALT. Cummins *et al.* (1988a-b) have observed an increase in the weight and cell content in MLNs, degranulation of jejunal mucosal mast cells (MMC), and an exponential increase in jejunal IEL that co-incides with weaning in rats (Babicky *et al.*, 1973; Thompson *et al.*, 1996). The MLN weight increases seven-fold from days 12 to 22 of life in rats before stabilizing (Cummins *et al.*, 1988b). Expression of IL-2R by MLN $CD5^+$ and $CD4^+$ T-cells peaks at day 22 of life (Cummins *et al.*, 1990). Several studies indicate that changes in the weight and number of cells per MLN parallel changes in activity elsewhere in the GALT (Seeling and Billingham, 1981; Thompson *et al.*, 1996). This activity in the MLN and the gut mucosa occurs concurrently with increases in the villus area, crypt length, crypt proliferation, and other changes that are characteristics of this period of development (Herbst and Sunshine, 1969; Cummins *et al.*, 1988a-b; Thompson *et al.*, 1996).

It has been suggested that MLN T-cells in nonhuman primates and humans proliferate in response to specific antigens moderately (++), whereas LP T-cells do not proliferate. In

addition, MLN T-cells express IL-2R α at low intensity, while LP T-cells express this activation marker at higher intensity (++ in **Table 1.4**). In conclusion, MLN T-cells have characteristics that suggest that they are comprised primarily of 'naive' T-cells, whereas most of the properties of LP T-cells are similar to those ascribed to 'memory' T-cells (James, 1991).

1.11. Intestinal LP cells

Much less work has been done on the LP cells. The intestinal LP consists of true lymphocytes (both T and B cells), plasma cells, macrophages, eosinophils, mast cells, fibroblasts, small unmyelinated nerve fibers, lymph vessels disseminated in a rich connective tissue and vascular network (Klein, 1991; Gautreaux *et al.*, 1994). Estimates of the proportion of T cells among the lamina propria lymphocytes (LPL) vary from 40% to 90% in various reports; however, most (65%-80%) of the $CD3^+$ LPL are $CD4^+$ (Selby *et al.*,1981; Elson, 1988), a proportion similar to that found in the peripheral blood. Recent evidence suggest that $CD4^+$ T cells have undergone prior activation. LP T-cells have mRNA for IL-2R, IL-2, γ-IFN, IL-4 and IL-5, and express increased class II MHC and IL-2R antigens (**Table 1.4**) (James, 1991; Strober and James, 1994). The T-helper cells collaborate with B lymphocytes and probably also with cytotoxic T-cells. In fact, T-helper cells in the LP provide more help and less suppression than do the $CD4^+$ cells in other sites. These findings and recent data that CD4 LP T-cells respond to specific antigen by secreting 'helper' lymphokines rather than by proliferation (**Table 1.4**), have led to the understanding that LP T cells are a class of memory cells, as about 96% of them in humans have the $CD45RO^+$ $CD45RA^-$ phenotype (James, 1991; Schieferdecker *et al.*, 1992; Strober and James, 1994).

1.11.1. Activation status of LPL

T-cells in the gut effector sites express IL-2R and appear to be at a higher stage of activation than T cells isolated from lymphoid tissues such as MLN complex, as has been shown by the cytokine secretion profile of LPL (**Table 1.4**). In fact, LP T cells are a committed subset which produce a unique cytokine secretion profile (Zieitz *et al.*, 1988; Hurst *et al.*, 1995).

Experimental studies on lymphocytes isolated from normal, non-human primates have demonstrated that intestinal LPL have a high capacity to produce IL-2, γ–IFN, IL-4 and IL-5 mRNA, while MLN complex cells have much lower capacity to express IL-2 and γ–IFN but have a high capacity to express IL-4 and IL-5 mRNA when stimulated (**Table 1.4**). These results are thought to indicate that LPL are in a more highly activated state than are the corresponding cells in other lymphoid areas. The data also indicate that mucosal T-cells may have differentiated through either the Th_1 type (IL-2 and γ–IFN) or Th_2 type (IL-4 and IL-5) pathways (McGhee *et al.*, 1993). However, Groux *et al.* (1998) have shown that repetitive antigenic stimulations of human and murine $CD4^+$ cells in the presence of IL-10 leads to the generation of a T-cell subset with low proliferative capacity which secrets high levels of IL-10 and γ-IFN and has immune regulatory activities both *in vitro* and *in vivo*. These cells, which the authors call T-regulatory 1 cells (Tr1), exhibit a cytokine profile distinct from Th_1, as they do produce γ-IFN, but not IL-2.

1.11.2. Distinction between LP and peripheral blood T-cells

The LP cell population is composed of both CD4 and CD8 cells, with a similar proportion of CD4 and CD8 lymphocytes to that in peripheral blood (Strober and James, 1994). The T cells in the intestinal LP, however, probably differ from the lymphocyte population in the circulation in several ways as follows;

(1) Most $CD4^+$ T cells in the intestinal LP are in an increased state of activation, when compared with lymphocytes in other regions. They lack $CD45RA^+$, and they resemble

memory cells (CD45RO+) in their ability to provide high helper activity for immunoglobulin synthesis by B-cells. This probably results from continuous exposure to luminal antigens (Selby *et al.*, 1984; Zeitz *et al.*, 1988).

(**2**) A higher proportion of T-cells in the intestinal LP express IL-2R, compared to circulating lymphocytes, indicating that an increased proportion LP cells are activated (James, 1991; James, 1993).

(**3**) One of the significant differences between LPL from non-human primates and T-lymphocytes from other sites is the increased expression or copy numbers of IL-2R α chain (p55) on T-cells. This probably results from continous exposure to luminal antigens (food and bacterial antigens).

(**4**) Resting LP T cells from normal non-human primates express MHC class II antigen, a marker of T-cell activation, whereas resting T cells from other sites do not express this activation marker (James, 1991).

(**5**) Finally, LPL express HML-1, αEβ7, mucosa-specific T-cell integrin antigen (Schieferdecker *et al.*, 1990; Schieferdecker *et al.*, 1991). Thus, these data collectively indicate that mucosal associated tissues, including intestinal LP, are important sources of T cells that are more activated than T cells in other sites (McGhee *et al.*, 1993).

Table 1.4. Evidence for specialized T-cell function in the mucosal immune system

	Mesenteric node	LP	IEL
Proliferation			
specific antigen	++	-	+
mitogen	++++	+++	±
Surface glycoproteins			
CD45RA+	++++	±	+
CD45RO+	ND¶	++++	+++
Leu-8 §	++++	±	++++
Activation			
IL-2R α chain	±	++∂	++∂
Lymphokine production			
IL-2	++	++++	+
γ-IFN	++	++++	+
IL-4	++++	++++	+
IL-5	++++	++++	+
Lymphokine utilization			
IL-2	++	++++	+ (*in vitro*)
IL-4	++++	-	unknown
Regulatory function			
Helper activity	++	++++	+
CD4 suppressor activity (Tr1)	ND	+/-	unknown

(James *et al.*, 1986; Cerf-Bensussan *et al.*, 1987; James, 1991; Senju et *et al.*, 1991; Schieferdecker *et al.*, 1992; Targan *et al.*, 1995)

¶ Not done

§ Leu-8 is a human leucocyte antigen (Mr 80 K daltons) (Jackson *et al.*, unpublished observations; Michie *et al.*, 1987); it is present on 68 ± 7% of peripheral blood lymphocytes, approximately 70-80% of T-cells, 10% of thymocytes, and most B-cells (Lanier *et al.*, 1983; Kansas *et al.*, 1985)

∂ 15-30% of IEL and LPL express IL-2R antigen

1.11. 3. LP macrophages

Macrophages make up approximately 10% of LP mononuclear cell isolates in humans (Bull and Bookman, 1979). Cells with typical macrophage features are widely disseminated in many tissues, especially in the lungs and the gut. In the latter regions, they tend to be localized in the superficial part of the mucosa just below the epithelial layer. It is not known whether they are specialized for this tissue or whether they specifically home to them. However, they may be derived from Peyer's patches (as do mucosal lymphocytes, LPL and IEL), because cells with monocyte morphology are found in draining lymph. A high proportion of LP macrophages express class II MHC antigens and other surface markers related to phagocytic cell activity. This suggests that LP macrophages are in a higher state of activation than are macrophages in other lymphoid tissues. The relative roles of macrophages as APCs is unknown; however, it appears increasingly possible that these cells may be involved in various aspects of local immune responses, such as determining whether active immunity or tolerance is induced (Mowat and Viney, 1997).

1.12. Mucosal mast cells (MMC)

Mast cells fall between innate (nonspecific) and acquired (specific) immune responses because of the presence of membrane-bound, polyclonal, antigen-specific IgE, which mediates degranulation via antigen recognition. They are present in abundance and in similar locations to T-cells in the gastrointestinal mucosa, an area subjected to chronic antigenic exposure (Smith and Weis, 1996).

MMC are not end-stage cells, but instead represent a phenotypic continuum that can be changed depending upon the microenvironment in which the cells are present. They arise from the bone marrow as immature cells and further differentiate within specific tissues in response to exogenous cytokines. MMC are dependent on the T-cell-derived cytokines interleukin 3 (IL-3=mast cell growth factor) and IL-4 for their proliferation, and are thus

named 'T-cell dependent' (Ruitenberg and Elgersma, 1976; Elson and Beagley, 1994; Smith and Weis, 1996). For instance, IL-3 promotes growth and differentiation of MMC from bone marrow by action on local precursors (Irani *et al*., 1987).

MMC are interspersed within the intestinal mucosa (LP and epithelial layer) as well as the submucosa. In the latter area, however, they are rare. Following a helminth infection, there is an expansion of MMC numbers (mastocytosis) within the LP and a concurrent migration of these cells into the epithelial region (Befus and Bienenstock, 1979; Arizono and Nakao, 1988; Smith and Weis, 1996). Studies of helminth infections in nude mice and rats suggests that this expansion is T-cell dependent, although the preferential migration of peripheral mast cells to the LP and intraepithelial space has not been addressed (Madden *et al*., 1991; Smith and Weis, 1996).

Although MMC have been described in several mammalian species, the fundamental properties of these cells has been largely obtained from studies in rats (Watkins *et al*., 1976; Ellen *et al*., 1984). Woodbury and Neurath (1978) investigated the development of MMC in the small intestine of rats. MMC were present in the intestine before birth in low numbers. MMC counts remained low in the first 3-4 weeks of life with weakly staining 'immature' granules present in the cells. After 4 weeks of life, MMC numbers increased five-fold to normal adult levels at approximately 9-13 weeks, after which MMC counts decreased slowly. In conclusion, these investigators have described an age-associated increase of MMC numbers.

Maturation of the small intestine during weaning has features in common with immunological reactions involving the gut mucosa. MMC activation is a prominent feature of both IgE and cell-mediated mucosal immune responses. MMC in rats are activated during weaning, as evidencd by substantial increase in serum rat mast cell protease II (RMCPII) at day 21 of life, by a lag in the age-dependent rise of jejunal RMCPII from 2 to 4 weeks, and by the

degranulated appearance of MMC. By 4 weeks, MMC granules appear normal again and serum RMPCII levels begin to fall to adult levels. This MMC activation and degranulation coincides with the mid-point of the indices of intestinal maturation during weaning (Cummins *et al.*, 1988a-b).

1.13. Intraepithelial lymphocytes of small intestine (IEL)

Small spherical cells residing between the intestinal columnar epithelial cells (enterocytes) lining the villous surface are called IEL. They constitute a sizeable proportion of lymphocytes in the gut (Rocha *et al.*, 1991). IEL comprise a special lymphoid population that is morphologically, functionally and phenotypically heterogenous. For example, they differ remarkably in phenotype, morphology and function from peripheral blood and LP T-lymphocytes. Furthermore, IEL are the closest lymphocytes in the body to various antigens (Cerf-Bensussan *et al.*, 1987; Lefrancois, 1987; Guy-Grand and Vassalli, 1988; Gyorffy *et al.*, 1992; Fujihashi *et al.*, 1993; Mowat and Viney, 1997) .

1.13.1. Historical aspects of IEL

The presence of IEL has been appreciated for over 100 years (Poussier *et al.*, 1994). Ebert was the first person to identified these cells as leucocytes within the intestinal layer. Until the late 1970s, the epithelium of the small bowel had been largely ignored as a tissue contributing directly in the induction and regulation of responses of the systemic and mucosal immune systems, and had been considered by investigators, including Weber (1847), only in terms of its absorptive role in nutrition (Ferguson, 1977; Bland *et al.,* 1991). Before the end of 19th century, there were several detailed descriptions of IEL in human and other vertebrates. Historically, IEL were thought to rejuvenate enterocyte nuclei by a phenomenon which used to be termed ‘caryoanabiosis’ (Ferguson, 1977).

1.13.2. Morphology of IEL

IEL are adjacent to the basement membrane in the basal part of the epithelium. Approximately one-sixth (1/6) of cells which cover the intestinal surface are IEL. IEL are variable in size by light and electron microscopy (Kagnoff, 1981) and are divided into two distinct subpopulations morphologically:

(**1**) Large-sized lymphocytes containing cytoplasmic basophilic granules (granulated intraepithelial lymphocytes, GIEL),

(**2**) Smaller lymphocytes with dense nuclei and sparse cytoplasm lacking granules (non-granulated intraepitelial lymphocytes, NGIEL).

These subpopulations are present in man, rabbits, rodents and pigs (Van Garderen *et al.*, 1991). In man, between 20-70% of IEL contain basophilic intracytoplasmic granules, which contain sulfated mucopolysaccharides. Because of the similarity of the metachromatic granules of GIEL and MMC, initial studies suggested that IEL are MMC. However, this is now thought to be unlikely; firstly, because the proteases in GIEL and in MMC are antigenically distinct (Huntley *et al.*, 1984), and secondly, mast cell deficient W/W^{v} (c-kit receptor null) mice have normal numbers of GIEL (Tagliabue *et al.*, 1982).

1.13.3. Distribution of IEL in various mucosal surfaces

IEL represent a heterogeneous population of lymphocytes situated in the epithelial layer of all mucosae. They are found in the mucosal epithelia of organs such as the intestines and stomach, the tongue, female reproductive tract (uterus and vagina), the breast epithelium during pregnancy and lactation, the bronchial airways and possibly the skin (Cerf-Bensussan *et al.*, 1991; Cheng *et al.*, 1991; Van Garden *et al.*, 1991; Kagnoff, 1993; Roitt *et al.*, 1996).

1.13.4. Intestinal distribution of IEL in mucosa

The epithelium of the villi in the small intestine contains large number of lymphocytes that vary in distribution in various parts of small bowel. In normal adult rats, the mean number of IEL is 14-16 per 100 epithelial cells. The normal human jejunum has approximately 20 IEL per 100 epithelial cells (Austin, 1986). The number of IEL/100 epithelial cells is lower in the distal parts of the digestive tract, resulting in a ratio of 13/100 in the normal ileum and a ratio of 5/100 in the normal colon. The reason for the higher abundance of IEL in the proximal parts of the digestive tract is probably due to the higher concentrations of food antigens in these regions, whereas the continuing digestion of the intraluminal mass results in decreased antigenicity of dietary proteins in the distal intestine (Marsh, 1980).

1.13.5. Quantification of IEL

Two methods for the quantification of IEL are used. In the first method, the number of IEL are counted as a proportion of the number of epithelial cells (Ferguson *et al.*, 1971). In the second method, the number of IEL are expressed as a proportion of mucosal volume (areal density) as specified by the length of the underlying muscularis mucosae (Marsh, 1980). The first method has been advocated as an indicator of local epithelial microenvironment, whereas the second method is thought to be more accurate as it uses a fixed reference as a denominator (Marsh and Cummins, 1989).

1.13.6. Significant factors which influence the numbers of IEL

1.13.6.1. Age

At birth, the rat small bowel is as mature as that of a mouse of three or four days of age, whereas the human species goes through that stage of development at about week 12 or 14 of gestation with regard to villi, enterocyte development and appearance of lymphocytes in the gut (Spencer *et al.*, 1986; Spencer *et al.*, 1987; Ferguson, 1992). These changes occur at

various times in different species (Ferguson, 1992); for some species, that stage of development may be complete before birth. The number of lymphocytes in the intestinal mucosa increases substantially after birth and during the weaning period. In the rat jejunum and human ileum, lymphocytes of both the helper/inducer and cytotoxic/suppressor phenotypes increase postnatally (Cerf-Bensussan *et al.*, 1984; Spencer *et al.*, 1986; Cummins *et al.*, 1988). At birth, the major stimulus for the induction of mucosal T cells is antigenic stimulation by food and bacterial antigens (Spencer *et al.*,1989; Bandeira *et al.*, 1990; Cerf-Bensussan and Guy-Grand, 1991,). In fact, for the expansion of intestinal mucosal lymphocytes, multiple immunologic events are envisaged. The proportion of α/βTCR expressing IEL rises dramatically with age, with a compensatory decline in the fraction of γ/δTCR IEL (the ratio of α/βTCR/γ/δTCR changes remarkably). In the case of the mouse, the absolute number of total IEL increases five to ten times between the ages of 6 and 20 weeks (Ferguson and Parrott, 1972); therefore, the absolute number of γ/δTCR IEL differs little within this schedule time.

It is important to emphasize that the age-dependent increase in both the number of IEL and the proportion that express α/βTCR is absolutely dependent on exposure of the animal to a conventional diet (food antigens) and environment. This has been documented in weanling and germ-free mice which have few IEL, most of which appear to be γ/δTCR. In fact, the ratio of IEL expressing α/βTCR and γ/δTCR in young germ-free animals is much lower than older animals, whereas in older animals the proportion of thymus-independent α/βTCR cells in the gut is increased (Viney and Guy-Grand, 1990; Bandeira *et al.*, 1990; Latthe *et al.*, 1994).

Thus, the proportion of α/βTCR$^+$ IEL is influenced by several factors including age, food and exposure to bacterial antigens (Lefrancois, 1991; Poussier & Julius, 1994). Conversely, the accumulation of IEL during fetal life may be an antigen-independent event (Spencer *et al.*, 1989), although this assumes that food antigens are absent in fetal life which may not be true.

1.13.6.2. Microbial antigens

In germ-free animals, many lymphoid organs including GALT are small (immature) and the LP contains few plasma cells (Ferguson, 1979; Cummins & Roberts-Thomson, 1994). Likewise, the number of IEL harvested from germ-free mice is remarkably smaller than that from specified pathogen-free animals (Nagasawai *et al.*, 1993). IEL numbers are low in germ-free animals or germ-free intestine and increase after introducing germ-free animals into standard housing (Jarry *et al.*, 1990; Lefrancois *et al.*, 1991; Trejdosiewicz, 1992; Nagasawa *et al.*, 1993). This suggests that expansion of IEL is influenced by bacterial flora and that IEL may participate in the mucosal immune defence (Cerf-Bensussan *et al.*, 1985).

Although germ-free mice have only few IEL, most of them are $\gamma/\delta TCR^+$, indicating that the tropism of $\gamma/\delta TCR^+$ T-cells for the gut epithelium is largely bacterial antigen-independent (Bandeira *et al.*, 1990). This may be the case in humans, since higher frequencies of γ/δ^+ IEL are seen in human fetal gut than in the post-natal intestine (Spencer *et al.*, 1989). Although $\alpha/\beta TCR^+$ T cells are present in the human fetal gut mucosa, their number is low compared to the antigenically stimulated post-natal gut. This suggests that the expansion of $\alpha/\beta TCR^+$ IEL is largely antigen-dependent. One possibility for the expansion of $\alpha/\beta TCR^+$ IEL in germ-free animals, when exposed to standard housing, is that antigenic stimulation gives rise to recruitment of IEL from the bone marrow (but not from Peyer's patches, MLN/ LP, since the CD8 α/α α/βTCR cells were not detected in these areas), or that clonal expansion occurs from resident IEL (Bandeira *et al.*,1990; Lefrancois and Goodman, 1991; Umesaki *et al.*, 1993). However, there is no evidence of a direct IEL-bacterial antigen interaction (Lefrancois and Goodman, 1989; Huleatt and Lefrancois, 1995). The nature of antigen-processing mechanisms required for recognition by IEL has yet to be defined (Bandeira *et al.*, 1990; Cheng *et al.*, 1991; Huleatt *et al.*, 1995).

1.13.6.3. Food antigens

Soluble proteins constitute the greatest immunological source of antigens. The higher IEL counts in the proximal (food-exposed) small intestine suggest that IEL respond to food antigens. Thus, IEL are increased approximately two-fold in the duodenum compared to the ileum (Ferguson, 1987). Following administration of cyclophosphamide to mice, ingestion of ovalbumin (a soluble protein) sensitizes T-cells in the MLN and gives rise to an increase in IEL numbers and in epithelial cell renewal (Mowat and Ferguson, 1981; Mowat, 1987). This suggests that food antigenic stimulation causes recruitment of IEL from MLN. In conclusion, there is substantial evidence that the number of $\gamma/\delta TCR^+$ IEL is determined by the antigenic composition of the diet, rather than by the presence of local bacteria (Imaoka *et al.*, 1996; Kawaguchi-Miyashita *et al.*, 1996), and that activated $\gamma/\delta TCR^+$ IEL appear to localise preferentially in the upper small intestine, where food antigens are most abundant (Penney *et al.*, 1995). This is further supported by recent findings, where the proportion of $\gamma/\delta TCR^+$ IEL in the jejunum of small intestine in the PVG/c rats at 6 months of age is five times greater than ileum, a site of higher concentrations of bacterial antigens (Helgeland *et al*. 1997).

There is no direct IEL-dietary protein antigen interaction. The antigenic specificity of human IEL is unknown. Even in coeliac disease, where the antigen is known, gluten specificity in IEL-derived T cell lines and clones has not been established (Trejdosiewicz, 1993). However, proliferative activation (Ki-67^+) of $\alpha/\beta TCR^+$ and $\gamma/\delta TCR^+$ $CD8^+$ IEL (but not $CD4^+$) has been observed (Halstensen and Brandtzaeg, 1993). This suggests that gluten (food antigen) in coeliac disease, somehow gives rise to proliferative activation of both subsets of IEL.

1.13.6.4. Suppressor elements in breast milk

Bovine and human breast milk are immunosuppressive and contain high concentrations of the immunosuppressive cytokine, transfoming growth factor-beta2 (TGF-β2) (Tokuyama H, 1989; Tokuyama Y., 1989; Jin *et al.*, 1991; Cox and Burk, 1991; Saito *et al.*, 1993, Cummins *et al.*, 1997).

1.13.7. Expansion of IEL during weaning

Several studies have shown that IEL are low before weaning and increase during infancy (Ferguson, 1977; Lyscom, 1983; Cummins *et al.*, 1988b). Weaning is gradual in the rat and occurs over a 2 week period (days 15 to 28 of life) with a decline in milk suckling and an increase in solid food consumption. This expansion of IEL appears to be immune-mediated, as the immunosuppressive, cyclosporine A, suppresses the rise in IEL (Cummins *et al.*, 1989a; Williams, 1994). Colonization of commensal bacteria, and a diversified diet all occur concomitantly during weaning (Bandeira et el., 1991). Therefore, weaning is associated with exposure of the intestinal mucosa to food and bacterial antigens. It is probably impossible to separate the contribution of food or bacterial antigens in expanding the size of the GALT. Bacterial lipopolysaccharide (LPS) is important in priming the GALT by up-regulating the localization, numbers and activation of dendritic cells (expressing MHC class II) that could potentially respond to food antigens (Mowat and Viney, 1997).

The peak of IEL is not entirely concomitant with the peak of mucosal immune activation in the LP, because expansion of IEL is postponed by approximately 1 week with respect to degranulation of MMC and release of serum RMCPII, and to the peak of activated T-cells (as shown by increased expression of IL-2R) in the MLN (Cummins *et al.*, 1988a-b). Thus, there appears to be a lag in immune reactivity between the LP and IEL during the weaning phase.

1.13.8. Phenotype of IEL

Numerous studies have shown that 80% to 90% of IEL in adult humans, rats and mice are $CD3^+$, and thus are T-cells (Selby *et al.*, 1981; Selby *et al.*, 1983; Ernst *et al.*, 1985; Goodman and Lefrancois, 1988; Lefrancois, 1991; Helgeland *et al.*, 1997). This is also corroborated by other investigations including cell transfer studies, immunofluorescence studies, and studies in T-deprived mice which show that a large fraction of IEL are T-cells (Kagnoff, 1981). In addition, approximately 12% (5-25%) of IEL in humans are non-T cells representing $CD2^-$ $CD3^-$ $CD7^+$. However, there is marked variation among individuals. For instance, Spencer and MacDonald (1989 and 1990) and Klein (1991) have reported that nearly 50 and 85% IEL in some children and adults are non-T-cells, respectively. There are plausible reasons for these discrepancies as the frequency of $\alpha/\beta TCR^+$ IEL, varies widely. For example, in animals the number of $\alpha/\beta TCR^+$ IEL depends on age and the level of antigenic stimulation in the intestine and can reach 75% in adult mice reared under conventional conditions (Bonneville *et al.*, 1988; Goodman and Lefrancois, 1988; Jarry *et al.*, 1990; Mosley *et al.*, 1991; Taguchi *et al.*, 1991; Fujihashi *et al.*, 1992).

The phenotype of IEL prior to weaning in rats and mice is mostly α/βTCR, although the numbers are low. However, several studies in mice and rats have shown that the total number of IEL or IEL with the α/βTCR phenotype, expand upon antigenic stimulation during weaning. This is also seen when germ-free animals become exposed to conventional conditions of housing and feeding (Cummins *et al.*, 1988b; Guy-Grand, 1990; Viney *et al.*, 1990; Guy-Grand *et al.*, 1991).

In contrast, the number of thymus-dependent $\gamma/\delta TCR^+$ IEL is determined by antigenic composition of the diet, but not by bacterial antigens. Thus, γ/δ thymus-dependent IEL do not change at the time of weaning in mice (Bandeira *et al.*, 1991). This is further supported by the preferential localisation of γ/δ-IEL in the upper small intestine, where food antigens are most abundant (Penney *et al.*, 1995). By contrast, the numbers of thymus-independent γ/δ IEL is

influenced by bacterial colonization of the intestine (Bonneville *et al.*, 1988; Goodman and Lefrancois, 1988; Mosley *et al.*, 1991; Taguchi *et al.*, 1991; Fujihashi *et al.*, 1992).

Most (60-90%) IEL in adult rats (and humans) have the CD8α/β and CD3 α/βTCR phenotype (Vaage *et al.*, 1990; Cerf-Bensussan and Guy-Grand, 1991). The CD3$^+$ and α/βTCR$^+$ CD8α/β^+ cells are thymus-dependent. Another population of CD3$^+$ cells is bone marrow-derived and has a CD5$^-$ phenotype (Mayrhofer, 1980; Mayrhofer and Whately 1983). CD5 is normally expressed by mature peripheral blood T-cells (maturation marker). This CD5$^-$ IEL population expresses the γ/δTCR in approximately 50% of the subset and accounts for the majority, if not all, γ/δTCR$^+$ IEL (Cerf-Bensussan and Guy-Grand, 1991).

Hunig *et al.* (1992) have suggested that CD8α/β expression on rat IEL follows activation of a pre-existing homodimeric CD8α/α chains. This is consistent with previous studies which show that CD8α/α is present in suckling and germ-free mice, and hence does not require antigenic stimulation for its expression (Guy-Grand *et al.*, 1991). There is no direct study of the shift from CD8α/α to CD8α/β expression amongst IEL during weaning.

Table 1.5. Phenotype of jejunal IEL in the adult rat.

Lymphocyte marker	% positive IEL
CD2	<5
CD3	84
α/βTCR	60-70
γ/δTCR	5
CD4	5
CD5	30-75¶
CD8	90
CD8 α/α	50-100
$CD4^{+}$ $CD8^{+}$	30
CD45RA (specific for B-cells)	<0.5
$CD45RB^{high}$	ND
IL-2R antigen	ND§

(Vaage *et al.*, 1990; Fangmann *et al.*, 1991; Lefrancois, 1991; Trejdosiewicz, 1992; Helgeland *et al.*, 1997).

¶ >50% of $CD8^{+}$ IEL appear to be CD5-. However, flow cytometric analysis has revealed that most $CD8^{+}$ IEL express CD5, but at a substantially reduced density.

§ ND, Not determined.

1.13.9. Phenotypic differences between IEL, LPL, and peripheral blood lymphocytes

As noticed above, IEL are quite distinct from LPL in several features including many of the phenotypic characteristics as follows:

(**1**) The predominant phenotype of IEL is $CD8^+$, while peripheral and LP T-cells are mainly $CD4^+$ (**Table 1.4**) (Lefrancois, 1991; Roitt *et al.*, 1993; Sydora *et al.*, 1993).

(**2**) By immunohistological criteria, all classical mature peripheral T-cells express CD5 (pan-T marker of mature cells), while more than 50% of human $CD8^+$ IEL appear to be $CD5^-$ (Malizia *et al.*, 1985; Trejdosiewicz *et al.*, 1987; Trejdosiewicz *et al.*, 1989), or express CD5 at a substantially reduced density (Ebert, 1989; Jarry *et al.*, 1990).

(**3**) Unlike intestinal LP and peripheral T-cells, $CD8^+$ IEL have low expression of CD5, CD6, and CD28 implying little interaction with B cells (Trijdosiewicz, 1993).

(**4**) Approximately 13% of human IEL express the γ/δTCR, a percentage which is significantly higher than that found in blood and LP. About 20% to 40% of IEL with γ/δTCR express CD8α/α. The expression of CD8 on γ/δ IELs in the immune system of the gut has been associated with an activated state (Spencer *et al.*, 1989; Halstensen *et al.*, 1989; Jarry *et al.*, 1990; Cerf-Bensussan and Guy-Grand, 1991; Roitt *et al.*, 1996).

(**5**) In contrast to γ/δ TCR peripheral blood lymphocytes, γ/δ TCR IEL never express CD4 (Groh *et al.*, 1989; Bucy, 1989) In addition, nearly 10% of human IEL have a unique phenotype of immature T cells ($CD3^-$ $CD7^+$), which is only rarely present in the LP. Those cells (approximately 10-15%) with the phenotype of $CD3^-$ $CD7^+$ $CD16^+$ in the blood are NK cells (Preffer *et al.*, 1989; Jarry *et al.*, 1990).

(**6**) Finally, a larger fraction of IEL (nearly 55%) in the adult mouse and a smaller proportion of IEL (10% to 40%, mean 10%) in the human express the thymus independent CD8α/α chains without the β chain, while LPL and peripheral blood lymphocytes express the thymus-dependent CD8α/β marker (Vaage *et al.*, 1990; Jarry *et al.*, 1990; Guy-Grand and Malassis-Seris, 1991; Cerf-Bensussan and Guy-Grand, 1991).

Table 1.6. Phenotypic differences between human LPLs and IELs. The phenotype of LPL is similar to peripheral T-cells. A higher proportion of IEL express γ/δTCR and more of this subset are $CD8^+$.

Cell type	α/βTCR	γ/δTCR	CD4	CD8	$CD45RA^-/CD45RO^+$
LPL	>95%	<5.0%	70%	30%	96%
IEL	60-90%	<10%¶	<10%	70%	90% (66-100%)§

¶ Larger proportions of $\gamma/\delta TCR^+$ IEL (10-40%) are found in some normal individuals. (Spencer, *et al.*, 1989; Viney, *et al.*, 1990; Schieferdecker *et al.*, 1992; Farstad *et al.*, 1993; Gross *et al.*, 1994; Roitt *et al.*, 1996).

§ There is no direct evidence of the proportion of α/β IEL T-cells with the phenotype of *memory cells*, hence the proportion of γ/δ IEL T-cells expressing CD45RO was included.

1.13.10. Comparison between proliferative responses of human IEL and peripheral blood lymphocytes

The *in vivo* proliferative response of IEL has been investigated in tissue sections using the measure of mitotic index. In spite of continuous contact with a large variety of antigens in the gut lumen, they exhibit a low mitotic index (Marsh, 1980). Unlike peripheral T-cells, *isolated* human IEL show a poor proliferative response to conventional T-cell mitogens (such as phytohaemagglutinin and concavalin A), to alloantigen and to immobilized anti-CD3 mAb (**Table 1.7,** Greenwood *et al.*, 1983; Ebert *et al.*, 1986; Ebert, 1989; Mowat, 1990). This demonstrates that IEL have impaired proliferation compared to T-cells in blood. The low levels of high affinity IL-2R expression could be a crucial element in causing the weak proliferative response of IEL (Sydora *et al.*, 1993).

However, isolated human IEL proliferate briskly in response to mitogens combined with sheep red blood cells. The sheep red blood cells markedly enhance the mitogen-induced response of IEL by augmenting events of activation, both IL-2 production and IL-2R expression (Ebert, 1989). Furthermore, isolated human IEL proliferate vigorously in response to a mixture of phorbol ester and ionomycin which bypass all the membrane steps of T-cell activation (Cerf-Bensussan and Guy-Grand, 1991).

In conclusion, there are several possible explanations for the poor proliferative responses of human IEL to mitogens and nominal antigens *in vitro*. IEL may require unusual co-stimulatory pathways, such as CD2 or the $\alpha_E\beta_7$ integrin (Ebert, 1989; Russell, *et al.*, 1994). Alternatively, IEL may be already fully differentiated effector cells that cannot be induced to proliferate. This is corroborated by the fact that most IEL have the morphological and phenotypic characteristics of cells which have been exposed to antigen, including the expression of the CD45 isoforms associated with memory or activated T-cells (CD45RO) (Mowat, 1990; Halstensen *et al.*, 1990; Guy-Grand, *et al.*, 1993; Abbas *et al.*, 1994). Furthermore, IEL are larger than normal resting T-lymphocytes, are constitutively highly motile and possess cytoplasmic *granules* containing perforin and granzyme A, which are markers of differentiated cells (Guy-Grand, *et al.*, 1991).

1.13.11. Functional differences between IEL and peripheral blood lymphocytes

In contrast to peripheral blood lymphocytes, no functional activity has yet been ascribed to human IEL conclusively, indicating that they neither provide helper nor suppressor functions for immunoglobulin synthesis by B-cells, and do not mediate spontaneous cytotoxicity (MacDonald *et al.*, 1990; Trejdosiewicz, 1992). However, characterization of IEL in rodents has revealed that a variable proportion of IEL contain cytoplasmic granules (in the rat, 50% of IEL have these granules), and exhibit cytotoxic activity *in vitro* (**Table 1.7**) (Flexman *et al.*, 1983; Mowat, 1990; Cerf-Bensussan *et al.*, 1991; Gyorffy *et al.*, 1992). In contrast to *in vitro*

studies in animals, which clearly show the cytotoxic properties of IEL, the cytotoxic activities of human GIEL, have been hardly studied. They do not exert spontaneous cytotoxicity against two tumor cells lines-namely, Chang and K562. Cerf-Bensussan and Guy-Grand (1991) showed that human IEL isolated from normal gut had poor cytotoxicity, although this needs further confirmation. It is important to emphasize that spontaneous cytotoxicity of IEL, either from humans or laboratory animals, has not been demonstrated.

Table 1.7. Functional properties of IEL

Present	Cytotoxic T-cell activity
	Lymphokine secretion (IFN-γ, IL-2, IL-3, IL-4,
	IL-5, IL-6, TNF-α^{δ}, TGF-β^{ϕ}, GM-CSF$^{£}$
	Local DTH responses
Low or absent	inducible NK cell activity
	Proliferation in response to mitogen.
Uncertain	Helper/suppressor T-cell activity

δ Tumor necrosis factor-α;

ϕ Transforming growth factor-β;

£ Granulocyte/macrophage-colony-stimulating factor

(Mowat, 1990; Barrett, *et al*., 1992; Fujihashi, *et al*., 1993; Elson and Beagley, 1994).

1.14. Lymphocyte-filled villi (LFV)

Villi are the finger-like processes that greatly increase the surface area of the small intestine. Approximately, 1 percent of villi have a modified structure. These villi are filled with closely packed lymphocytes and dendritic cells. The covering epithelium consists of specialised antigen-absorbing cells.

Recent work in Mayrhofer's laboratory (Department of Microbiology and Immunology, University of Adelaide) has classified these villi as organised lymphoid structures. LFV are detected at the second week of life in the mucosa of the rat small intestine. They exist in both conventional and specific-pathogen free rats, suggesting that they are not inflammatory foci.

LFV are also present in congenitally athymic rats, indicating they are not thymus-dependent. LFV do not receive lymphocytes from the recirculating pool, suggesting that they might be sites of primary lymphopoiesis (Mayrhofer, unpublished data).

1.14.1. Distinction between LFV and Peyer's patches

LFV vary from Peyer's patches (clustered lymphoid follicles) in several ways:

(1) In contrast to Peyer's patches, LFV do not contain significant numbers of B-cells (they lack follicles and germinal centres) and there is only a relatively minor subpopulation that expresses either TCR or CD3.

(2) High-endothelial venules are not obvious in LFV, but are usually obvious in the interfollicular areas (T-dependent zones) of Peyer's patches.

(3) LFV occur at all points on the gut circumference from pylorus to ileocaecal valve, whereas Peyer's patches are located on the antimesenteric wall of the small bowel, and in rats, are most common in the ileum.

(4) LFV have a specialised epithelium that absorbs macromolecules (e.g. horseradish peroxidase) and are distinct ultrastructually from M cells (Mayrhofer, unpublished data).

1.14.2. Phenotype and activation status of LFV

LFV lymphocytes express CD45, CD43, class I, and IL-2Rα chain (CD25) antigens. In young animals, they contain a population of lymphocytes which may be pre-T-cells. With increasing age in both normal and nude rats, a population of $\alpha/\beta TCR^{+}$ cells appear. Autoradiographic studies using tritiated thymidine (^{3}H-Tdr) indicate that LFV contain dividing lymphocytes in the base (Brooks and Mayrhofer, unpublished observations).

1.15. Clusters of crypto-patch lymphoid cells

Crypto-patches are a newly identified component of the GALT which are located in crypt LP in mice and are filled with closely-packed lymphoid cells. Less than 2% express either surface immunoglobulin (IgM) or TCR antigen and are, therefore, not conventional B or T lymphocytes (Kanamori, *et al.*, 1996). They are present throughout the small and large intestine of mice, are found in athymic and SCID (severe combined immunodeficiency) animals and have a comparatively high proliferative activity. They also express recombination activating gene-2 (RAG-2), and markers of early T-lymphocytes, including heat-stable antigen (HSA; bone marrow precursors express high levels of this antigen shortly after reaching the thymus) and CD44 (Pgp-1=phagocytic glycoprotein) (Zugic, 1991; Abbas, *et al*., 1994; Kanamori *et al.*, 1996). They are not detectable until the second week of postnatal life-namely, at the commencement of weaning (Gordon and Hermiston, 1994). Crypto-patches are absolutely dependent on IL-7, a crucial factor in early T-cell maturation (Peschon *et al.*, 1994) and one of the cytokines generated by intestinal epithelial cells (Watanabe *et al.*, 1995). Hence, it has been suggested that crypto-patches are the source of progenitors for epithelial and probably other mucosal T-lymphocytes in mice. The phenotype of crypto-patch cells also agrees with the precursors of several other cell types, including mast cells, B-cells, and dendritic cells.

1.16. Aims of the thesis

This study aimed to address the hypothesis that physiological inflammation in the small intestine and MLN complex is upregulated during the weaning period. The overall aim of this study was to characterize the phenotype and activation status of the immune cells in the GALT during and after weaning. There are few studies concerning the phenotype and activation status of these cells during weaning.

Specific aims:

(1) To identify changes in the number, phenotype and activation status of IEL and LP cells during weaning. The phenotypic markers which were studied included various T and B-cell markers, NK cell marker, and markers of non-lymphoid cells, by either the indirect immunoperoxidase or alkaline phosphatse staining methods. To better understand the role of IEL and LP immune cells in the maturation of the small intestine, this study investigated which subsets of IEL and LP cells expand during weaning, and whether this expansion is due to the local proliferation of these cells or to recruitment of cells from other sites.

(2) To determine changes in the number, phenotype and activation status of LFV cells during this time. To also determine whether crypto-patches are present in the rat small intestine, and if so, to investigate the number, phenotype and activation status of this novel lymphoid organ.

(3) The Peyer's patches and MLN complex were studied to determine whether there is a correlation between the activation status of inductive and effector sites of the GALT (LP and IEL).

To address these aims, rat litters were killed at given intervals, jejunal sections and MLN were obtained and frozen in OCT before cryostat histological sections were fixed in acetone. These samples were analysed by immunohistochemistry for expression of different cell surface markers.

These studies were designed to further our understanding of inflammatory and infectious conditions in the small intestine, and ultimately for the design of vaccines and more effective therapies. In addition, this study may provide further information on the development and maturation of small intestine, especially during infancy.

Chapter 2

General Materials and Methods

2.1. Animals

Specific pathogen-free (SPF) DA and PVG/c parental rats were obtained from the Animal Resource Centre (ARC), Perth, Western Australia. DA female rats were mated with PVG/c males or PVG/c females with DA males to obtain [DAxPVG/c or PVGxDA] F_1 litters. Animals were reared and mated under conventional conditions in the Animal House of The Queen Elizabeth Hospital (TQEH) to obtain litters from days 7 to 70 of life. With a maximum of 6 litters for any given dam, pups of each litter were approximately the same weight, and runted animals were excluded. Day 0 was designated as the day of birth. Litters were housed with the dams and were allowed to wean naturally.

2.2. Procedure for killing animals

Animals were killed under ether anaesthesia in a closed chamber in a laminar flow hood. Jejunal samples, MLN, and splenic tissue were collected as follows:

2.2.1. Collection of small intestine (upper jejunum)

Once anaesthetized, the skin was moistened with 70% alcohol. A 'V' shaped incision was made in the lower abdomen using blunt-nosed, sharp-bladed scissors. The skin was pulled back to expose the small bowel and spleen. The entire length of the small intestine was dissected from the mesentry. Care was taken not to perforate the gut. After dividing it into thirds, the mid-third of jejunum was flushed with ice-cold PBS (at pH 7.4, containing 13.2 mM sodium phosphate and 154 mM NaCl) and transferred into a petri-dish containing ice-cold PBS for preparation of frozen sections. Segments of jejunum were taken from the proximal end of the intestine.

2.2.2. Collection of MLN

For collection of MLN, a midline incision was made in the abdomine and abdominal muscles were pinned back. The chain of superior nodes (**Fig. 2.1**) was identified, starting at the junction between the small and large intestine (ileo-caecal junction) and running parallel to the ileum. The slightly yellowish, encapsulated nodes were distinguished from the softer whiter fat. By using dagger forceps, the lymph node chain was grasped, gently torn and mobilized free of fat. They were transferred through a rinse of ice-cold-PBS into a petri-dish. Care was taken not to crush or damage the nodes during the procedure.

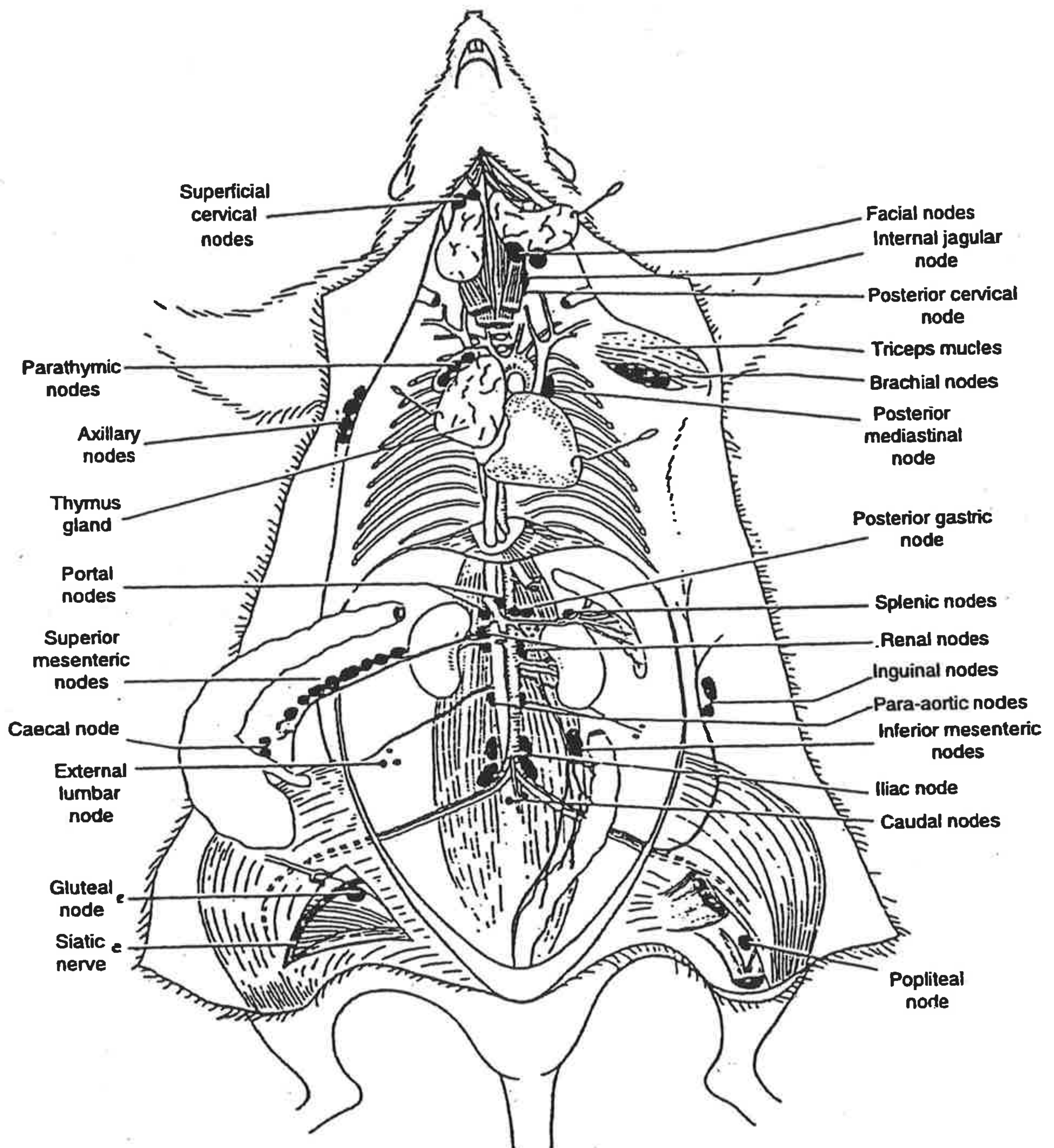


Fig. 2.1 Diagram showing the location of lymph nodes in the rat (copied from Hunt, 1978).

2.2.3. Collection of spleens

The spleen is the largest single aggregate of secondary lymphoid tissue in the body. The small, bright red spleen which is located in the upper left quadrant of the abdomen was grasped with dagger forceps. The spleen was gently dissected from connective tissue and the splenic vessels in the hilum. Finally, it was transferred through a rinse of ice-cold PBS into a petri-dish for preparation of transverse frozen sections.

2.3. Preparation of tissue sections

2.3.1. Frozen sections

Jejunal segments (0.5-1 cm in length) were opened lengthwise along their mesenteric border and laid serosal-side down on to a Millipore filter (type HA, 0.45 µm pore size, Millipore, Bedford, MA). Jejunal samples were carefully orientated on to filter paper, a drop of OCT compound (Tissue-Tek, Miles Laboratories, Elkhart, IN, USA) was applied, and tissue was embedded on an edgewise orientation in an aluminum foil boat containing OCT. MLN were placed in ice-cold PBS, and submerged directly into OCT. Spleen sections were cut transversely, and placed into OCT.

Tissues in OCT compound were frozen in a beaker of isopentane (GPR grade, BDH Laboratory Supplies, Poole, England) cooled by liquid nitrogen. They were inserted in sealed jars with ice to minimize dryness of tissues and to preserve antigenicity. Finally, they were stored at -70° C until required. Tissue was sectioned and mounted at 5 µm onto slides pre-coated with HistoGrip (Zymed, San Francisco, USA) using a Microtome Cryostat (HM 500 OM, Microm Heidelberg) operated at -22° C. Initial jejunal and MLN sections were stained rapidly (30 sec) with Lillie Mayer's haematoxylin to examine their morphology under the microscope prior to immunostaining. Upon assurance of morphology and orientation, further sections were cut for specific immunostaining.

Jejunal sections were fixed immediately in acetone (4° C) for 10 mins and dried, while MLN sections were dried unfixed. Acetone was used as a fixative for leucocyte surface antigens, because it preserves immunoreactive sites. Although it is very poor tissue penerator, it is nevertheless appropriate for thin cryostat sections (3-5 µm) and smears (Farmilo and Stead, 1998). Accordingly, acetone was used to fix the jejunal cryostat sections.

2.3.1.1. Preservation and drying of jejunal cryostat sections prior to immunostaining

While other investigators do not usually fix gut tissues prior to air-drying, it was found essential to completely fix gut tissues to maintain optimal cellular morphology for cell counts in the LP. Indeed, unfixed tissues showed marked deterioration in tissue morphology, autolysis, and nuclear changes, compared to correctly fixed gut tissues in parallel. Therefore, following cutting, jejunal sections were fixed in pre-cooled acetone (4° C) for 10 mins, and were air-dried overnight at room temperature prior to immunostaining.

Spleen and the MLN sections did not need full fixation to maintain optimal morphology, because they contained lesser amount of lysosomal enzymes and, therefore, they were air-dried unfixed, for 24-48 h at room temperature. It has been shown that thin sections and extended drying prevents the artifacts often seen in immunostains of lymphoid tissues fixed in acetone (Farmilo, and Stead, 1989).

2.3.2. Paraffin wax embedded sections

Jejunal segments were opened along their mesenteric border and laid serosal side down on cardbord. Strips approximately 1 mm x 4 mm were cut and trimmed with scissors, with the long axis in the orientation of the axis of the gut lumen. One strip was placed into a *glass* container of Carnoy's fixative (60% ethanol, 30% chloroform, 10% glacial acetic acid),

accompanied by a single shake to straighten the villi and remove mucus. Sections were left in this fixative for 30 mins, and transferred for storage into 95% ethanol. Tissues were embedded in paraffin. Histological sections were cut (5 µm), and left on a hot-plate at 50-56° C for 1 hr to melt the wax and to let sections adhere firmly onto the slides (heat-fix). Five µm sections, while they were still warm, were de-waxed in xylene for 2x10 mins, and rehydrated through 100% ethanol into distilled water prior to staining.

2.4. Immunohistochemistry

2.4.1. Antibodies

Mouse anti-rat monoclonal antibodies (mAbs) or turkey anti-human TGF-β polyclonal antibody, used in immunostaining studies are listed in **Table 2.1**. In addition, the secondary antibodies used to detect mouse monoclonal antibodies or turkey polyclonal antibody were as follows:-

(**a**) Affinity purified $(Fab')_2$ sheep anti-mouse IgG conjugated to horseradish peroxidase (Amersham Australia, Sydney, Australia) was used in the indirect immunoperoxidase technique. This antibody has no cross-reactivity to rat IgG.

(**b**) Sheep anti-mouse IgG3: peroxidase (Serotec Ltd, Oxford, UK, Code no : AAC 05P) was used in the indirect immunoperoxidase technique to detect anti-rat CD3 monoclonal antibody (G4.18, IgG3 isotype).

(**c**) Unconjugated rabbit anti-mouse Ig (pre-diluted link antibody) was used in the indirect alkaline phosphatase, anti-alkaline phosphatase system (K0670, APAAP System 40 kit, Dako, Carpinteria, CA, USA).

(**d**) Peroxidase-rabbit anti-chicken/turkey IgG (H+L) was used in the indirect immunoperoxidase method to detect anti-human TGF-β IgG, turkey (Zymed Laboratories, INC. San Francisco, CA, USA)

The negative control antibodies were:-

(**a**) Purified mouse IgG1 (Beckton-Dickinson, 50 μg/ml);

(**b**) Purified mouse IgG (Sigma Immuno Chemicals, 1 mg/ml, mixed isotypes);

(**c**) Turkey IgG, whole molecule (Rockland, 2 mg/ml)

Except in the case of link antibody and mouse anti-calf alkaline phosphatase imune complex, 10% normal rat serum (innocuous protein) was used to inhibit non-specific binding through Fc receptors (eg., mature B-lymphocytes and macrophages possess Fc receptor proteins), and to absorb any residual cross-reactivity by secondary antibodies for rat immunoglobulin (Basten *et al.*, 1972). As noticed above, rat serum was excluded from the link antibody, because it already contained normal blocking swine serum.

Table 2.1. Determinants of monoclonal mouse antibodies used for immunostaining.

Determinant	Clone	isotype	Dilution	Source
CD3	G4.18	IgG3	undiluted	Mayrhofer*
α/βTCR	R73	IgG1	1/1000, ascites	Serotec
γ/δTCR	V65	IgG1	1/40, purified	PharMingen
CD4	W3/25	IgG1	1/7000, ascites	Serotec
CD5	OX-19	IgG1	1/1500,ascites	Serotec
CD8α	OX-8	IgG1	1/1000, asites	Serotec
CD8β	341	IgG1	1/50, purified	Serotec
CD25 (IL-2Rα)	NDS-61	IgG1	1/250, ascites	Serotec
CD43	W3/13	IgG1	1/500, ascites	Serotec
CD45	OX-1	IgG1	1/500, ascites	Serotec
CD45RA (B-cell)	OX-33	IgG1	1/1000, purified	Serotec
NKR-P1	3.2.3	IgG1	1/1000, purified	Serotec
Macrophage	ED2	IgG1	1/1000, ascites	Serotec
Ki-67	Ki-67	IgG1	1/50, purified	Dako

*Graham Mayrhofer, Department of Microbiology and Immunology, University of Adelaide

Serotec Ltd, Oxford, UK

PharMingen, San Diego, CA, USA

Dako Australia, Sydney, Australia

Becton-Dickinson, Bedford, MA, USA

2.4.2. Enumeration of IEL, MLN, and LFV cells by the indirect immunoperoxidase staining method

Sections were removed from storage (-20 or -70° C) and brought to room temperature (30 minutes) before transfer to slide racks. Slides were fixed in pre-cooled acetone (4° C) for 10 mins to fully inactivate lysosomal enzymes and prevent autolysis, which is otherwise often seen in gut sections, and slides were allowed to dry for 15 sec. Unfortunately, the fixation by acetone was not complete; frozen sections subject to extended immunochemical procedures often showed deleterious morphological changes, including chromatolysis and apparent loss of cellular membranes. It was found that tissue morphology is preserved by ensuring that the sections are thoroughly dried both before and after fixation in acetone. Slides were rehydrated with ice-cold TBS (containing 0.05 M Tris-HCl, 0.15 M NaCl at pH 7.6) for 5 minutes. After addition of primary antibody (50 µl), slides were incubated in a covered humidified chamber to prevent evaporation of the antibody solution, and the chamber was left undisturbed for 1 h at 4° C. It was found that this incubation time and ambient temperature were optimal for most of leucocyte surface markers in this study (with the exception of the CD3 antigen which needed 3 h incubation at room temperature). It was also important that all sections remain hydrated during immunostaining, because drying leads to shrivelling and fragmentation, essentially destroying the section. After this time, slides were washed three times in ice-cold TBS (3 minutes per wash) and then incubated with 30 µl of secondary antibody (1/20 dilution of Amersham $(Fab')_2$ sheep anti-mouse IgG-horseradish peroxidase conjugate) for 1 h at 4° C. Following this incubation, slides were washed three times in ice-cold TBS (3 minutes per wash) and moved to room temperature. Slides were incubated with 0.05% 3,3' diaminobenzidine tetrahydrochloride substrate (DAB) (10 mg DAB tablets, Sigma, St Louis, MI, product No. D-5905)/3% hydrogen peroxide for 6 mins at room temperature. After this incubation, slides were left in warm tap water, with occasional agitation, for 5 mins before counterstaining.

2.4.3. Differentiation of brown staining cells (positive) and unstained cells (identified by their blue nuclei) by counterstaining with haematoxylin

Counterstaining was carried out by light staining of slides with undiluted Lillie Mayer's haematoxylin (alcohol containing counterstain, **Table 2.2**) for 2 minutes followed by a rinse in PBS, 2 fast dips in 0.6% acid-alcohol (for differentiation until the nuclei are blue), and a brief wash in water for 30 sec. Slides were blued in Scott's solution (**Table 2.3**) for 10 sec, followed by a quick dip in tap water to remove excess salts from the Scott's solution prior to dehydration and clearing. This involved 2 minutes in each of two 50%, 70%, 95%, and 100% ethanol baths followed by 10 minutes in each of two Histo-clear II (National Diagnostic, Atlanta, GA) baths. Finally, slides were mounted using Histomount neutral pH mounting medium (National Diagnostic, Atlanta, GA) and viewed under a microscope fitted with an eye-piece graticule for cell density counting of IEL, MLN T-cells and LFV cells.

Table 2.2. Lillie Mayer's haematoxylin

1-To 1000 mls of distilled water add 200 g of aluminum ammonium sulphate. Heat well until dissolved (do not boil).

2-Cool to room temperature, and add 1800 mls of distilled water.

3-Dissolve 20 g of haematoxylin (certified, Sigma, Cat No. H 3136) in the smallest amount of alcohol, and add to the solution.

4-Add 4 g of sodium iodate, 80 mls glacial acetic acid, and 1200 mls glycerol.

5-Filter.

Leave for at least a week before use.

Table 2.3. Scott's Tap water substitute

(Used as a substitute for ammonium water for blueing sections after haematoxylin).

Magnesium sulphate hydrated	100 g
Sodium hydrogen carbonate (Sodium bicarbonate)	10 g
Tap water	5000 mls
Sodium azide (preservative, 0.01%)	5.0 g

Sections in Scott'a tap water were not left for a long period of time (over 10 sec) to prevent the haematoxylin turning black.

With minor modifications, Ajax Chemical 1982-89, and Lillie, R. D. 1977

2.4.4. Trasforming growth factor-β (TGF-β) detection in the small intestine by the indirect immunoperoxidase staining method

Rat paraffin gut sections were fixed in fresh 10% buffered formalin overnight at room temperature. Five μm sections were cut and dewaxed twice in xylene (10 mins each time). Slides were hydrated and pre-treated with freshly prepared 0.6% H_2O_2 in methanol for 30 mins to remove endogenous peroxidase activity. Sections were then treated with hyaluronidase for 30 mins at 37° C, to permeabilize tissue (Boehringer, 0.5 mg/ml). Sections were then incubated overnight (12 h) with anti-human TGF-β IgG, turkey (Becton-Dickinson, 40091) at a concentration of 3.3 μg/ml (1/300 dilution). Control sections were treated with turkey IgG (3.3 μg/ml). Slides were washed three times with ice-cold TBS (three mins per wash). Sections were then incubated with secondary antibody, peroxidase-rabbit anti-chicken, turkey IgG (H+L) in the ratio of 1/20 (Zymed Laboratories, San Francisco, CA, USA, 94080) for 1 h at 4° C. Slides were washed three times in ice-cold TBS (3 mins per wash). Colour was developed with DAB and Sections were counterstained with Lillie Mayer's haematoxylin. They were dehydrated in sequential alcohol solutions. Finally, slides were mounted using Histo-mount neutral pH mounting medium and coverslipped to view TGF-β expression in the gut sections.

2.4.5. Enumeration of LP, LFV, and crypto-patch cells by the indirect alkaline phosphatase anti-alkaline phosphatase (APAAP) staining method

Slides were rehydrated in ice-cold TBS for 5 mins. It was noted that the use of phosphate buffered saline reduced the APAAP staining reaction and quality of cellular morphology. It was found that using Fast Red chromogen alone gave high background staining of the LP region, caused by diffusion of the red stain from the endogenous alkaline phosphatase of the brush border (microvilli). Therefore, substrate solution containing Fast Blue chromogen (Vector Laboratories, Burlingame, CA, Cat No. SK-5300, USA) was added to the sections for 2 mins at room temperature to visualise staining of endogenous alkaline phosphatase in the intestinal

brush border and LP cells (presumably macrophages and mast cells). In fact, addition of the Fast Blue chromogen served as an agent to bind endogenous alkaline phosphatase and prohibit heavy diffusion of the red staining of this isoenzyme from the microvilli into the LP. A further staining step using the usual substrate solution containing Fast Red chromogen was then used to visualise antibodies binding to the LP cells.

The addition of this one extra substrate solution containing Fast Blue helped greatly to make this technique useful for staining of gut sections which contain endogenous alkaline phosphatase, and in turn, enabled proper interpretation of results. It is of note that intestinal alkaline phosphatase activity peaks during weaning and cannot be quenched by levamisole. An attempt to block intestinal alkaline phosphatase by using 20% acetic acid for 20 minutes after application of secondary antibody was *not* successful. It only partly blocked enzyme activity. In addition, it distorted the brush border microvilli, and eliminated the APAAP staining reaction, perhaps due to denaturation of antibody. Arsenic acid also blocks enzyme activity of alkaline phosphatase effectively (Cathala *et al.*, 1975; Lopez *et al.*, 1967), but further investigations are required to find out the optimal concentration and incubation time, as inappropriate use of chemical agents may destroy antigenic sites, particularly IL-2R.

Slides were placed in a warm tap water bath for 5 mins, decanted and incubated with appropriately diluted primary antibody or negative control reagent (mouse IgG1) in a humid chamber for 1 h at 4° C. Slides were washed three times in ice-cold TBS (3 mins each wash) and adequate amounts of pre-diluted secondary antibody (1/50 dilution, unlabelled rabbit anti-mouse Ig) were added, and incubated in a covered humidified chamber for 45 mins at room temperature. It is of note that the secondary antibody must be in excess so that one of its Fab sites binds to the primary antibody leaving the other binding site free to bind antibody from the enzyme immune complex (Boenisch, 1989). Following that incubation, slides were washed three times in ice-cold TBS (3 mins per wash). Slides were incubated with sufficient amounts of soluble calf intestinal alkaline phosphatase-mouse, anti-calf intestinal alkaline phosphatase

complex (APAAP complex) for a further 45 mins at room temperature. Slides were washed three times in ice-cold TBS (3 mins per wash), and adequate amounts of filtered substrate solution containing naphtol AS-MX phosphate substrate and Fast Red TR chromogen were added to localize antibody binding, after which sections were incubated for 8 mins in the dark at room temperature. After this incubation, slides were left in a warm tap water bath, with occasional agitation, for 5 mins prior to counterstaining.

2.4.6. Differential counts of cells staining red (positive) to unstained cells (identified by their blue nuclei) by counterstaining with haematoxylin

Sections were counterstained with undiluted fresh Mayer's haematoxylin (aqueous-based counterstain, **Table 2.4**) for 5 mins. Slides were then washed quickly in two warm tap water baths followed by a 30 sec rinse in another warm tap water bath. Slides were blued in Scott's solution for 10 sec followed by a quick dip in tap water to remove excess salt from Scott's solution prior to mounting. Finally, they were mounted using an aqueous mounting medium of neutral pH (M02, Biomeda Corporation, Foster City, CA, USA). This was essential to prevent leaching of the nuclei staining and to increase staining preservation, as another mounting medium, Hydromount (National Diagnostic, Atlanta, GA; HS-106), leached the nuclei totally.

Table 2.4. Mayer's haematoxylin

Mayer's haematoxlin is a progressive stain for nuclei, and is used to stain where differentiation has a deleterious effect on previous stages.

1-Haematoxylin (Certified, Sigma, H 3136)	1.5 g
2-Distilled water	1000 mls
3-Heat to dissolve, then add:	
4-Sodium iodate	0.2 g
5-Alumminum ammonium sulphate	50 g
6-Heat to boiling point, cool and allow to stand overnight. Then add:	
7-Chloral hydrate	50 g
8-Citric acid	1.0 g
9-Warm gently to dissolve. Cool and filter.	

Ajax Chemical 1982-89, and Lillie, R. D. 1977

2.4.7. Quantification of IEL and LP cell counts

Positively stained cells were enumerated per 389 μm of mucosa, using a calibrated linear microscopic graticule (389 μm, x25 objective lens) aligned along the muscularis mucosae from two successive intestinal segments per animal. The average of ten counts was calculated and the results were expressed as density of cells per mm of muscularis mucosae. For immunoperoxidase staining, it was fairly easy to distinguish endogenous activity as coarse, dark-brown granular cytoplasmic staining in both eosinophils and macrophages while positive staining cells had less intense staining (**Fig. 2.2**). For APAAP staining, attention had to be given to carefully distinguish true positive staining, as membrane staining of nucleated cells from false rim staining of lymph and blood capillaries (endothelial cells lined) in the LP (Chapter 6; **Fig. 6.2**), as well as endogeneous cytoplasmic staining of presumed macrophages

and mast cells. This emphasised the importance of negative controls alongside unknown specimens. For Alcian blue staining, it was fairly easy to distinguish MMC with prominent blue granules from artifact.

2.4.8 Quantification of MLN cell counts

Positively stained cells in the MLN complex were counted using a calibrated ocular grid (square eyepiece) in five or more T-cell areas. Under x40 objective lens, we counted positive cells in a 0.058 mm^2 area (the area of a large graticule square was first calculated as μm^2). Then, the number of positive cells was multiplied by a conversion factor (17.24) to obtain the number of positive cells/mm^2. For instance, if the count of positive cells in a big square is 10, then the total number of positive cells is 172.4. The values were expressed as mean percentage ratios of IL-2R (CD25) positive cells to α/βTCR expressing cells as follows:

$$\frac{\text{Mean density of IL-2R}^{+}\text{ cells in the T-cell zone x100}}{\text{Mean density of }\alpha/\beta\text{ T-cells in the T-cell zone}\P}$$

¶ Assuming that all IL-2R$^+$ cells are α/βTCR$^+$

The basis for this assumption is that all MLN cells in the T-cell zone (para-corttical zone) express α/βTCR (Thompson *et al.*, 1996).

2.4.9. Quantification of Peyer's patch cell counts

IL-2R$^+$ activated cells in the T-dependent interfollicular area were enumerated in one or two T-cell zones, using a microscope equipped with a calibrated ocular grid (square eyepiece). The values were expressed as the ratio of IL-2R$^+$ cells/total counts of cells in a defined area of the grid for each sample.

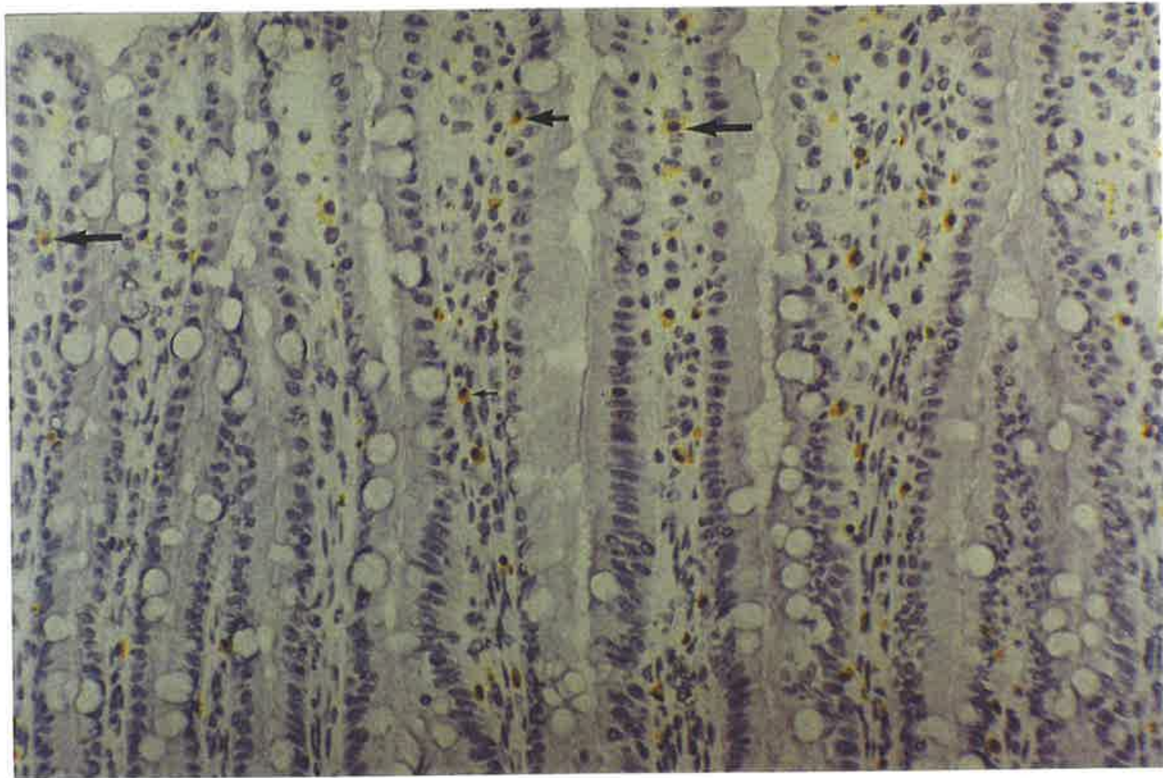


Fig. 2.2 Photomicrograph of rat small intestine frozen section stained with the indirect immunoperoxidase technique.

The small arrow shows one eosinophil in the LP, whereas the medium arrow demonstrates another eosinophil in the epithelial layer of a rat at day 28 of age. Note that eosinophils are stained densely, compared to $\alpha/\beta TCR^{+}$ LP cells (stained by mAb R73), which show lighter staining (the large arrow). Section is counterstained with Lillie Mayer's haematoxylin, Magnification X50.

2.4.10. Quantification of LFV cell counts

LFV were identified as villi usually wider than adjacent villi with a core, more evident basally, of mononuclear cells. The phenotype or activation of cells in LFV was determined by counting positive staining cells/total nucleated cells per LFV, after either immunoperoxidase or immunoalkaline phosphatase staining in a 5 μm tissue section. The results were expressed as the mean percentage ± SE of LFV expressing the different cell surface markers.

2.5. Measures of mucosal immune activity

2.5.1. Quantification of MMC counts as indicator of mucosal immune activity by Alcian blue staining method

After brief rehydration in distilled water, sections were placed in 0.7 N HCl for 5 mins, followed by staining in 0.1% Alcian blue in 0.7 N HCl (Table 2.5) for 30 mins. Finally, they were washed in 0.7 N HCl for 10 mins prior to counterstaining.

2.5.2. Differential counts of cells staining blue (blue granules) to unstained cells (identified by their red nuclei) by counterstaining with safranin O

Counterstaining of sections was performed with 0.5% Safranin O (**Table 2.5**) in 0.125 N HCl for 2 mins. Slides were rinsed briefly in distilled water, followed by dehydration in absolute tertiary butyl alcohol (2-Methyl-Propan-2-ol) for 5 mins. Finally, they were cleared in each of two Histo- clear II baths for 2x10 mins and then mounted in Histomount neutral pH mounting medium.

Table 2.5. Preparation of histological stains

Alcian blue 8 GN	1.0 g/100 ml of 0.7 N HCl (pH adjusted to 0.6)
Safranin (pH 2.5)	0.5 g/100 ml in 3% (vol/vol) acetic acid

Mix until dissolved and add a crystal of thymol as a preservative. Alcian blue usually has a normal shelf life of about 3 months but one must be aware of rapid deterioration of solutions when large numbers of slides are stained.

2.5.3. Quantification of MMC counts

MMC were counted in sections stained with Alcian blue (pH 0.6)/safranin. Cells of both the epithelial layer and LP compatrment were counted using a linear graticule (389 µm) and 25x objective lens. Cell conts were expressed as cells/mm of muscularis mucosae of each sample.

2.6. Statistics

Data are expressed as mean ± SE of the mean and statistical comparisons made using the Student's unpaired t-test.

Chapter 3

Intraepithelial lymphocytes and mucosal mast cells of the small intestine: changes in number and phenotype during weaning

3.1. General introduction

The intestinal mucosa has low antigen exposure prior to weaning, although there may be low levels of food protein antigens in amniotic fluid foetally and in maternal breast milk post-natally (Matsumura *et al*., 1967; Matsumura *et al*., 1974; Dahl *et al*., 1984; Cummins and Thompson, 1997). The gut-associated lymphoid tissues (IEL, LFV, and Peyer's patches) have low immunological activity before weaning and increased activity co-inciding with weaning in both rats (Mayrhofer *et al*., 1983; Cummins *et al*., 1988a-b; Thompson *et al*., 1996) and human infants (Cummins *et al*., 1994; Cummins and Thompson, 1997). This heightened immunological activity at weaning is evident by expansion of IEL and by activation and degranulation of MMC.

3.1.1. Intraepithelial lymphocytes (IEL)

IEL are a heterogeneous group of lymphocytes that reside above the basement membrane between and beneath epithelial cells of the small intestine. This may explain why cell-mediated immune responses occur in the wall of the intestine (Tomasi, 1995). They constitute approximately 10% of the total lymphocyte pool (Cerf-Bensussan and Guy-Grand, 1991). Although, much progress has been made in the areas of IEL phenotypic characterization, *in vitro* functional assays, and TCR expression, the specificity and function of IEL *in vivo* remain unknown. At least some $\alpha/\beta TCR^+$ IEL respond to antigens in a classical MHC-restricted cytotoxic T-lymphocytes response. The function of α/β T-cell subsets that are unique to the IEL-for example, the $CD4^+$ $CD8^+$ IEL remain to be determined. No defined 'conventional' responses for $\gamma/\delta TCR^+$ IEL are known (Lefrancois, 1994).

The numbers of IEL are influenced by food antigens, bacterial flora and immunological activity. In the duodenum, where food antigens are present in large quantities, IEL are increased about 2-fold when compared to the ileum; a finding which suggests that they respond to food antigens (Cerf-Bensussan and Guy-Grand, 1991; Penney *et al*., 1995). IEL remain low in germ-free animals with most, or perhaps all, being $\gamma/\delta TCR^+$ T-cells (Guy-

Grand *et al.*, 1990; Bandeira *et al.*, 1990). Furthermore, their numbers return to normal if animals are conventionalized (Crabbe *et al.*, 1968 and 1970; Glaister, 1973) with an increase in $\alpha/\beta TCR^+$ IEL and a decrease in $\gamma/\delta TCR^+$ IEL (Viney *et al.*, 1990). IEL are present in hypothymic (nude) rodents (Lefrancois, 1991) but in lower numbers which suggests a proportion of IEL are thymus-dependent. Cyclosporin A, which inhibits production of IL-2 and other cytokines in T-lymphocytes, also inhibits any rise in IEL during weaning (Cummins *et al.*, 1989a; Williams, 1994).

Previous studies in this laboratory (by use of haematoxylin and eosin staining method) have shown that number of IEL increase exponentially during the weaning period (Cummins *et al.*, 1988b), but have not examined the IEL phenotype. The information concerning the phenotype of rat IEL have been largely obtained by flow cytometric analysis of dispersed cells (Vaage *et al.*, 1990; Fangmann *et al.*, 1991; Takimoto *et al.*, 1992; Torres-Nagel *et al.*, 1992). However, flow cytometry may be inferior to *in situ* studies using immunohistochemical methods, since the latter gives a better picture of the anatomical distribution of IEL with a given phenotype and avoids the possibility of contamination with LP cells (Woodward *et al.*, 1998). Thus, the present study was performed to determine the phenotype of IEL *in situ*. The first purpose of this research was to identify changes in the phenotype (particularly the $\alpha/\beta TCR^+$ subset), numbers of IEL, and to compare IEL counts with MMC numbers during weaning. The second aim was to characterize the time-course of the expanding population of IEL.

3.1.2. Mucosal mast cells (MMC)

Mast cells have high affinity IgE receptors on their surface (Chapter 1) and their immunological activity is mediated through these IgE receptors (Metzger *et al.*, 1986). Binding of antigens to IgE molecules results in cross-linking of IgE receptors, that leads to

the release of the granules, or production of chemical mediators in the granules [Ishizaka (T) and Ishizaka (K) 1984].

Although MMC have been described in several mammalian species, the fundamental properties of these cells has been largely derived from studies in rats (Watkins *et al.*, 1976; Ellen *et al.*, 1984). Woodbury and Neurath (1978) investigated the development of MMC in the small intestine of these animals. MMC were present in the intestine before birth, but their numbers were generally low. MMC counts remained low for the first 3-4 weeks of life with weakly staining 'immature' granules present in the cells. After 4 weeks of life, MMC numbers increased five-fold to normal adult levels at approximately 9-13 weeks after which MMC counts decreased slowly. In conclusion, these investigators described an age-associated increase of MMC numbers.

As jejunal MMC contain few granules and stain weakly in animals at 21 days of life, this was interpreted as a marker of immaturity (Woodbury and Neurath, 1978). However, this appearance is also consistent with activation and degranulation of MMC. Maturation of the small bowel during weaning is accompanied by:

(**1**) Lengthening of intestinal crypts

(**2**) An increase in crypt cell proageration, and

(**3**) Increased IEL numbers

(Herbert and Sunshine, 1969; Orlic *et al.*, 1981).

These characteristics are also indicators of immunologically-mediated reactions in the small intestine (Ferguson and Jarrett, 1975; Mowat and Ferguson, 1982; Ferguson, 1987), and would suggest that mucosal immune activity may increase during weaning, with activation of MMC. This may explain the "immature" appearance of MMC at this time.

3.2. Experimental design

This work was done with the assistance of Miss Helen Bermingham. The GALT were studied in litters of DAxPVG/c rats. These litters were aged from 14 to 42 days of life with day 0 being designated as the day of birth. There were 6 to 8 rat pups in each litter and runted animals were excluded. Each rat pup was anaesthetized with ether, the axillary artery cut and allowed to bleed out. The small intestine was isolated and jejunal samples collected from the upper one-sixth of the small intestine. Jejunal samples were taken and oriented onto cardboard and placed in Carnoy's fixative for MMC counts, whereas a further sample was embedded in OCT for preparation of frozen sections and were stored for approximately 12-18 months at -70°C. In this study, they were cut for immunohistochemical analysis. The sections were stained by an indirect immunoperoxidase technique using a variety of monoclonal antibodies directed against T cell markers. Counts were expressed as the number of positively stained cells per mm of muscularis mucosae.

3.3. Results

3.3.1. Total IEL counts

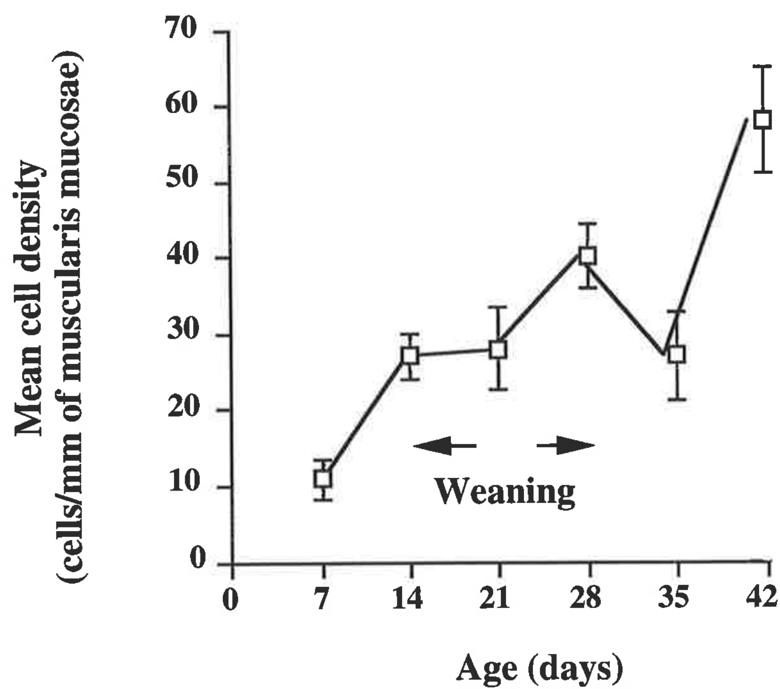
The cell density of IEL stained with anti-rat CD45 mAb (all isoforms of CD45, present on all cells of haemopoietic origin), was made by counting numbers of stained IEL per mm of muscularis mucosae. Total IEL increased ($P<0.005$) nearly five-fold from days 7 to 42 (**Fig. 3.1** and **Table 3.1**).

3.3.2. Expression of CD3/TCR on IEL T- cells

Cryostat sections were fixed in 95% ethanol and immunostained with anti-rat CD3 (G4.18, IgG3 isotype) antibody for 1 hr incubation at 4°C and detected by anti-mouse IgG F $(ab')_2$ secondary antibody linked to horseradish peroxidase. The staining intensity was poor. Therefore, cryostat sections were fixed in acetone at 4° C or a different secondary antibody was used. This was horseradish peroxidase-conjugated sheep anti-mouse IgG3. It was found that replacement of fixative, and not secondary antibody, improved the staining intensity modestly.

Accordingly, by using acetone as a fixative, 9% of IEL at day 42 were labelled by anti-CD3 antibody, compared to 41% of IEL which were stained by anti-α/βTCR (R73) and anti-γ/δTCR (V65) separately (**Table 3.2**). It should be noted that Wilcoxon-van Elteren test has shown that the proportion of α/βTCR^{+} T-cells is significantly different ($P<0.05$) from the proportion of CD3^{+} T-cells. This suggests that the remainder are γ/δTCR (Vaage *et al.*, 1990). Anti-rat CD3 mAb (G4.18) yielded good staining of T-cells in the spleen unlike its poor performance for jejunal sections. This is demonstrated in **Fig. 3.2**. Acetone is a better fixative, because acetone removes lipid from the cell membrane better than alcohol. This may facilitate the antibody binding to the CD3 molecule, as the CD3 chains do not have a large extracellular domain (Stites and Imboden, 1994; Cotran, 1994). Nevertheless, as anti-CD3 antibody failed to perform properly, the sum of α/βTCR+γ/δTCR was employed to estimate total CD3^{+} T-cells, because the CD3^{+} chains are in a close association with the α/βTCR or the γ/δTCR. Therefore, the number and proportion of α/βTCR^{+} and γ/δTCR^{+} T-cells were determined (**Table 3.3**). The number of α/βTCR expressing IEL increased ($P<0.05$) approximately 2.5 fold from days 14 to 42, whereas γ/δTCR expressing IEL T-cells did not change. The proportion of IEL T-cells expressing α/βTCR or γ/δTCR remained approximately the same at 26-55% of total CD45^{+} cells. Importantly, there was a major population (45-74%) of phenotypic null cells.

Fig. 3.1. Expansion of CD45+ IEL in the jejunum of healthy DAxPVG/c rats from pre-weaning to post-weaning life.



Data indicate the mean ± SE density counts of cells per mm of muscularis mucosae in litters from 6-10 animals.

Eosinophils were excluded from these counts.

Table 3.1. Phenotype of IEL cells.

Age (days)	CD4	CD5	CD8α¶	CD43	CD45
14	4 (0.9)	5 (0.3)	15 (3.1)	5 (0.9)	27 (2.9)
21	8 (1.5)	8 (2.4)	14 (3.4)	10 (1.6)	28 (5.3)
28	4 (0.6)	4 (0.8)	12 (1.9)	8 (0.9)	40* (4.2)
35	3 (0.5)	1 (0.2)	10 (1.4)	9 (1.4)	27 (5.7)
42	10* (2.2)	6 (0.82)	17 (3.4)	14* (2.4)	58** (7.0)

Data represent the mean (SE) density counts of cells per mm of muscularis mucosae in litters from 6-8 rats.

¶ OX-8 mAb stains the α chain of the CD8 molecule.

* $P<0.05$

** $P<0.005$

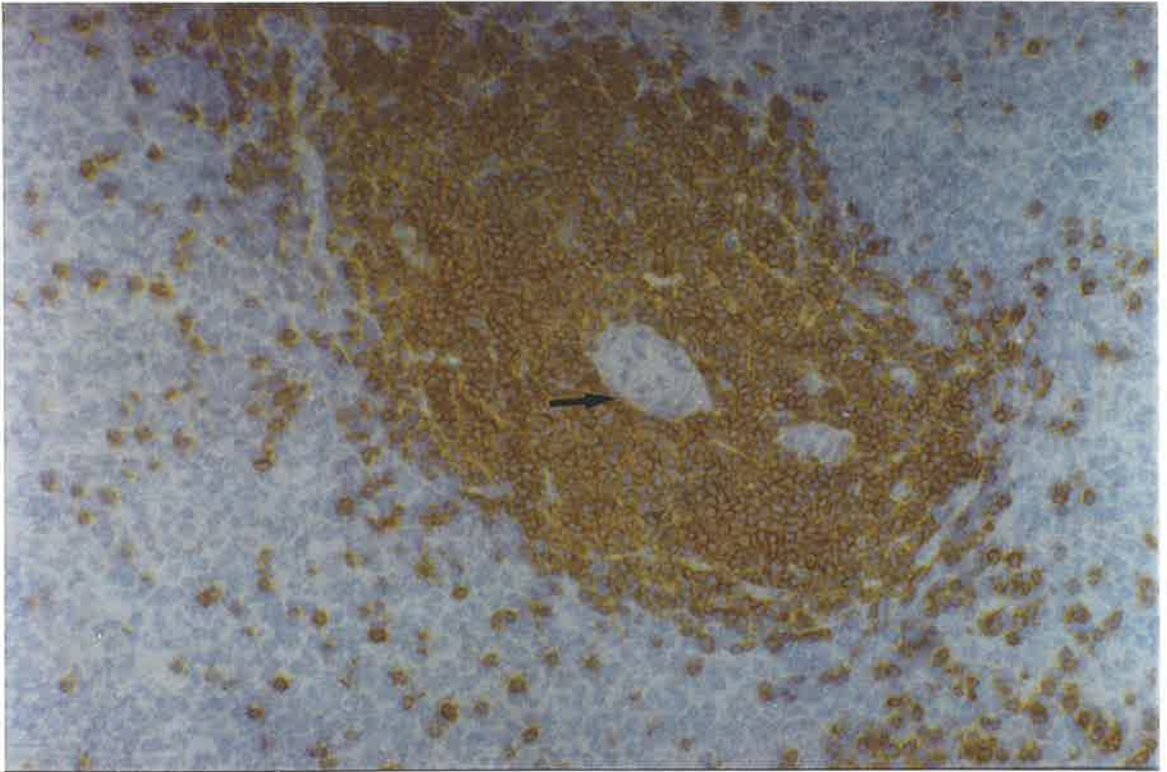
Means of IEL cells expressing the given markers at day 14 were compared with those at day 28 (end of weaning), to determine whether significant changes occured during weaning. Means were also compared between days 21 (mid-weaning) and 42 (post-weaning), to determine whether significant changes occured post-weaning.

Table 3.2. Marked discrepancy between the number of $CD3^+$ IEL T-cells and the sum of $\alpha/\beta TCR^+$ plus $\gamma/\delta TCR^+$ cells at day 42.

Phenotypic marker	Mean	$\%CD45^+$ cells
$\alpha/\beta+\gamma/\delta$ TCR	24¶	41§
CD3	5	9

¶ Data represent cell density counts per mm of muscularis in a litter of 7 rats.

§ Figures represent the percentage of $CD45^+$ cells which were positive for α/βTCR or γ/δTCR.



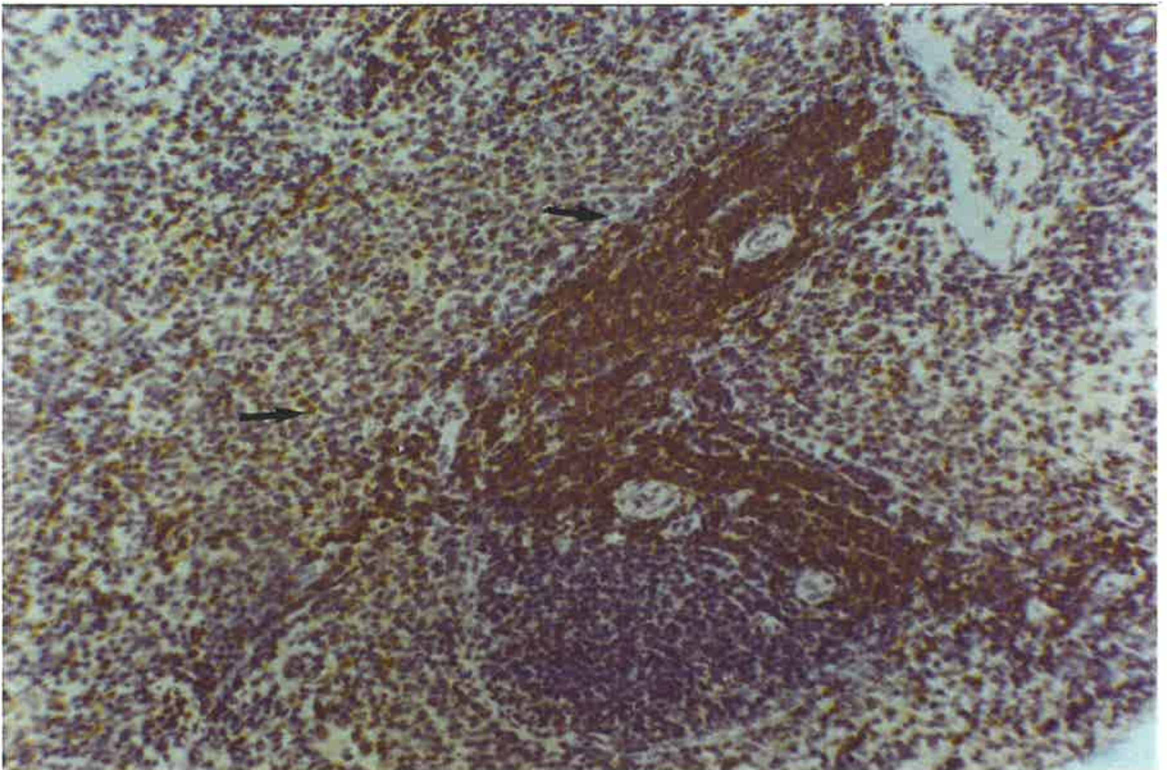


Fig. 3.2 Rat spleen stained for α/βTCR$^+$ cells and CD3$^+$ cells.

Upper panel: This panel shows expression of α/βTCR (detected by mAb R73) in the T-dependent area of spleen at 4 weeks of life (**positive control specimen**). Sections were incubated with this antibody and subsequently incubated with a peroxidase-labelled anti-mouse Ig reagent, and developed with DAB. Arrow points to small arterioles that are surrounded by cuffs of α/βT-cells (splenic periarteriolar lymphoid sheaths, PALS). Immunoperoxidase, sections were counterstained with Lillie Mayer's haematoxylin, X50.

Lower panel: This panel shows expression of CD3 (with an antibody G4.18) specific for T-cells in the PALS (arrowed). CD3$^+$ T-cells are also scattered throughout the spleen (arrowed). Note the G4.18 mAb performed well on spleen frozen section, whereas positive cells were few on gut frozen sections. Immunoperoxidase, X50.

Table 3.3. Percentages of IEL T-cells in weanling and post-weanling rats.

Age (days)	α/βTCR+	γ/δTCR+	Sum (α/βTCR+γ/δTCR)	%CD45+ cells§
14	7¶ (0.8)	4 (0.7)	11	40
21	9 (1.9)	6 (1.3)	15	55
28	8 (2)	3 (0.9)	11	26
35	8 (1.4)	6 (1)	14	52
42	18* (4.2)	6 (1.1)	24	41

Each group contained 6-8 rat pups.

¶ Data represent the mean (SE) density counts of cells per mm of muscularis mucosae.

§ Figures indicate the percentage of $CD45^+$ cells which are positive for α/β or γ/δTCR.

* $P<0.05$

The significance refers to comparison with day 14.

The absolute count of $\alpha/\beta TCR^+$ IEL increased 2.5-fold from days 14-42 ($P<0.05$, **Table 3.3**). The majority of this increase occurred during the post-weaning period. For example, there was a greater than 2-fold increase ($P<0.05$) in the second week of the post-weaning period (**Table 3.3**). However, $\gamma/\delta TCR^+$ IEL did not change. There was a large discrepancy between $CD45^+$ cell and IEL expressing CD3 or CD8α during and after weaning (**Table 3.4**). There was not a perfect overlap between these $CD3^+$ and $CD8\alpha^+$ IEL. For instance, at the beginning of weaning (day 14), 55% of IEL expressed CD8α, whereas approximately 40% of IEL were T-cells ($\alpha/\beta TCR^+$ plus $\gamma/\delta TCR^+$). These data show that most $CD8\alpha^+$ cells were T-cells. The remaining were probably NK cells, because rat NK cells express CD8α, but not CD3-for example, 13% at day 14 (55% minus the sum of 16% and 26%, **Table 3.5**).

3.3.3 Expression of CD5 and CD43

$CD5^+$ cells did not change, whereas the number of $CD43^+$ (pan-T-cell marker) cells increased approximately three-fold fom pre-to post-weaning ($P<0.05$) (**Table 3.1**). Taken together, about 13% and 27% of IEL stained for the CD5 and CD43 molecules, respectively. This shows that a higher proportion of IEL expressed CD43, but not CD5, although almost all of $CD43^+$ IEL expressed α/βTCR (**Table 3.5**). This also indicates that a lower proportion of IEL in younger rats are positive for these two markers as, in adult rats, approximately 100% and 29% of IEL are positive for the CD43 and CD5 markers, respectively (Vaage *et al.*, 1990; Fangmann *et al.*, 1991).

3.3.4. Counts of $CD4^{+}$ $CD8^{+}$ double-positive and $CD4^{-}$ $CD8^{-}$ double-negative IEL

Changes in $CD4^{+}$ and $CD8^{+}$ cells are given in **Tables 3.6**. The mean density counts of $CD8\alpha^{+}$ cells did not change, but the proportion of $CD45^{+}$ cells which expressed $CD8\alpha$, was prominent (55%) at the onset of weaning (day 14) and decreased to 29% over the course of the next four weeks of life. As same IEL could express CD4 and CD8, the counts of $CD4^{+}$, $CD8^{+}$ and counts with mixture of antibodies were compared. If the count of latter is less than the labelling experiments yielded CD4 and CD8 mean values of 4 and 12 cells/mm of muscularis, respectively, giving a sum of 16 which is similar to the value of 19 obtained with the dual stained cells. Similarly, at day 42, the sum was 27, which was not significantly different than the value of 18 obtained by the dual staining.

IEL are usually located between and beneath epithelial cells, but it was sometimes difficult to identify $CD4^{-}$ $CD8^{-}$ double-negative IEL in frozen sections. The criterion used was that they had small round dense nuclei compared to the more oval and less dense nuclei of enterocytes. Most $CD4^{-}$ $CD8^{-}$ double-negative IEL fall within the $\gamma/\delta TCR^{+}$ phenotype (Jarry *et al*., 1990). However, mixed labelling experiment would be required to confirm this.

Table 3.4. Comparison between $CD45^+$, $CD8\alpha^+$ and $CD3^+$ T-cell counts in the epithelial layer of small intestine of rats during infancy.

Age (days)	CD45	CD8α	Total T-cells §
14	27	15	11
21	28	14	15
28	40	12	11
42	58	17	24

Data represent the mean density counts of cells per mm of muscularis mucosae in litters from 7-8 rats.

§ Total T-cells were enumerated as the sum of $\alpha/\beta TCR^+$ and $\gamma/\delta TCR^+$ cells.

Table 3.5. Percentages of lymphocyte markers of IEL.

	phenotypic marker					
Age (days)	**α/βTCR**	**γ/δTCR**	**CD4**	**CD8α**	**CD5**	**CD43**
14	26¶ (3.0)	16 (2.5)	14 (3.5)	55 (11.5)	20 (1.0)	20 (3.5)
21	33 (7.0)	22 (5.0)	30 (5.5)	50 (12)	27 (8.5)	34 (6)
28	20 (5.0)	6 (2.0)	9 (1.5)	30 (5)	10 (2.0)	20 (2.0)
35	28 (5.0)	21 (4.0)	12 (2.0)	38 (5.0)	4 (1.0)	34 (5.0)
42	31 (7.0)	10 (2.0)	17 (4.0)	29 (6.0)	9 (1.5)	24 (4.0)

Each litter contained 6-8 rat pups.

¶ Mean (SE) percentage of IEL as a proportion of $CD45^+$ cells.

Table 3.6. Percentages of CD4+, CD8+ and dual stained£ CD4+CD8+ IEL.

Age (days)	CD4	CD8	CD4+CD8+	CD4-CD8-
14	¶14 (4±0.9)#	55 (15±3.1)	¥0 (26±3.2)	37 (10±1.7)
21	30 (8±1.5)	50 (14±3.4)	0 (24±3)	21 (6±1.3)
28	9 (4±0.6)	30 (12±1.9)	0 (19±5)	29 (11±3)
42	17 (10±2.2)	29 (17±3.4)	§16 (18±5.5)	22 (13±2.8)

£ IEL were double-stained with mAbs W3/25 (anti-CD4) and OX-8 (anti-CD8α)

¶ Numbers outside the brackets represent the percentages of CD45+ cells that are double labelled.

Numbers in the brackets indicate mean ± SE density counts of cells per mm of muscularis in litters of 7 rats.

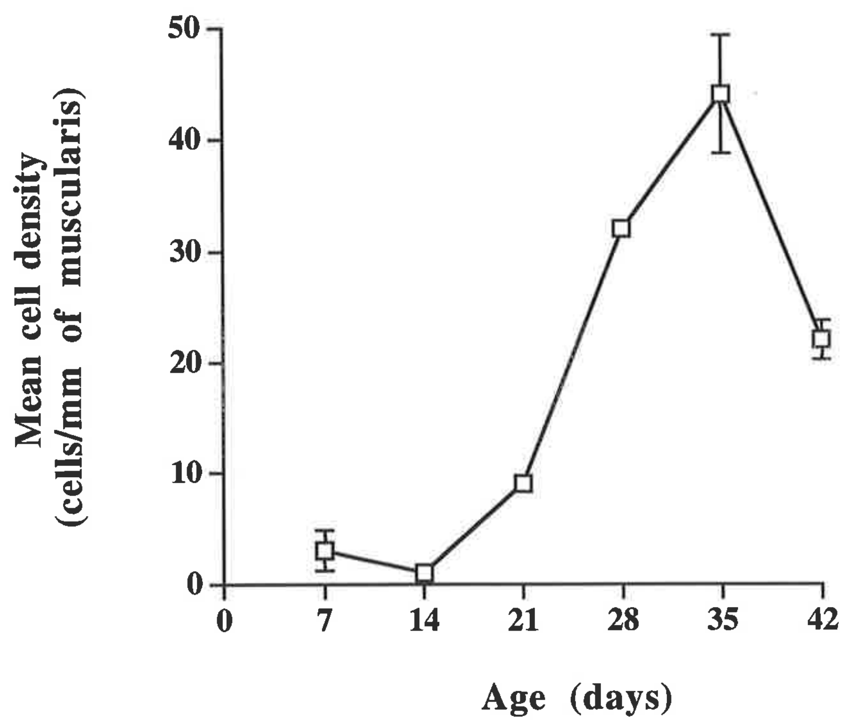
¥ Figure represents the estimated percentage of CD45+ cells which show double labelling for CD4 and CD8.

§ 9 cells/mm divided by 58 CD45+ IEL per mm gives 16% of CD45+ cells.

3.3.5. MMC

MMC showed no significant increase (P>0.05) from days 7 to 21, but there was a progressive loss of granule staining during this time, with fewer and smaller granules being present. However, MMC increased significantly (P<0.005) from days 14 to 35. After day 35, MMC counts decreased (P<0.005, **Fig. 3.3**). The overall results showed an age-associated increase in the number of intestinal mast cells.

Fig. 3.3. Expansion of MMC in rats from pre-weaning to post-weaning.



Each litter contained 6 to 10 animals.

SE for some data points were so tight, the symbol obscures the error bars.

3.4. Discussion

The purpose of this study was to characterise the phenotype of IEL as well as changes in number of IEL and MMC during weaning. The results showed that $CD45^+$, $\alpha/\beta TCR^+$ and $CD8\alpha^+$ IEL expanded during the weaning phase. However, $\alpha/\beta TCR^+$ cells were only a minority subset of $CD45^+$ cells. This finding corroborates a previous report which has demonstrated that the total number of IEL expand during weaning (Cummins, *et al.*, 1988b). Other studies have also shown that IEL with the α/βTCR phenotype expand during infancy in mice (Viney *et al.*, 1990; Guy-Grand *et al.*, 1991).

Anti-rat CD3 mAb worked poorly on frozen jejunal sections in detecting the $CD3^+$ T-cells, compared to frozen sections of spleen or Peyer's patch cells (Alisen Brooks, personal communication). The likely explanation is that the level or copy number of the CD3 molecule on intestinal IEL are lower than the concentration on other cells in other sites. Therefor, anti-rat α/βTCR and γ/δTCR were used to estimate $CD3^+$ T-cells in the epithelium of small intestine.

As described earlier, most (60-90%) of IEL in adult rats have the α/βTCR phenotype (Vaage *et al.*, 1990; Cerf-Bensussan and Guy-Grand, 1991), so that an increase in both the number of IEL and the proportion that express α/βTCR in animals is expected (Poussier *et al.*, 1994). This study found that $\alpha/\beta TCR^+$ IEL did increase during infancy (**Table 3.1**), although the proportion of $CD45^+$ IEL remained the same (**Table 3.5**). There was also a decline in $CD45^+$ IEL cells at day 35, probably due to biological variation from one litter to another litter (**Fig. 3.1**, litter effect). Subsequent studies described in Chapter 4 showed increased numbers of IEL at this age.

Flow cytometric analysis has also shown that 67% of IEL in adult rats express α/βTCR (Vaage *et al.* 1990). However, in this investigation IEL contained a large population of cells (70%) which were unlabelled by mAb anti-α/βTCR (R73) (**Table 3.5**). There are several

plausible reasons for this discrepancy. First, this study investigated weanling rats from 14-42 days of age, whereas Vaage *et al.* (1990) studied adults rats from 60 to 120 days of age. There is good evidence that IEL in weanling animals are immature or express lower surface levels of α/βTCR compared with those from adult animals (Poussier *et al.*, 1994). This would explain why approximately 44% of IEL in three-week-old rats in this study were CD3$^-$. Interestingly, subsequent studies in older rats descibed in the Chapter 4 agrees with that of Vaage *et al.* (1990) with approximately 60-70% of IEL in adult rats having the α/βTCR phenotype, despite the use of different methods.

At mid-weaning (day 21), the greatest number of IEL with the phenotype of α/βTCR$^+$ CD4$^+$ CD5$^+$ CD8α^+ CD43$^+$ was identified. These findings suggest that first , CD5$^+$ cells are likely to correspond to the α/βTCR$^+$ cells, because γ/δTCR$^+$ IEL lack surface expression of CD5 (Cheng *et al.*, 1991). Second, IEL appear to become mature phenotypically around mid-weaning, because CD3 and CD5 are associated with maturation (Beverly and Callard, 1981). However, it is unknown whether CD4$^+$ CD8$^+$ double-positive IEL are mature cells or precursors of IEL (Lefrancois, 1991).

This study showed that approximately 44% of IEL were non-classical T-cells. This finding agrees with that of Poussier *et al.* (1994), who showed that 25% of IEL are CD3$^-$ by flow cytometry in mice at 3 weeks of life. Both studies have shown that a significant proportion of IEL are CD3$^-$ and perhaps pre-T-cell (CD7$^+$ CD3$^-$ TCR$^-$ CD4$^-$ CD8$^-$).

At the start of weaning (day 14), 55% of cells expressed CD8α, yet nearly 40% of IEL were positive for α/βTCR and γ/δTCR. Most double-positive thymocytes express low TCR levels, whereas double-positive IEL express high, mature T-cell levels of TCR (Lefrancois, 1991), and this level can be detected by immunohistochemical staining. This excludes the possibility that the copy number of TCR was sufficiently low that it could not be detected by immunohistochemical staining. Thus, one possible reason for this discrepancy is that a

fraction of cells with the $CD3^{-}$ $CD8\alpha^{+}$ phenotype are NK cells, because a subpopulation of rat NK cells expresses the CD8α molecule (Cantrell *et al*., 1982).

The present study also showed that $CD43^{+}$ cells were in similar proportions, 20% and 24% , before and after weaning, respectively. This was different to the findings of Lyscom *et al*. (1983), in which $CD43^{+}$ IEL were approximately 8% at 4 weeks of life and 25% over the subsequent two weeks. There are several plausible reasons for this discrepancy. First, in the current study, the denominator of cell counts in the intestine was cell counts per mm of muscularis mucosae, whereas in the study of Lyscom *et al*. (1983) the denominator was cell counts per 100 epithelial cells nuclei. Second, this study used indirect immunoperoxidase staining of frozen sections, while Lyscom *et al*. (1983) used immunofluorescence staining of isolated IEL. The isolation technique may have biased the cell phenotype. Finally, the studies used different rat strains.

As animals aged (days 14 to 42), the absolute counts of IEL expressing CD8α remained approximately constant. There was a dramatic decrease in the proportion of $CD8\alpha^{+}$ cells, although during this time the proportion of $CD43^{+}$ cells remained almost constant (**Table 3.5**). These findings suggest that from mid-weaning (day 21) to post-weaning (day 42), most $CD8^{+}$ cells were $CD43^{+}$ as well. However, double labelling would be required to determine the precise overlap of $CD8^{+}$ and $CD43^{+}$ IEL.

It has been reported that the percentage of IEL sub-types ($CD8^{+}$ cells) in rats at 2 weeks of life is 38% and that this increases up to 52% over the course of the next four-weeks of life (Lyscom *et al*., 1983). However, our present study showed that the proportion of $CD8\alpha^{+}$ cells at day 14 was 55% and, that this decreased to 29% over the subsequent four weeks. However, the current study showed that the proportion of $CD8\alpha^{+}$ cells at day 14 was 55% and this decreased to 29% over the subsequent four weeks. The estimated number of dual positive

$CD4^+$ $CD8^+$ IEL was low during infancy and was only evident at day 42. This agrees with Takimoto *et al*. (1992) who found $CD4^+CD8^+$ IEL in 4 week old rats.

The results also revealed that the proportion of $CD4^-$ $CD8^-$ double-negative IEL at the onset of weaning was about 40%, and decreased to approximately 20% at post-weaning (day 42, **Table 3.6**). This is supported by other findings, where the proportion of $CD4^-$ $CD8^-$ double-negative IEL occurs with lower frequency in normal human and rat *adult* small intestine. For instance, it has been reported that approximately 1-20% and 7% of IEL in humans and rats, respectively, are $CD4^-$ $CD8^-$ double negative IEL (Spencer *et al*., 1989; Jarry *et al*., 1990; Fangmann *et al*., 1991). These findings suggest an age-associated decrease in the proportion of $CD4^-$ $CD8^-$ double-negative IEL. Furthermore, $CD4^-$ $CD8^-$ double-negative IEL in this study do not represent B-cells, because subsequent studies described in other rats in Chapter 4, showed that IEL did not react with anti-rat CD45RA mAb (specific for B-cells).

This study also examined the MMC morphology and population in the jejunum as indicators of immunological activity. MMC contained fewer and smaller cytoplasmic granules at days 14-21 of life. This is due to activation and intense degranulation of these cells. The discrepancy between this study which showed a significant decrease of MMC after day 35 of life (**Fig. 3.3**) is, in contrast to the study of Woodbury and Neurath (1978) who found a continous increase with age. The reason for this is not known, but may be due to different strains of rats used or other variables.

Expansion of MMC numbers co-incided with the broad peak of T-cell activation in the second week of weaning in the LP (Chapter 6). This finding is corroborated with earlier findings which showed T-lymphocyte dependence of MMC for proliferation both *in vivo* and *in vitro* (Ruitenberg and Elgersma, 1976; Nawa and Miller, 1979; Mayrhofer, 1979). These findings indicate that MMC increase in immunologically-mediated reactions in the small intestine. However, the apparent number of MMC may fall under intense stimulation. This is

supported in severe graft-versus-host reaction (GvHR) in rats and contrasts with increased MMC counts in mild GvHR (Cummins *et al*., 1989b).

3.5. Conclusion

The results presented in this Chapter showed that *during weaning*:

(**a**) The number of α/βTCR$^+$ IEL increased;

(**b**) CD4$^+$ IEL increased;

(**c**) CD43$^+$ IEL increased; but

(**d**) CD5$^+$ IEL, CD8α^+ IEL and γ/δTCR$^+$ IEL did not change with weaning;

(**e**) CD4$^+$CD8$^+$ double-positive IEL were only present after weaning;

(**f**) MMC expanded during the second week of weaning and thereafter;

(**g**) A significant proportion of IEL at any age was CD3$^-$, and may consist of either pre-T-cells or NK cells. This shows novel changes in the phenotype of IEL during weaning.

Chapter 4

Non-proliferative expansion of intestinal intraepithelial lymphocytes during weaning

4.1. General introduction

Weaning in the rat begins around day 15 of life and extends over the following 10-15 days with a gradual decline in suckling and a corresponding increase in solid food consumption (Babicky *et al.*, 1973). This process takes place spontaneously without the need to separate the pups from the dams.

Weaning in rats is associated with evidence of immune activity in the gut-associated lymphoid tissues (GALT). Activation of GALT could be due to different factors, including stimulation by food proteins, a change in the commensal bacteria of the gut, or to stimulation either directly or indirectly by cells acquired from maternal milk before weaning (Weiler *et al.*, 1983; Schnorr and Pearson, 1984; Kumar *et al.*, 1989; Bandeira *et al.*, 1991; Thompson *et al.*, 1996). Food comprises the principal source of exogenous (environmental) antigens and seems likely to be the major stimulus to the development of the intestinal immune system including quantitative and qualitative changes in IEL (Poussier and Julius, 1994).

4.1.1. IEL

IEL constitute a distinct phenotypically diverse population, whose origin, specificity and functions, remain largely unknown. The abundance of IEL, particularly in the small intestine, and the unique position of these cells in intimate contact with enterocytes and in close proximity to an environment containing a wide variety of luminal antigens, suggest that IEL play a significant role in mucosal immune defense (Ernst *et al.*, 1985; Dobbins, 1986; Lefrancois, 1991).

IEL consist of two major $CD8^+$ subsets based on TCR antigen expression: α/βTCR and γ/δTCR. Only a few cells express γ/δTCR. The CD8 molecule is an α/α homodimer rather than the usual α/β heterodimer. The significance of this is unknown, but it could indicate interaction with a non-classical class I MHC molecule. A recent study from Torres-Nagel *et al.* (1992) suggests that CD8α/β expression follows activation of a pre-existing CD8$\alpha(\alpha)$

population. The majority (60-90%) of IEL in adult rats (and humans) have the CD8α/β and CD3 α/βTCR phenotype (Cerf-Bensussan and Guy-Grand, 1991; Vaage *et al.*, 1990). The CD3 and α/βTCR CD8α/β cells are thymus-dependent. Another population of CD3 cells is bone marrow-derived and has a $CD5^-$ phenotype (Mayrhofer, 1980; Mayrhofer and Whately 1983). CD5 is normally expressed by mature peripheral blood T-cells (i.e., a maturation marker). This $CD5^-$ IEL population expresses the γ/δTCR in about 50% of the subset and accounts for the majority, if not all, $\gamma/\delta TCR^+$ IEL (Cerf-Bensussan and Guy-Grand, 1991).

In humans, IEL can be identified in the fetal intestine by 11 weeks gestation (Spencer *et al.*, 1986), whereas in mice, the appearance of IEL is delayed until the first week of life, ie-before weaning (Woodward *et al.*, 1998). This means that their appearence is not antigen-dependent. This is supported by the finding that IEL also occur in an allograft intestine under the kidney capsule (Ferguson and Parrott 1972b). On the other hand, the intestinal bacterial flora and food proteins influence the number and phenotype of IEL (Ferguson and Parrott, 1972a; Leigh *et al.*, 1985). Clearly, luminal antigens have a great influence on the development of several compartments of GALT in humans and in rodents. To better understand the role of IEL in the maturation of the small intestine, this study investigated which subset of IEL (particularly α/β T-cells) expands during weaning, and whether this expansion is due to the local proliferation of IEL cells or to recruitment of cells from other sites. Moreover, this research re-examined previous data on IEL counts, particularly those of $CD8\alpha^+$ cells which were unexpectedly low in the previous study.

4. 2. Experimental design

IEL were studied in litters of DAxPVG/c rats. These litters were aged from 7 to 70 days of life with day 0 being designated as the day of birth. Litter size varied from 7 to 11 animals and runted animals were excluded. Each rat pup was anaesthetized with ether, the axillary artery cut and allowed to bleed out. The small intestine was isolated and jejunal segments

collected at the first proximal one-sixth segment of the small intestine (corresponding to 15 cm from the pylorus in 12 week old animals). Rat jejunal and ileal sections were taken and oriented onto cardboard, and placed in formalin for TGF-β staining. Positive control tissue for TGF-β consisted of blocks of human colon that had been fixed in methacarn (60% methanol, 30% choloroform, and 10% glacial acetic acid). Sections were embedded in parrafin. Rat jejunal samples were embedded in OCT for preparation of frozen sections. For immunohistochemical analysis, sections were stained by an indirect immunoperoxidase staining method, using a variety of monoclonal and polyclonal antibodies directed against T cells, B-cells, macrophages, NK cells markers and TGF-β. Counts were expressed as the number of positively stained cells per mm of muscularis mucosae.

4. 3. Results

$CD45^+$ IEL were low on day 7, but subsequently increased ($P<0.005$) rapidly during weaning, particularly after day 21 (mid-weaning). There was a further increase after weaning. For instance, $CD45^+$ IEL increased ($P<0.005$) approximately 2.5-fold from days 28 to 70 (**Table 4.1** and **Fig. 4.1**).

$\alpha/\beta TCR^+$ IEL increased in numbers during weaning (**Table 4.1** and **Fig.4.2**). There was also a further increase of α/βTCR+ cells after weaning (days 28 to 70, $P<0.005$). The overall results showed that both $CD45^+$ cells and $\alpha/\beta TCR^+$ IEL expanded concomitantly, although numbers of α/βTCR+ IEL were lower (**Fig. 4.3**). For instance, approximately 30-50% of these expanding cells were $\alpha\beta TCR^+$ (**Table 4.2**). The cell *density* of $\alpha/\beta TCR^+$ IEL increased approximately 5-fold ($P<0.001$). However, the proportion of $CD3^+$ IEL did not change from days 14 to 70 (**Table 4.3**). In contrast to α/βTCR+ IEL, the number of γ/δTCR+ IEL remained almost constant during and after weaning, so that only a small minority (1-3%) of these expanding cells were $\gamma/\delta TCR^+$ IEL (**Table 4.2**).

A smaller percentage of IEL T-cells expressed the pan-T-cell marker (CD43), as compared to the α/βTCR marker. For example, at day 21, 30% of IEL were positive for α/βTCR, whereas only 6% of these cells expressed the CD43 molecule. This indicates that most α/βTCR expressing IEL were negative for CD43. Double labelling would indicate whether those CD43^{+} cells also express α/βTCR, γ/δTCR, or neither, as CD43 in rats is also expressed on plasma cells, polymorphs and some other cells (Williams *et al.*, 1977; Dyer and Hunt, 1981; Brown *et al.*, 1981).

The highest density of NK cells in the intestinal epithelium occurred at days 21 and 70. However, the highest proportion of IEL expressing the NK cell marker was at day 21. The reason for this discrepancy between NK cell density and proportion at days 21 and 70 was due to a 4-fold expansion of CD45+ IEL between the indicated ages, while the density of NK cells did not alter significantly (**Tables 4.1** and **4.2**). Neither macrophages nor B-cells were detected at the ages investigated as shown in **Table 4.1**. No detectable Ki-67 staining for IEL was seen from days 7 to 70. This indicates that expansion of IEL was not due to local proliferation.

Table 4.1. Phenotype and composition of IEL cells.

Age (days)	CD45	α/βTCR	γ/δTCR	CD8α	CD4	CD43	NK	CD45RA (B cell)	Macrophage
7	12 (3.2)	6 (1.3)	1 (0.1)	8 (1.3)	ND	0.5 (0.2)	ND	ND	ND
14	23 (2.7)	9 (1.3)	1 (0.2)	12 (4.4)	ND	2 (0.8)	ND	ND	ND
21	35 (1.8)	11 (2.4)	1 (0.1)	15 (5.7)	ND	2 (0.8)	7 (3.0)	ND	ND
28	55** (4.2)	19* (1.6)	1 (0.4)	24 (7.7)	ND	3 (0.8)	3* (0.4)	ND	0
35	64 (6.3)	21 (1.6)	2 (0.4)	27 (8.2)	ND	4 (0.9)	4 (0.9)	0	ND
70	128** (17.3)	44** (3.7)	1 (0.1)	34* (12.3)	2 (0.4)	6* (2.1)	8 (1.8)	0	ND

Data represent the mean (SE) density counts of cells per mm of muscularis mucosae in litters from 7-11 rats.

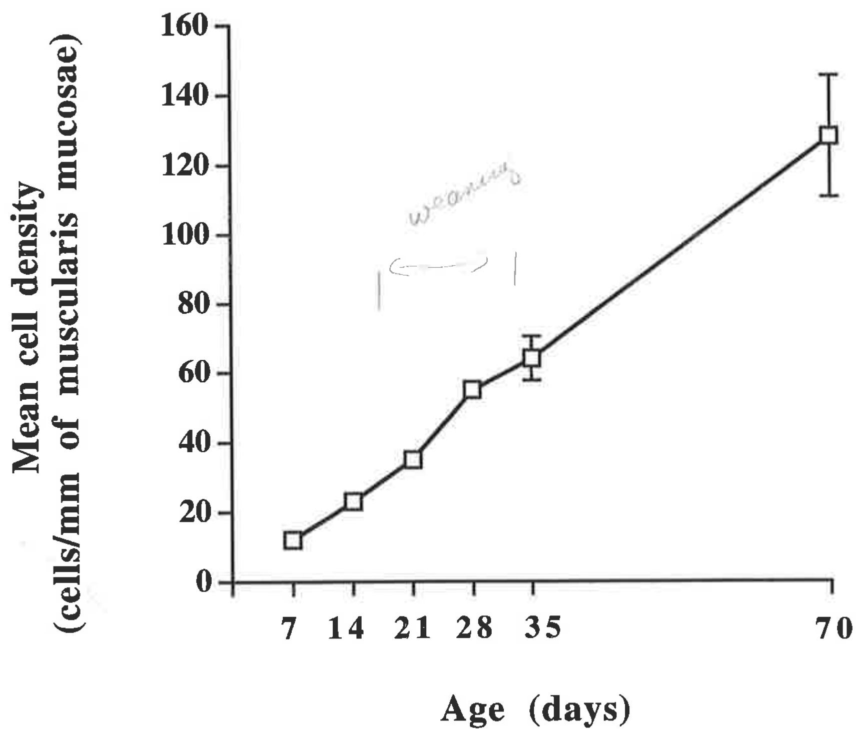
ND-not done

* $P<0.05$

** $P<0.005$

Means of α/βTCR+, γ/δTCR+, CD8α+, CD43+ and CD45+ cells at day 14 were compared with those at day 28 (end of weaning), to determine whether significant changes occured during weaning. Means were also compared between days 21 (mid-weaning) and 70 (post-weaning), to determine whether significant changes occured during the period from mid-weaning to adulthood. However, because of lack of data at day 14 for NK cells, comparison was made between days 21 and 28.

Fig. 4.1. Expansion of CD45+ IEL from pre-weaning to post-weaning in rats.



Each litter contained 7-11 rats.

Eosinophils were excluded from these counts.

SE for the pertinent data points was so tight, the symbol obscures the error bars.

Fig. 4.2 Rat jejunal IEL stained with anti-α/βTCR mAb (R73).

Upper panel: The larger arrows show a few $\alpha/\beta TCR^+$ IEL in pre-weaned rats (day 7 of life), whereas the smaller arrows demonstrate a few $\alpha/\beta TCR^-$ IEL in these animals. X50.

Lower panel: This panel shows an increase in the number of $\alpha/\beta TCR^+$ cells at the end of weaning (day 28, arrowed). Immunoperoxidase, sections were counterstained with Lillie Mayer's haematoxylin, X50.

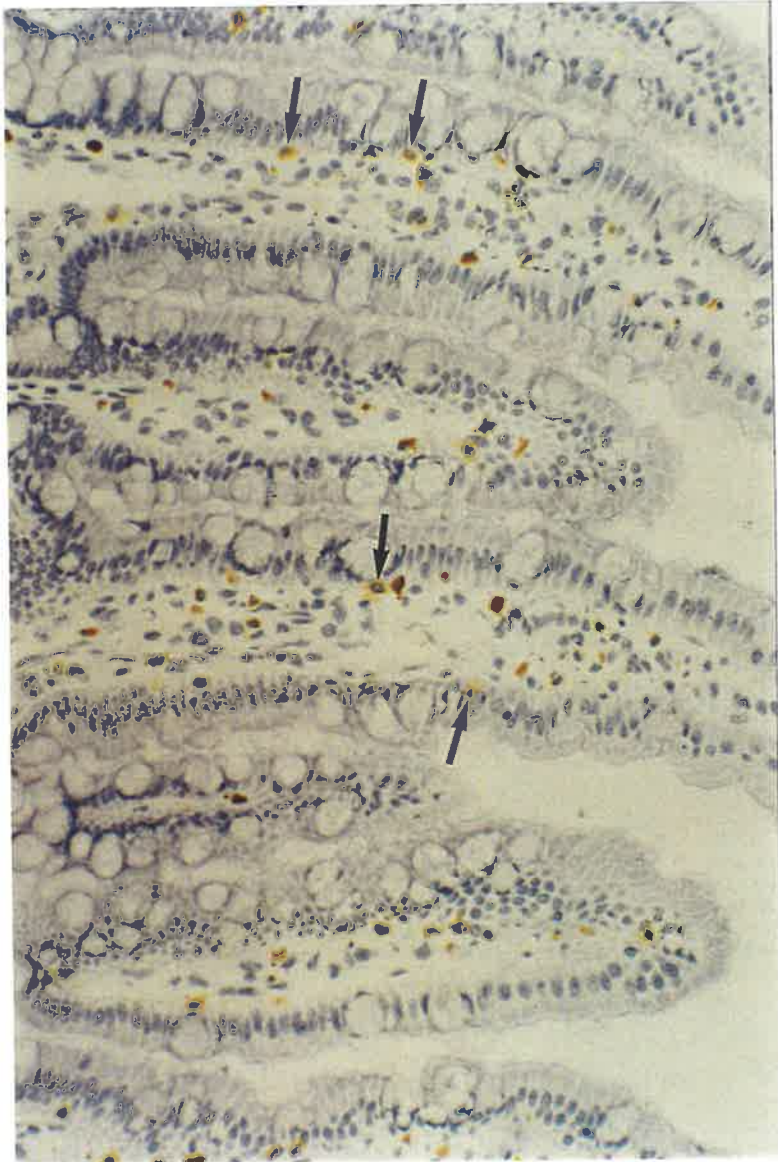
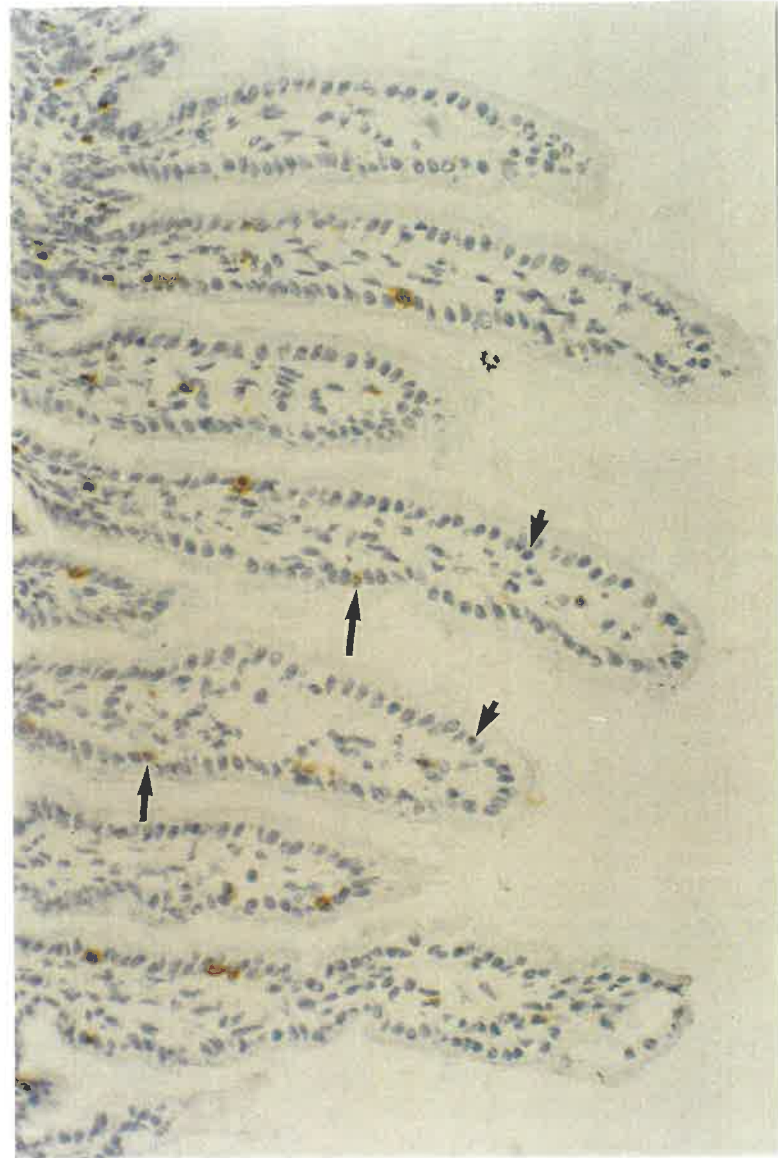
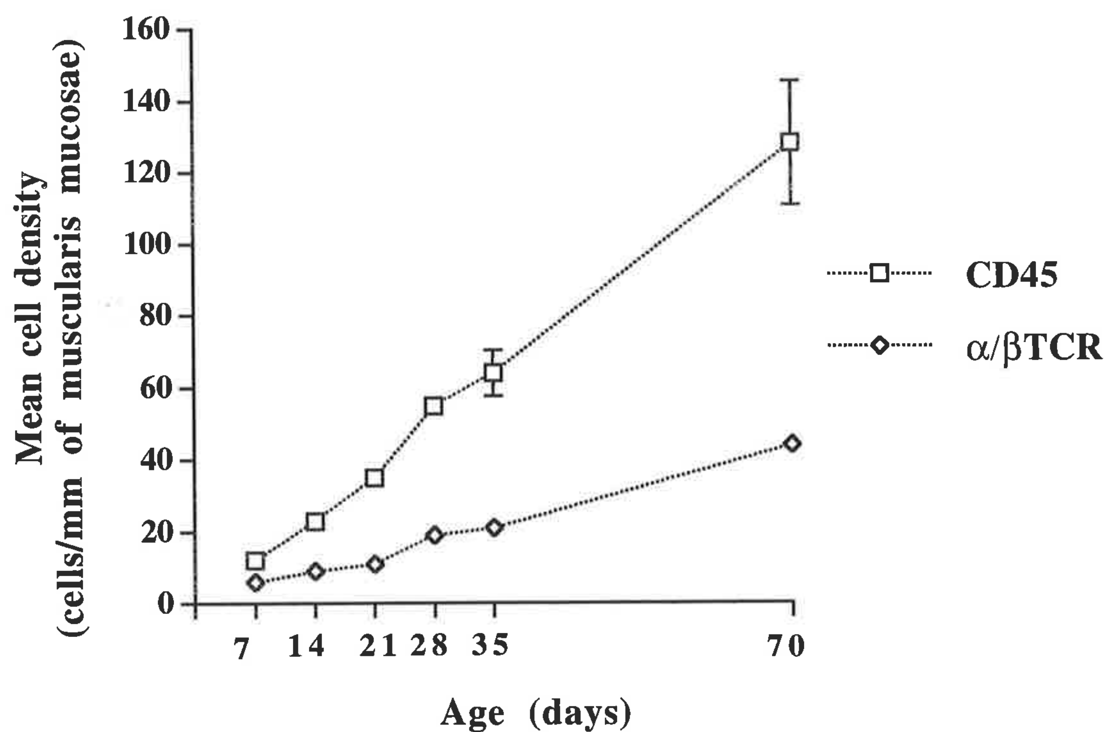



Fig. 4.3. Expansion of CD45+ and α/βTCR+ IEL in rats from pre- to post-weaning.



Each litter contained 7-11 rats.

Error bars of most data points were tight, so that the symbol obscures the SE.

Table 4.2. Expression of lymphocyte markers of IEL.

Phenotypic marker

Age (days)	α/βTCR	γ/δTCR	CD4	CD8α	CD43	NK
7	46§ (11)	3 (1.0)	ND	66 (11)	4 (2.0)	ND
14	40 (6.0)	3 (1.0)	ND	51 (19)	9 (3.5)	ND
21	30 (7.0)	3 (0.5)	ND	42 (16)	6 (2.0)	20 (9.0)
28	32 (3.0)	1 (1.0)	ND	44 (14)	5 (1.5)	5 (1.0)
35	33 (2.5)	3 (1.0)	ND	42 (13)	5 (1.5)	7 (1.5)
70	35 (3.0)	1 (0.1)	1 (0.5)	27 (10)	5 (2.0)	6 (1.5)

Each age group contained 1-2 litters (7-11 rats per litter).

§ Data indicate the mean (SE) percentage of IEL as a proportion of $CD45^+$ IEL.

The sum of CD43, α/βTCR, γ/δTCR (markers of classical T-cell) positive cells, and NK cells in the epithelial layer (43%) was very close to CD8α^+ cells (44%) at day 28. This indicates that there were two types of immune cells in the epithelium with the CD8α phenotype- namely, IEL T-cells and NK cells (**Tables 4.1** and **4.3**).

Table 4. 3. Percentages of IEL T-lymphocytes expressing CD3 in young rats from pre-to post-weaning.

Age (days)	CD3+ IEL T-cells§
14	43¶
21	34
28	36
35	36
70	36

Each litter contained 7-11 animals.
§ CD3+ IEL T-cells were calculated as the sum of α/βTCR+γ/δTCR.

¶ Figures represent the proportion of CD45+ cells expressing CD3.

$CD45^+$ and $CD8\alpha^+\alpha/\beta TCR^+$ IEL increased from pre-weaning to post-weaning by approximately 4-fold, particularly in the last week of weaning. However, there was a 2-fold discrepancy between $CD45^+$ cells and $CD8\alpha^+\alpha/\beta TCR^+$ IEL (**Table 4.2**). These findings suggest more heterogeneity in IEL subpopulations in weanling than adult rats.

As noted in Chapter 3, a large proportion of the expanding cells during weaning and thereafter did not have any clearly defined phenotype other than CD45 and were unexpectedly negative for the CD8 molecule. This was confirmed in this study, and a reasonable relationship was found between the proportions of $CD8\alpha^+$ and $\alpha/\beta TCR^+$ IEL comparing previous and current work (**Table 4.4**).

Table 4.4. Re-examination of low counts of $CD8\alpha^+$ $\alpha/\beta TCR^+$ IEL cells from previous and current work.

	Phenotypic marker			
	CD8α		α/βTCR	
Age (days)	**Study 1**	**Study 2**	**Study 1**	**Study 2**
14	75§ (11.5)	51 (19)	35 (3.0)	40 (6.0)
21	49 (12)	42 (16)	33 (7.0)	30 (7.0)
28	30 (5.0)	44 (14)	20 (5.0)	32 (3.0)
35	38 (5.0)	42 (13)	28 (5.0)	33 (2.5)

Each litter contained 7-11 rats.

Study 1 refers to experiments described in Chapters 3, whereas study 2 refers to the current Chapter.

§ Data represent the mean (SE) percentage of IEL as a proportion of $CD45^+$ cells.

A large proportion of $CD45^+CD3^-$ cells was identified at days 28 and 35, by using a panel of monoclonal antibodies against T-cells (α/βTCR, γ/δTCR, CD4, and CD8α), B-cells (CD45RA) and NK cells. As discussed earlier, unlabelled lymphoid cells (IEL) were distinguished from columnar enterocytes on the basis of morphology and their location; they were smaller, darker, and round. IEL are usually located between and underneath the enterocytes, but this could not be always readily distinguished on frozen sections due to damage to tissue by freezing and sectioning. Hence, in spite of some difficulty in distinguishing unstained IEL from the epithelial cells in frozen sections, the numbers of $CD45^+$ IEL equalled approximately the sum of T-cells, B-cells, NK cells, and negative cells (**Table 4.5**). These findings showed that almost 40% of $CD45^+$ IEL did not not have a clearly defined phenotype at days 28 and 35.

Table 4.5. Determination of surface phenotypes of $CD45^{+}$ IEL with an undefined nature at days 28 and 35, by using a panel of monoclonal antibodies against T-cells (α/βTCR, γ/δTCR, CD4, and CD8α), B-cells (CD45RA) and NK cells.

Age (days)	CD45§	Sum (T+B+NK cells)	Negative cells
28	55 (4)	31 (2)	20 (2)
35	64 (6)	29 (4)	24 (2)

§ Eosinophils were excluded from these counts.

Data are expressed as mean (SE) density counts of cells per mm of muscularis in litters of 9 rats.

4.3.1. Detection of TGF-β in the small intestine of rat

TGF-β staining was noted in the epithelial layer of pre-weaned rats (day 7 of life). Immunostaining in this site was uniform along the crypt-villus axis. The detected staining in the epithelium of small intestine was presumably due to presence of TGF-β in dam breast milk rather than endogenous TGF-β in the jejunum of thses animals.

In the ileal sections of rats at mid-weaning, some immunoreactivity was seen at villus tips. However, some low staining intensity was also observed in negative control sections. As discussed in the experimental protocol, human colon tissues were used as a positive control. The results showed that the LP tissue of colon cancer contained many positive cells with nuclear staining, whereas very few positive cells were detected in the LP of colon from a healthy subject.

4.4. Discussion

The purpose of this research was to identify the phenotype of IEL (particularly of α/β T-cells) and the time-course of the expanding population of IEL during weaning. The results showed an expansion of $CD45^+$ IEL during this time, which increased rapidly from mid-weaning (**Fig. 4.1**). Less than 50% of this expanding population consisted of $\alpha\beta TCR^+$ cells (**Table 4.2**). A new finding of this study was that approximately 50% of the expanding cells did not have a clearly defined phenotype other than $CD45^+$. There is a paucity of information on the phenotypic markers of IEL from pre-weaning to weaning. However, the total number of $CD45^+$ IEL (139 cells/mm of muscularis) in adult rats at 10 weeks of life (**Table 4.1**) were consistent with those in DA rats (116 IEL/mm of muscularis mucosae) at similar age (Lee and Mayrhofer, Hons. thesis, 1993).

In this study, $CD45^+$ IEL were found to be expanded during weaning. There was also a noticeable increase in the intensity of immunostaining after day 14 for both CD45 and $\alpha\beta TCR^+$ which would be compatible with increased numbers of both receptors. This expansion seems to have been mostly due to recruitment of cells from the LP rather than local proliferation, since Ki-67^+ (nuclear proliferation marker) was absent from days 7 to 70 of life except for intestinal crypts. IEL and LP cells from weanling rats are also known to have low proliferation *in vitro* after phytohaemaglutinin (PHA) stimulation (Ennulat, 1989).

Approximately 30-50% of IEL in weanling and post-weanling rats expressed the CD3 marker, and hence were mature T-cells (**Table 4.3**). This indicates that the remaining IEL are probably a population of pre-T-cells. There is little information elsewhere in the literature concerning the percentage of $\alpha/\beta TCR^+$ IEL in weanling and post-weanling rats. Nonetheless, these findings are in substantial agreement with those in humans reported by MacDonald and Spencer (1990) who found that about 50% of IEL in children were non-T-cells ($CD7^+$ $CD3^-$) or perhaps pre-T-cells. The remaining were $CD3^+$, and hence T-cells. Although no NK

activity has been reported for human IEL (Cerf-Bensussan *et al.*, 1985; Ebert 1989), a fraction of $CD7^+$, $CD3^-$ IEL in children could be NK cells.

The proportion of $\alpha/\beta TCR^+$ IEL T-cells did not increase, but the density of these cells increased approximately 5-fold from days 14 to 70. This was due to a similar increase in density of $CD45^+$ IEL cells (**Tables 4.2** and **4.1**). This means that the proportion of T-cells did not change during weaning despite a large increase in cell numbers. However, the data suggest that there is a compensatory relative decrease in the proportion of $\gamma/\delta TCR^+$ IEL and NK cells during and after weaning.

The proportion of $\alpha/\beta TCR^+$ or $\gamma/\delta TCR^+$ IEL is influenced by the microenvironment, and the strain and age of animals (Jarry *et al.*, 1990; Bonneville *et al.*, 1988; Goodman and Lefrancois, 1988; Mosley *et al.*, 1991; Taguchi *et al.*, 1991; Fujihashi *et al.*, 1992). Therefore, there are several possible explanations for the presence of a high fraction of $CD3^-$ IEL in this study. For example, the number of $\alpha/\beta TCR^+$ IEL depends on the level of antigenic stimulation in the intestine and can reach 75% in adult mice retained under conventional conditions (Jarry *et al.*, 1990). In addition, 20-80% of mouse IEL express γ/δTCR, depending on the microenvironment, the age and the strain of animals (Bonneville *et al.*, 1988; Goodman and Lefrancois, 1988; Mosley *et al.*, 1991; Taguchi *et al.*, 1991; Fujihashi *et al.*, 1992).

Earlier work (Chapter 3) on stored frozen sections showed that a significant proportion of IEL were $CD3^-$ from days 14 to 42 (~25-60%). This was confirmed in this study in which approximately 50-70% of IEL were $CD3^-$ (**Table 4.3**). These findings suggest that a large population of IEL are presumably a mixture of pre-T-cells, eosinophils and NK cells, as no B-cells or macrophages were detected in the epithelium (**Tables 4.1** and **4.2**).

Experimental results in Chapter 3 also showed that the proportion of $CD8\alpha^+$ cells was lower than expected for post-weanling rats (ie, ~30% at day 42 instead of 50%). This was also confirmed in this study where the proportions of $CD8\alpha^+$ cells were approximately 40% and 30% for post-weanling rats at days 35 and 70, respectively (**Table 4.2**). Since only a small proportion of IEL in the previous study were $CD4^+$, these results lend support to the assumption that a significant fraction of IEL have the $CD3^+ CD4^- CD8^-$ phenotype normally associated with γ/δ^+ T-cells in other sites (Cerf-Bensussan, *et al.*, 1987; Jarry *et al.*, 1988; Jenkins, *et al.*, 1986; Spencer *et al.*, 1989).

In rats, Lyscom *et al.* (1983) have reported low numbers of IEL at birth. This is in agreement with the observation in this thesis which showed a low IEL cell density (12 cells/mm of muscularis) prior to weaning (day 7). Ferguson and Parrott (1972a) used haematoxylin and eosin sections to count IEL in mice on morphological grounds. They found IEL around the time of weaning-namely, at the third week of life. Using the more sensitive assay of three colour immunofluorescence, murine $CD45^+$ IEL were detected 6 days after birth in the intestinal epithelium (Woodward *et al.*, 1989).

This study also showed that TGF-β is well-demonstrated in methacarn, paraffin embedded human colon sections, using polyclonal anti-human TGF-β. However, this antibody did not perform well on rat gut sections for the detection of of this immunosuppressive cytokine, using either methacarn or formalin fixatives. This is unlikely to be due to poor cross-reactivity between human and rat TGF-β, because there is 98% amino acid homology amongst species (Derynck *et al.*, 1986).

4.5. Conclusions

The data presented in this Chapter showed that:

(**a**) There were only relatively small numbers of $CD45^+$ IEL prior to weaning and they expanded during and after weaning;

(**b**) $\alpha/\beta TCR^+$ IEL increased towards the latter half of weaning, but $\gamma/\delta TCR^+$ IEL did not change during and after weaning;

(**c**) NK cells peaked at day 21; and

(**d**) Most IEL did not show any clearly defined phenotype except CD45.

These results strengthen the original hypothesis that weaning is associated with an increase in $CD45^+\alpha/\beta TCR^+$ cells in rats. However, no major changes in the proportion of different subsets of IEL were evident. Further investigations will be necessary to identify those $CD45^+$ $CD3^-$ IEL. Expansion of this population seems likely to be due to migration from the LP rather than by local proliferation in the mucosa. This unusual phenotype of IEL in the rat may reflect the presence of a discrete population of T-cells with a distinct developmental lineage. Time did not permit further investigation to identify the appropriate antibody for the detection of rat TGF-β. Thus, I would recommend further study in this area using chicken anti-recombinant human TGF-β1 (rhTGF-β1), IgY (R&D Systems, Minneapolis, MN, USA) to see if improved staining can be achieved. Alternatively, monoclonal mouse anti-TGF-β1,-β2-β3 (Massague, 1990) from Genzyme (Cambridge, MA, USA) is recommended.

Chapter 5

Phenotype and activation of intestinal intraepithelial lymphocytes with emphasis on eosinophils and natural killer cells during weaning

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5.1. General introduction

Under normal circumstances, approximately 50%-70% of IEL express CD3 in *adult* rats, indicating the T-cell nature of this cell population (Vaage *et al*, 1990; Lefrancois *et al*, 1991; Kearsey *et al*, 1996). However, the composition of IEL is strongly dependent on antigenic stimulation by environmental factors including food intake and microbial antigens (Banderia *et al*, 1990; Poussier *et al*, 1994). Weaning (physiological stress) is associated with an increase in food and bacterial antigens and is associated with infiltration of other immune non-T-cells (eosinophils, NK cells) into the intestinal epithelium.

During weaning, there is upregulation of physiological inflammation in the intestine with activation of T-cells which express IL-2R (Thompson *et al.*, 1996). This is associated with an accelerated period of intestinal proliferation, and maturation of digestive functions (Cummins and Thompson, 1997). The concept of 'physiological inflammation' of the small intestine comes from the increase in inflammatory cells in the intestinal mucosa of germ-free mice after exposure to a conventional environment (Sprinz, 1962; Abrams, *et al.*, 1963), but has been broadened to include the notion that the intestine has a background level of inflammation because of food and bacterial antigens (Fiocchi, 1997). Food antigen stimulation is probably important in mediating physiological inflammation, because the number of γ-IFN producing cells, MMC and IEL are higher in the proximal (food exposed) small bowel than in the distal (bacteria-exposed) intestine (Cerf-Bensussan and Guy-Grand, 1991; Brett, *et al.*, 1992), and because there are reduced numbers of immune cells in animals fed hypoallogenic diets (Li, *et al.*, 1995).

5.1.1. Eosinophils

Eosinophils and neutrophils are rare in the epithelium of small intestine, but their numbers may increase in inflammatory conditions of the digestive tract (Austin and Dobbins, 1982). The mechanisms by which eosinophils become activated are not well understood. However, these cells appear to have at least two types of receptors for IgE; namely, low-affinity FcεR2

receptors and carbohydrate binding protein, MAC-2, that also occurs on macrophages (Cherayil *et al*, 1989). In addition, receptors for IgG (FcγR2), IgA (FCαR), and complement have been identified on the surface of human eosinophils. Different Fc receptors (FcεR, FCγR and FCαR) mediate the release of different mediators (Tomassini *et al*, 1991). Activation of eosinophils is also induced by IL-3 (Rothenberg *et al*., 1988), IL-5 (Lopez *et al*., 1988), and GM-CSF (Owen *et al*., 1987).

5.1.2. NK cells

NK cells are non-T, non-B large granular lymphocytes that constitute part of the innate, non-antigen dependent immune system. Their lineage remains uncertain, because they express some markers of T-lymphocytes (such as CD2 and CD7) as well as markers of monocytes and granulocytes. NK cells are the only cytotoxic effectors with a CD8$\alpha^{+}\beta^{-}$ phenotype (Torres-Nagel, 1994). Furthermore, different NK cells express different sets of membrane molecules. It is not known whether this heterogeneity reflects subpopulations of NK cells or various stages in their activation or maturation (Versteeg, 1992; Kuby, 1994).

NK cells in human peripheral blood have been extensively studied and have a typical morphology (Grossi *et al*, 1982) and phenotype (Lanier *et al*, 1986). Morphologically, these cells are homogenous cells with cytoplasmic azurophilic granules, but phenotypically they are heterogeneous as demonstrated by the presence or absence of the CD3 complex (Lanier *et al*, 1986).

In the human gut, approximately 2% of mononuclear cells in the LP are NK cells with less NK activity than blood cells (Shanahan *et al*, 1988). Little is known about this mucosal cell population, although it has been recently reported that these cells play an important role in the induction and regulation of inflammation and immunity in the human intestinal mucosa (Pang *et al*, 1993). The goal of this research was to investigate which subsets of IEL T-cells/or non T-cells (NK cells and eosinophils) expand during weaning, and to evaluate phenotypic

changes. The second goal of this study was to characterize the activation and proliferation status of IEL using IL-2R and Ki-67 antigens, respectively.

5. 2. Experimental protocol

IEL were studied in litters of PVG/cxDA rats.These litters were aged from 17 to 70 days of life (every 2 or 3 days) with day 0 being designated as the day of birth. Litter size varied from three to eleven animals and runted animals were excluded. Each rat pup was anaesthetized with ether, the axillary artery cut and allowed to bleed out. The small intestine was isolated and jejunal segments collected at the first proximal one-sixth segment of the small intestine (corresponding to 15 cm from the pylorus in 12 week old animals). Intestinal samples were embedded in OCT, frozen in isopentane cooled by liquid nitrogen and placed on dry-ice until stored at -70° C. The sections for immunohistochemical analysis were stained by an indirect immunoperoxidase method, using a variety of monoclonal antibodies directed against T cells, B-cells, NK cells and granulocytes. Counts were expressed as the number of positively stained cells per mm of muscularis mucosae.

5. 3. Results

5.3.1. Changes in IEL T-cells and NK cells

CD45^{+} cells in the epithelial layer expanded during the weaning period by approximately 23-fold (P<0.005, **Table 5.1** and **Fig. 5.1**). Interestingly, the majority of this expansion occurred in the last week of weaning (day 25). Subsequently, CD45^{+} IEL began to stabilise. α/β TCR^{+} IEL T-cells also expanded during weaning-namely, from days 15 to 25 by approximately 11-fold (P<0.005). Similar to CD45^{+} cells, α/βTCR^{+} IEL increased equally from days 15 to 19, but these cells showed a lesser increase from days 19 to 25, which almost represents the second week of weaning (**Table 5.1** and **Fig. 5.1**). Similar to CD45^{+} IEL, α/βTCR^{+} IEL T-cells began to stabilise gradually, after day 25. The CD43 (pan-T-cell marker) was infrequent

(**Table 5.1**). The overall results demonstrated that from days 17 to 44, approximately 20%-40% of expanding $CD45^+$ cells were $CD3^+$ IEL. However, this proportion increased to 65% by day 70, indicating the T-cell nature of IEL in adult rats. The density of $CD3^+$ cells increased greater than 2-fold from days 19 to 25, but the proportion of $CD3^+$ IEL dropped from approximately 40% to 20%, relative to the larger proportional expansion of $CD45^+$ cells (**Table 5.2**). Thus, both $\alpha/\beta TCR^+$ and $\alpha/\beta TCR^-$ IEL were observed during infancy (**Fig. 5.2**).

NK cells increased significantly from days 15 to 19 ($p<0.05$) (**Table 5.1** and **Fig. 5.3**). At day 19, 72% of $CD45^+$ IEL were positive for the NK cell marker (NKR-P1). However, the proportion of CD45+ cells expressing the NK cell marker was not sustained by late weaning (day 25), being less than 10% of $CD45^+$ IEL ($P>0.05$). The value at day 25 was not significantly different from the start of weaning (day 15) (**Table 5.2**). However, the staining intensity of cells expressing the NK cell marker increased with age (**Fig. 5.4.**). The $CD8\alpha$ is expressed on cytotoxic-suppressor T-cells and on a subpopulation of rat NK cells (Cantrell *et al*, 1982; Norment and Littman 1988). The results showed that from days 15 to 44, $CD8\alpha^+$ cells counts approximately equalled the sum of T-cells (expressing either α/βTCR or γ/δTCR) plus NK cells in the intestinal epithelium (**Table 5.3**). However, by late weaning (day 25), approximately 30% of $CD8\alpha^+$ cells were neither T-cells nor NK cells. These 'null' cells may be pre-T-cells in an an unusual stage of differentiation, where $CD8\alpha$ expression precedes α/βTCR expression.

There was a large increase in the number of $CD45^+$ cells from days 19 to 44. However, the proportion of these expressing either T or NK cell markers was much lower than expected. For example, only 25% of the $CD45^+$ cells expressed either T cell or NK cell markers by late weaning (day 25) (**Table 5.2**). The remaining $CD3^-$ cells included very few macrophages or B-cells. (**Table 5.1**).

Table 5.1. Phenotype of IEL cells.

Age (days)	Surface antigens						
	CD45¶	**CD8α**	**CD43**	**CD4**	**NK**	**Macrophage**	**CD45RA (B cells)**
15	5† (0.9)	12 (3.1)	ND	0.2 (0.07)	6 (1.7)	0.2 (0.1)	0.06 (0.04)
17	13* (3.3)	13 (1.8)	1 (0.2)	ND	10 (1.7)	1 (0.08)	ND
19	26** (4.3)	24* (3.1)	ND	ND	11* (1.3)	ND	ND
25	113** (13.0)	50** (5.4)	8 (1.6)	ND	8 (1.4)	1 (0.2)	0
44	117** (10.7)	37** (3.2)	5 (0.3)	ND	3 (0.5)	0	0

Data are expressed as mean (SE) density counts of cells per mm of muscularis mucosae in litters from 7-11 rats.

¶ Eosinophils (peroxidase positive) were excluded from these counts.

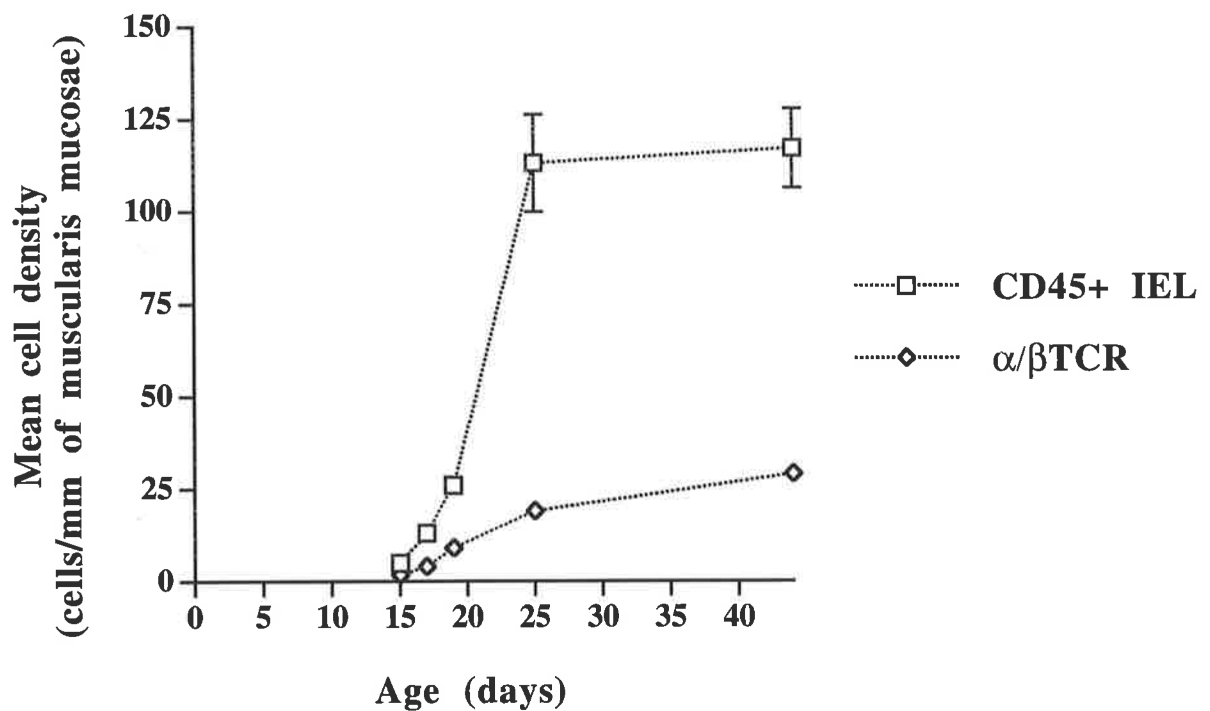
† $CD45^+$ IEL had very low levels of staining, and some IEL cells were negative for this marker. This may indicate the immaturity of these cells.

* $P<0.05$

** $P<0.005$

The significance refers to comparison with day 15.

Fig. 5.1. Expansion of CD45+ and α/βTCR+ IEL from weaning to post-weaning (day 44).



Data are expressed as mean ± SE density counts of cells per mm of muscularis mucosae in litters from 7 to 11 animals.

Error bars for some data points were obscured by the symbols.

Table 5.2. Percentages of $CD45^+$ IEL cells.

Proportion of IEL positive (%)

Age§ (days)	α/βTCR	γ/δTCR	CD8α	CD43	NK
17	28 (7.0)	0	99 (14)	4 (2.0)	75 (13)
19	35 (3.0)	3 (1.0)	93 (12)	ND	42 (5.0)
25	17 (3.0)	1 (0.5)	45 (5.0)	7 (1.5)	7 (1.0)
44	25 (3.0)	1 (0.1)	31 (3.0)	4 (0.5)	2 (0.5)
70	64 (11)	1 (0.1)	ND	ND	ND

Each litter contained 7-11 rats.

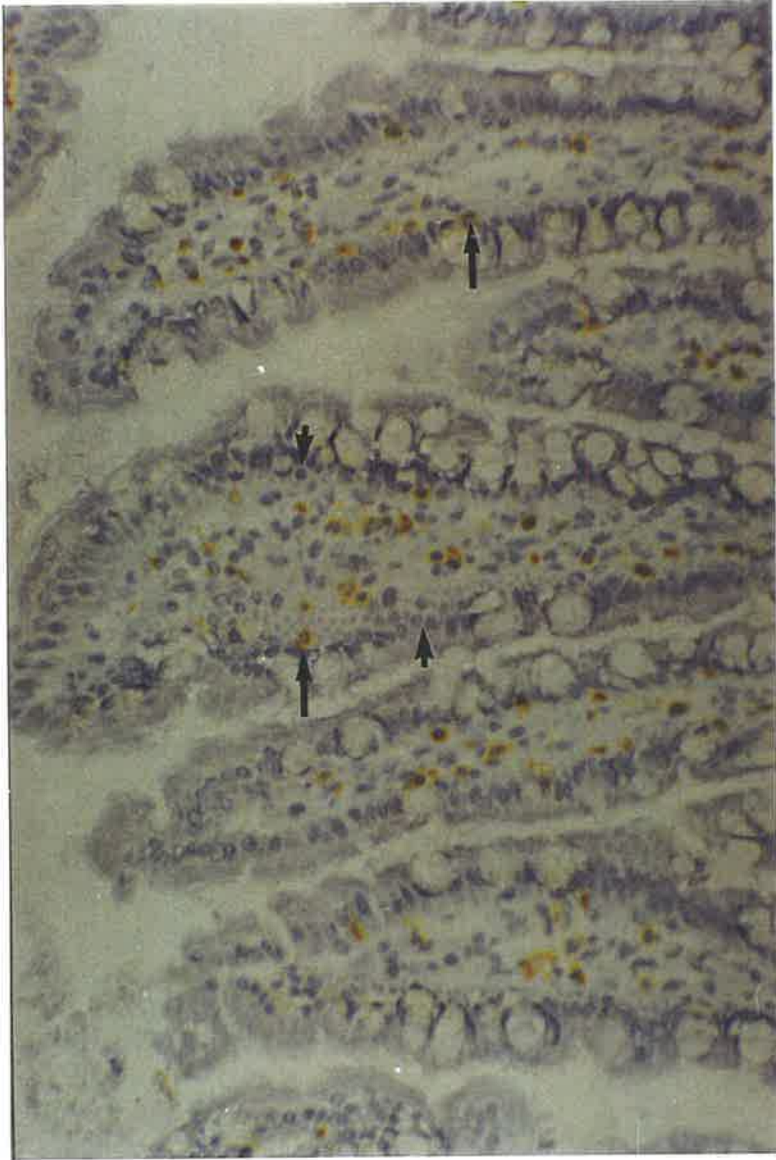
Data indicate the mean (SE) percentage of IEL as a proportion of $CD45^+$ cells.

§ Percentage of IEL cells at day 15 of life was not calculated due to a lower density of $CD45^+$ cells as compared to $CD8\alpha^+$ cells.

Fig. 5.2 Rat jejunal IEL stained by anti-α/βTCR mAb (R73).

Upper panel: The smaller arrows demonstrate several $\alpha/\beta TCR^-$ IEL at day 25 of life, whereas the larger arrows display $\alpha/\beta TCR^+$ expressing IEL at this stage. X50.

Lower panel: The smaller arrows show that in more mature rats (post-weaning, day 44), there are also several IEL which have not reacted with this mAb. Immunoperoxidase, X50.



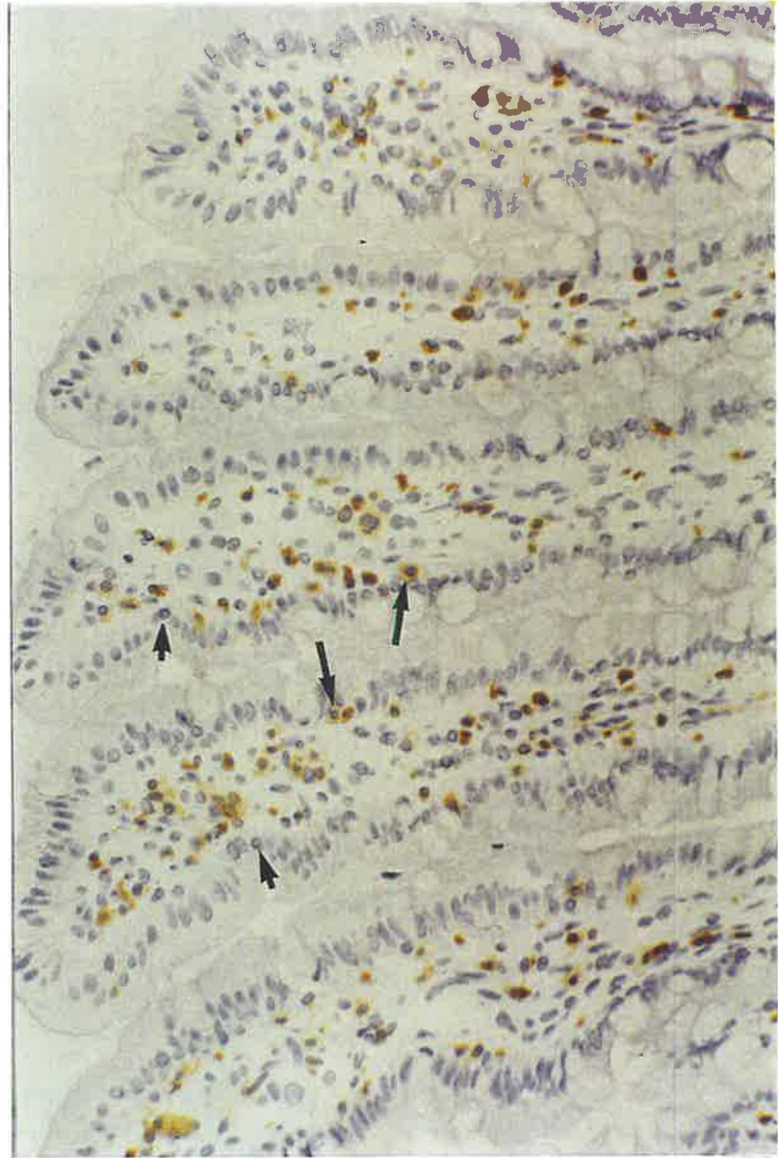
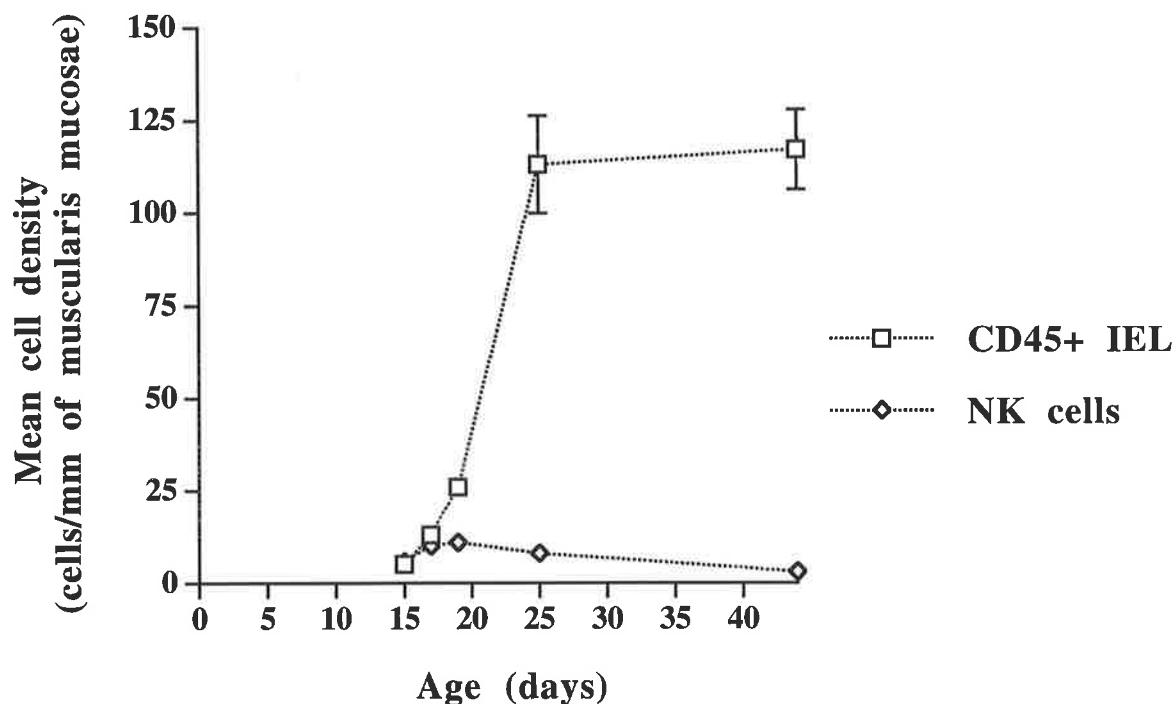


Fig. 5.3. Changes in CD45+ and NK cell counts in the epithelial layer of small intestine from weaning to post-weaning (day 44) in rats.



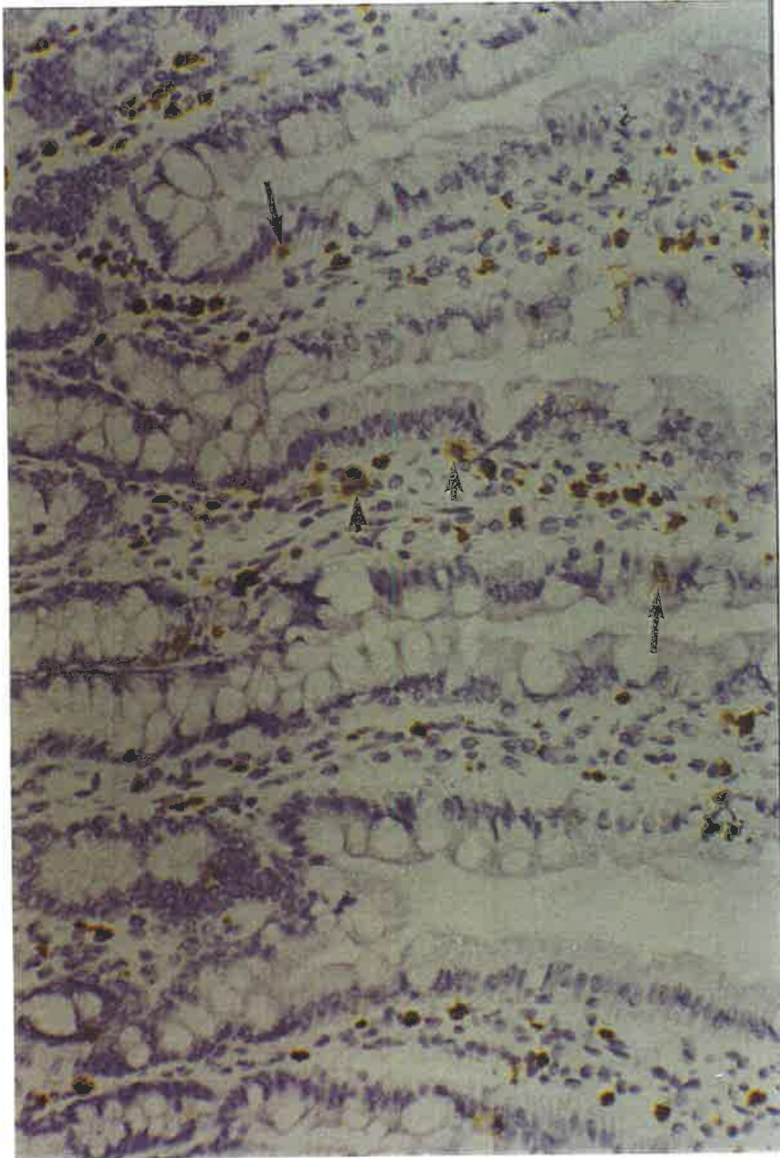
Data are expressed as mean ± SE density counts of cells per mm of muscularis mucosae in litters from 7 to 11 animals.

Error bars for most data points were obscured by the symbols.

Fig. 5.4. Rat jejunal NK cells stained with anti-NK marker mAb (3.2.3) .

Upper panel: The larger arrows show NK cells in the epithelial layer at the end of weaning, the smaller arrows demonstrate NK cells in the LP compartment. Note that NK cells at this stage display intermediate staining. X50.

Lower panel: This panel demonstrates an increase in the staining intensity of large NK cells at post-weaning (day 44). Arrows indicate the same as for the upper panel. Immunoperoxidase, X50.



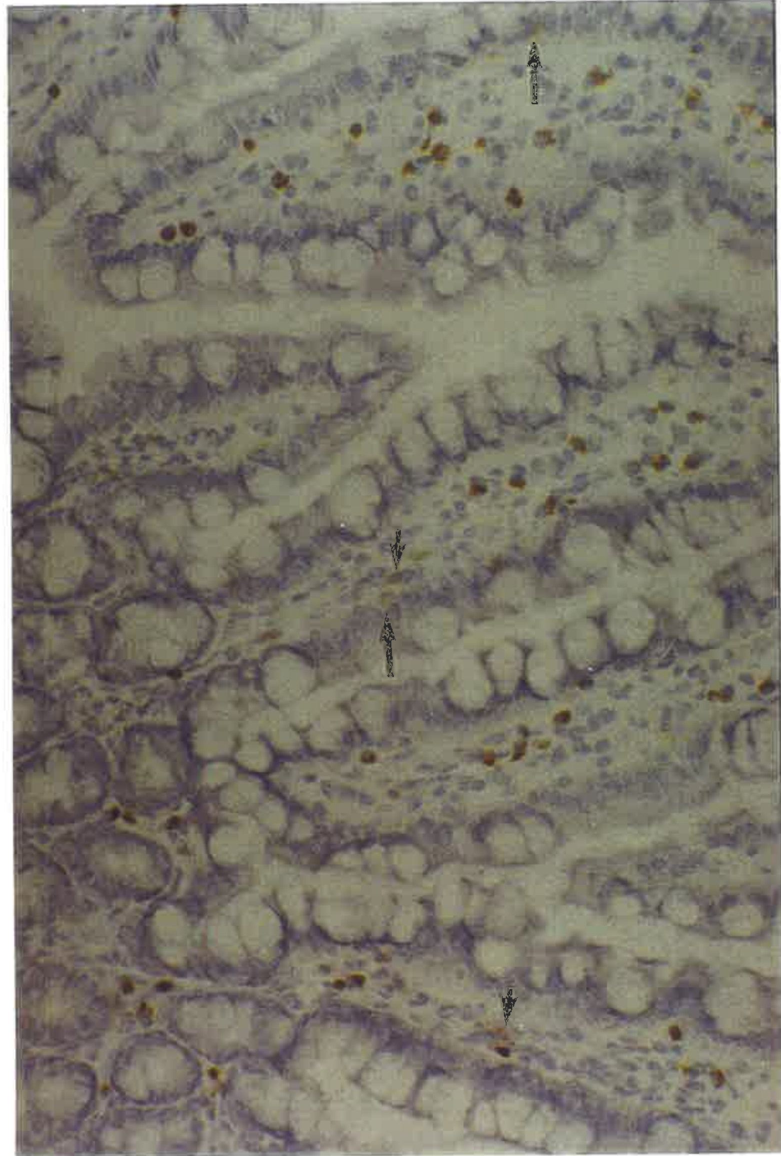


Table 5.3. Comparison between CD8α^+ cell counts to sum of (α/βTCR+γ/δTCR+NK cells) in the intestinal epithelium of rats from weaning to post-weaning.

Age (days)	CD8α	α/βTCR+γ/δTCR+NK cells
15	12¶	8¶
17	13	14
19	24	21
25	50	28
42	37	33

Data indicate the mean density counts of cells per mm of muscularis mucosae in litters from 7-11 animals.

¶ Some data for different groups were derived from **Table 5.1**.

The CD45$^+$ CD3$^-$ 'null' phenotype was further investigated using a panel of monoclonal antibodies against T-cells (α/βTCR, γ/δTCR, CD4, CD8), B-cells (CD45RA), NK cells, macrophages and granulocytes. CD45$^+$ IEL counts approximately equalled the sum of T-cells, B-cells, NK cells, eosinophils and granulocytes (**Table 5.4**). These findings indicate that: first, all of the immune cells of the epithelial compartment were CD45$^+$; second, CD45$^+$ cells are nearly the sum of NK cells, eosinophils (which are CD3$^-$) and T-cells (which are always CD3$^+$). A fraction of NK cells are probably NK-T-cells, and express both NK and T-cell markers.

Table 5.4 Phenotype of IEL at day 19, which were either CD45+, or positive to a panel of monoclonal antibodies to T-cells (α/βTCR, γ/δTCR, CD4, CD8α), B cells (CD45RA), NK cells, macrophages and granulocytes.

CD45+ cells ¶	Sum(T-cells+B-cells+NKcells+macrophages+granulocytes∂)	Eosinophils
39 (3) §	25 (2)	14 (2)

¶ Eosinophils (peroxidase positive cells) were included for these counts.

∂ Null CD45+ cells by default express the 'granulocyte' marker.

§ Data represent the mean (SE) density counts of cells per mm muscularis in a litter of 9 rats.

5.3.2. Changes in eosinophil (peroxidase positive) counts in the epithelium of small intestine

Eosinophil counts in the epithelium are given in **Table 5.5**. They were present in an intraepithelial position even before weaning. For instance, at day 7, 11 cells/mm of muscularis mucosae were detected. They increased during weaning; from days 15 to 25 ($P<0.05$). Interestingly, they showed an early peak at day 17 ($P<0.001$). After weaning (day 44), counts dropped to pre-weaning levels (day 14, $P>0.05$). However, eosinophils numbers increased again ($P<0.005$) reaching normal adult levels at day 70. Appreciable numbers of eosinophils, which were observed in an intraepithelial position at days 17 and 25, were more widely distributed along the whole villus at day 70.

Table 5.5. Changes in eosinophil counts in the epithelium of rat small intestine from pre-weaning to post-weaning.

Age (days)	cell density
7	11 (1.2)**
14	9 (1.6)**
15	10 (1.4)**
17	34 (4.0)
19	14 (2.1)**
21	16 (4.8)*
25	25 (4.0)**
28	15 (2.1)**
35	15 (1.7)**
44	8 (1.3)**
70	20 (3.7)*

Data are given as mean (SE) density counts of cells per mm of muscularis mucosae in litters from 6 to 11 animals.

The significance refers to comparison with day 17.

* $P<0.05$

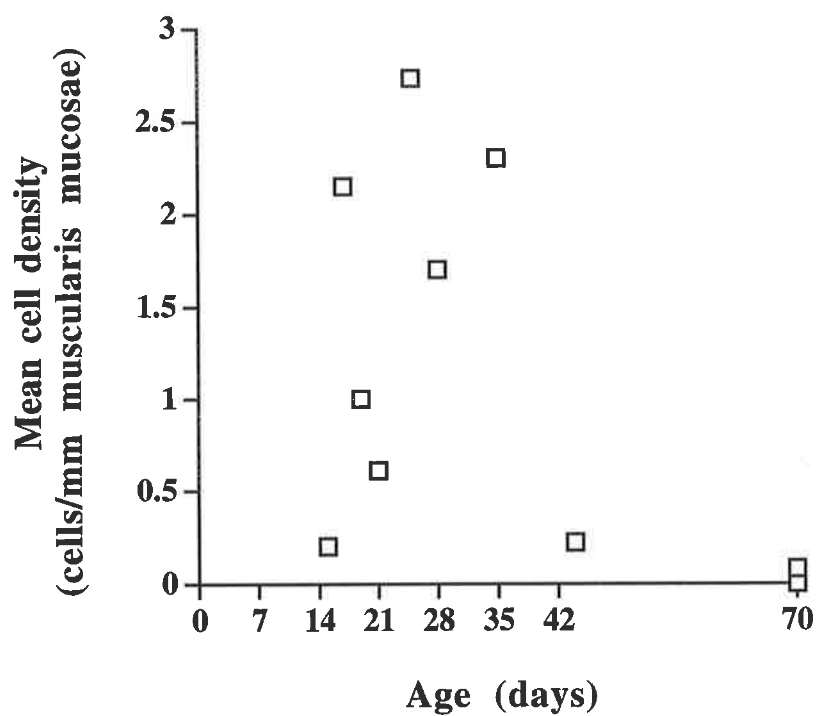
** $P<0.005$

5.3.3. Changes in activation and proliferation with expression of IL-2R and Ki-67 antigens

A proportion of IEL expressed IL-2R at low density with a broad peak probably slightly later than that observed for similar cells in the LP (Chapter 6) (**Fig. 5.4**). The results demonstrated that IEL at day 21 were in a more activated state than the corresponding cells at post-weaning (day 70, $P<0.0001$).

At day 19, 1.4% of $CD45^+$ IEL were positive for Ki-67, although a moderate proportion of intestinal crypt cells showed positive staining. However, in other weanling or post-weanling rats, IEL showed no detectable proliferation. These findings indicate a low proliferation of IEL at day 19.

Fig. 5.4. Changes in IL-2R activation status of IEL in rats from weaning (day 15) to post-weaning.



Each litter contained 3 to 12 animals.

5.4. Discussion

This study has shown that there is upregulation of physiological inflammation in the small intestine of the rat during weaning. $CD45^+$ cells were present in the intestinal epithelium at the time of weaning, but their numbers were quite low. Their counts remained low in the first week of weaning, but thereafter rapidly increased in number. $CD45^+$ IEL expanded in the intestinal epithelium sharply. An identifiable but minor subset of this expanding population consisted of $\alpha\beta TCR^+$ cells. The majority of the expanding cells did not have a clearly defined phenotype other than $CD45^+$. The late expansion of $CD45^+$ IEL raises the possibility that IEL increase because of longer-term exposure to food antigens.

There was a noticeable increase in the *intensity* of immunostaining after day 19 for both CD45 and $\alpha\beta TCR^+$, which would be compatible with increased copy numbers of both receptors. On the other hand, there is a collaboration between α/βTCR and CD45 markers in T-cell activation, so that T-cells that lack CD45 cannot respond to antigen, even though they express normal levels of the TCR. CD45, a tyrosine phosphatase, is required for TCR signaling (Imboden and Stites, 1994).

The current study has also shown that expression of T-cell markers on IEL is similar to that occuring during T-cell development in the thymus as expression of CD8 on both thymocytes and IEL precedes expression of α/βTCR (Chapter1, **Fig. 1.2**). For instance, at day 17, a smaller percentage of IEL labelled for α/βTCR compared to $CD8\alpha^+$ cells (**Table 5.2**). This may indicate an extrathymic development pathway for IEL, or at least an influence of the gut microenvironment on the maturation of thymic-independent IEL.

Heterogeneity of IEL subpopulations was also shown during weaning. For example, around the mid-weaning (day 19), the intestinal epithelium was composed of different populations of immunological cell types-namely, NK cells (42%), eosinophils (35%) and α/β T-cells (35%) (**Tables 5.2** and **5.4**). These findings suggest that first; weaning which is accompanied by

intense antigenic stimulation, allows access of other immunological cell types into the epithelium; and second, since the sum of these cell exceeds 100%, a fraction of NK cells may be NK-T-cells, which express both NKR-P1 and CD3 markers.

NK cells were also present in the intestinal epithelium at the start of weaning, and their numbers peaked around mid-weaning (day 19). Eosinophils were detected prior to weaning (day 7), and their counts peaked at an early stage of weaning (day 17). Taken together, this study demonstrated an early peak of eosinophil and NK cells (inflammatory cells) in the intestinal epithelium during the first week of weaning. These findings indicate that physiological inflammation in the intestinal epithelium occurs around mid-weaning. One possible explanation for heavy infiltration of the intestinal epithelium by eosinophils is degranulation of MMC by a major basic protein released by activated eosinophils (Cummins *et al*, 1988a-b). This may cause release of eosinophil chemotactic factor of anaphylaxis (ECF-A) from activated MMC (Bach, 1982; Roitt *et al.*, 1996).

IEL had a peak of IL-2R$^+$ cells similar to that of LP cells (Chapter 6) which would be consistent with migration of activated T-cells from the LP to the epithelium. The stimulus for migration of IEL from the LP probably arises from the intestinal epithelium. A variety of chemoattractant and pro-inflammatory factors have been described that are produced from the intestinal epithelium, at least during pathological immunological reactions, some of which could influence immune cells. These include TNFα, GM-CSF, IL-1α, IL-1β, IL-7, stem cell factor, thyroid-stimulating hormone (TSH), C-X-C or α family chemokines such as IL-8, GROα, GROβ, GROγ, ENA-78, and C-C, or β family chemolines such as MCP-1, MIP-1β, and RANTES (Puddington *et al*, 1994; Watanabe *et al*, 1995; Wang *et al*, 1997; Kagnof and Eckmann, 1997). Using *in vitro* migration assays, IEL and to a lesser extent LP cells have been shown to migrate to the α-chemokines IL-8 and GRO and to the β-chemokines RANTES, MIP and MCP (Ebert, 1995; Ebert, 1997). What has not been shown is whether a lesser quantity of any of these is produced during physiological inflammation associated with

weaning. Nevertheless, the present study would suggest that migration of lymphocytes occurs from the LP into the epithelium and it would be reasonable to propose that this migration is mediated by chemokines.

5.5. Conclusions

This study showed that:

(**a**) $CD45^+$ cells were present in low numbers at the time of weaning, and their numbers increased dramatically in the latter half of weaning;

(**b**) NK cells constituted a major component of IEL cells in the first week of weaning;

(**c**) Eosinophils were present in the intestinal epithelium prior to weaning and peaked at an early stage of weaning; and

(**d**) IEL at day 21 were in a more activated state than the corresponding cells at post-weaning.

These findings support the hypothesis that physiological inflammation of the small intestine is closely associated with weaning. Expansion of $CD45^+$ $CD3^-$ IEL towards the end of weaning, might relate to influx or proliferation of precursor cells. The heterogeneity of IEL during weaning may reflect a situation where weaning is accompanied by intense antigenic stimulation, with the subsequent attraction of other immunological cell types into the epithelium.

Chapter 6

Phenotype and activation status of intestinal lamina propria cells during weaning

6.1. LP T-cells

The intestinal LP contains mainly B-cells (including plasma cells), T-cells, and various accessory cells such as eosinophils, macrophages and mast cells, which are all dependent for activation by $CD4^{+}$ T-cells (MacDonald and Spencer, 1988; Lee *et al.*, 1988; Fiocchi, 1990; Lionetti *et al.*, 1993). Unlike IEL, virtually all LP T-cells in humans and rodents express the $\alpha/\beta TCR^{+}$ and have the phenotype of memory/activated T-cells, being $CD45R0^{+}/CD45RB^{lo}$ (Farstad *et al.*, 1996, James *et al.*, 1986; Schieferdecker *et al.*, 1992; James and Zeitz, 1994; Hurst and Barrett, 1996; Abreu-Martin and Torgon, 1996). The fact that LP T-cells have very weak proliferative responses to antigen or other TCR dependent stimuli, but release large amounts of effector cytokines such as γ-IFN, IL-4 and IL-5, suggests that this population is under steady antigenic stimulation *in situ* (Abreu-Martin and Torgon, 1996, Pirzer *et al.*, 1990; James and Zeitz, 1994; Taguchi *et al.*, 1990). This unusual activation state of low proliferation, but high cytokine production of LP T-cells is associated with a unique requirement for the CD2-dependent pathways of T-cell activation (Targan *et al.*, 1995). This may either reflect the advanced differentiation status of the cells *in situ*, or could be due to a requirement for co-stimulation via B7 in this compartment of the mucosal immune system (Hurst *et al.*, 1995).

Concerning T-lymphocyte functions in the intestinal human LP, *in vitro* studies have shown that T-cell activation leads to several events as follows:

(1) lymphokine release,

(2) an increase in crypt cell proliferation,

(3) villous atrophy,

(4) increased HLA-DR expression on the villous and crypt enterocytes,

(5) increased IEL numbers,

(6) macrophage activation.

(MacDonald and Spencer, 1988; MacDonald and Spencer, 1990).

All of these characteristics are observed in T-cell activation to food antigens (e.g., coeliac disease and cow's milk sensitive enteropathy) (MacDonald and Spencer, 1990; Ferreira *et al.*,

All of these characteristics are observed in T-cell activation to food antigens (e.g., coeliac disease and cow's milk sensitive enteropathy) (MacDonald and Spencer, 1990; Ferreira *et al.*, 1990; MacDonald, 1990). Since T-cell activation can change intestinal architecture pathologically, it has been proposed that T-cell activation during weaning can also promote mucosal development physiologically (Thompson *et al.*, 1996; Cummins and Thompson, 1997).

Most studies of LP T cells have been done in humans, and there is paucity of information in other species including rats. The information that does exist has largely been obtained by immunofluorescence staining and flow cytometry. The present study was undertaken to determine the phenotype and the activation status of LP cells *in situ.* The purpose of this research was to characterize the phenotype and particularly the activation status of LP lymphocytes using IL-2R expression, and how LP cells change phenotypically and numerically during weaning.

6.2. Experimental design

LP cells were studied in litters of DAxPVG/c or PVG/cxDA rats. These litters were aged from 7 to 70 days of life with day 0 being designated as the day of birth. Litter size varied from 3 to 12 animals and runted animals were excluded. Each rat pup was anaesthetized with ether, the axillary artery cut and allowed to bleed out. The small intestine was isolated and jejunal segments were collected from the proximal one-sixth of the small intestine (corresponding to 15 cm from the pylorus in 12 week old animals). Intestinal samples were embedded in OCT, frozen in isopentane cooled by liquid nitrogen, remained on dry ice during the procedure and were stored at -70° C. Phenotype and activation status of LP cells were identified immunohistochemically by using the indirect alkaline phosphatase staining method, with a variety of monoclonal antibodies directed against T cells, B-cells, macrophages, and NK cells markers. Moreover, counts of eosinophils, based on endogenous peroxidase activity, was

6.3. Results

6.3.1. Expansion of LP cells

CD45+ LP cells were relatively low at the onset of weaning and increased approximately 5-fold during weaning (P<0.005). There was approximately a 2-fold increase from mid-weaning (day 21) to post-weaning (day 42) (P<0.005, **Table 6.1** and **Fig. 6.1**). The expression of CD45 by LP cells, using alkaline phosphatase staining method, is demonstrated in **Fig. 6.2**. The mean density of α/βTCR+ cells in the LP was low in pre-weaned rats, but their counts increased approximately 7-fold (P<0.005) during the weaning period. There was a further increase after weaning (days 28 to 35, P<0.05), and then it declined by day 42. Note that the initial increase of α/βTCR+ cells preceded the dramatic increase in the total number of CD45+ cells. Expansion of both CD45+ and α/βTCR+ cells in the LP is demonstrated in **Fig. 6.1.** The phenotype of LP cells was also examined in adult rats (e.g., day 70). However, because the number of available rats were restricted, data was pooled from five separate litters-namely, two DAxPVG/c rats and three PVG/cxDA rats. These data are shown in **Table 6.2**. For instance, the mean density counts of α/βTCR+ cells was approximately 4.0 and 11 cells/mm of muscularis at days 21 and 70, respectively (**Tables 6.1** and **6.2**). These values were significantly different (P<0.005). The staining intensity of both CD45+ and α/βTCR+ cells in the LP was lower in animals before day 19 and increased after this age. There was a tendency for α/βTCR+cells to be located basally in the LP around the crypts up to day 19, but they showed a more uniform distribution after this age in both the pericryptal regions and in the LP of villi. T-cells bearing γ/δTCR constituted a minority of LP during and after weaning (Tables **6.1**, **6.2**, and **6.3**). However, both the density and the proportion of these cells increased rapidly at the end of weaning (day 28) and subsequently dropped back to almost weaning levels (**Tables 6.1** and **6.3**). The predominant phenotype of LP cells at day 70, was CD4+ cells (~55%). Some CD4+ cells could be macrophages, because these cells also express the CD4 marker. However, at this stage, approximately 35% of LP cells expressed the CD8 molecule (**Table 6.4**). These results show that LP T-cell population in rats is composed of both CD4 and CD8 cells.

NK cells were detected at day 17 and both their absolute counts and proportion showed an early peak at day 19 ($P<0.05$). At this time, approximately 70% of $CD45^+$ LP cells were positive for the NK cell marker. However, by late weaning, these had fallen to 5% of $CD45^+$ cells (**Tables 6.1** and **6.3**). At day 19, the mean (SE) density counts of NK cells was 5 (0.7) cells/mm of muscularis mucosae, whereas at day 42 (post-weaning), it was 1 (0.3) (**Table 6.1**). The differences were highly significant ($P<0.005$). However, there was not a significant difference between the mean values of NK cells at days 21 and 70 (post-weaning).

A major population of LP cells was negative for the NK cell marker, γ/δTCR, macrophage, or B cells, and thus had a null phenotype. Some of these cells could be plasma cells which were not enumerated. However, it is suspected that most of these null cells were pre-T-cells.

At day 19, 2.6% of LP cells showed detectable staining for Ki-67, along with staining of some cells in intestinal crypts. In spite of detectable Ki-67 staining on intestinal crypts, no staining was evident on days 21, 25, or 28. Thus, these findings indicate a low proliferation of LP cells to luminal antigens during the weaning phase.

Table 6.1. Phenotype of LP cells in rats.

Age (days)	CD45	α/βTCR	γ/δTCR	NK	Macrophage
7	14 (3.7)	0.5 (0.2)	0	ND	ND
14	21 (3.7)	3 (0.9)	0	ND	ND
17	12 (3)	1 (0.3)	0.2 (0.1)	2 (0.6)	1 (0.2)
19	7 (1.6)	5 (0.4)	0.1 (0.06)	5 (0.7)	1 (0.1)
21	46 (4)	4 (1)	0.1 (0.04)	4 (0.6)	0.5 (0.1)
23	49 (2.7)	4 (0.5)	ND	2 (0.5)	ND
25	35 (3.9)	11 (1.9)	1 (0.25)	2* (0.4)	1 (0.2)
28	101** (6.8)	20** (1)	10** (1.5)	ND	ND
35	54 (4.7)	32 (2.4)	3 (0.5)	ND	ND
42	97** (6.4)	9* (1.3)	1** (0.1)	1** (0.3)	0

Data are represented as mean (SE) density counts of cells per mm of muscularis mucosae in litters of 6-12 animals.

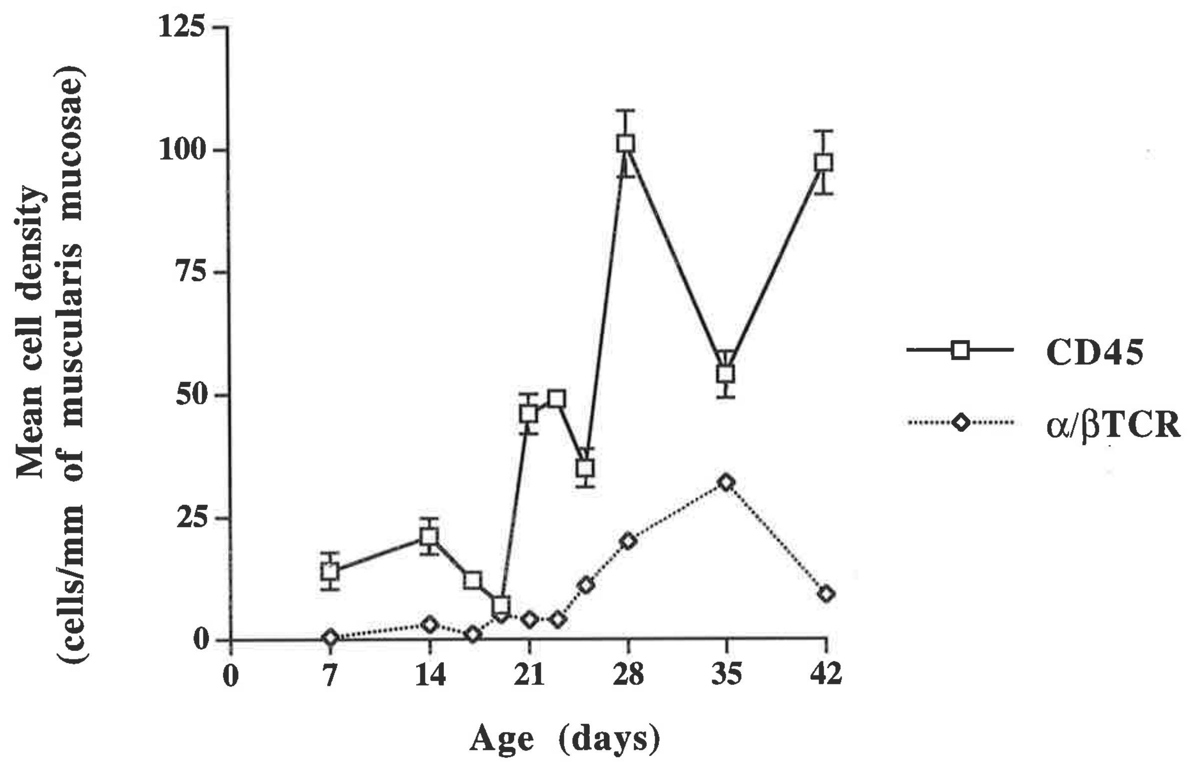
The significances at day 28 refer to comparison with day 14 for what occurred during weaning. However, NK cells were compared between days 17 and 25. The significances at day 42 refer to comparison with day 21 for what occurred after mid-weaning.

* $P<0.05$

** $P<0.005$

The data for CD45 and α/βTCR markers are also graphed in **Fig. 6.1**.

Fig. 6.1. Expansion of CD45+ and α/βTCR+ LP cells from pre-weaning to post-weaning.



Each litter contained 6-12 rats.

Error bars for most data points were tight, so that the symbol obscures the SE.

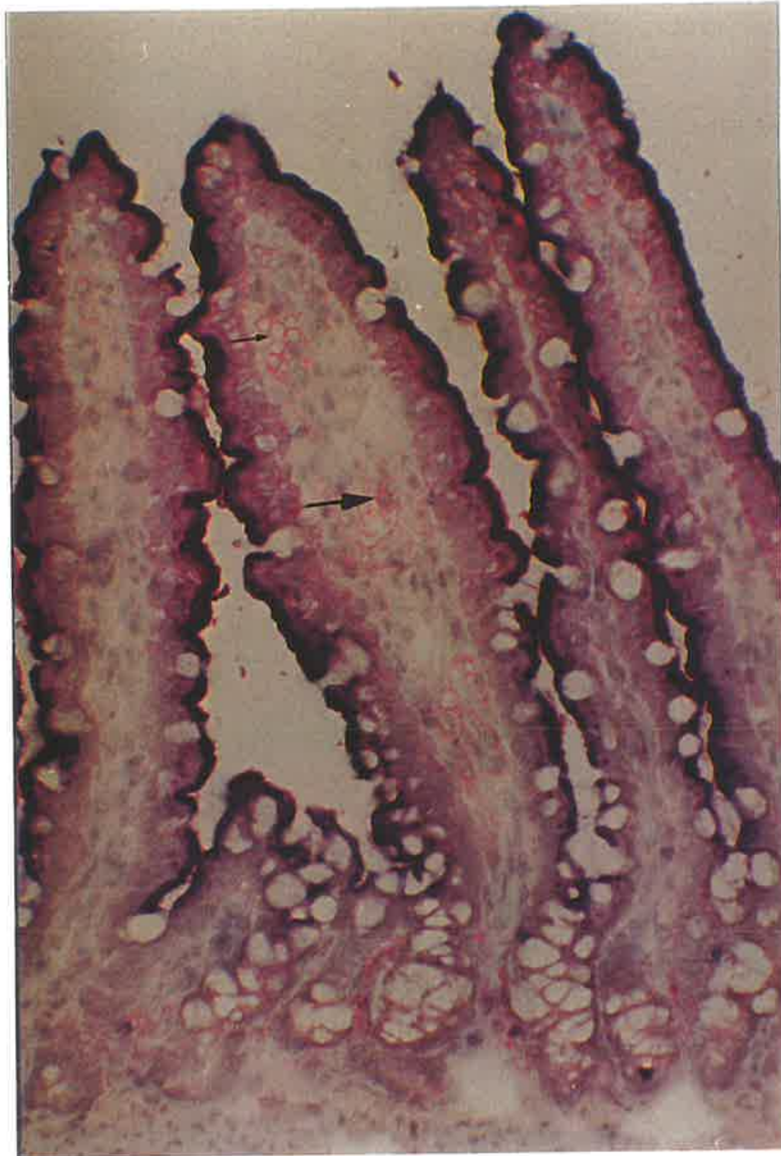


Fig. 6.2 Rat jejunal $CD45^{+}$ LP cells stained by anti-CD45 mAb (OX-1), using the indirect alkaline phosphatase anti-alkaline phosphatase technique.

The larger arrow shows one $CD45^{+}$ cell in the LP compartment with a bright red precipitate at the surface. The smaller arrow displays "small" blood vessels with red surface staining (lined-up) X50.

Table 6.2. Phenotype of LP cells in rats.

Age (days)	CD45	α/βTCR	γ/δTCR	CD4	CD8	NK	CD45RA	Macrophage
21¶	46 (4)	4 (1)	0.1 (0.04)	ND	ND	4 (0.6)	ND	0.5 (0.1)
70§	58 (8.2)	11** (2.2)	2** (0.4)	32 (6.6)	18 (2.5)	3 (0.6)	2 (0.4)	4** (0.8)

Data are represented as mean (SE) density counts of cells per mm of muscularis mucosae in litters of 3-5 animals§.

¶ Pooled data from two separate litters (seven DAxPVG/c and 12 PVG/cxDA rats, n=19).

§ Pooled data from five separate litters (two litters of DAxPVG/c rats, three litters of PVG/cxDA rats, n=19).

** $P<0.005$

Table 6.3. Percentages of LP mononuclear cells from pre-weaning to post-weaning in rats.

Age (days)	α/βTCR	γ/δTCR	NK	Macrophage
7	4 (1.5)	0	ND	ND
14	14 (4.0)	0	ND	ND
17	6 (2.5)	2 (1.0)	13 (5.0)	6 (2.0)
19	62¶ (6.0)	2 (1.0)	67 (10)	11 (1.5)
21	8 (2.0)	0.2 (0.1)	8 (1.5)	1 (0.5)
23	9 (1.0)	ND	5 (1.0)	ND
25	30 (5.5)	2 (1.0)	5 (1.0)	3 (0.5)
28	20 (1.0)	9 (1.5)	ND	ND
35	59 (4.5)	5 (0.9)	ND	ND
42	9 (1.5)	1 (0.1)	2 (0.5)	0

Data indicate the mean (SE) percentage of LP immune cells as a proportion of total $CD45^+$ cells.

Each litter contained 6-12 rats.

¶ Because of relatively low total number of $CD45^+$ cells at day 19, compared with day 21, the percentages of $CD45^+$ cells which expressed other markers at this age appear to be greatly increased.

Table 6.4. Percentages of LP T- cells and other mononuclear cells in rats at post-weaning.

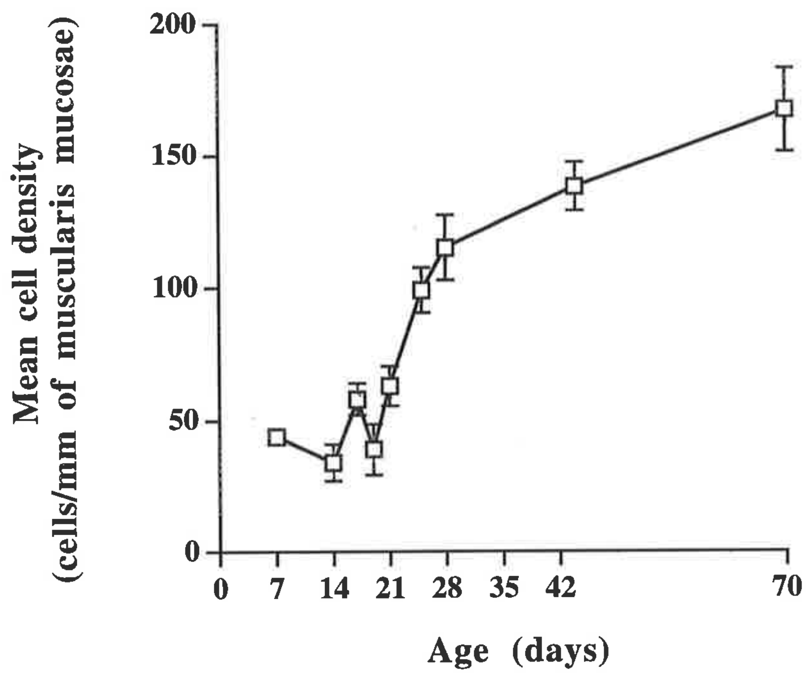
Age (days)	α/βTCR	γ/δTCR	CD4	CD8	NK	CD45RA	Macrophage
70	19 (4.0)	3 (1.0)	55 (11)	32 (4.0)	5 (1.0)	3 (1.0)	6 (1.0)

Data represent the mean (SE) percentage of LP cells as a proportion of $CD45^{+}$ cells. Pooled data from five separate litters (two litters of DAxPVG/c rats, three litters of PVG/cxDA rats, n=19).

6.3.2. Changes in eosinophil (peroxidase positive) counts in the intestinal LP

Eosinophil counts in the LP are given in **Fig. 6.3**. They were found in substantial numbers (44 cells/mm of muscularis) in pre-weaned animals. They began to expand after day 14 and had increased 2-fold by day 28 ($P<0.005$). An early peak was also observed at day 17 and correlates with the presence of eosinophils in the epithelial layer (Chapter 5). After weaning, eosinophil numbers continued to increase reaching normal adult levels at day 70 ($P<0.005$).

Fig. 6.3. Changes in eosinophil counts in the LP from pre-weaning to post-weaning.



Data indicate the mean ± SE density counts of cells per mm of muscularis mucosae in litters from 4 to 11 rats.

6.3.3. Changes in activation status of LP cells with expression of IL-2R

Changes in IL-2R activation of LP cells from pre to post-weaning in rats are given in **Fig. 6.4**. The mean (SE) density counts of IL-2R$^+$ LP cells per mm of muscularis mucosae at mid-weaning was 2.1 (0.3), whereas at post-weaning (day 70) it was 0.66 (0.1). Differences were highly significant ($P<0.005$). There was also a significant difference between IL-2R in the LP during weaning (days 15 to 28) when compared to rats after weaning ($P<0.05$). At day 21, this IL-2R peak represented approximately 4% of CD45$^+$ LP cells (**Table 6.5**). Similar to α/βTCR$^+$ T-cells, IL-2R$^+$ cells were basally located in the deeper regions of mucosa (pericryptal regions) in animals until 19 days of life, but had a more uniform distribution in both the pericryptal and villous LP in older rats.

Fig. 6.4. Changes in IL-2R activation of LP cells in rats from pre-weaning to post-weaning.

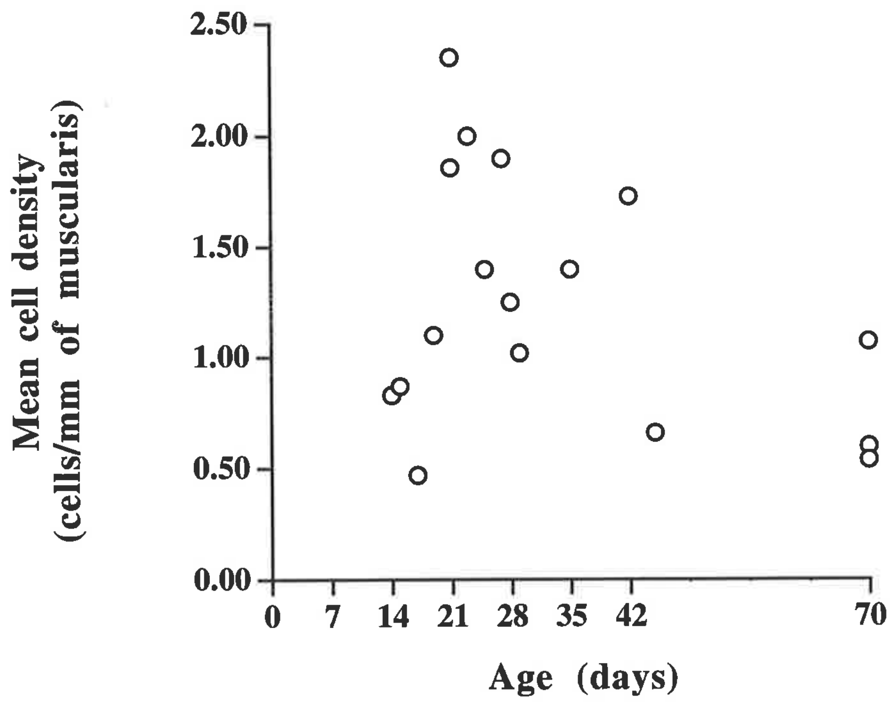


Table 6. 5. Mean of individual percentages of α/β T-cells and $IL\text{-}2R^+$ activated cells in the intestinal LP of rats at mid- and post-weaning.

Age (days)	α/β T-cells	$IL\text{-}2R^+$ cells
21¶	8 (1.8)	3.9 (1.3)
70§	25 (4.0)**	0.7 (0.1)*

Data are given as a proportions (SE) of total $CD45^+$ LP immune cells.

§ Pooled data from 5 separate litters (2 DAxPVG/c rats and 3 PVG/cxDA rats, n=19).

¶ Pooled data from 2 separate litters (7 DAxPVG/c and 12 PVG/cxDA rats, n=19).

* $P<0.05$

** $P<0.005$

The distribution of $IL\text{-}2R^+$ cells throughout the intestinal wall (serosa, muscularis, submucosa, and mucosa) at mid-weaning and post-weaning is shown in **Table 6.6**. $IL\text{-}2R^+$ cells were distributed equally between mucosa (LP) and submucosa on day 21. No positive cells were identified in the deeper layers of muscularis externa and serosa. However, at day 35, essentially all of the $IL\text{-}2R^+$ cells were present in the mucosa.

Table 6.6. IL-2R$^+$ cell density in the gut wall of rats at mid and post-weaning.

Age (days)	Mucosa (LP)	Submucosa	Muscularis externa	Serosa
21	0.75*	0.75	0	0
35	1.40	0.03	0	0

Data are represented as the means in litters from 7 to 11 rats.

6.3.4. Nature of the IL-2R+ cells in the LP

The morphology of activated LP (IL-2R$^+$) cells was diverse; some were large and others were small. Smaller cells were located deeper in the mucosa, and around the crypts, and were probably NK cells or T-cells. A few were large cells with the morphological appearance of macrophages, located largely underneath the epithelium. The cellular origin of IL-2R$^+$ activated cells was also investigated at day 21, by using a combined cocktail of monoclonal antibodies against IL-2R+α/βTCR, IL-2R+macrophage, IL-2R+NK cell marker, or IL-2R+γ/δTCR (**Table 6.7**). LP cells could express both IL-2R and either α/βTCR, γ/δTCR, macrophage related antigen or NK cell marker. Therefore, LP cells were counted after either single or mixed labelling experiments. If the count of the mixed antibodies is equal to the sum of IL-2R$^+$ cells and one of the above mononuclear cells, this would infer mutually exclusive positive cell populations. An incomplete sum would indicate overlap of the two populations, inferrring the existence of dual positive cells. However, the results showed that there was not any overlap between IL-2R$^+$ cells and α/β or γ/δTCR$^+$ cells. For example, single labelling yielded a IL-2R$^+$ mean value of 0.7, and a sum of 2.78 for IL-2R$^+$ cells plus α/β-T-cells. However, single labelling for the macrophage marker yielded a mean value of 0.5, and double labelling for macrophages and IL-2R$^+$ cells gave a value of 1.00. Since the sum of IL-2R$^+$ and single macrophage marker labelled cells is 1.2 cells/mm of muscularis, the difference between this and

the double-labelling experiment implies that there may be a small overlap between these two populations. In addition, single labelling for NK cells yielded a mean value of 3.57, and double-labelling for IL-2R$^+$ and NK cells gave a sum of 2.64 which was lower than for NK cells. This is anomalous and could be explained by steric hindrance. In conclusion, the relatively small frequencies of these labelled populations made comparison of these frequencies difficult. This experiment should be repeated using another litter in which the density of IL-2R$^+$ cells is higher.

Table 6.7. Cellular origin of IL-2R$^+$ activated cells in the intestinal LP at mid-weaning (day 21 of life) by dual labelling.

Marker	Cell density
IL-2R	0.7 (0.11)
α/βTCR$^+$ T-cells	2.12 (0.37)
α/β-T-cell+IL-2R$^+$ cells	2.78 (0.5)
γ/δTCR$^+$ T-cells	0.02 (0.02)
γ/δ-T cells+IL-2R$^+$ cells	0.75 (0.2)
NK cells	3.57 (0.56)
NK cells+IL-2R$^+$ cells	2.62 (0.92)
Macrophage	0.5 (0.1)
Macrophage+IL-2R$^+$ cells	1.00 (0.24)

Data represent the mean (SE) density counts of cells per mm of muscularis mucosae in a litter of 12 rat pups.

6.4. Discussion

The immunohistological study (alkaline phosphatase anti-alkaline phosphatase and immunoperoxidase staining methods) of the mucosa of the rat small intestine was undertaken to investigate the phenotype and activation status of LP cells during weaning. This study has shown that there is upregulation of physiological inflammation in the small intestine of rats during weaning with an increase in IL-2R$^+$ cells in the LP compared to either pre-weaned or adult rats. There was also expansion of CD45$^+$ cells in the LP. Expansion of these cells has not been reported previously. An identifiable, but minority subset of this expanding population consisted of α/βTCR$^+$ cells in the LP, just as there was in the epithelial compartment (Chapter 5). α/β-T-cells were present in low numbers before weaning. Their counts increased during the weaning phase with an early peak around mid-weaning (day 19). After weaning, α/βT-cells continued to increase slightly up to day 35 (**Table 6.1** and **Fig. 6.1**). After this time, α/βTCR$^+$ cells in the LP decreased. The reason for this decline remains obscure, although biological variation from one litter to another one cannot be ruled out.

There was also a noticeable increase in the intensity of immunostaining after day 19 for both α/βTCR and CD45 markers which would be consistent with an increased numbers of both receptors, presumably a consequence of immune stimulation. α/βTCR$^+$ CD4$^+$ LP T-cells respond to specific antigens by secreting 'helper' lymphokines rather than by proliferation (Strober *et al.*, 1994; Chapter 1, **Table 1.6**). In this study, a small percentage of LP cells were positive for the Ki-67 proliferation marker. These results suggest that a proportion of α/βTCR$^+$ CD8$^+$ LP T-cells may proliferate in response to food antigens during weaning since LP cells from non-human primates do not proliferate in response to bacterial antigens. For example, Zeitz and co-workers (1988) showed that T-cells isolated from the LP of monkeys infected with *Chlamydia trachomatis* produce large amounts of IL-2, but do not multiply.

T-cells with the γ/δTCR phenotype were absent in the LP prior to weaning, and they appeared around the time of weaning (day 17), but numbers did not change till the end of weaning (day

28). At this stage, there was a rapid increase of γ/δ LP T-cells. The late expansion of these cells raises the possibility that γ/δ LP T-cells expand due to longer-term exposure to food antigens when compared to α/βTCR+ LP T-cells. Since, some γ/δTCR+ IEL may originate from the LP and these cells respond to the antigenic composition of the diet rather than bacterial antigens (Imaoka *et al.*, 1996; Kawaguchi-Miyashita *et al.*, 1996), it would be reasonable to propose that γ/δ LP T-cells have already responded to food antigens and expanded. However, further investigations should be carried out to find out the specificities or biological function of γ/δ intestinal LP T-cells in gut maturation.

This study showed that LP T-cell population in adult rats (at 10 weeks of life) consisted of both CD4+ and CD8+ cells, with the former being twice as numerous as the latter, just as in peripheral blood (**Table 6.2**). However, Lyscom *et al.* (1982) using immunofluorescence staining, reported that CD4+ cells and CD8+ cells constituted approximately 16% and 30% of LP cells in (PVG/Ola) adult rats at 8-15 weeks of life. A possible reason for obtaining lower percentages of CD4 and CD8 cells in the study of lyscom *et al.* is that immunofluorescence is a less sensitive method (MacDonald *et al.*, 1990). However, the reason for the higher percentage of CD8+ cells, compared to CD4+ cells is difficult to explain. The results of current study are corroborated by other findings in humans in which CD4+ LP cells are 2-fold higher, compared to CD8+ cells (Strober *et al.*, 1994).

NK cells were present in low numbers in the early stage of weaning (day 17), and reached a peak at day 19 in both the LP and epithelial compartments (Chapter 5). Subsequently, NK cells in the LP declined significantly in the second week of weaning. It would appear that these cells are probably NK-T-cells which express α/βTCR for antigen recognition and subsequent expansion.

Eosinophils were present in high numbers in the LP prior to weaning, and counts increased in the early stage of weaning (day 17). Eosinophil numbers increased approximately 2-fold during

the weaning phase, and continued to increase until day 70 (**Fig. 6.3**). These findings are consistent with previous findings in which eosinophils in the mucosa of rat small bowel showed an early peak during the weaning period, and increased 3-fold by day 24 (Cummins *et al.*, 1988). In conclusion, both studies have shown that weaning is associated with expansion of eosinophils.

A new finding from this study was that that most of the expanding cells did not have a clearly defined phenotype other than $CD45^+$. These null cells in the LP have the phenotype of $CD45^+$ $CD3^-$ $CD4^-$ $CD8^-$ and are probably a mixture of mast cells, plasma cells, and pre-T-cells. Other cell populations such as NK cells, macrophages and B-cells were excluded. I am unaware of any phenotypic surface markers to confirm which proportion of these cells are pre-T-cells, because the CD7 pre-T-cell early marker has not heen described in the rat.

A unique characteristic of this study is the comparison of both LP cells and IEL. Expansion of $CD45^+$ cells in both compartments was demonstrated during weaning (Chapter 5). This expansion appears to have been predominantly from migration rather than *in situ* proliferation, because at day 19 (but not at other measured times) only 2.6% of LP cells were positive for Ki-67, despite positive staining of crypt enterocytes. IEL and LP cells from weanling rats have low proliferation after PHA (mitogen) stimulation (Ennulat, 1989). However, we could not rule out some contribution of increased IEL numbers from proliferation that was below the level of detection by Ki-67 staining.

As noted above, expansion of $CD45^+$ LP cells occurred without appreciable proliferation during weaning. This could be due to endogenous anti-proliferative cytokines-namely, TGF-β1 and IL-10 in the LP (McMabe *et al.*, 1993). This notion is supported by McMabe *et al.* who suggested that non-proliferative activation of LP cells in Crohn's disease (in which there is a T-cell activation to *unidentified* luminal antigens) could be due to presence of immunosuppressive cytokines of TGF-β1, IL-4 and IL-10.

The results also showed that IL-2R+ LP cells reached a peak during weaning (days 15-28 of life). Most of these activated cells were resident in the mucosa. This pattern of distribution may indicate that levels of luminal antigens in deeper layers of the gut wall are not adequate to stimulate immune cells. Although antigens may persist in this submucosal site (Fell *et al.*, 1996), antigen presenting cells such as macrophages are rare in this region (Segal and Petras, 1992).

Previous studies (Cummins *et al.*, 1988a-b; Thompson *et al.*, 1996) as well as this study show an association of physiological inflammation in the small intestine with weaning. These authors have already shown that weaning is associated with jejunal MMC degranulation, expansion of eosinophil counts, an increase in weight, cellularity and expression of IL-2R T-cells in the MLN complex, and with an increase in total IEL numbers. This physiological inflammation has now been further characterized by the phenotype and activation of LP cells.

6.5. Conclusion

This study showed that:

(**a**) CD45+ cells were present in the intestinal LP prior to weaning phase and their numbers expanded approximately 7-fold during and after this time;

(**b**) Similar to the epithelial layer, α/βTCR+ cells constituted a small sub-group of the expanding population;

(**c**) NK cells were present in low numbers in early stage of weaning and their counts peaked at around mid-weaning;

(**d**) Eosinophils were detected at high levels prior to weaning and their numbers continued to expand during the weaning; and

(**e**) IL-2R+ activated LP cells peaked during weaning. Collectively, the results presented in this Chapter confirm that physiological inflammation is closely associated with weaning.

Chapter 7

Changes in number, phenotype and activation of lymphocyte-filled villi during weaning

7.1 General Introduction

LFV are unique lymphoid organs in the mucosa of the rat small intestine and are distinguished by the presence of packed lymphocytes occupying the entire LP (**Fig. 7.1**). They constitute approximately 1 per 100 villi along the length of the small bowel. LFV are present in both normal and athymic nude rats. Moreover, they are present in equivalent numbers in both conventional and specific pathogen-free (SPF) rats suggesting that they are not reactive foci of chronic inflammation, although the gut in these animals is still exposed to food and microbial antigens. They are recognisable in suckling rats prior to weaning at 9 days of life (Mayrhofer, unpublished observations). Thus, food antigens are not necessary for LFV development.

As discussed in Chapter 1, very few studies have been done on LFV cells. In several unpublished studies by Mayrhofer, it was found that LFV in young animals contained a population of lymphocytes which may be pre-T-cells. With increasing age in both normal and nude rats, a population of $\alpha/\beta TCR^+$ T-cells appeared. Furthermore, he found that LFV cells in euthymic adult rats exhibited an extensive network of class II MHC^+ dendritic cells. LFV contained few or no B-cells. A minor subpopulation of lymphocytes expressed T-cell markers and were predominantly subepithelial. This subpopulation had the phenotype $\alpha/\beta TCR^+$, $CD2^+$, $CD5^+$, $CD25^+$, $CD43^+$, LFA-1^+, $CD45RC^+$ $CD54^-$, MHC class II and RGL-2^-. Most of this population were $CD4^+$, whereas a smaller number were $CD8^+$. Autoradiographic studies using tritiated thymidine (^{3}H-TdR) indicated that LFV possessed dividing lymphocytes, mostly occuring in TCR^- cells in the base of LFV.

As noted in Chapter 1, Kanamori *et al.* (1996) described recently the presence of novel lymphoid structures ('crypto-patches') in the mucosa of the mouse small bowel. The differentiation markers expressed by the lymphoid cells in crypto-patches (e.g. c-kit, IL-7R) suggest that these structures may have a primary generative function. This concept is

corroborated by the presence of cryto-patches in both severe combined immuno-deficient (SCID) and recombinase activating gene-1 (RAG-1) knockout mice. The crypto-patch occupies the LP in the crypt region of the mucosa and it was estimated that the small intestine contains approximately 1500 of these structures. Less than 2% express either surface immunoglobulin (IgM) or TCR antigen and are, therefore, not conventional B or T lymphocytes (Kanamori, *et al.*, 1996). They are not detectable until the second week of postnatal life-namely, at the commencement of weaning (Gordon and Hermiston, 1994). It seems that LFV are more numerous than crypto-patches (about 1 per 100 villi), and that the packed lymphocytes occupy most of the LP of the villus and extend to the muscularis mucosae (Mayrhofer, unpublished observations).

The above studies with LFV were done in young adult rats, and studies with crypto-patches were done in mice. There has been no previous study of LFV lymphoid cells during the weaning period. Neither have there been any reports of crypto-patches in the rat, although Mayrhofer has suggested that LFV in rats are the corresponding structures for crypto-patches in the mouse. The goals of this research were to study changes in the number, phenotype and activation status of lymphoid cells in LFV, and to compare these cells with those in the epithelium (IEL) and LP compartments. The second aim of this study was to determine whether crypto-patches were present in the rat small intestine during weaning. This information would further our understanding of LFV and crypto-patch association with other GALT.

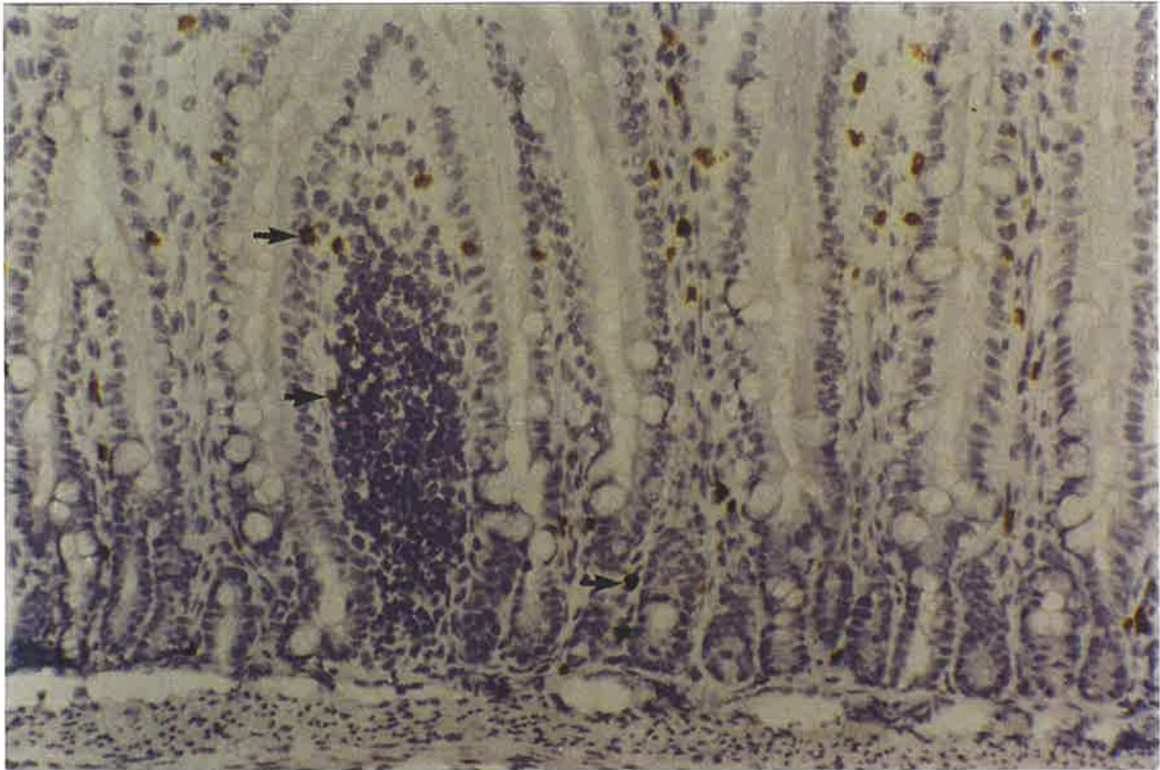


Fig. 7.1 LFV in the rat jejunal mucosa.

The villus is broader than surrounding classical villi, the lamina propria is packed with lymphocytes and the epithelium contains a greater density of intraepithelial lymphocytes. All reaction product in this Figure is associated with endogenous peroxidase activity of eosinophils (arrowed). Immunoperoxidase, section is counterstained with Lillie Mayer's haematoxylin, X50.

7.2 Experimental design

LFV cells were studied in litters of DAxPVG/c or PVG/cxDA rats. These litters were aged from 7 to 70 days of life with day 0 being designated as the day of birth. Litter size varied from 3 to 12 animals and runted animals were excluded. Each rat pup was anaesthetized with ether, the axillary artery cut and allowed to bleed out. The small intestine was isolated and jejunal segments collected at the first proximal one-sixth segment of the small intestine (corresponding to 15 cm from the pylorus in 12 week old animals). Intestinal samples were embedded in OCT, frozen in isopentane cooled by liquid nitrogen, and remained on dry-ice until stored at -70° C. Non-serial, 5 μm frozen sections were cut parallel to the axis of the gut, and the phenotype or activation of LFV cells was determined by counting positive staining cells/total nucleated cells per LFV, after either the immunoperoxidase or immunoalkaline phosphatase technique. As described previously, the frequency of LFV is low (1 out of 100 villi); therefore, to obtain substantial data at different ages is difficult and the data in the result section are compiled from analysis of many LFV. The results were expressed as the mean percentage (SE) of positive cells related to total nucleated lymphoid cells in a LFV/or crypto-patch.

7.3 Results

Two different techniques were used to enumerate phenotypic markers in LFV. There were no significant differences, for instance, between IL-2R$^+$ cells stained by the alkaline phosphatase (49±2.5, n=25) or immunoperoxidase methods (41±3.7, n=27) indicating similar sensitivity ($P>0.05$).

The earliest LFV was observed at day 7 of life as a small villus which contained a core of mononuclear cells in the LP (**Fig. 7.2**). Total number of LFV cells were identified by

haematoxylin staining. LFV cells were present in substantial numbers in pre-weaned rats (67 cells/LFV), and their counts expanded 2-fold from days 14 to 70 ($P<0.005$, **Fig. 7.3**).

The $CD45^+$ status could not be determined accurately at day 7 due to hazy weak staining, but they were enumerated at day 14 due to higher levels of this marker. The proportion of $CD45^+$ cells and other positive cells in LFV was made by counting numbers of stained cells divided by total number of nucleated lymphoid cells. The proportion of $CD45^+$ cells increased nearly 3-fold from days 14 to 42 (**Table 7.1**). For example, as it can be seen in **Fig. 7.4**, there is a large increase in $CD45^+$ cells during weaning (days 15 to 28). Similar to $CD45^+$cells in LFV, $CD43^+$ cells were first stained at day 14. The density of $CD43^+$ cells was not counted.

$\alpha/\beta TCR^+$ T-cells appeared in LFV at day 19. Their proportion increased greater than 3-fold during weaning (days 19 to 25, $P<0.05$, **Table 7.1**). For instance, there was an increase in $\alpha/\beta TCR^+$ cells in LFV from days 21 to 25 (**Fig. 7.5**). There was a further increase from days 25 to 35 ($P<0.005$). $\gamma/\delta TCR^+$ T-cells were first detected at day 70, and constituted 0.1% of LFV cells. NK cells were first seen at day 17 and their counts reached an early peak (~50%) at day 19 ($P<0.05$). After that, their proportion dropped and remained almost constant at 30%-40% from mid-weaning to post-weaning (**Table 7.1**). This is further demonstrated in **Fig. 7.6**. The proportion of NK cells was higher than α/β TCR^+ cells. This is also illustrated in **Fig. 7.7**. The phenotype of LFV cells at day 70 consisted of 57% $CD45^+$, 29% $\alpha/\beta TCR^+$, 36% NK cells and 33% IL-2R^+ cells.

B-cells ($CD45RA^+$ cells) were first seen at day 25 and were infrequent. Macrophages were first seen infrequently at day 17 and were located in the base of LFV (**Fig. 7.8**). Their numbers at most ages were infrequent or absent; for instance at days 21 and 28, macrophages were absent from LFV. Following day 21, the staining intensity of cells expressing IL-2R, CD45 and α/βTCR antigens increased. There was also a demonstrated increase in the

proportion of $\alpha/\beta TCR^+$ cells after day 23 (**Table 7.1**). Eosinophils were also observed in LFV especially from days 17 to 23.

IL-2R bearing cells were present in suckling rats (day 7, **Fig. 7.2**) and their percentage peaked at day 19-i.e., days 17 *vs.* 19 ($P<0.05$). The mean proportion of $IL\text{-}2R^+$ cells at day 19 was 55 (± 8.2), whereas at day 70 it was 33 (±6.0). The values were significantly different ($P<0.05$). Despite an increase in both total count of LFV cells and $CD45^+$ cells, very few $Ki\text{-}67^+$ cells were found at any age. For example, at days 14, only one $Ki\text{-}67^+$ cell was found in 17 LFV (mean number of 66 cells/LFV). At day 70, no Ki-67 cells were found in 41 LFV (mean number of 139 cells/LFV). These data do not exclude the possibility of a small amount of proliferation.

This study also reports the presence of a novel lymphoid aggregate, resembling the crypto-patch, first observed in the mucosa of the mouse small intestine by Kanamori *et al*. (1996). It was first detected at day 28 of life in rats, but may have been present at an earlier time. This needs further investigation. The morphological features that distinguish rat crypto-patch from classical LFV are the basal extension of the LP at the level of the intestinal crypts. It is also dissimilar to LFV by being lined by shallow epithelium, and being wider and shorter than classical LFV (**Fig. 7.9**). However, preliminary work revealed that the phenotype and activation of cryto-patch cells in rats were almost identical to LFV cells. For instance, at day 70, a higher proportion of crypto-patch cells was positive for CD4, rather than CD8, and most crypto-patch cells expressed IL-2R activation marker (e.g. at day 45, **Table 7.2**).

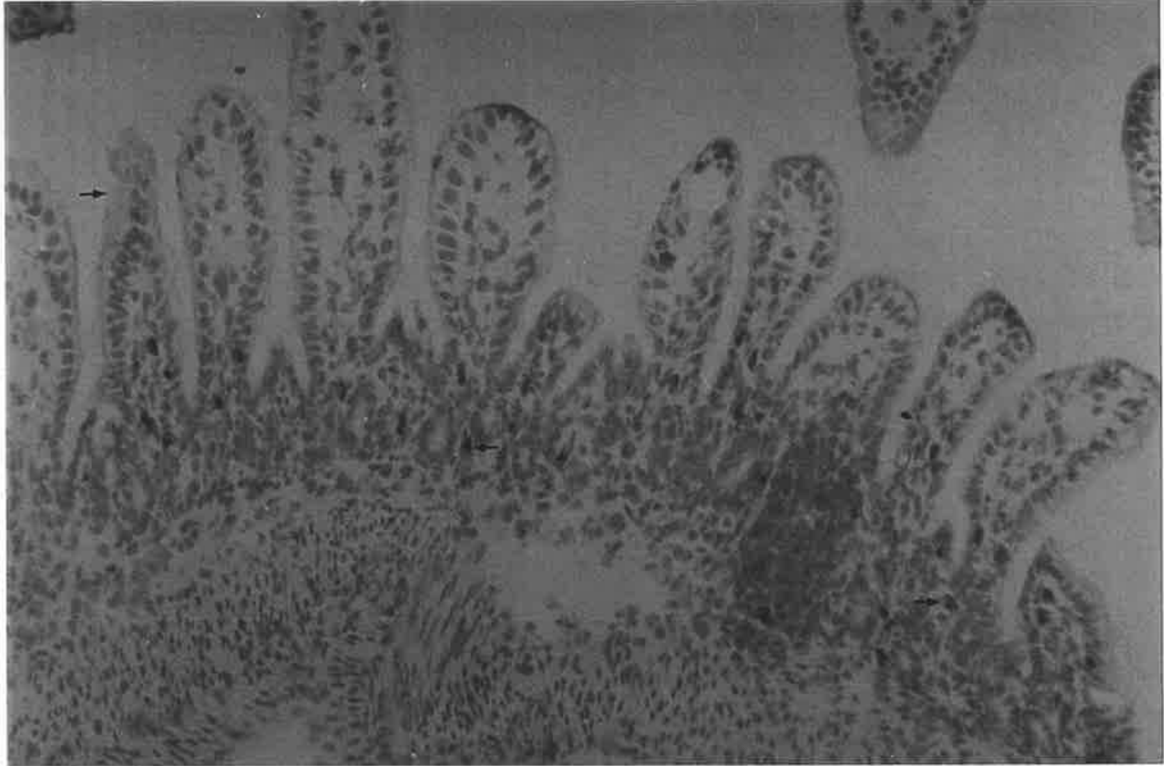
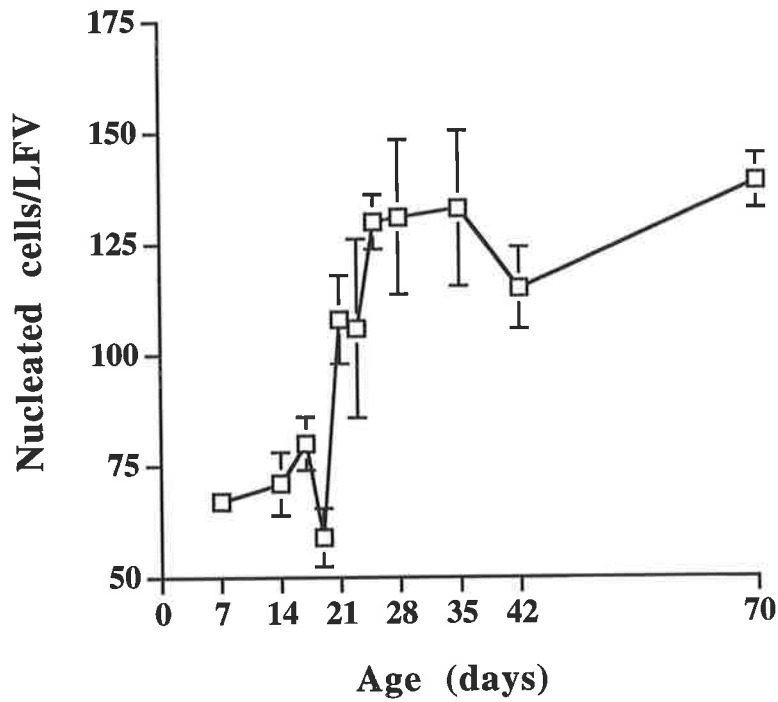


Fig. 7.2 Expression of IL-2R on LFV cells in pre-weaned rats (day 7 of age). Immunoperoxidase staining of LFV was carried out on non-serial frozen section with a monoclonal mouse anti-rat IL-2R (NDS-61). Densely stained cells (eosinophils) are also located in the LP, around the basal portion of the villi or in the pericryptal region (arrowed). Note also the tall and slender villi and shallow crypts in pre-weaned animals (immature small intestine, arrowed). X50.

Fig. 7.3. Changes in the number of nucleated cells in rat LFV from pre-to post-weaning.



Data represent the mean ± SE of total number of nuclei in 41 LFV in five μm frozen sections. Haematoxylin was used to identify nucleated cells.

Fig. 7.4 LFV stained with anti-CD45 mAb (OX-1).

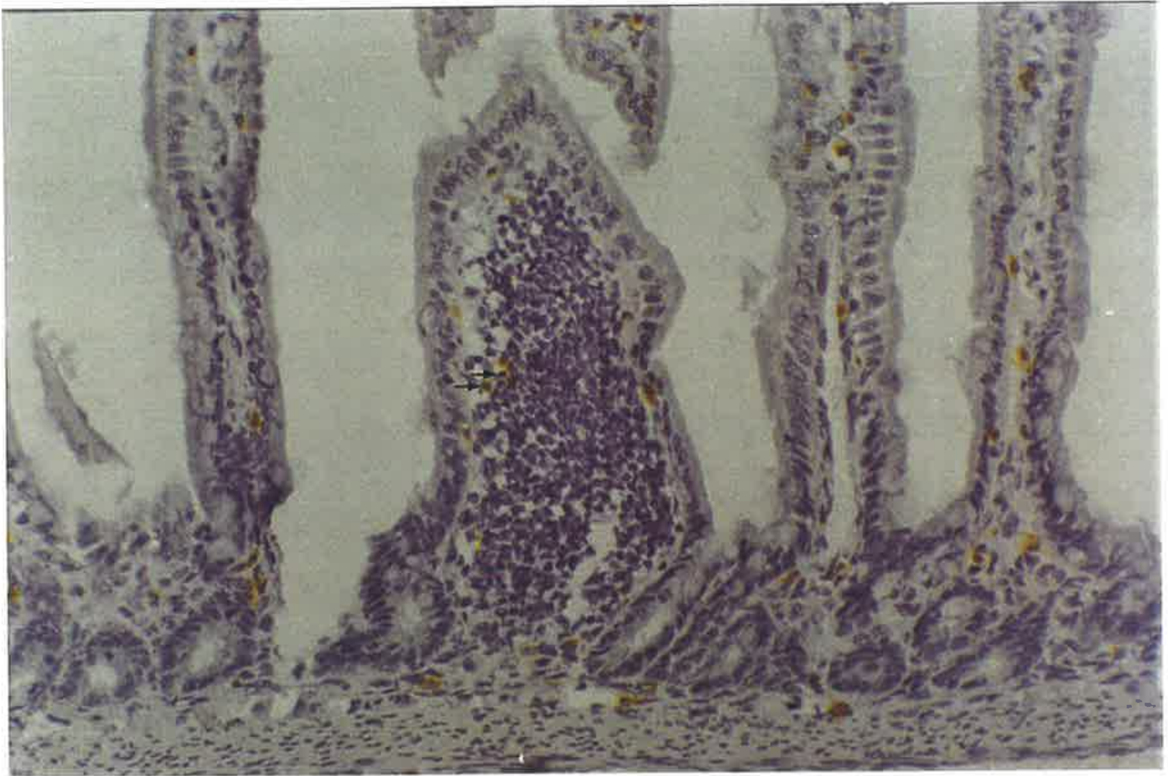
Upper panel: The smaller arrows show expression of CD45 on LFV cells at the start of weaning (day 15), while the larger arrow displays one endogenous peroxidase-positive cell amongst LFV cells. X50.

Lower panel: This panel demonstrates a marked increase in both the number and staining intensity at the end of weaning. Darkly stained cell is an endogenous peroxidase positive cell (eosinophil, arrowed). Immunoperoxidase, X50.

Fig. 7.5 Rat LFV stained with anti-α/βTCR mAb (R73).

Upper panel: This panel shows few α/βTCR^{+} cells at mid-weaning (arrowed).

Lower panel: This panel shows an increase in the number of α/βTCR^{+} cells towards the latter half of weaning (day 25). Note the subepithelial position of several α/βTCR^{+} cells at this stage (arrowed). α/β T-cells in LFV increased during weaning. Immunoperoxidase, X50.



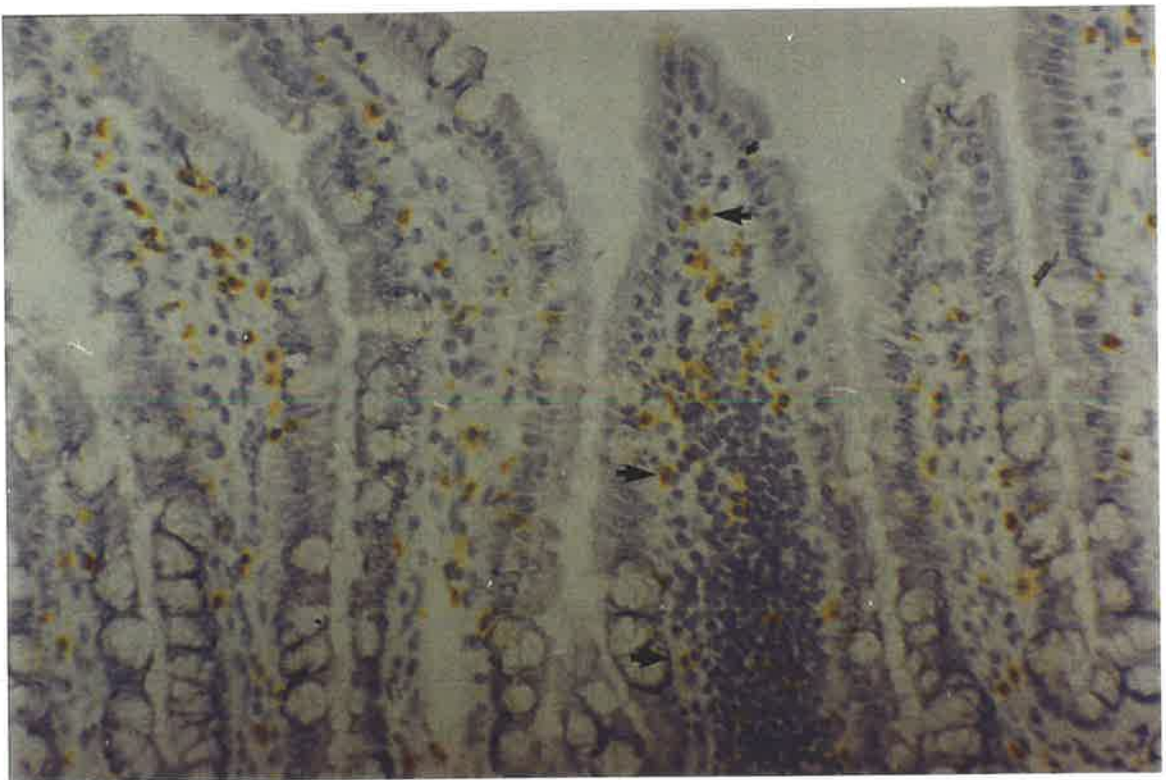
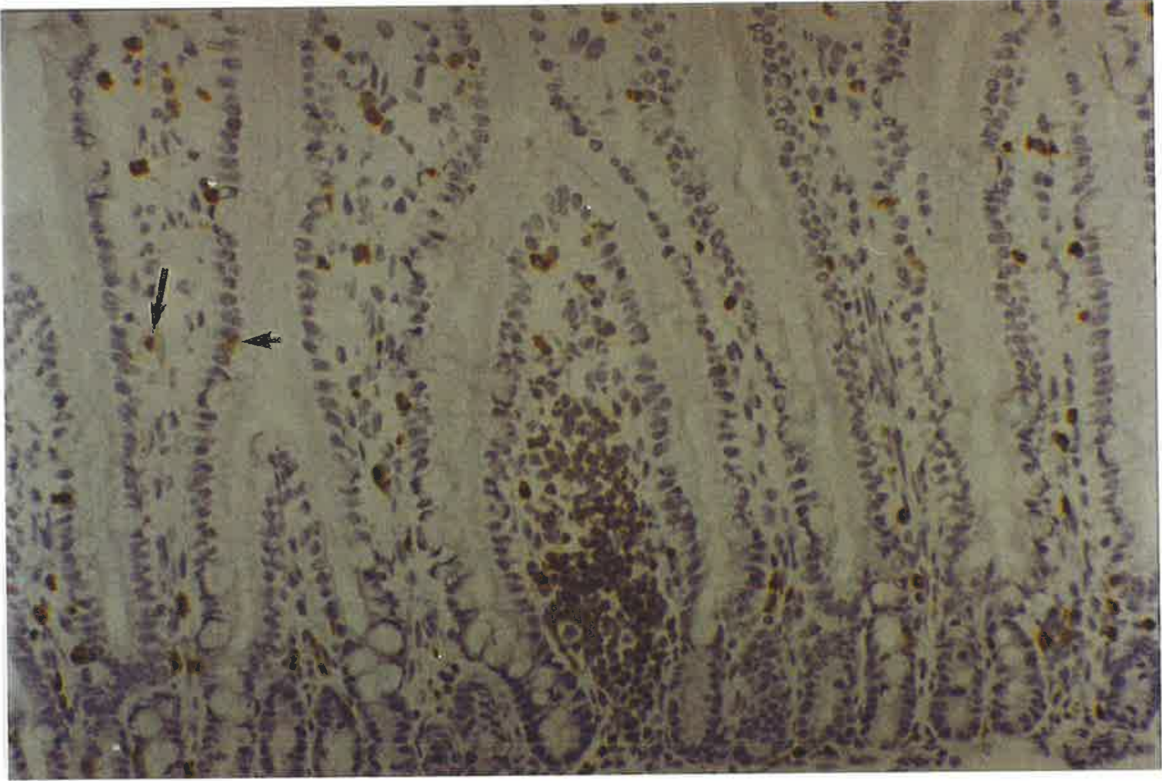


Fig. 7.6 Rat LFV stained for NK cells.

Upper panel: This panel shows expression of the NK marker on LFV cells at mid-weaning. X50.

Lower panel: This panel demonstrates that no change in NK cells is obvious at post-weaning (day 70). The smaller arrows display NK cells both in the villus epithelium of LFV as well as the epithelial layer of classical villi, while the larger arrows demonstrate NK cells in the LP. Immunoperoxidase, X50.



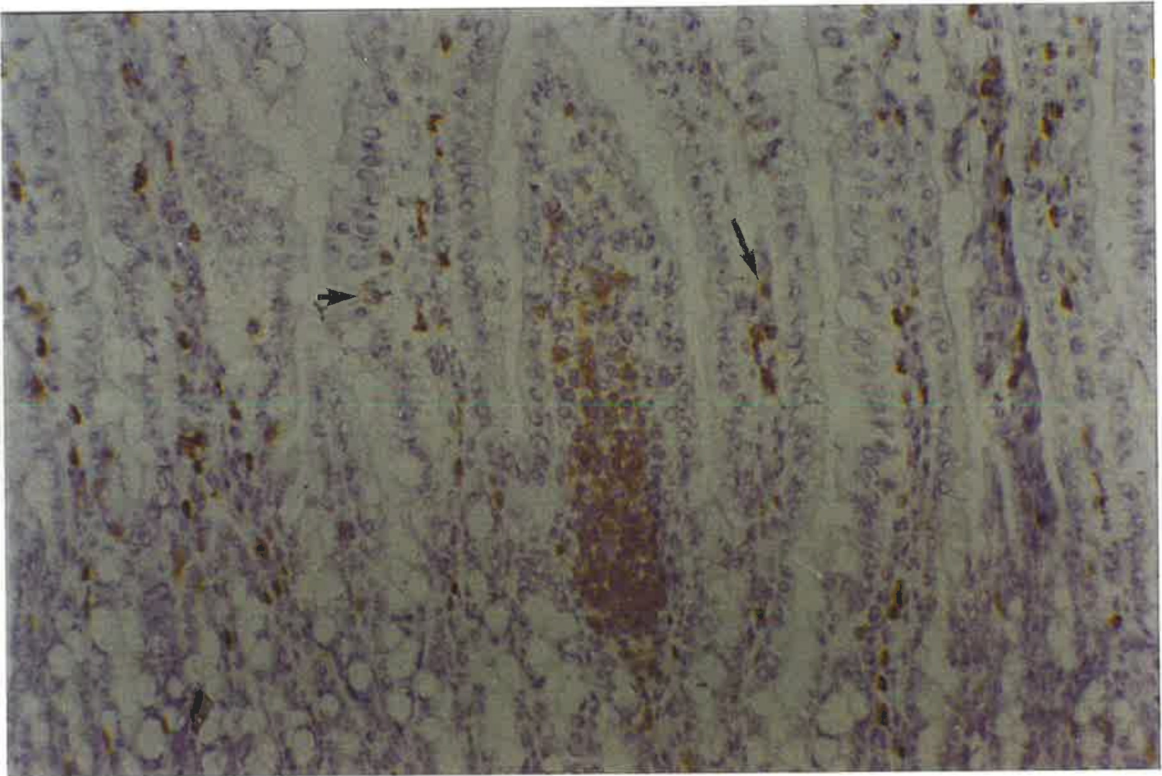
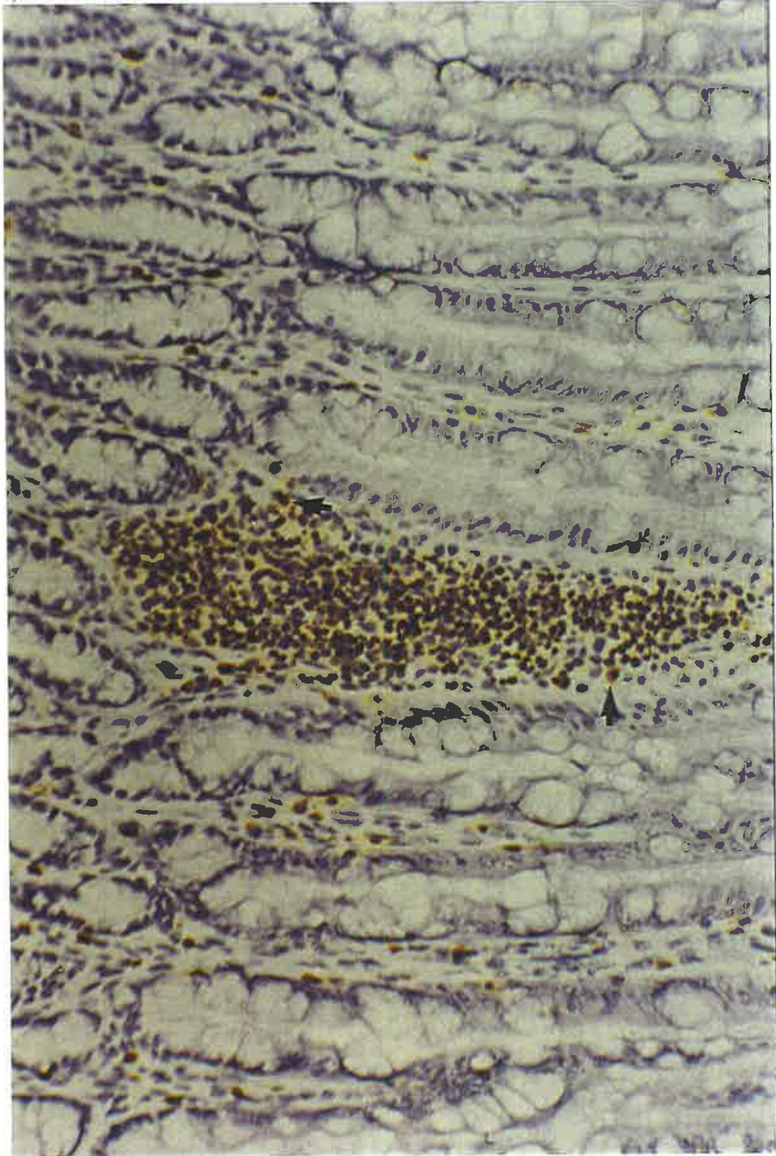
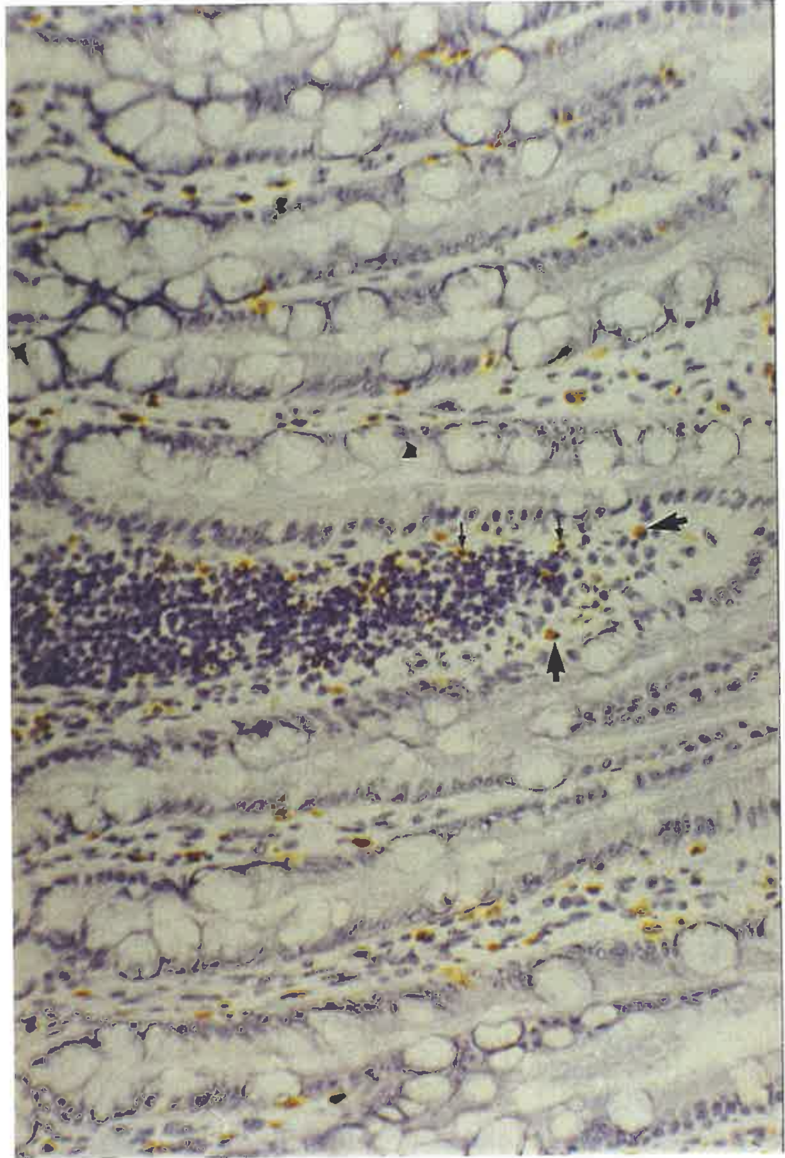


Fig. 7.7 Rat LFV stained for α/β T-cells and NK cells.

Upper panel: The smaller arrows show expression of $\alpha/\beta TCR^+$ cells on LFV cells at the end of weaning. X50.

Lower panel: This panel shows expression of the NK marker on these cells at the same age. Note a major population of LFV cells is positive for the NK cell marker, whereas a small population of these cells expresses α/βTCR. The larger arrows demonstrate peroxidase-positive cells (eosinophils) in both figures. Immunoperoxidase, X50.





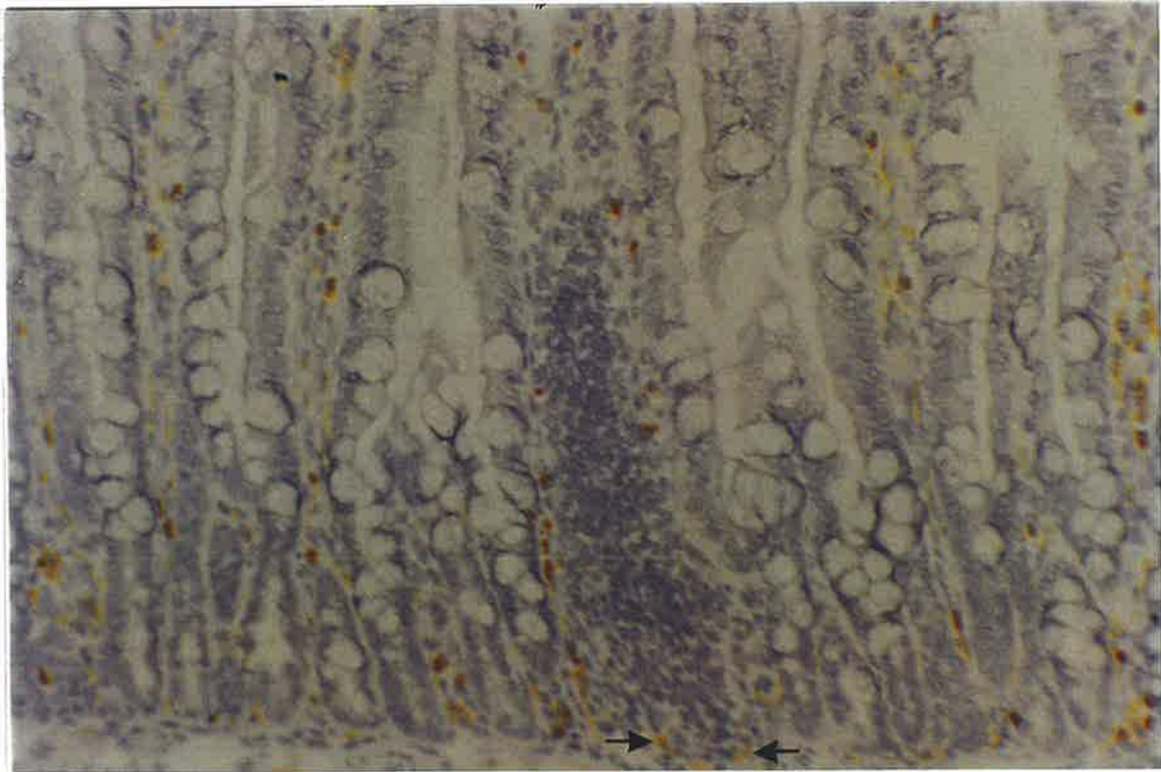


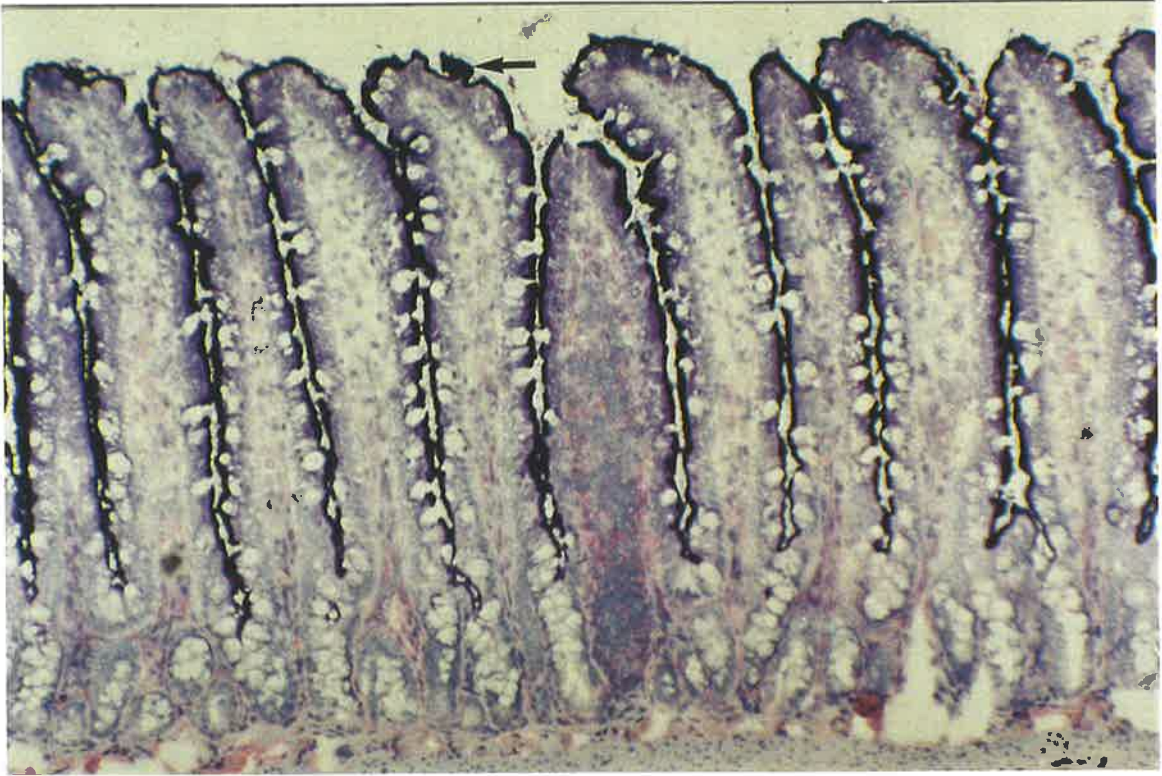
Fig. 7.8 LFV stained with mAb ED2 (anti-macrophages).

Macrophages are located at the base of this lymphoid organ from a 25 day old rat (arrowed). Immunoperoxidase, X50.

Fig. 7.9 Rat LFV stained by anti-CD4 mAb (W3/25) and presence of an aggregate of mononuclear cells (probably crypto-patch) in the rat jejunum at 10 weeks of life.

Upper panel: This panel shows one LFV. Section was stained with this antibody followed by incubation with a rabbit anti-mouse Ig reagent, then incubated with soluble calf intestinal alkaline phosphatase-mouse anti-calf intestinal alkaline phosphatase complex (APAAP complex). The purple staining of brush border microvilli (arrowed) is the outcome of using two different substrate solutions containing fast-blue and fast-red chromogens and reaction with endogenous alkaline phosphatase. X25.

Lower panel: This panel shows an aggregate of mononuclear cells (analogus to the mouse crypto-patch found in mouse small intestine). This structure is shorter and wider than adjacent villi, it extends into the basal LP at the level of the intestinal crypts (arrowed), and contains shallow epithelium. Sections were counterstained with Mayer's haematoxylin, X50.



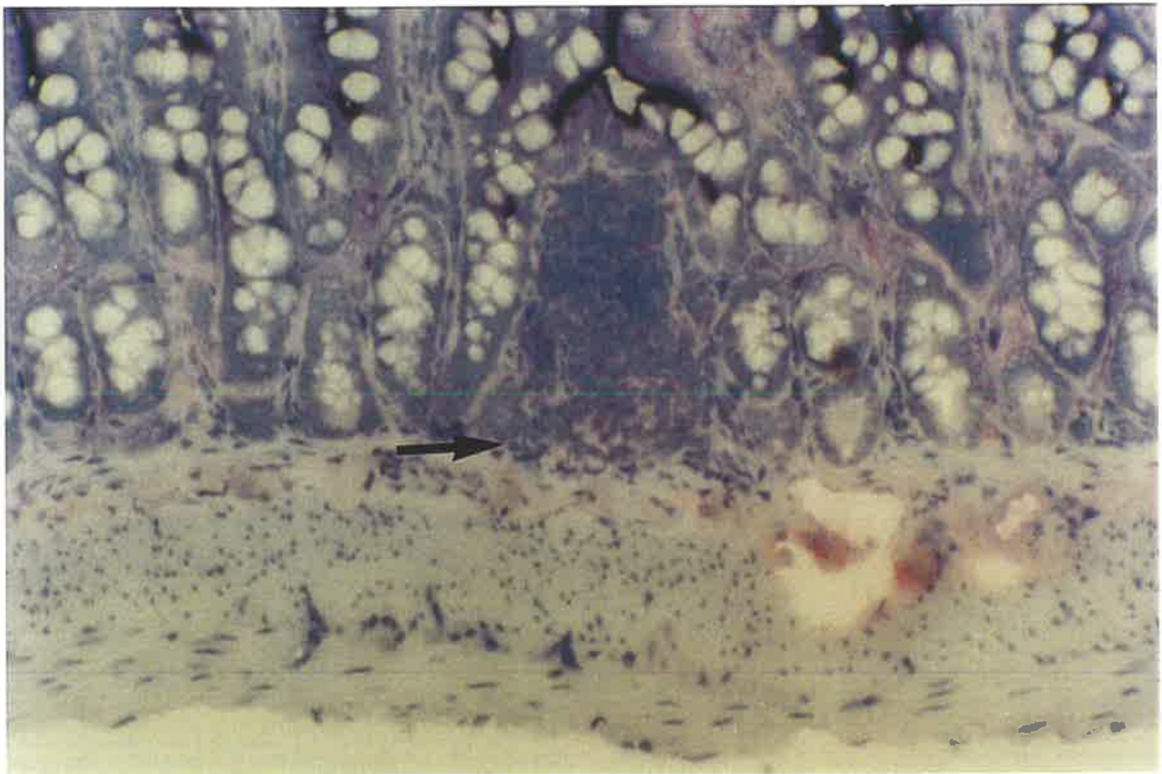


Table 7.1 Phenotype and activation status of LFV cells in rats from days 7 to 70 of life.

Age (days)	CD45	α/βTCR§	NK cells	CD45RA(B cell)	IL-2R
7	ND¥	ND	ND	ND	39∂
14, 15	27±22	1±0.4	30±7	ND	40±3.4
17	48±41.4®	0	11±2.8	ND	20±9 *
19	51±2.3	4±4.3	49±7.8*	0	55±8.2
21	78±5.3 *	5±3.5	33±9.5	ND	40±17.1
23	70	6	ND	ND	56
25	56±8	13±1*	50±4.1	2	35±5.8
28, 29	62±6.5	9±1.8	31±9.4	2±2.3	47±4.9
35	65±17.8	37±0.7 *	38±5.6	1	49±4.7
42, 44, 45	73±6.5	10±3.3	28	ND	50±4.2
70	57±16	29±7.5	36±10	1.2±0.7	33±6 *

Data represent the mean ± SE percentage of nucleated cells of the LP in a 5 μm tissue section.

¥ No LFV was detected.

® The large SE at day 17 may be explained by differences in maturity

The counts for γ/δTCR+ cells, CD4+ cells, CD8α+ cells, and macrophages at day 70 were 0.1 ± 0.1, 37 ± 8.2, 28 ± 16, and 1.3 ± 1, respectively.

* P<0.05

The significance refers to comparison with day 17 for NK cells, and day 19 for CD45, α/βTCR and IL-2R. The reasons, for choosing these days have been discussed previously.

Table 7.2 Change in number and phenotype of rat small intestinal crypto-patch cells¶ from weaning to post-weaning.

Age (days)	CD45	CD4	CD8	γ/δTCR	CD45RA (B-cell)	Macrophage	IL-2R	Nucleated cells/CP†
28	100 (0.3)‡	ND§	ND	ND	ND	ND	ND	60 (12.7)
45	ND	ND	ND	ND	ND	ND	99	111
70	47	24	1	1(1)	0	0	ND	81 (24.3)

¶ 1-6 crypto-patch (es) were examined.

‡ Results indicate the mean percentage (SE) of nucleated cells expressing the indicated markers.

§ Not detected

† Data represent the mean (SE) number of nucleated cells/crypto-patch (CP).

7.4 Discussion

This histological and immunohistological study of the mucosa of the rat small bowel was undertaken to search changes in the numbers, phenotype, and activation of LFV and crypto-patch cells during the weaning phase. As described in Chapter 1, structures of LFV are different from Peyer's patches, because most lymphocytes in LFV express neither surface immunoglobulin nor the T-cell receptor. Since LFV contain very few or no B-cells, few T-cells and are present in athymic (nude) mice and rats, it has been suggested that they may be specialized sites that support primary T-cell differentiation.

The results showed that the weaning period (days 15-28 of life) is preceded by the development of recognisable LFV in suckling rats at day 7. This shows that development of LFV is essentially independent of food antigens-stimulation which coincide with weaning. At day 7, LFV contained approximately 70 cells and these expanded approximately 2-fold by day 70 (**Fig. 7.3**).

LFV and crypto-patches are rare (approximately 1 LFV per 100 villi, although crypto-patches are rarer) and substantial efforts are going to be needed to obtain significant data at various ages during the weaning and post-weaning periods. Therefore, the results are only meant to provide the first data on these structures in rats; findings which may stimulate further investigations into this very fascinating area.

The major findings in this study were as follows. $CD45^+$ cells in LFV were first seen at the start of weaning and their counts expanded approximately three-fold from pre-to post-weaning (day 42) (**Table. 7.1**). The largest identifiable subset of the expanding population of nucleated lymphoid cells, leaving aside $CD45^+$ cells, were cells expressing the NK cell marker (NKR-P1), which comprised approximately 10-40% of cells (**Table 7.1**). NK cells were detected at an early stage of weaning and their counts peaked around mid-weaning (day

19). Interestingly, NK cell counts in three compartments-namely, LP, epithelium and LFV, exhibited an early peak at day 19. Moreover, NK expression in LFV preceded the appearance cells bearing α/βTCR. This is consistent with an early peak of NK expression in the epithelial and LP compartments. It is difficult to know what the relationship is between the various cells expressing NK marker in the three compartments of gut mucosa. A small proportion of $\alpha/\beta TCR^+$ cells appeared at day 19. Their proportion increased during and after weaning (**Table 7.1**). Similar to the epithelial and LP compartments (Chapters 5 and 6), a proportion of the expanding population of $CD45^+$ cells did not display any other clearly defined phenotype and were presumably precursors of T cells.

This study showed that IL- $2R^+$ cells were present in a relatively high proportion of nucleated lymphoid cells (~40%) in pre-weaned rats at day 7, and that they peaked around mid-weaning. After that period, the proportion of IL-$2R^+$ cells remained constant approximately at 40-50% of total nucleated lymphoid cells. However, the *intensity* of staining for IL-2R increased after day 19 of life. The presence of a high proportion of IL-$2R^+$ cells during the pre-weaning period and its rise at around mid-weaning (day 19) suggest that this compartment is dependent on both endogenous (self) and luminal antigens. Interestingly, both IL-$2R^+$ cells and NK cells showed a concomitant early peak at day 19. These findings suggest that most IL-$2R^+$ cells were NK cells. However, dual labelling would be required to confirm this.

Despite an increase in the number of LFV cells and $CD45^+$ cells, no detectable Ki-67 staining was seen, except at day 14. However, preliminary work by Brooks and Mayrhofer (unpublished data) suggests that when ^{3}H-thymidine is injected intravenously into DA rats, some DNA synthesis is found in the LFV of animals killed within one hour. This suggests that they are labelled in *situ*, although it is impossible to determine whether these cells

divided before or after entry into LFV. An alternative explanation for the increasing cell numbers in LFV with age is by migration from other sites.

Histological and immunohistological examination of human intestinal biopsy specimens collected from seven normal individuals has revealed that LFV are also present in humans (Moghaddami, Mayrhofer and Cummins, unpublished observations). Similar to LFV cells in rats, they found occasional CD19+ B-cells. Conversely, the main population of these cells was T-cells, with a predominance of CD4+ memory cells (CD54RO). In addition, LFV cells in adult humans did not express IL-2R. Thus, LFV in humans are structurally identical to those in rats.

7.5 Conclusion

The results presented in this Chapter showed that:

(**a**) α/βTCR+ T-cells appeared in LFV in the third week of life, and their proportion increased greater than 9-fold by day 35 of life;

(**b**) An unexpected finding in LFV was an early increase of NK cells (at day 19) that remained greater than the proportion of α/βTCR+ T-cells;

(**c**) LFV cells also contained a much higher proportion of IL-2R+ bearing cells in suckling rats compared to cells in the epithelial and LP compartments (Chapters 5 and 6), and that their proportion remained almost constant after mid-weaning; and

(**d**) LFV are distinct lymphoid organs of the GALT, phenotypically different from both LP and IEL cells.

A high proportion of IL-2R expression by LFV cells, an early T-cell activation marker, and low expression of α/βTCR suggest that LFV may be primary sites for T-cell differentiation and maturation in the gut. Furthermore, they could be also involved in the deletion of antigen-reactive α/β T-cells and could therefore be involved in oral tolerance.

Chapter 8

Changes in activation status of inductive sites of gut-associated lymphoid tissues: mesenteric lymph nodes and Peyer's patches during weaning

8.1. General introduction

Previous studies have indicated that weaning in the rat is associated with increased immunological activity in the GALT. Cummins *et al.* (1988a-b) have already observed an increase in the weight and cell content in the MLNs, degranulation of jejunal MMC, and an exponential increase in jejunal IEL that co-incides with weaning (Babicky *et al.*, 1973; Thompson *et al.*, 1996). Since similar changes are also present during mucosal graft-*vs.*-host reactions, investigators have suggested that changes during weaning are also associated with activation of T-cells. The MLN weight increases seven-fold from days 12 to 22 of life in rats before stabilizing (Cummins *et al.*, 1988b). Expression of IL-2R by MLN $CD5^{+}$ and $CD4^{+}$ T-cells peaks at day 22 of life (Cummins *et al.*, 1990). Several studies indicate that changes in the weight and cellularity of the MLN parallel changes in activity elsewhere in the GALT (Seeling and Billingham, 1981; Thompson *et al.*, 1996). This activity in the MLN and the gut mucosa occurs concurrently with increases in the villus area, crypt length, crypt proliferation, and other changes that are characteristics of this period of development (Herbst and Sunshine, 1969; Cummins *et al.*, 1988a-b; Thompson *et al.*, 1996).

Peyer's patches are secondary lymphoid organs with clearly defined T-and B-cell-dependent regions (**Fig. 8.1**). They reside in the submucosal layer and are separated from the intestinal lumen by a single layer of specialized cuboidal epithelial cells, the follicle-associated epithelium which are derived from adjacent crypts. This epithelial layer consists of many lymphoid cells of all types, together with a unique population of specialised epithelial cells (M cells) whose function seems to be the uptake and delivery of antigen to T-and B-cells. This close interaction between epithelial and immune cells found in the Peyer's patches is characteristic of the mucosal immune system and is also seen in corresponding tissues such as the tonsil and appendix (Mowat and Viney, 1997).

It is generally believed that Peyer's patches are the inductive site of the mucosal immune system, because they are the main site for priming of T and B cells in the intestine, and

luminal antigens gain access to the GALT via M cells in the Peyer's patch epithelium (Trier, 1991; Gebert *et al.*, 1996). Unlike normal enterocytes, M cells have a decreased number of small apical microvilli. The microvilli actually are replaced by microfolds, to which microorganisms and other luminal antigens can bind preferentially. M-cell-dependent uptake of antigen into Peyer's patch has been demonstrated for several microorganisms and particulate antigens and is accepted to account for the specialised ability of some invasive bacteria (e.g., Salmonella, Yersinia, Shigella and other invasive species) to grow in the Peyer's patch (Keren, 1992; Kato and Owen, 1994). However, the overall importance of M cells for the uptake of the full range of intestinal antigens is unclear. M cells probably do not express class II MHC antigens and cannot process antigen and are believed to deliver intact antigen to APC which are abundant both in the follicle-associated epithelium and in the underlying areas of the Peyer's patches (Keren, 1992; Kato and Owen, 1994). Many dendritic-like cells are found in Peyer's patches (Wilders, 1983; Ruedl *et al.*, 1996; Kelsall and Strober, 1996) and these have been shown to prime T cells after exposure to antigen both *in vitro* and *in vivo* (Kelsall and Strober, 1996; Richman *et al.*, 1981; Liu and MacPherson, 1995).

T cells primed in this way migrate to the LP and probably other mucosal surfaces via the MLNs, thoracic duct and bloodstream. Although this approach is assumed to result in conventional T-cell priming to intestinal antigen, dendritic cells in Peyer's patches may vary from their peripheral counterparts phenotypically and functionally (Ruedl *et al* ., 1996; Kelsall and Strober, 1996; Richman, 1981; Liu and MacPherson, 1995; Spalding *et al.*, 1984), suggesting that presentation by these cells could have unusual immunological consequences *in vivo*. For example, they may induce tolerance to food proteins and commensal bacteria.

Most information concerning the activation status of MLN T-cells, has been obtained by flow cytometry. The present study was undertaken to determine the activation status of MLN T-cells *in situ*. The second aim of this study was to characterise the phenotype and activation status of the Peyer's patch T-cells during the weaning phase.

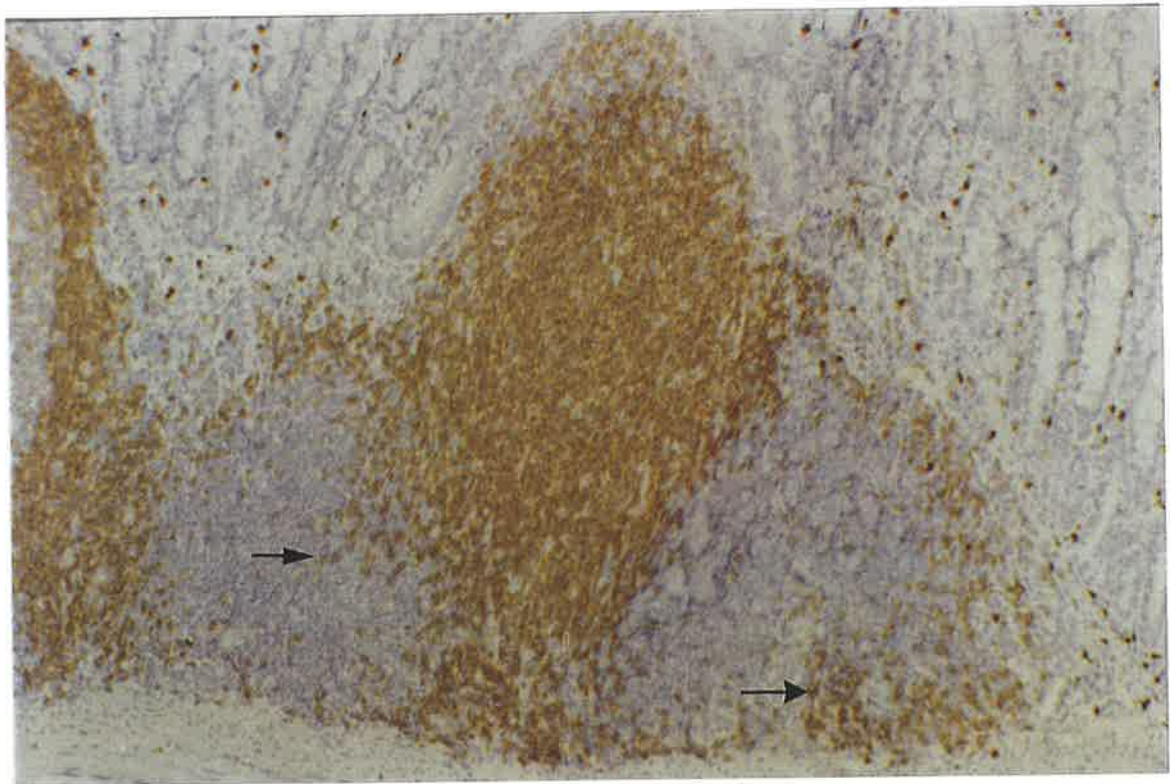


Fig. 8.1. Immunohistochemical localization of B-cells (CD45RA) in rat Peyer's patch at 10 weeks of age.

Most B-cells are in the follicle (B-cell zones), and the remaining are scattered in the interfollicular zone (T-cell areas, arrowed). Immunoperoxidase, X25.

8.2. Experimental protocol

Litters of DAxPVG/c or PVG/cxDA rats were used between 14 and 70 days of age with day 0 being designated as the day of birth. Litter size varied from 7 to 12 animals and runted animals were excluded. Collection of MLN has already been described in Chapter 2. For Peyer's patches, jejunal specimens containing lymphoid structures were identified on the *anti-mesenteric* wall of the small bowel. Tissues were snap frozen in isopentane, cooled by liquid nitrogen, and stored at -70° C in a sealed container with ice to impede drying of tissues and to preserve antigenicity. Finally, MLN sections or Peyer's patches were sectioned at 5 µm and mounted onto slides pre-coated with HistoGrip for immunostaining either by the alkaline phosphatase or immunoperoxidase staining method.

8.3. Results

8. 3. 1. Changes in activation status of MLN

The alkaline phosphatase staining method was used for the detection of IL-2R^{+} cells in the T-dependent region (paracortex) of the MLN complex. However, high background staining with some of the cells in the T-cell areas was observed (**Fig. 8.2**). Therefore, this method was switched to a peroxidase staining method to count IL-2R^{+} cells and α/βTCR^{+} cells in the T-dependent areas. Immunoperoxidase staining of the MLN complex showed that some of the IL-2R expressing cells were large and showed the morphology of a macrophage, but most were presumably T-cells in the T-cell interfollicular zone. Since anti-CD3 (1F4, G4.18) antibodies for the rat do not perform well for immunostaining, total T-cells in the MLN complex were assessed as α/β T- cells constitute 95% of the total MLN T-cells even at day 21 (Thompson *et al.*, 1996). The results showed that the mean proportion of IL-2R bearing cells in the T-region of the MLN complex (paracortex) at day 21 was approximately 7%, compared to 2% at day 42 (**Table 8.1**). Most positive cells expressed IL-2R at high intensity. This is demonstrated in **Fig. 8.3.** A comparison was also made between the activation status of MLN and other GALT at mid and post-weaning (**Table 8.2**). The results demonstrated that the

GALT were in a higher state of immunological activity at mid-weaning, when compared to either pre-weaned or post-weaned animlas.

MLN T-cells in the T-dependent regions displayed moderate (++) proliferation at day 21, and modest (+) proliferation at day 42, as determined by Ki-67 staining. However, B-cells in the germinal centres of secondary follicles (cortex area) exhibited modest proliferation (+) at day 21 and extensive proliferation (++++) at day 42.

Fig. 8.2 High yields of IL-2R bearing cells in the T-dependent interfollicular area of rat MLN at mid-weaning.

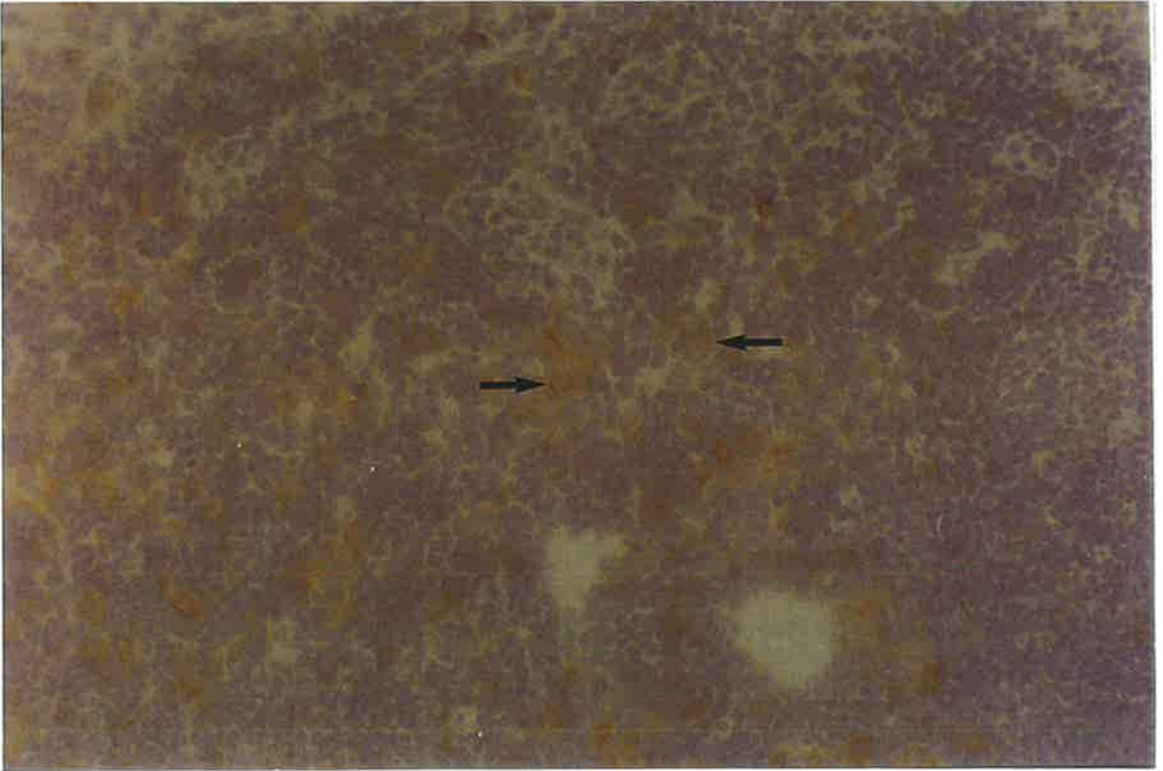
The Alkaline phosphatase staining of MLN was performed on frozen sections with mAb NDS-61. The larger arrow points to labelled cells. Note formation of a bright red precipitate at the site of the target antigen. The quantification of IL-2R^{+} activated cells could not be determined with certainty by use of this technique, because the sections stain non-specifically (smaller arrow). X50.

Table 8.1. Activation status of MLN T-cells at mid-weaning (day 21) and post-weaning (day 42) in rats.

Age (days)	%IL-2R$^+$ cells
21	6.8±1.3
42	2.0±0.3**

Data are given as mean proportion ± SE of IL-2R$^+$/α/βTCR T-cells in litters of rats from 6 to 8 animals.

** P<0.005



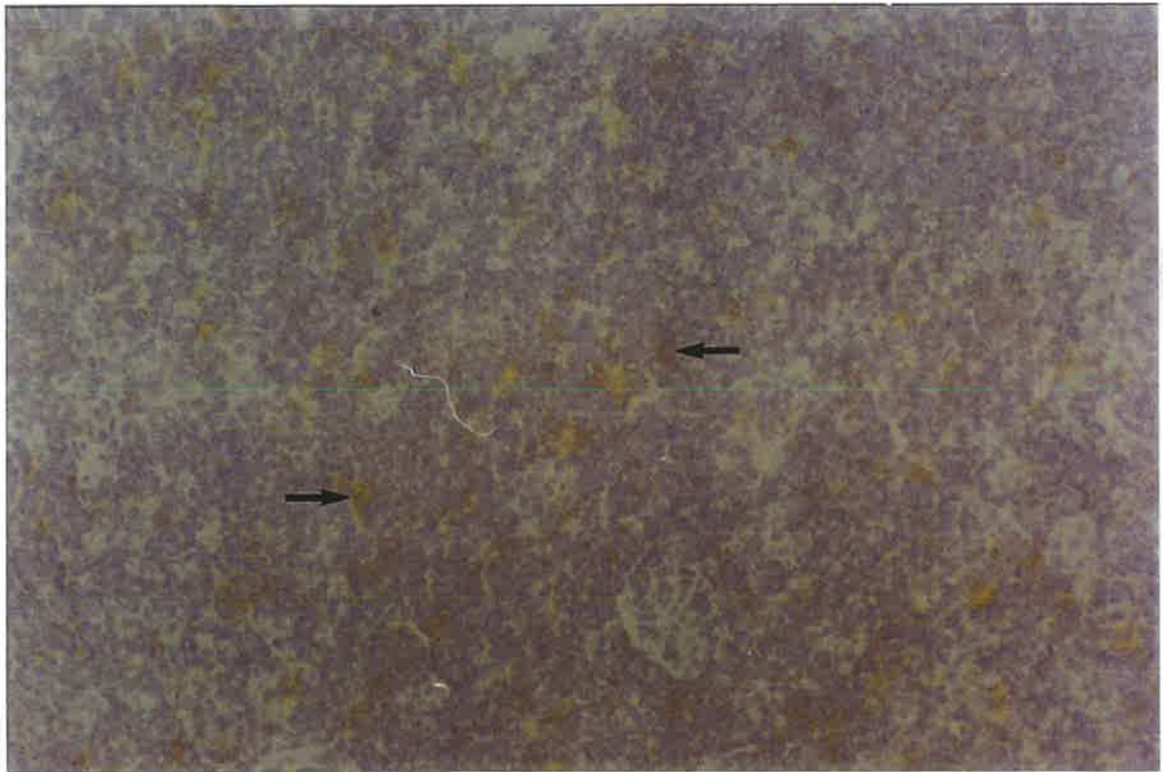


Fig. 8.3 Rat MLN stained with anti-IL-2R mAb (NDS-61).

The upper panel shows expression of IL-2R in the paracortex region of the MLN T-cell area at mid-weaning (arrowed). A marked *decrease* in the number of IL-2R$^+$ activated cells is seen in the T-dependent area of MLN at post-weaning (day 42, lower panel). X50.

Table 8.2. Comparison of activation status, as determined by IL-2R expression, of jejunal Peyer's patches (T-dependent interfollicular region), MLN (paracortex zone), LP, IEL and LFV cells at mid-weaning (day 21) and post-weaning (days 42, 45 or 70).

	Mid-weaning	**Post-weaning**
Peyer's patches (per total cell count)	0.19 (2)¶	0.08 (2)¶
MLN (per T-cells)	6.8% ± 1.35 (6)*	2.0% ± 0.3 (8)*
LP (per mm of muscularis)	2.1±0.3 (16)**	0.6±0.1 (15)**§
IEL (per mm of muscularis)	0.61±0.15 (5)**	0.05±0.03 (11)**§
LFV (pre total cell count)	40%±17 (2)¶	52%±7.8 (2)¶

Data are given as mean ± SE;

The figures inside the brackets represent the number of animals.

¶ These were not analysed statistically because there were only two values in each group.

* P<0.05

** P<0.005

** P<0.005

§ Day 70

8. 3. 2. Changes in phenotype of Peyer's patch cells

T-cells in the T-cell zones of Peyer's patch first expressed CD4 and CD43 at day 14, but not CD5, CD8 or CD45. CD45, CD5 and CD8 appeared at days 17, 19 and 21, respectively. For CD45, this is demonstrated in **Fig. 8.4**. $CD4^+$ cells were predominant over $CD8^+$. The predominant phenotype of $CD8^+$ cells was $CD8\alpha/\beta$ rather than $CD8\alpha/\alpha$. At day 42, T-cells in the T-dependent regions exhibited the phenotype of $CD45^+$, $CD43^+$, $CD25^+$, $CD8^+$, $CD5^+$, $CD4^+$, $\alpha/\beta TCR^+$, and $\gamma/\delta TCR^-$.

8.3.3. Changes in activation of Peyer's patch cells with expression of IL-2R

At the onset of weaning (day 14), moderate numbers of $IL\text{-}2R^+$ cells were detected in the follicular zone (B-cell area) of Peyer's patches. However, at the end of weaning (day 28) very few positive cells were seen in this region. In addition, at this stage, several cells in the T-dependent interfollicular and dome zones expressed IL-2R strongly. In conclusion, the highest ratio of $IL\text{-}2R^+$ cells/total cells in the T-dependent interfollicular area was found at mid-weaning (day 21, **Fig. 8.5**).

8.3.4. Changes in proliferation of Peyer's patch cells with expression of Ki-67 antigen

At the start of weaning, no detectable Ki-67 staining in the follicular zone of Peyer's patches was observed, suggesting that the germinal centres in this region are rudimentary at this stage (as germinal centres are the site of active proliferation in the Peyer's patches). However, at day 21, several cells (B-cells) positive for this proliferation marker were seen, even though fewer positive cells in the T-cell region were evident. A marked increase in the number of dividing cells in the germinal centre was obvious at the end of weaning (**Fig. 8.6**). Therefore, proliferation of B-cells was most evident after weaning.

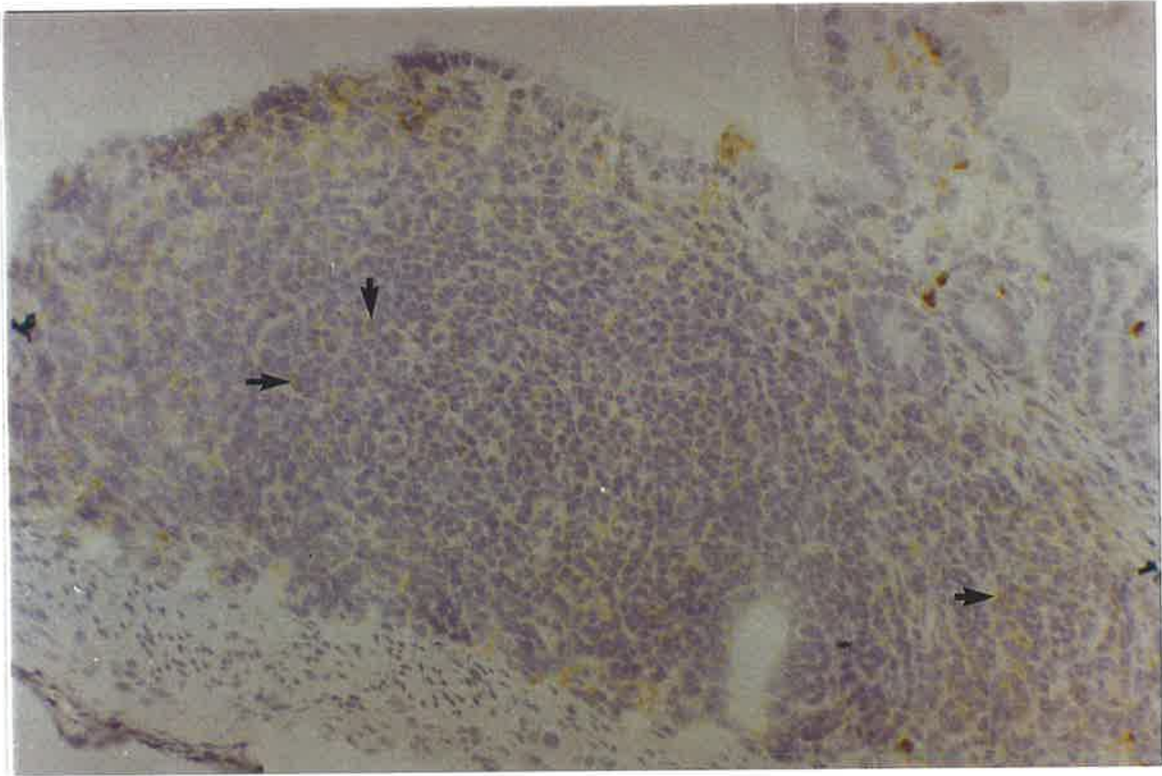
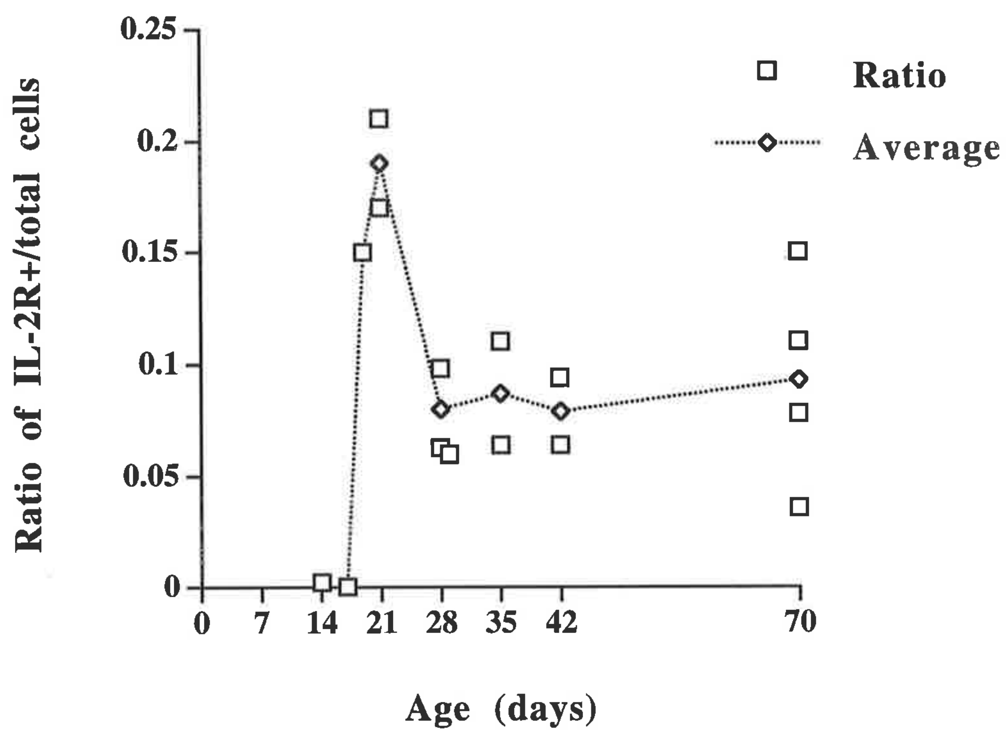


Fig. 8.4 Rat jejunal Peyer's patch stained with mAb OX-1 (CD45) at day 17 of age.

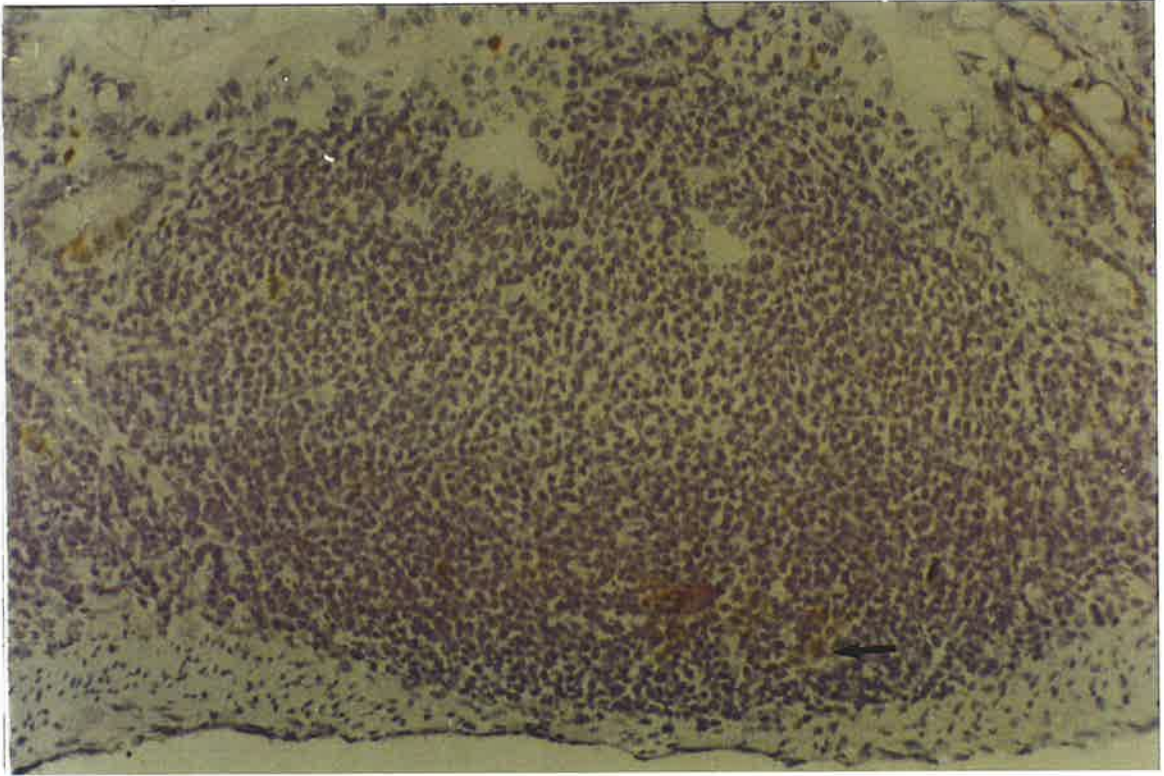
This was the earliest stage at which this marker was detected with faint staining. Immunoperoxidase, X50.

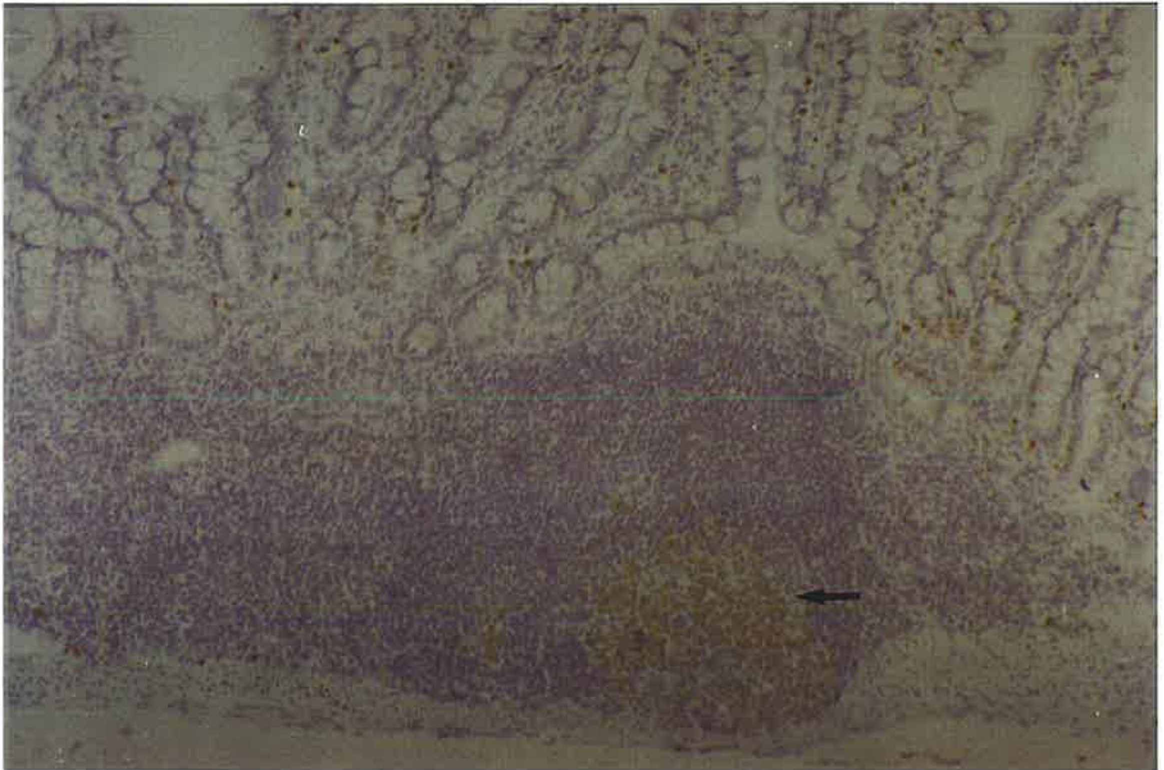
Fig. 8.5. Changes in IL-2R activation of Peyer's patch cells from days 14 to 70 of life.



Data represent the ratio of IL-2R+ cells/total cells in the T-dependent interfollicular area of 16 rat jejunal Peyer's patches.

Fig. 8.6 Rat jejunal Peyer's patch follicle stained for Ki-67$^+$ cells. The upper panel demonstrates that few B-cells in the germinal centre of follicle are positive for the Ki-67 proliferation maker at mid-weaning (formation of germinal centre, arrowed). A marked increase in the number of B-cells in the germinal centre is seen at the end of weaning (lower panel, arrowed). X50.





8.4. Discussion

As discussed earlier, luminal antigens in the gastrointestinal tract consist of food antigens, especially proteins, and bacteria, both of which seem likely to stimulate the mucosal immune system. Food proteins, in particular, would be expected to increase exponentially with weaning (Cummins and Thompson, 1997). Thus, there is the possibility that food antigens in the small intestine are more important than bacterial antigens. On the other hand, it has been shown that gram-positive organisms are found in the jejunum during milk feeding up to day 18 of life, after which gram-negative organisms and yeasts begin to appear with weaning (Cummins *et al.*, 1988b; Cummins and Thompson, 1997). As the proliferative response of B-cells to food antigens (thymus-dependent antigens) is weaker than T-cells, and as food-antigens may be more important than bacterial antigens, this may explain that why fewer B cells in the germinal centres of MLN underwent proliferation at day 21, compared to day 42 (Kuby *et al.*, 1994; Tizard, 1995).

Interestingly, approximately 95% of MLN T-cells are $\alpha/\beta TCR^+$ $CD3^+$ (Thompson *et al.*, 1996), even though only 30% of LP or IEL (pre)-T cells are $\alpha/\beta TCR^+$ at day 21. A recent study has shown a similar discrepancy between IEL and MLN in 2 month old rats which had 75% and 98% $CD3^+$ cells, respectively (Helgeland *et al.*, 1997). The current model for migration of mucosal gut lymphoblasts would suggest that lymphoblasts from Peyer's patches pass to the regional MLN, where they undergo expansion under an antigenic stimulus from mucosally-derived and antigen-primed dendritic cells (Mowat and Viney, 1997). What this model does not explain is the presence of $CD45^+$ $CD3^-$ cells (presumably pre-T cells) in the LP which presumably expand locally. The $\alpha/\beta TCR^+$ cells in the LP could have been derived from the MLN complex.

The present study showed that immunological activity in the MLN reflected activity in Peyer's patches and in the LP of the mucosa. Thus, activated IL-$2R^+$ cells were increased 2 to 3-fold at mid-weaning (day 21) in the MLN, in Peyer's patches and in the LP, compared to

post-weaned animals (day 42, **Table 8.2**). The present study also gave approximately identical values for IL-2R+ MLN T-cells by immunostaining at days 21 and 42 of life compared to the previous study which used flow cytometry (Thompson *et al.*, 1996). Therefore, the mean percentage of IL-2R+ activated T-cells at day 21 was 6.8% by immunostaining and 7.2% by flow cytometry, and at day 42 was 2% by immunostaining compared with 2.4% by flow cytometry. The results of current study have now shown that Peyer's patches, MLN, LP cells and LFV cells were in higher activation status at mid-weaning and were presumably responding to luminal antigens (**Table 8.2**).

This suggests that increased expression of IL-2R in the Peyer's patches, MLN and LP of the intestine would explain the mid-weaning (4 months of age) peak of soluble IL-2R levels in the sera of human infants (Cummins *et al.*, 1994; Chan *et al.*, 1995) because the source of these is likely from membrane-bound IL-2R on activated immune cells (Rubin *et al.*, 1985). Serum IL-2R is unlikely to be derived from systemic immune cells as expression of IL-2R on peripheral blood cells decreased to a low level at this age (Cummins *et al.*, 1994) as does IL-2R expression on spleen cells, at least in rats (Cummins *et al.*, 1990).

The results showed a small number of IL-2R+ cells in the dome area of Peyer's patches at day 28. The dome area of Peyer's patch is rich in cells expressing class II MHC antigens-namely, macrophages, dendritic cells, B-cells, and T-cells. Since B-cells, and probably dendritic cells do not usually express this marker in the gut (Stites and Terr, 1994), IL-2R bearing cells in the dome area are probably T-cells and macrophages.

The results revealed detectable Ki-67 staining in the germinal centres of secondary follicules of Peyer's patches at day 21. This suggests that germinal centres of Peyer's patches appear at this stage in the rat, since there was no detectable staining for this proliferation marker prior to day 21. However, germinal centres of Peyer's patches in the mouse appear between 4 and 5 weeks of life (Ferguson and Parrott, 1972).

As discussed earlier, the present study showed that physiological inflammation of the small bowel is closely associated with weaning. This physiological inflammation is immunologically mediated as indicated by the peak of IL-2R$^+$ cells in the MLN and LP cells at mid-weaning, and by other studies that showed that expansion of IEL is reduced by immunosuppressive treatment with either cyclosporin A (Cummis *et al.*, 1989a) or anti-IL-2R antibody (which is known to prevent binding of the IL-2 to the rat IL-2R (Thompson *et al.*, 1996).

8.5. Conclusion

The persented results in this Chapter have shown that physiological inflammation of the small bowel peaks during weaning in the rat. Peyer's patch cells reached the highest activation status with expression of IL-2R$^+$ at mid-weaning, which occured simultaneously with a peak of activation of α/βTCR$^+$ T-cells in the MLN.

Chapter 9

General Discussion

9.1. Physiological inflammation of the small intestine during weaning

Physiological inflammation of small bowel was investigated in infant rats during weaning. Weaning is associated with exposure of the intestinal mucosa to food and bacterial antigens. The original hypothesis was that the immune system of the small intestine upregulates physiological inflammation during weaning. This physiological inflammation was investigated by the number, phenotype and activation of IEL, MMC, LP cells, LFV cells, Peyer's patch and MLN cells. This thesis focused on the jejunum, where IEL at least, are known to be in reasonable abundance (20 IEL/100 epithelial cells, Marsh, 1980). Future studies should compare the number and phenotype of IEL and other lymphoid cells within other regions of the small intestine. These studies were performed because very little is known about the phenotype and activation of lymphoid cells in the small bowel during weaning or the relationship between lymphoid cells in different compartments of the GALT.

Physiological inflammation was first shown by expansion of CD45^{+} cells in both the LP and IEL compartments. Phenotypic studies of the expanding CD45^{+} cells before day 70, showed that the largest identifiable subset was α/βTCR^{+}, although these comprised only 10-50% of cells, depending on age. By day 70, approximately 65% of CD45^{+} IEL were α/βTCR^{+}, which is consistent with the values of 50-70% reported in adult rats (Vaage *et al*., 1990; Lefrancois *et al*., 1991; Kearsey *et al.* 1996). Expansion of IEL in this study seems to have been mainly from migration rather than *in situ* proliferation, as Ki-67 positivity of IEL was low or absent throughout weaning, despite positive staining of crypt epithelial cells.

The APAAP staining method has not previously been a useful approach for staining gut sections that contain endogeneous alkaline phosphatase in the brush border microvilli, as this isoenzyme cannot be quenched by levamisole which is present in the substrate solution. In addition, other inhibitors of this isoenzyme may denature membrane-bound antigens, particularly IL-2R. However, by use of two different chromogens and by generating a good contrast between the staining of endogenous alkaline phosphatase of microvilli and antibody

bound alkaline phosphatase which may label the LP cells, this study was able to quantify the LP cells. This dual chromogen technique detailed in Chapter 2, used an initial stain with Fast Blue which stained the epithelial endogenous alkaline phosphatase. This binding of endogenous alkaline phosphatase appeared to prevent the diffusion of this isoenzyme into the LP region. A second stain (Fast Red) was used to demonstrate the presence of alkaline phosphatase bound to a complex of antibodies linked to the LP cells. It is of note that Fast Red staining would be indistinguishable from the diffuse background staining of loose connective tissue of LP if Fast Red is used on its own. Thus, this dual chromogen method premitted to use that for gut sections which contain endogenous alkaline phosphatase, and enabled proper interpretation of results. Thus, I would recommend this method for the future study of LP immune cells.

$\alpha/\beta TCR^+$ T-cells were relatively more abundant in the epithelial layer than the LP (means of 30% versus 8% of $CD45^+$ cells at mid-weaning). A ten-fold increase of IEL with the $\alpha/\beta TCR^+$ phenotype, has been shown during the first two years of life in humans which is consistent with the data in this study (Cerf-Bensussan and Guy-Grand, 1991). There was also a noticeable increase in the intensity of immunostaining after day 19 for both CD45 and α/βTCR markers which would be compatible with increased numbers of both receptors, presumably due to immune stimulation. On the other hand, there is a collaboration between α/βTCR and CD45 in T-cell activation, so that T cells that lack CD45 cannot respond to antigen, even though they express normal levels of the TCR. CD45, a tyrosine phosphatase, is required for TCR signalling (Imboden and Stites, 1994).

Physiological inflammation was also shown by elevated NK cell numbers in the early phase of weaning, both in the epithelial, LP, and LFV compartments. It is difficult to know what the relationship is between the various cells expressing the NK cell marker in each of the compartments of the GALT. However, LFV could be a site of extrathymic differentiation and

maturation, with NK cells originating there and circulating to the LP and epithelial compartments.

Eosinophil counts were also elevated in the early phase of weaning, both in the epithelial layer and LP. Eosinophil infiltration into the epithelial layer was also remarkable towards the end of weaning (Chapter 5). These findings confirm a previous report in which eosinophils in the LP and epithelium of rat small intestine expand during the weaning phase (Cummins *et al.*, 1988b). Since the study of Cummins *et al.* (1988b), there have been no further reports of eosinophils counts during weaning. Eosinophils were also found in LFV during early and mid-weaning-i.e., the same age at as they were present in the mucosa. In conclusion, the elevated number of eosinophils in the early phase of weaning may be a response to food antigens without any obvious involvement of an IgE-mediated mechanism (Knutson, *et al.*, 1993). It is not yet known whether eosinophils are influenced by mucosal T-cells in the small intestine.

There was also a significant mastocytosis from mid-weaning (day 21) to post-weaning (day 35). This co-incided with a broad peak of T-cell activation in the LP (Chapter 6) in the second week of weaning. This is of interest, because weaning activates the mucosal T-cells, and these cells are known to activate and degranulate MMC (Ruitenberg and Elgersma, 1976; Nawa and Miller, 1979; Mayrhofer, 1979).

IL-2R is expressed shortly after T-cell activation and is, therefore, a marker of T-cells that have recently encountered their specific antigen (Reed *et al.*, 1986). Physiological inflammation in the small intestine during weaning was upregulated 2 to 3-fold with a peak of IL-2R in the MLN, in jejunal Peyer's patches and in the LP compared to either pre-weaned or adult animals (**Table 8.2**). These findings show that IL-2R$^+$ activated cells in these lymphoid tissues respond to food and microbial antigens in the gut lumen. This increased activity is in spite of a prevailing notion that the immune system of the infant is immature, presumably

with defective immune reactivity (Pabst and Kreth, 1980). The argument is that the immune system is compartmentalised into a mucosal system that is activated and sequesters reactive immune cells, and a systemic compartment which appears to have low reactivity but is not representative of the total immune system. This heightened mucosal activity should engender caution in immunostimulation of mucosal surfaces in the young, such as with mucosal vaccines containing cytokine genes, as this could lead to pathological tissue damage.

LFV were identified in suckling rats prior to weaning at day 7 of life, suggesting that *solid* food antigens are not necessary for LFV development. However, the effect of bacterial antigens could not be ruled out, as gram-positive organisms are present in the jejunum during milk feeding (Cummins *et al.*, 1988b; Cummins and Thompson, 1997).

Weaning also influences the LFV compartment, as the total number of nucleated lymphoid cells as well as $\alpha/\beta TCR^+$ T-cells expanded approximately 2 and 3.5-fold, respectively during this time (**Fig. 7.3** and **Table 7.1**). The staining intensity of CD45 and IL-2R markers also increased after day 19 of life. These findings support the conclusion that weaning is also closely associated with physiological inflammation in this novel gut-associated lymphoid organ.

The presence of a considerable proportion of $IL\text{-}2R^+$ cells (~40%) in LFV in pre-weaned rats (day 7) and its peak around mid-weaning, suggest that they were activated by both endogenous (self) and luminal antigens. Studies are required to determine how the luminal antigens are absorbed (if this is the case) by the specialised epithelial cells of LFV, as these are distinct ultrastructurally from M cells in the leaky epithelium of Peyer's patches (Mayrhofer, unpublished observations).

Despite an increase in both LFV cell numbers and $CD45^+$ cells, no detectable Ki-67 staining was seen, except at day 14. Therefore, one possible explanation for the increasing cell

numbers in LFV with age is by migration from other sites. However, preliminary work by Brooks and Mayrhofer (unpublished data) suggested that proliferation was present in the LFV of rats as determined by ^{3}H-thymidine (^{3}HTdR) incorporation. This suggests that they are labelled *in situ*, although it is impossible to determine whether these cells divided before or after entry into LFV.

α/βTCR^{+} T-cells appeared in LFV at day 19 of life, whereas these cells were present at an earlier stage in the intestinal epithelium and LP (Chapters 5, 6, and 7). α/βT-cells in these three lymphoid tissues expand during weaning. These findings suggest that α/βT-cells in these structures respond to intestinal antigens during weaning, though not by proliferation. In conclusion, two unexpected findings related to LFV were:

(**1**) An early increase of NK cells with counts that remained higher than counts of α/βTCR^{+} T-cells, and;

(**2**) A higher proportion of IL-2R^{+} cells than in other GALT in suckling rats.

This thesis also reports the presence of a novel lymphoid aggregate, resembling the crypto-patch, first observed in the mucosa of the mouse small intestine by Kanamori *et al*. (1996). It was first detected at the end of weaning phase (day 28) in rats, but may have been present at an earlier time. This needs further investigation. Preliminary work in the current study revealed that the phenotype of crypto-patch cells in rats is probably different from that of crypto-patch cells in mice as less than 2% of crypto-patch cells in mice are conventional T-cells (Kanamori *et al.*, 1996) when compared to 25% (sum of CD4^{+} and CD8^{+} cells) in rats in the current study (**Table 7.2**).

The morphological features that distinguish the rat crypto-patch from classical LFV are the basal extension of the LP at the level of the intestinal crypts. It also varies from LFV by being lined by shallow epithelium, and being wider and shorter than classical LFV (**Fig. 7.9**). However, preliminary work revealed that the phenotype and activation of rat cryto-patch cells

were almost identical to LFV cells. For instance, at day 70, a higher proportion of crypto-patch cells in rats was positive for CD4 rather than CD8, and most crypto-patch cells expressed IL-2R (e.g. at day 45, **Table 7.2**).

9.2. Factors involved in activation of the mucosal immune system

Possible stimuli for immune activation include changes in colonisation with bacterial flora, introduction of food antigens and engraftment of milk-derived lymphocytes. It is very difficult to distinguish the relative effects of food and bacteria in activating the intestinal immune system and promoting intestinal growth, because they are not mutually exclusive. The small intestine of germ-free animals, which contains very few IEL (mostly with the phenotype of γ/δTCR), has some characteristics of an immature gut (Abrams *et al.*, 1963; Viney *et al.*, 1990). The study of the activation of the intestinal immune system may permit the assessment of the relative effects of food and bacterial factors. Germ-free animal facilities were not available for this study.

Another possibility is that, rather than supplying a stimulus to the intestinal immune system, milk feeding may be immunosuppressive. Hence, withdrawal of breast-feeding (removal of suppression) may have a permissive role in allowing activation of the intestinal immune system. This possibility is suggested by the observation that maternal $CD8^+$ milk T cells have a high expression of the CD11b marker consistent with suppressor T-cells, and that there are high levels of the immunosuppressive cytokine, TGF-β2, in milk (Saito, 1993).

9.3. Future prospects

Studies are required to identify the phenotype of $CD45^+$ null cells in the epithelial layer and LP compartments. These null cells could be pre-T-cells. Presently, there is no monoclonal antibody against the pre-T-cell marker of CD7 in rats. Monoclonal antibodies which

recognize pre-T-cells in rats, need to be developed. Studies are also required to determine the time-course and cellular origin (T-cell, NK cell, NK-Tcell) of IL-2 in the intestinal mucosa of the rat. Methods are also needed to induce down-regulation of inflammation and to determine the cellular origin (T-cells, NK-T-cells, mast cells, eosinophils) of immunosuppressive cytokines (IL-4, IL-10, and TGF-β1). Since this study showed an increase in the number of eosinophils, it may be relevant to study eosinophils in a rat model in inflammatory bowel disease (IBD).

As discussed earlier, LFV are recognisable in suckling rats prior to weaning at day 7 of life. In addition, LFV are shown to be present in normal numbers in conventional and specific pathogen-free rats. However, the small intestine in latter animals is still exposed to food and microbial antigens. Therefore, studies are required to examine whether development of LFV is antigen-independent. Either germ-free rats or foetal gut implants (which will provide antigen-free tissue) will be useful approaches to this investigation. The procedure for the latter one is that a segment of small intestine is defunctioned by end-to-end graft into a horn of the uterus. To investigate this, light microscopy is required to examine the morphology of LFV, followed by immunohistochemistry to define lymphocyte subsets.

Very little is presently known about the role of local antigen in the development of LFV or the phenotype of lymphocytes in LFV. It is, therefore, important to attempt to use a panel of monoclonal antibodies against all mononuclear cell surface markers to define lymphocyte subsets. In addition, for those cells that are unstained by a cocktail of antibodies, it will be necessary to produce new monoclonal antibodies that define further surface antigens unique to LFV, and to determine the functions of these markers. As a proportion of LFV cells may be NK T-cells, this could be investigated by dual labelling to determine those cells which are mainly positive for the NK cell marker.

9. 4. Conclusion

(**1**) These studies have shown that weaning influences all compartments of the GALT physiologically. This suggests that activation and expansion of lymphoid and non-lymphoid cells may be a response to weaning, perhaps due to immune activation by food and microbial antigens.

(**2**) Weaning which is associated with intense antigenic stimulation (physiological stress), causes access of other non-lymphoid white blood cells (eg, granulocytes) to the epithelial layer of small intestine.

(**3**) As discussed in Chapter 4, an alternative antibody should be used for the detection of rat TGF-β.

(**4**) Weaning is associated with a peak of physiological intestinal inflammation of IL-2R$^+$ cells and with expansion of both α/βT-cells and 'null' CD45$^+$ cells. The stimulus responsible for this physiological inflammation is unknown, but presumably is food and/or bacterial antigens.

(**5**) Expansion of IEL appears to be mediated by migration rather than by proliferation as Ki-67 staining was rarely detected.

(**6**) Expansion of MMC during weaning also indicated heightened immunological activity.

(**7**) Development of LFV is independent of solid food antigens, although it appears that these antigens are involved in expanding the size of LFV during weaning.

(**8**) Histological and immunohistochemical characterization of lymphocytes in LFV demonstrated that they were phenotypically different from populations in the epithelial and

LP compartments. They contained lymphocytes with similarities in phenotype to immature T-cells. LFV were also sites of intense lymphocyte activation which peaked at mid-weaning, but apparently without proliferation. These findings suggest that LFV may be primary sites for T cell generation and maturation.

(9) Peyer's patch and MLN cells reached the highest activation status with expression of IL-2R at mid-weaning.

Bibliography

Abbas AK, Lichman AH, and Pober JS. (1994). T-cell maturation in the thymus. eds. *Cellular and Molecular Immunology*. Philadelphia: W.B. Saunders Company, 168-185.

Aberu-Martin MT and Torgon SR. (1996). Regulation of immune responses of the intestinal mucosa. *Critical Rev in Immunol*; **16**: 277-308.

Abrams G, Bauer H, and Sprinz H (1963). Influence of the normal flora on mucosal morphology and cellular remewal in the ileum. A comparison of germ-free and conventional mice. *Lab Invest.* **12**: 355-364.

Adleer W H, Takiguchi T, and Smith R T (1971). Effect of age upon pronary allountigen recognition by mouse spleen cells. *J Immunol;* **107**: 1357-1362.

Ajax chemicals (1982-89), Material safety data sheets, Ajax Chemicals Pty Ltd, Auburn, New South Wales, Australia.

Allison JP and Havran WL. (1991). The immunobiology of T-cells with invariant γ/δ antigen receptors. *Annu Rev Immunol*, **9**: 679-705

Arase H, Arase N, Nagakawa K, Good RA, and Onoe K. (1993). NK1.1^{+} CD4^{+} CD8^{-} thymocytes with specific lymphokine secretion. *Eur J Immunol*; **23**: 307-310.

Arase H, Arase N, Ogasawara K, Good RA, and Onoe K. (1992). An NK1.1^{+} CD4^{+} CD8^{-} single-positive thymocyte subpopulation that expresses a highly skewed T-cell antigen receptor Vβ family. *Proc Natl Acad Sci USA*; **89**: 6506-6510.

Arase H, Arase-Fukushi N, Good RA, and Onoe K. (1993). Lymphokine-activated killer cell activity of CD4^{-} CD8^{-} TCRα/β^{+} thymocytes. *J Immunol*; **151**: 546-555.

Arizono N and Nakao S. (1988). Kinetics and staining properties of mast cells proliferating in rat small intestine tunica muscularis and subserosa following infection with *Nippostrongylus brasiliensis*. *APMIS*; **96**: 964-970.

Austin LL and Dobbins WO. (1982). Intraepithelial leucocytes of the intestinal mucosa in normal man and in Whipple's disease: a light and electron microscopic study. *Dig Dis Sci*; **27:** 311-320.

Austrup F, Rebstock S, Kilshaw PJ, and Hamann A (1995). Transforming growth factor-β1-induced expression of the mucosa-related integrin αE on lymphocytes is not associated with mucosa-specific homing. *Eur J Immunol*; **25**: 1487-1491.

Babicky A, Parizek J, Ostadalova I, and Kolar J. (1973). Initial solid food intake and growth of young rats in nests of different sizes. *Physiol Bohemoslov*; **22:** 557-566.

Bach MK. (1982). Mediators of anaphylaxis and inflammation. *Annu Rev Microbiol*; **36**: 371-413.

Bandeira A, Itohara S, Bonneville M, *et al.* (1991). Extrathymic origin of intestinal intraepithelial lymphocytes bearing T-cell antigen receptor γ/δ. *Proc Natl Acad Sci*; **88**: 43-47.

Bandeira A, Mota-Santos T, Itohara S, *et al.* (1990). Localization of γ/δ T cells to the intestinal epithelium is independent of normal microbial colonization. *J Exp Med*; **172**: 239-244.

Barnard JA, Beauchmp RD, Coffey RJ, and Moses HL. (1989). Regulation of intestinal epithelial cell growth by transforming growth factor type-β. *Proc Natl Acad Sci USA*; **86**: 1578-1582.

Barnard JA, Warwick GJ, and Gold LI. (1993). Localization of transforming growth factor β isoforms in the normal murine small intestine and colon. *Gastroenterology*; **105**: 67-73.

Barral-Netto M, Barral A, Brownell CE, *et al.* (1992). Transforming growth factor-β in leishmanial infectioin: a parasite escape mechanism. *Science*; **257**: 545-548.

Barrett TA, Gajewski TS, Danielpour D, Eugene BC, Beagley KW, and Bluestone JA. (1992). Differential function of intestinal intraepithelial lymphocyte subsets. *J Immunol*; **149:** 1124-1130.

Barth RJ, Mule JJ, Spiess PJ, and Rosenberg SA. (1991). Interferon γ and tumor necrosis factor have a role in tumor regressions mediated by murine $CD8^+$ tumor-infiltrating lymphocytes. *J Exp Med*; **173**: 647-658.

Basten A, Warner NL, and Mandel T. (1972). A receptor for antibody on B-lymphocytes. II. Immunochemical and electron microscopy characteristics. *J Exp Med*; **135**: 627-42

Beagley KW and Elson. (1992). Cells and cytokine in mucosal immunity and inflammation. *Gastroenterol Clin North Am*; **21**: 347-366.

Befus D and Bienenstock J. (1979). Immunologically mediated intestinal mastocytosis in *Nippostrongylus brasiliensis*-infected rats. *Immunology*; **38**: 95-101.

Bendelac A, Killeen N, Littman DR, and Schwartz RH. (1994). A subset of $CD4^+$ thymocytes selected by MHC class I molecules. *Science*; **263**: 1774-1778.

Bendelac A, Matzinger P, Seder RA, Paul WE, and Schwartz RH. (1992). Activation events during thymic selection. *J Exp Med*; **175**: 731-742.

Bendelac A, Rivera MV, Park SH, and Roark JH. (1997). Mouse CD1-specific NK1 T-cells: development, specificity, and function-*Annu Rev Immunol*; **15**: 535-62.

Bendelac A. (1995). Mouse Nk1+ T-cells. *Curr Opin Immunol*; **7**: 367-374.

Beverley PCL and Callard RE. (1981). Distinctive functional characteristics of human 'T' lymphocytes defined by E rosetting or by a monoclonal anti-T cell antibody. *Eur J Immunol*; **11**: 329.

Bland PW and Whiting CV. (1989). Antigen processing by isolated rat intestinal villus enterocytes. *Immunology*; **68**: 503-506.

Bonneville M, Janeway CA, JR, Ito K, *et al.* (1988). Intestinal intraepithelial lymphocytes are a ditinct set of γδ T cells. *Nature* (London); **336**: 479-481.

Brett F, Mowat A, Farquharson MA, *et al.* (1992). The distribution of immunoreactive interferon-γ- containing cells in normal human tissues. *Immunology*; **77**: 515-519.

Brown WRA, Barclay AN, Sunderland, CA, and Williams AF. (1981). Identification of a glycophorin-like molecule at the cell surface of rat thymocytes. *Nature*; **289**: 456-460.

Bull DM and Bookman MA. (1977). Isolation and functional characterization of human intestinal mucosal lymphoid cells. *J Clin Invest*; **59**: 966-74.

Cantrell DA, Robins RA, Brooks CG, and Baldwin RW. (1982). Phenotype of rat NK cells defined by monoclonal antibodies marking rat lymphocyte subsets. *Immunology*; **45**: 97-103

Cerf-Bensussan N and Guy-Grand D. (1991). *Intraepithelial lymphocytes*, In *Mucosal Immunology* I: *Basic principles*, MacDermott RP and Elson C. Editor WB Saunders: Philadelphia. 549-576.

Cerf-Bensussan N, Guy-Grand D, and Griscelli C. (1985). Intraepithelial lymphocytes of human gut: isolation, characterization and study of natural killer cell activity. *Gut*; **26**: 81-88.

Cerf-Bensussan N, Jarry A, Brousse N, Lisowska-Grospierre B, Guy-Grand DL, and Griscelli C. (1987). A monoclonal antibody (HML-1) defining a novel membrane molecule present on human intestinal lymphocytes. *Eur J Immunol*; **17**: 1279-1285.

Cerf-Bensussan N, Quaroni A, Kurnick JT, *et al.* (1984). Intraepithelial lymphocytes modulate Ia expression by intestinal epithalial cells. *J Immunol*; **132**: 2244-2252.

Cerf-Bensussan N, Schneeberger EE, and Bhan AK. (1983). Immunohistologic and immunoelectron microscopic characterization of the mucosal lymphocytes of the human small intestine by the use of monoclonal antibodies. *J Immunol*; **130**: 2615-2622.

Chan KN, Phillips AD, Walker-Smith JA, and MacDonald TT. (1995). Serum interleukin-2 receptor in infants and young children. *Acta Paediatr*; **84**: 151-155.

Cheng SH, Penninger JM, Ferrick DA, *et al.* (1991). Biology of murine γ/δ T-cells. *Crit Rev Immunol*; **11**: 145-166.

Cherayil BJ, Weiner SJ, and Pilliai S. (1989). The Mac-2 antigen is a galactose-specific lectin that binds IgE. *J Exp Med*; **170**: 1959-1972.

Choy MY, Walker-Smith JA, Williams CB, *et al.* (1991). Differential expression of CD25 (interleukin-2 receptor) on lamina propria T-cells and macrophages in the intestinal lesions in Crohn's disease and ulcerative colitis. *Gut;* **31**: 1365-1370.

Coffman RL, Lebman DA, and Shrader B. (1989). TGF-β specifically enhances IgA production by lipopolysacharide-stimulating murine B-cells. *J Exp Med*; **170**: 1039-1044.

Cotran RS, Kumar V, and Robbins SL. (1994). Diseases of immunity. In: Robbins SL. eds. *Pathologic Basis of Disease*. Philadelphia, London, Toronto: W.B. Saunders Company; **5th ed**: 171-240.

Cox DA and Burk RR. (1991). Isolation and characterization of milk growth factor, a transforming-growth-factor-β2-related polypeptide, from bovine milk. *Eur J Biochem;* **197**: 353-8.

Crabbe PA, Bazin H, Eyssen H, and Heremans J. (1968). The normal microbial flora as a major stimulus for proliferation of plasma cells synthesizing IgA in the gut. The grem-free intestinal tract. *Int Arch Allergy Appl Immunol*; **34**: 362-75.

Crabbe PA, Nash DR, Bazin H, Eyssen H, and Heremans JF. (1970). Immunohistochemical observation on lymphoid tissue from conventionaland germ-free mice. *Lab Invest;* **22**: 448-57

Crabbe PA, Nash DR, Bazin H, Eyssen H, and Heremans JF. (1970). Immunohistochemical observations on lymphoid tissues from conventional and germ-free mice. *Lab Invest*; **22**: 448-456.

Cummins AG and Roberts-Thomson IC. (1994). Role of the epithelium in defence of the small and large intestine. In: Goldie R. eds. *The Handbook of Immunopharmacology; Immunopharmacology of Epithelial Barriers*. London, San Diego: Academic Press Inc., 213-223.

Cummins AG and Thompson FM. (1997). Postnatal changes in mucosal immune response: A physiological perspective of breast feeding and weaning. *Immunol Cell Biol*; **75**: 419-429.

Cummins AG, Eglinton BA, Gonsalez A, and Roberton DM. (1994). Immune activation during infancy in healthy humans. *J Clin Immunol*; **14**: 107-115.

Cummins AG, LaBrooy JT, and Shearman DJC. (1989a). Effect of cyclosporin A in delaying maturation of the small intestine during weaning in the rat. *Clin Exp Immunol*; **75**: 451-456.

Cummins AG, Munro GH, Miller HRP, and Ferguson A. (1988a). Association of maturation of the small intestine at weaning with mucosal mast cell activation in the rat. *Immunol Cell Biol*; **66**: 417-422.

Cummins AG, Munro GH, Miller HRP, and Ferguson A. (1989b). Effect of cyclosporin A treatment on the enteropathy of graft-versus-host-reaction in the rat: A quantitative study of intestinal morphology, epithelial cell kinetics and mucosal immune activity. *Immunol Cell Biol*; **67:** 153-160.

Cummins AG, Steele TW, LaBrooy JT, and Shearman DJC. (1988b). Maturation of the rat small intestine at weaning: changes in epithelial cell kinetics, bacterial flora, and mucosal immune activity. *Gut*; **29**: 1672-1679

Cummins AG, Thompson FM, and Mayrhofer G. (1990). A flow cytometric study of activation of mesenteric lymph node cells at weaning in the rat. *Advances in Mucosal Immunology*, ed. MacDonald TT. Lancaster: Kluwer Academic Publishers. 477-478.

Cummins AG. (1989c). Maturation of the small intestine in the rat at weaning. In: Cummins, AG. PhD thesis, Interaction of the mucosal immune system with the epithelium of the small intestine. Adelaide, South Australia; 53-90.

Cunningham-Rundles C, Brandeis WE, and Good RA. (1978). Milk precipitins, circulating immune complexes and IgA deficiency. *Pro Natl Acad Sci (USA)*; **75**: 3387-3389.

Dahl GM, Telemo E, Westrom BR, Jakobsson I, Lindberg T, and Karlsson BW. (1984). The passage of orally fed proteins from mother to fetus in the rat. *Comp Biochem Physiol*; **77**: 199-201.

De Geus B, Van den Eden M, Coolen C, Nagelkerken L, Van der Heijden P, and Rozing J. (1990). Phenotype of intraepithelial lymphocytes in euthymic and athymic mice: implications for dofferentiation of cells bearing a CD3-associated γ/δTCR. *Eur J Immunol;* **20**: 291-298.

Derynck R, Jarrett JA, Chen EY, and Goeddel DV. (1986). The murine transforming growth factor-β precursor. *J Biol Chem*; **261**: 4377-4379.

Dobbins WO. (1986). Human intestinal intraepithelial lymphocytes. *Gut*; **27** (8): 972-85

Dyer MJS and Hunt SV. (1981). Committed T-lymphocyte stem cells of rats. *J Exp Med;* **154**: 1164-1177.

Ebert E. (1995). Human intestinal intraepithelial lymphocytes have potent chemotactic activity. *Gastroenterology*; **109**: 1154-1159.

Ebert EC, Roberts AI, Brolin RE, and Raska K. (1986). Examination of the low proliferative capacity of human jejunal intraepithelial lymphocytes. *Clin Exp Immunol;* **60**: 148-157.

Ebert EC. (1989). Proliferative responses of human intraepithelial lymphocytes to various T cell stimuli. *Gastroenterology*; **97**: 1372-1381.

Ebert EC. (1990). Intra-epithelial lymphocytes: IFN-γ production and suppressor/cytotoxic activities. *Clin Exp Immunol*; **82**: 81-85.

Ebert EC. (1997). Response of mucosal T-lymphocytes to chemokines. *Mucosal Immunology Update*; **5**: 39-41.

Ellen E, Jarrett E, and Haig DM. (1984). Mucosal mast cells *in vivo* and *in vitro*. *Immunol Today*; **5**: 115-119.

Elson CO and Beagley KW. (1994). Cytokines and immune mediators. In: Johnson LR. eds. *Physiology of the Gastrointestinal tract,* **3rd edi**. NY: Raven Press, 243-265.

Ennulat NM, Tice DG, and Reinitz ER. (1989). Immune response of rat small bowel lymphocytes. Transplant Proc; **21**: 2898-2899.

Ernst PB, Befus DA, and Bienenstock J. (1985). Leukocytes in the intestinal epithelium: an unusual immunological compartment. *Immunol Today*; **6**: 50.

Espevik T, Waage A, Faxvaag A, and Shalaby MR. (1990). Regulation of IL-2 and IL-6 production from T-cells: involvement of IL-1β and TGF-β. *Cell Immunol*; **126**: 47-56.

Fangmann J, Schwinzer R, and Wonigeit K. (1991). Unusual phenotype of intestinal intraepithelial lymphocytes in the rat: predominance of T cell receptor $\alpha/\beta^+/CD2^-$ cells and high expression of the RT6 alloantigen. *Eur J Immunol*; **21**: 753-760.

Farmilo AJ and Stead RH. (1989). Fixation in immunocytochemistry. *Handbook of Immunochemical Staining Methods*. Dako Corporation, Carpinteria, California.

Farstad I, Halstensen TS, Lien B, Kilshaw PJ, Lazarovitz AI, and Brandtzaeg P. (1996). Distribution of β7 integrins in human intestinal mucosa and organized gut-associated lymphoid tissue. *Immunology*; **89**: 227-237.

Farstad IN, Halstensen TS, Fausa O, and Brandtzaeg P. (1993). Do human Peyer's patches contribute to the intestinal intraepithelia γ/δ T-cell population? *Scand J immunol*; **38**: 451-458.

Fell JME, Walker-Smith JA, Spencer J, and MacDonald TT. (1996). The distribution of dividing T-cells throughout the intestinal wall in inflammatory bowel disease (IBD). *Clin Exp Immunol*; **104**: 280-285.

Ferguson A and Jarrett EEE. (1975). Hypersensitivity reactions in the small intestine. I. Thymus dependence of experimental 'partial villus atrophy'. *Gut*; **16**: 114-117.

Ferguson A and Parrott DMV. (1972a). The effect of antigen deprivation on thymus-dependant and thymus-independant lymphocytes in the small intestine of the mouse. *Clin Exp Immunol*; **12:** 477-488.

Ferguson A and Parrott DMV. (1972b). Growth and development of "antigen-free' grafts of foetal mouse intestine. *J Pathol;* **106**: 95-101.

Ferguson A. (1977). Intraepithelial lymphocytes of the small intestine. *Gut*; **18**: 921- 937.

Ferguson A. (1987). Models of immunologically driven small intestinal damage. In: Marsh MN. eds. *Immunopathology of the Small Intestine*. Chichester: John Wiley and Co Ltd, 225-252.

Ferguson A. (1992). Dietary manipulation and induction of tolerance. Discussion IV. *J Pediatr;* **121**: S101-S102.

Ferguson A. (1994). Immunological function of the gut in relation to nutritional state and mode of delivery of nutrients. *Gut (Supp)*; **1**: S10-S12.

Ferreira RdC, Forsyth LE, Richman PI, Wells C, Spencer J, and MacDonald TT. (1990). Changes in the rate of crypt epithelial cell proliferation and mucosal morphology induced by a T-cell-mediated response in human small intestine. *Gastroenterology*; **98**: 1255-1263.

Ferreira Rdc, Forsyth LE, Richman PI, Wells C, Spencer J, and MacDonald TT. (1990). Changes in the rate of crypt epithelial cell proliferation and mucosal morphology induced by a T-cell-mediated response in human small intestine *Gastroenterology*; **98**: 1255-1263.

Fiocchi C. (1990). Mucosal cellular immunity. In: Targan SR and Shanahan F. eds. *Immunology and immunopathology of the liver and gastrointestinal tract.* NY: Igaku-Shoin, 107-138.

Fiocchi C. (1997). Intestinal inflammation: a complex interplay of immune and nonimmune cell interactions. *Am. J Physiol*; **273** (Gastrointest Liver Physiol); **36**: G769-G775.

Flexman JP, Shellam GR, and Mayrhofer G. (1983). Natural cytotoxicity, responsivenes to interferon and morphology of intraepithelial lymphocytes from the small intestine of the rat. *Immunology*; **48**: 733-741.

Fujihashi K, Taguchi T, Aicher W K, *et al.* (1992). Immunoregulatory functions for murine intraepithelial lymphocytes: γ/δT cell receptor-positive (TCR$^+$) T cells abrogate oral tolerance, while α/βTCR$^+$ T cells provide B cell help. *J Exp Med;* **175**: 695-707.

Fujihashi K, Yamamoto M, McGhee JR, Beagley KW, and Kiyono H. (1993). Function of α/β TCR$^+$ intestinal intraepithelial lymphocytes: Th_1 and Th_2-type cytokine production by CD4$^+$ CD8$^-$ and CD4$^+$CD8$^+$ T-cells for helper activity. *Int Immunolo;* **5**: 1473-1481.

Gebert A, Rothkotter HJ, and Pabst R. (1996). M cells in Peyer's patches of the intestine *Int Rev Cytol*; **167**: 91-159.

Glaister JR. (1973). Factors affecting the lymphoid cells in the small intestinal epithelium of the mouse. *Int Arch Allergy Appl Immunol*; **45**: 719-730.

Goodman T and Lefrancois L. (1988). Expression of the γ/δ T-cell receptor on intestinal CD8$^+$ intraepthelial lymphocytes. *Nature* (London); **333:** 855-858.

Gorrell MD, Willis G, Brandon MR, and Lascelles AK. (1988). Lymphocyte phenotypes in the intestinal mucosa of sheep infected with *Trichostrongylus colubriformis*. *Clin Exp Immunol;* **72**: 274-279.

Greenwood JH, Austin LL, and Dobbins WO. (1983). *In vitro* characterization of human intestinal intraepithelial lymphocytes. *Gastroenterology;* **85**: 1023-1035.

Groh V, Porcelli S, Fabbi M, and Lanier L. (1989). Human lymphocytes bearing the T cell receptor γ/δ are phenotypically diverse and evenly distributed throughout lymphoid system. *J Exp Medp;* **169**: 1277-1294.

Gross GG, Schwartz VL, Stevens C, *et al.* (1994). Distribution of dominant T-cell receptor β chains in human intestinal mucosa. *J Exp Med*; **180**: 1337-1344.

Grossi CE and Ferrarini M. (1982). Morphological and cytochemistry of human large granular lymphocytes. In: Heberman RB. eds. NK Cells and Other Natural Effector Cells. NY: Academic Press Inc, 11.

Groux H, *et al.* (1998). Interleukin 10 is a growth factor for a population of regulatory T-cells. *Gut*; **42**: 157-8.

Groux H, O' Garra A, Bigler M, *et al.* (1997). CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature*; **389:** 737-41.

Guy-Grand D and Vassalli P. (1993). Gut intraepithelial T lymphocytes. *Curr Opin Immunol;* **5**: 247-252.

Guy-Grand D, Cerf-Bensussan N, Malissen B, Malassis-Seris M, Briottet C, and Vassalli P. (1991). Two gut intraepitrelial CD8+ lymphocyte populations with different T cell receptors: a role for the gut epithelium in T cell differentiation. *J Exp Med;* **173**: 471-481.

Guy-Grand D, Griscelli C, and Vassalli P. (1978). The mouse gut T-lymphocyte, a novel type of T-cell. Nature, origin, and traffic in mice in normal and graft-versus-host conditions. *J Exp Med*; **148** : 1661-77.

Guy-Grand D, Malassis-Seris M, Briottet C, and Vassalli P. (1991). Cytotoxic differentiation of mouse gut thymodependent and independent intraepithelial T lymphocytes is induced locally. Correlation between functional assays, presence of perforin and granzyme transcripts, and cytoplasmic granules. *J Exp Med*; **173**: 1549-1552.

Gyorffy EJ, Glogaver M, Kennedy L, *et al.*. (1992). T-cell receptor-γ/δ association with lymphocyte population in sheep intestinal mucosa. *Immunology*; **77**: 25-30.

Halstensen TS and Brandtzaeg P. (1993). Activated T lymphocytes in celiac lesion: non-proliferative activation (CD25) of $CD4^+$ α/β cells in the lamina propria, but proliferation (Ki-67) of α/β and γ/δ cells in the epithelium. *Eur J Immunol*; **23**: 505-510.

Halstensen TS and Brandtzaeg P. (1993). Activated T-lymphocytes in the celiac lesion:non-proliferative activation (CD25) of $CD4^+$ α/β cells in the lamina propria but proliferation (Ki-67) of α/β and γ/δ cells in the epithelium. *Eur J Immunol;* **23**: 505-510.

Halstensen TS, Farstad IN, Scott H, Fausa O, and Brandtzaeg P. (1990). Intraepithelial TcR α/β^+ lymphocytes express more CD45R0 than the TcR γ/δ^+ counterparts in coeliac disease. *Immunology*; **71**: 460-466.

Halstensen TS, Scott H, and Brandtzaeg P. (1989): Intraepithelial T cells of the TCR $\gamma\delta^+$ $CD8^-$ and$V\delta 1/J\delta 1^+$ phenotypes are increased in coeliac disease. *Scand J .Immunol*; **30**: 665-672.

Han D, Plaisance S, Rubinstein E, Alileche A, *et al.* (1995). Non random activation of endogenous IL-2, IL-2Rα and IL-2Rβ genes after transfection of mouse fibroblasts with a cDNA for the α chain of the human IL-2R. *Eur J Immunol*; **25**: 1905-1912.

Hayakawa K, Lin BT, and Hardy RR. (1992). Murine thymic $CD4^+$ T-cell subsets: A subset (Thy^0) that secrets diverse cytokines and overexpresses the Vβ8 T cell receptor gene family. *J Exp Med*; **176**: 269-274.

Helgeland L, Brandtzaeg P, Roland B, and Vaage JT. (1997). Sequential development of intraepithelial γ/δ and α/β T lymphocytes expressing CD8αβ in neonatal rat intestine: requirement for the thymus. *Immunology;* **92**: 447-456.

Herbert JJ and Sunshine P. (1969). Postnatal development of the small intestine of the rat. Changes in morphology of weaning . *Pediatr Res*; **3**: 27-33.

Herbst JJ and Sunshine P. (1969). Postnatal development of the small intestine of the rat. Changes in mucosal morphology at weaning. *Pediatr Res*; **3**: 27-33.

Herman A, kappler JW, Marrack P, and Pullen AM. (1991). Superantigens: mechanism of T-cell stimulation and role in immune responses. *Annu Rev immunol*; **9**: 745-772.

Hirokawa K, Sato K, and Makinodan T. (1982). Influence of age of thymic grafts on the differentiation of T-cells in nude mice. *Clin Immunol Immunopathol*; **24**: 251-262.

Hoang P, Dalton HR, and Jewell DP. (1991). Human colonic intraepithelial lymphocytes are suppressor cells. *Clin Exp Immunol;* **85**: 498-503.

Huleatt JW and Lefrancois L. (1995). Antigen-driven induction of CD 11c on intestinal intraepithelial lymphocytes and CD8+ T-cells *in vivo*. *J Immunol*; 5684-5693.

Hunt SV. (1987). "preparation of lymphocytes and accessory cells" In: Klaus GGB. eds. Lymphocytes a practical approach. Oxford: IRL Press, 1-34.

Huntley JF, McGorum B, Newlands GFJ, and Miller HRP. (1984). Granulated intraepithelial lymphocytes; their relationship to mucosal mast cells and globule leukocytes in the rat. *Immunology*; **53**: 525-35.

Hurst S and Barrett TA. (1996). T-cell responses to enteric antigens. *Semin Gastrointest Dis*. **7**: 118-123.

Hurst SD, Linsley PS, and Barrett TA. (1995). Lamina propria T-cells are a committed subset which produce a unique cytokine profile and are highly dependent upon costimulation via B7. *Clinical Immunology and Immunopathology*; **76**: S80-S119.

Imaoka A, Matsumoto S, Setoyama H, Okada Y, and Umesaki Y (1996). Proliferative recruitment of intestinal intraepithelial lymphocytes after microbial colonization of germ-free mice. *Eur J Immunol*; **26**: 945-948.

Imboden JB. (1994). Lymphocytes and lymphoid tissues as well as T-lymphocytes and natural killer cells. In: Stites DP, Terr AI, and Parslow TG. eds. *Basic and Clinical Immunology*; **8th ed**. London: Prentice-Hall International Inc., Chs. 2 and 8; 26-29 and 94-104.

Irani AA, Craig SS, DeBlois G, Elson CO, Schechter NM and Schwartz LB. (1987). Deficiency of the tryptase-positive, chymase-negative mast cell type in gastrointestinal mucosa of patients with defective T-cell function. *J Immunol*; **138:** 4381-4386.

Ishida A, Yasunobu Y, Shinji M, Kubo C, Hidaka Y, and Nomoto K. (1992). Consumption of milk from cows immunzed wih intestinal bacteria influences age related changes in immune competence in mice. *J nutr*; **122**: 1875-1883.

Ishikawa H, Li T, Abeliovich A, Yamamoto S, *et al.*. (1993). Cytotoxic and IFN-γ-producing activities of γ/δ T-cells in the mouse intestinal epithelium are strain dependent. *Proc Natl Acad Sci* (USA); **90**: 8204-8.

Ishizaka T and Ishizaka K. (1984). Activation of mast cells for mediator release through IgE receptors. *Prog Allergy*; **34**: 188-235.

Ito K, Van Kaer L, Bonneville M, *et al.* (1990). Recognition of the product of a novel MHC TL region gene (27b) by a mouse γ/β T-cell receptor. *Cell*; **62**: 549-561.

James SP and Zeitz M. (1994). Human gastrointestinal mucosal T-cells. In: Ogra PL, Mestecky J, Lamm ME, Strober W, McGhee JR, and Bienenstock J. eds. *Handbook of Mucosal Immunology.* San Diego: Academic Press Inc; 275-285.

James SP, Fiocchi C, Graeff AS, and Strober W. (1985). Immunoregulatory function of lamina propria T-cells in Crchn's disease. *Gastroenterology*; **88**: 1143-1150.

James SP, Fiocchi C, Graeff AS, and Strober W. (1986). Phenotypic analysis of lamina propria lymphocytes. Predominance of helper-inducer and cytolytic T-cell phenotypes and deficiency of suppressor-inducer phenotypes in Crohn's disease and control patients. *Gastroenterology*; **91**: 1483-1489.

James SP. (1991). Mucosal T-cell function. *Gastroenterol Clin North Am*; **20**: 597-612.

Janeway CA, Jones B, and Hayday A. (1988). Specificity and function of T cells bearing γ/δ receptors. *Immunol Today*; **9**: 73-76.

Janis EM, Kaufmann SHE, Schwartz RH, and Pardoll DM. (1989). Activation of γ/δ T-cells in the primary immune response to Mycobacterium tuberculosis. *Science;* **244** (4905): 713-716.

Jarry A, Cerf-Bensussan N, Brousse N, Francoise S, and Guy-Grand D. (1990). Subsets of $CD3^+$ (T-cell receptor α/β or γ/δ) and $CD3^-$ lymphocytes isolated from normal human gut epithelium display phenotypical features different from their counter parts in peripheral blood. *Eur J Immunol*; **20**: 1097-1103.

Jarry A, Cerf-Bensussan N, Guy-Grand D, Muzeau F, and Potet F. (1988). Same peculiar subset of HML-1+ lymphocytes present within normal intestinal epithelium is associated with tumoral epithelium of gastrointestinal carcinomas. *Gut*; **29**: 1632-1638.

Jenkins D, Goodall A, and Scott BB. (1986). T-lymphocyte populations in normal and coeliac small intestinal mucosa defined by monoclonal antibodies. *Gut*; **27**: 1330-1337.

Kagnoff MF and Eckmann L. (1997). Epithelial cells as sensors for microbial infection. *J Clin invet*; **100**: 6-10.

Kagnoff MF. (1987). Immunology of the digestive system. In: Johnson LR, Christensen J, Grossman MI, Jacobson ED, and Schultz SG. eds. *Physiology of the Gastrointestinal Tract.* NY: Raven Press, **2**: 1699-1728.

Kagnoff MF. (1993). Immunology of the intestinal tract. *Gastroenterology*; **105**: 1275-1280.

Kanamori Y, *et al.* (1996). Identification of novel lymphoid tissues in murine intestinal mucosa where clusters of c-kit$^+$ IL-7R$^+$ Thy1$^-$ lympho-hemopoietic progenitors develop. *J Exp Med*; **184**: 1449-1459.

Kansas GS, Wood GS, Fishwild Dm, and Engleman EG. (1985). Functional Characterization of Human T Lymphocyte Subsets Distinguished by Monoclonal Anti-Leu-8. *J Immunol*; **134** (5): 2995-3002.

Kanzaki T, Olofsson A, Moren A, *et al.* (1990). TGF-β1 binding protein:a component of the large latent complex of TGF-β1 with multiple repeat sequences. *Cell*; **61**: 1051-61.

Kato T and Owen RL. (1994). Structure and function of intestinal mucosal epithelium. In: Ogra PL, Mestecky J, Lamm ME, Strober W, McGhee JR, and Bienenstock J. eds. *Handbook of mucosal immunology*. San Diego: Academic Press Inc, 11-26.

Kawaguchi M, Nanno M, Umesaki Y, *et al.* (1993). Cytolytic activity of intestinal intraepithelial lymphocytes in germ-free mice is strain dependent and determined by T-cells expressing γ/δ T-cell antigen receptors. *Proc Natl Acad Sci (USA)*; **90**: 8591-4.

Kawaguchi-Miyashita M, *et al* (1996). Development and cytolytic fumction of intestinal intraepithelial T lymphocytes in antigen-minimized mice. *Immunology*; **89**: 268-273.

Kearsey JA and Stadnyk AW. (1996). Isolation and characterization of highly purified rat intestinal intraepithelial lymphocytes. *J Immunol Methods*; **194**: 35-48.

Kehrl JH, Wakefield LM, Roberts AB, Jakowlew S, *et al.* (1986). Production of TGF-β by human T-lymphocytes and its potential role in the regulation of T-cell growth. *J Exp Med* **163**: 1037-1050.

Kelsall B and Strober W. (1996). Distinct populations of dendritic cells are present in the subepithelial dome and T cells regions of the murine Peyer's patch. *J Exp Med*; **183**: 237-247.

Keren DF. (1992). Antigen processing in the mucosal immune system. *Semin Immnol;* **4**: 217-226.

Kikly K and Dennert G. (1992). Evidence for extrathymic developnent of TNK cells $NK1^+$ $CD3^+$ cells responsible for acute marrow graft rejection are present in thymus-deficient mice. *J Immunol;* **149**: 403-412.

Kitamura Y, Kasugai T, Arizono N, and Matsuda H. (1993). Development of mast cells and basophils: Process and regulation mechanisms *Am J Med Sci*; **306**: 185-191.

Kitamura Y. (1989). Heterogeneity of mast cells and phenotypic change between subpopulations. *Annu Rev Immunol*; **7**: 59-76.

Klein J. (1991). Lymphocytes and the rest. *Immunology*. In: Klein J. eds. *Immunology*. Boston, Oxford, London: Blackwell Scientific Publications, 29-49.

Klein J. (1991). Lymphokines and their receptors. In: Klein J eds. *Immunology*. Boston, Oxford, London: Blackwell Scientific Publications, 227-245.

Komano H, Fujiura Y, Kawaguchi M, *et al.* (1995). Homeostatic regulation of intestinal epithelia by intraepithelial γ/δ T-cells. *Proc Natl Acad Sci USA Immunol*; **92**: 6147-6151.

Koyasu S. (1994). $CD3^{+}$ $CD16^{+}$ $NK1.1^{+}$ $B220^{+}$ Large granular lymphocytes arise from both $\alpha/\beta TCR^{+}$ $CD4^{-}$ $CD8^{-}$ and $\gamma/\delta TCR^{+}$ $CD4^{-}$ $CD8^{-}$ cells. *J Exp Med;* **179**: 57-1972.

Kuby J. (1994). Cells and organs of the immune system. In: Kuby J. eds. *Immunology*. NY: W. H. Freeman and Company, 61.

Kulkarni AB, Chang-Goo H, Becker D, *et al.* (1993). Transforming growth factor-β1 null mutation in mice causes excessive inflammatory response and early death. *Proc Natl Acad Sci (USA)*; **90**: 770-774.

Kumar P and Clark M. (1994). Molecular biology, genetic disorders and immunology as well as Gastroenterology. In: Kumar P and Ckark M. eds. *Clinical Medicine*. London: Bailliere, **3rd ed**: 129-140 and 200-204.

Kumar SN, Stewart GL, Steven WM, and Seelig LL. (1989). Maternal to neonatal transmissiion of T cell mediated immunity to *Trichinella spiralis* during lactation. *Immunology*; **68**: 87-92.

Kusumi A, Fujihashi K, Yamamoto M, *et al.* (1995). Characterization of cytokine mRNA expression by $CD8^{+}$, γ/δ T-cells in murine intestinal epithelium. Clinical immunology and immunopathology. The molecular and cellular basis of immunological disease. **76**:S55.

Kutlu T, Cerf-Bensussan N, Brousse N, *et al* (1990): T-cell subpopulations in coeliac disease; Long term follow up with a gluten containing diet. In Mearin EL. Mulder CJJ (eds): International Dicke Symposium. Coeliac disease: 40 years gluten-free. Leiden, The Netherlands; Dordrecht, Kluwer Academic Publishers, 65-70.

Lanier LL, Engleman EG, Gatenby P, Babcock GF, Warner NL, and Herzenberg LA. (1983). Correlation of functional properties of human lymphoid cell subsets and surface marker phenotypes using multiparameter analysis and flow cytometry. *Immun Rev*; **74**: 143-160.

Lanier LL, Philips JH, Tutt M, and Kymar V. (1986). Natural Killer cells: definition of a cell type rather than a function. *J Immunol*; **137**: 2735-2739.

Lantz O and Bendelac A. (1994). An invariant T cell receptor α chain is used by a unique subset of major histocompatibility complex class I- specific $CD4^+$ and $CD4^-8^-$ T cells in mice and humans. *J Exp Med*; **180**: 1097-1106.

Latthe M, Terry L, and MacDonals TT. (1994). High frequency of $CD8\alpha/\alpha$ homodimer-bearing T-cells in human fetal intestine. *Eur J Immunol*; **24**: 1703-1705.

Lee E, Schiller LR, and Fordtran JS. (1988). Quantification of colonic lamina propria cells by means morphometric point counting method. *Gastroenterology*; **94**: 409-418.

Lefrancois L and Goodman T. (1989). *In vivo* modulation of cytolytic activity and Thy-1 expression in TcR-γ/δ^+ intraepithelial lymphocytes. *Science (Wash. Dc)*; **243** (4899): 1716-1718.

Lefrancois L. (1991). Intraepithelial lymphocytes of the intestinal mucosa:curiouser and curiouer. *Semi mmunol*; **3**: 99-108.

Lefrancois L. (1991). Phenotypic complexity of intraepithelial lymphocytes of the small intestinae. *J. Immunol*; **147**: 1746-1751.

Lefrancois L. (1994). Basic aspects of intraepithelial lymphocyte immunobiology. In: Ogra PL, Lamm ME, McGhee JR, Mestecky J, Strober W, and Bienenstock J. eds. *Handbook of mucosal immunology*. San Diego: Academic Press Inc, 287-297.

Leigh RJ, Marsh MN, Crowe P, Kelly C, Garner V and Gordon D. (1985). Studies of intestinal lymphoid tissue IX. Dose dependent, gluten-induced lymphoid infiltration of coeliac jejunal epithelium. *Scand J Gastroenterol;* **20**: 715-719.

Li J, Kudsk KA, Gocinski B, Dent D, Glezer J, and Langkamp-Henken B. (1995). Effects of parenteral and enteral nutrition on gut-associated lymphoid tissue. *J Trauma*; **39**: 44-51.

Lillehoj HS and Bacon LD. (1991). Increase of intestinal intraepithelial lymphocytes expressing CD8 antigen following challenge infection with *Eimeria acervulina*. *Avian Dis;* **35**: 294-301.

Lillie RD. (1977). H. J. Conn's Biological Stains, **9th ed**.; Williams and Wilkins.

Lionetti P, Spencer J, Breese EJ, Murch SH, Taylor J, and MacDonald TT. (1993). Activation of mucosal $V\beta 3^{+}$ T cells and tissue damage in human small intestine by the bacterial superantigen, Staphylococcus aureus enterotoxin B. *Eur J Immunol*; **23**: 664-668.

Liu L and MacPherson GG. (1995). Rat intestinal dendritic cells: Immunostimulatory potency and phenotypic characterization. *Immunology*; **85**: 88-93.

London SD, Ceba JJ, and Rubin DH. (1989). Intraepithelial lymphocytes contain virus-specific, MHC-restricted cytotoxic cell precursors after gut mucosal immunization with Reovirus serotype 1/Lang. *Regional Immunol;* **2**: 98-102.

Lopez AF, Sanderson CJ, Gamble JR, Campbell HD, Young IG, and Vadas MA. (1988). Recombinant human interleukin 5 is a selective activator of human eosinophil function. *J Exp Med*; **167**: 219-224.

Lundqvist C, Hammarstrom ML, Athlin L, and Hammarstrom S. (1992). Isolation of functionally active intraepithelial lymphocytes and enterocytes from human small and large intestine. *J Immunological Methods*; **152**: 253-263.

Lyscom N and Brueton MJ. (1982). Intraepithelial, lamina propria and Peyer's patch lymphocytes of the rat small intestine: isolation and characterization in terms of immunoglobulin markers and receptors for monoclonal antibodies. *Immunology;* **45**: 775-783.

Lyscom N and Brueton MJ. (1983). The development of intraepithelial and Peyer's patch lymphocyte sub-types in the small intestine of newborn rats. *Clin Exp Immunol*; **54**: 158-162.

MacDonal TT and Spencer J. (1992). Cell-mediated immune injury in the intestine. *Gastro Clins of Nth America*; **21**: 368-371.

MacDonald TT and Spencer J. (1988). Evidence that activated mucosal T cells play a role in the pathogenesis of enteropathy in human small intestine. *J Exp Med*; **167**: 1341-1349.

MacDonald TT and Spencer J. (1990). Gut immunology. *Baillieres Clin Gastroenterol;* **4**: 291-313.

MacDonald TT and Spencer J. (1990). Ontogeny of the mucosal immune response. *Springer. Immunopathol*; **12**: 129-137.

MacDonald TT and Spencer J. (1990). The role of activated T-cells in transformed intestinal mucosa. *Digestion*; **46** (Suppl 2): 290-296.

MacDonald TT, Choy M, Hutchings P, and Cooke A. (1990). Activated T-cells and macrophages in the intestinal mucosa of children with inflammatory bowel disease. In: MacDonald TT. *Advances in Mucosal* Immunology. London: The eight International Congress of Mucosal Immunology, **S211:** 683-690.

MacDonald TT. (1990). The role of activated T-lymphocytes in gastrointestinal disease. *Clin Exp Allergy*; **20**: 247-252.

Madden K, Urban J, Zittener H, Schrader J, Finkelman F, and Katona I. (1991). Antibodies to IL-3 and IL-4 suppress helminth-induced intestinal mastocytosis. *J Immunol;* **147**: 1387-1391.

Mahida YR, Patel S, Wu k, *et al.* (1988). Interleukin 2-receptor expression by macrophages in inflammatory bowel disease. *Clin. Exp Immunol*; **74**: 382-386.

Makinodan T and Kay MMB. (1980). Age influence on the immune system. *Adv Immunol;* **29**: 287- 330.

Marrack P and Kappler J. (1990). The Staphylococcal enterotoxins and their relatives. *Science*; **248**: 705-711.

Marsh MN and Cummins AG. (1993). The interactive role of mucosal T-lymphocytes in intestinal growth, development and enteropathy. *J Gastro Hepato*; **8**: 270-278.

Marsh MN. (1980). Studies of intestinal lymphoid tissue. Quantitative analysis of epithelial lymphocytes in the small intestine of human control subjects and of patients with celiac sprue. *Gastroenterology*; **79**: 481-92.

Massague J. (1990). THE TRANSFORMING GROWTH FACTOR-β FAMILY. *Ann Rev Cell Biol*; **6**: 597-641.

Matsumura T, Kuroume T, Iwasaki I, Oguri M, and Matsumoto T. (1967). Studies on congenital sensitization. I. Detection of food antigens and antibodies in the maternal serum, umbilical cord serum, and the 1st voided urine of newborn infants, amniotic fluid and meconium. *Arerugi*; **16**: 858-865.

Matsumura T, Kuroume T, Oguri M, *et al.* (1974). Demonstration of haemagglutinating antibody against food antigens in the amniotic fluid and cord blood. *Allerg Immunol;* **20-21** (4): 373-384.

Mayer L and Schlien R. (1987). Evidence for function of Ia molecules on gut epithelial cells in man. *J Exp Med;* **166**: 1471-1483.

Mayer L, Panja A, Li Y, *et al.* (1992). Unique features of antigen presentation in the intestine *Ann NY Acad Sci*; **664:** 39-46.

Mayrhofer G and Fisher R. (1979). Mast cells in severely T-cell depleted rats and the response to infestation with *Nippostrongylus brasiliensis. Immunology*; **37**: 145-55.

Mayrhofer G and Whately RJ. (1983). Granular intraepithelial lympocytes of the rat small intestine. I. Isolation, presence in T lymphocyte-deficient rats and bone marrow origin. *Int Arch Allergy Appl Immunol*; **71**: 317-327.

Mayrhofer G, Pugh C, and Barclay A. (1983). The distribution, ontogeny and origin in the rat of Ia-positive cells with dendritic morphology and Ia antigen in epithelia, with special reference to the intestine. *Eur J Immunol*; **13:** 112-122.

Mayrhofer G. (1979). The nature of the thymus dependency of mucosal mast cells. II. The effect of thymectomy and of depleting recirculating lymphocytes on the response to *Nippostrongylus brasiliensis*. *Cell Immunol;* **47**: 312-322.

Mayrhofer G. (1980). Thymus-dependent and thymus-independent subpopulations of intestinal intraepothelial lymphocytes: a granular subpopulation and of probable bone marrow origin and realtionship to mucosal mast cells. *Blood*; **55**: 532-535.

Mayrhofer G. (1994). Epithelial Disposition of Antigen. In: Goldie R. eds. *The Handbook of Immunopharmacology; Immunopharmacology of Epithelial Barriers*. London, San Diego: Academic Press Inc., 19-70.

McGhee JR and Kiyono H. (1994). Effective mucosal immunity. Current concepts for vaccine delivery and immune response analysis. *Int J Technol Assessment in Health Care*; **10**: 93-106.

McGhee JR, Beagley KW, Fujihashi K, *et al.* (1993). Regulatory mechanisms in mucosal immunity: roles for T-helper cell subsets and derived cytokines in the induction of IgA response. *Local immunity in reproductive tract tissues*; 17-51.

McMabe RP, Secrist H, Botney M, Egan M, and Peters MG. (1993). Cytokine mRNA expression in intestine from normal and inflammatory bowel disease patients. *Clin Immunol .Immunopathol*; **66**: 52-58.

Metzger H, Alcaraz G Hohman R, Kinet JP, Pribluda V, and Quarto R. (1986). The receptor with high affinity for immunoglobulin E. *Ann Rev Immunol;* **4**: 419-70

Michie SA, Garcia CF, Strickler JG, Dailey MO, Rouse RV, and Warnke RA. (1987). Expression of the Leu-8 Antigen by B-cell Lymphomas. *Am J pathol*; **88**: 486.

Miescher GC, Howe RC, Lees RK, and MacDonald HR. (1988). CD3 associated alpha/beta and gamma/ delta heterodimeric receptors are expressed by distinct populations of $CD4^-$ $CD8^-$ thymocytes. *J Immunol*; **140b**: 1779-1782.

Miller A, Lider O, Roberts AB, Sporn MB, and Weiner HL. (1992). Suppressor T-cells generated by oral tolerization to myelin basic protein suppress both *in vitro* and *in vivo* immune responses by the release of transforming growth factor–β after antigen-specific triggering. *Proc Natl Acad Sci (USA)*; **89**: 421-425.

Mosley RL and Klein JR. (1992). A rapid method for isolating murine intestine intraepithelial lymphocytes with high yield and purity. *J Immunological Methods*; **156**:19-25.

Mosley RL, Whetsell M, and Klein JR. (1991). Proliferative properties of murine intestinal intraepithelial lymphocytes (IEL): IEL expressing TcRα/β or TcR γ/δ are largely unresponsive to prolrative signals mediated via conventional stimulation of the CD3-TcR complex. *Int Immunol*; **3**: 563-569.

Mossman T, and Coffman R. (1989). T_H1 and T_H2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol;* **7**: 145-173.

Mowat AM and Ferguson A. (1981). Hypersensitivity in the small intestinal mucosa. V. Induction of cell-mediated immunity to a dietary antigen. *Clin Exp Immunol*; **43**: 574-582.

Mowat AM and Ferguson A. (1982). Intraepithelial lymphocyte count and crypt hyperplasia measure the mucosal component of the graft-versus-host reaction in mouse small intestine. *Gastroenterology*; **83**: 417-423.

Mowat AM and Viney JL. (1997). The anatomical basis of intestinal immunity. *Immunol Rev*; **156**: 145-166.

Mowat AM, Mackenzie S, Baca ME, Felstein MV, and Parrott DMV. (1986). Functional characteristics of IEL from mouse small intestine. II. *In vivo* and *in vitro* responses of IEL to mitogenic and allogeneic stimuli. *Immunology*; **58**: 627-634.

Mowat AM, McInnos IB, and Parrott DMV. (1989). Functional properties of IEL from mouse small intestines IV. Investigation of the proliferative capacity of IEL using phorbol ester and calcium ionophore. *Immunology*; **66**: 398-403.

Mowat AM. (1987). The regulation of immune responses to dietary protein antigens. *Immunol Today*; **8**: 93-98.

Mowat AM. (1990). Human intraepithelial lymphocytes. *Springer Semin Immunopathol;* **12**: 165-190.

Mowat AM. (1990). Human intraepithelial lymphocytes. *Springer Semin Immunopathol*; **12**: 165-190.

Nadine AJ, Cerf-Bensussan, Brousse N, etal. (1990). Subsets of CD3+ (T cell receptor α/β or γ/δ) and CD3- lymphocytes isolated from normal human gut epithelium display phenotypical features different from their counterparts in peripheral blood. *Eur J Immunol*; **20**: 1097-1103

Nagasawa T, Sano K, and IKe F, *et al.* (1993). Negative selection of thymus-dependent CD4$^+$ CD8$^+$ intestinal intraepithelial lymphocytes by internal superantigens. *Cell Immunol*; **147:** 158-166.

Nawa Y and Miller HRP. (1979). Adoptive transfer of the intestinal mast cell response in rats infected with Nippostrongylus brasiliensis. *Cell Immunol*; **42**: 225-239.

Ness JC, Morgan L, Outzen HC, and Tapper D. (1991). Specific growth factor activity identifies and predicts murine mammary tumor. *J Surg Res*; **50**: 6-14.

Norment AM and Littman DL. (1988). A second subunit of CD8 is expressed in human T-cells. *EMBO J*; **7**: 3433-3439.

O' Brien RL and Born W. (1991). Heat shock proteins as antigens for γ/δ T cells. *Semi Immunol*; **3**: 81-7.

Ohteki T and MacDonald HR. (1994). Major histocompatibility complex class I related molecules control the development of CD4$^+$ 8$^-$ and CD4$^-$ 8$^-$ subsets of natural killer 1.1$^+$ T-cell receptor α/β$^+$ cells in the liver of mice. *J Exp Med*; **180**: 699-704.

Ohteki T, Okuyama R, Seki S, *et al.* (1992). Age-dependent increase of extrathymic T cells in the liver and their appearance in the periphery of older mice. *J Immunol;* **149**: 1562-1570.

Oppenheim JJ, Ruscetti FW, and Faltynek C. (1994). Cytokines. In: Stites DP, Terr AI, and Parslow TG. eds. *Basic and Clinical Immunology*; **8th ed**. London: Prentice-Hall Intrenational Inc., 112-113.

Orlic D, Gray BA, Carmichael RD, and Baron P. (1981). Lymphocyte infiltration of gut epithelium in fetal and neonatal rabbits. *Biol Neonate;* **40**: 91-98.

Owen JR, WF, Rothenberg ME, Silberstein DS, Gasson JC, Stevens RL, Austen KF, and Soberman RJ. (1987). Regulation of human eosinophil viability, density and function by granulocyte/macrophage colony-stimulating factor in the presence of 3T3 fibroblasts. *J Exp Med*; **166**: 129-141.

Pabst HF and Kreth HW. (1980). Ontogeny of the immune response as a basis of childhood disease. *J. Pediatrics*; **97**: 519-534.

Pang G, Buret A, Batey RT, Chen QY, Couch L, Cripps A, and Clancy R. (1993). Morphological, phenotypic and functional characteristics of a pure population of $CD56^+$ $CD16^-$ $CD3^-$ large granular lymphocytes generated from human duodenal mucosa. *Immunology*; **79**: 498-505.

Penney L, Kilshaw PJ, and MacDonald TT. (1995). Regional variation in the proliferation and lifespan of $\alpha\beta TCR^+$ and $\gamma\delta TCR^+$ intraepithelial lymphocytes in murine small intestine. *Immunology*; **86**: 212-218.

Perkkio M and Savilahti E. (1980). Time of appearance of immunoglobulin-containing cells in the mucosa of the neonatal intestine. *Pediatr Res;* **14**: 953-5.

Peschon J, *et al.* (1994). Early lymphocyte expansiion is severely impaired in interleukin 7 receptor-deficient mice. *J Exp Med;* **180** :1955-1960.

Peters M, Secrist H, Anders KR, *et al*: (1986). Increased expression of cell surface activation markers by intestinal mononuclear cells. *Clin Res*.; **35**: 462.

Picker LJ and Butcher EC. (1992). Physiological and molecular mechanisms of lymphocyte homing. *Annu Rev Immunol;* **10** : 561-591.

Poussier P and Julius M. (1994). Thymus independent T-cell development and selection in the intestinal epithelium. *Annu Rev Immunol;* **12**: 521-53.

Price GB and Makinodan T. (1972) Immunologic in Spnescence II: Characterization of extrinsic deficiencies *J Immounol;* **108**: 413-417.

Prizer UC, Schuurman G, Post S, Betzler M, and Meuer SC. (1990). Differential responsiveness to CD3 Ti *vs*. CD2-dependent activation of human intestinal T-lymphocytes. *Eur J Immunol;* **20** : 2339-2344.

Puddington L, Olson S, and Lefrancois L. (1994). Interactions between stem cell factor and c-kit are required for intestinal immune system homeostasis. *Immunity*; **1**: 733-739.

Raulet DH. (1989). The structure, function, and molecular genetics of the γ/δ TcR. *Annu Rev Immunol;* **7**: 175-207.

Reed JC, Abidi AH, Alpers JD, Hoover RG, Robb RJ, and Nowell PC. (1989) Effect of cyclosporin A and dexamethasone on IL-2R gene expression. *J Immunol*; **137**: 150-154.

Reynolds JD, and Morris B. (1983). The evolution and involution of Peyer's patches in fetal and postnatal sheep. *Eur J Immunol*; **13**: 627-631.

Richman L, Graeff AS, and Strober W. (1981). Antigen presentation by macrophage-enriched cells from the mouse Peyer's Patch. *Cell Immunol*; **62**: 110-118.

Roberts AB and Sporn MB. (1988). Transforming growth factor β. *Adv Cancer Res*; **51**: 107-45.

Rocha B, Guy-Grand D, and Vassalli P. (1995). Extrathymic T-cell differentiation. *Curr Opin Immunol*; **7**: 235-242.

Rocha B, Vassalli P, and Guy-Grand D. (1991). The Vβ repertoire of mouse gut homodimeric αCD8$^+$ intraepithelial αβTcR$^+$ lymphocytes reveals a major extrathymic pathway of T-cell differentiation. *J Exp Med;* **173**: 483-486.

Rocha B, Vassalli P, and Guy-Grand D. (1992). The extrathymic T-cell development pathway. *Immunol Today*; **13**: 449-454.

Roitt IV, Brostoff J, and Male DK. (1996). The lymphoid system. eds. *Immunology*, **4th ed**. London: Mosby Inc, 3.1-3.12.

Rothenberg ME, Owen JR, WF, Silberstein DS, Woods J, Rothenberg RJ, Austen KR, and Stavens RL. (1988). Human eosinophils have prolonged survival, enhanced functional properties, and become hypodense when exposed to human interleukin 3. *J Clin Invest*; **81**: 1986-1992.

Rubin LA, Kurman CC, Fritz ME, *et al* (1985). Soluble interleukin-2 receptors are released from activated human lymphoid cells *in vitro*. *J Immunol*; **135**: 3172-3177.

Ruedl C, Rieser C, Bock G, Wick G, and Wolf H. (1996). Phenotypic and functional characterization of CD11c- dendritic cell population in mouse Peyer's patches. *Eur J Immunol*; **26**: 1801-1806.

Ruitenberg EJ and Elgersma A. (1976). Absence of intestinal mast cell response in congenitally athymic mice during *Trichinella spiralis* infection. *Nature (London);* **264**: 258-260.

Russell GJ, *et al.* (1994). Distinct structural and functional epitopes of the $\alpha_E\beta_7$ integrin. *Eur J Immunol*; **24**: 2832-2841.

Rust CJ, Verreck F, Victor H, *et al.* (1990). Specific recognition of Staphylococcal enterotoxin A by human T-cells bearing receptors with the Vγ9 region. *Nature*; **346**: 572-574.

Saito S, Yoshida M, Ichijo M, *et al.* (1993). Transforming growth factor-beta (TGF-β) in human milk. *Clin Exp Immunol*; **94**: 220-224.

Sato K, Ohtsuka K, Haegawa K, *et al.* (1995). Evidence for extrathymic generation of intermediate T cell receptor cells in the liver revealed in thymectomized, irradiated mice subjected to bone marrow transplantation. *J Exp Med*; **182**: 759-767.

Schieferdecker HL, Ullrich R, and Zeitz M. (1991). Phenotype of HML-1 positive T-cells in the human intestinal lamina propria. *Immunol Res*; **10**: 207-10.

Schieferdecker HL, Ullrich R, Hirseland H, and Zeitz M. (1992). T cell differentiation antigens on lymphocytes in the human intestinal lamina propria. *J Immunol;* **149**: 2816-2822.

Schieferdecker HL, Ullrich R, Hirseland H, and Zeitz M. (1992). T-cell differentiation antigens on lymphocytes in the human intestinal lamina propria. *J Immunol*; **149**: 2816-2822.

Schieferdecker HL, Ullrich, R, Weiss-Breckwoldt AN, Schwarting R, Stein H, Riecken EO, and Zeitz M. (1990). The HML-1 antigen of intestinal lymphocytes is an activation antigen. *J Immunol;* **144**: 2541-2549.

Schnorr KL, and Pearson LD. (1984). Intestinal absorption of maternal leukocytes by newborn lambs. *J Reprod Immunol*; **6**: 329-337.

Seelig LL and Billingham RE. (1981). Concerning the natural transplantation of maternal leucocytes in milk. *Transplant Proc*; **13**: 1245-1249.

Segal GH and Petras RE. (1992). small intestine. In: Sternberg SS. eds. *Histology for pathologists*. NY: Raven Press, Ltd., 547-571.

Selby WS, Janossy G, and Jewell DP. (1981). Immunohistological characterization of intraepithelial lymphocytes of the human gastrointestinal tract. *Gut*; **22**: 169-76.

Selby WS, Janossy G, Bofill M, and Jewell DP. (1984). Intestinal lymphocyte subpopulation in inflammatory bowel disease: an analysis by immunohistological and cell isolation techniques. *Gut*; **25**: 32-40.

Selby WS, Janossy G, Bofill M, *et al.* (1983). Lymphocyte subpopulations in the human small intestine. The findings in normal mucosa and in the mucosa of patients with adult coeliac disease. *Clin Exp Immunol*; **52**: 219-228.

Selby WS, Janossy G, Goldstein G, *et al.* (1981). T-lymphocyte subsets in human intestinal mucosa: the distribution and relationship to MHC-derived antigens. *Clin Exp Immunol;* **44**: 453-458.

Senju M, Wu KC, Mahida YR, and Jewell DP. (1991). Tow-color immunofluorescence and flow cytometric analysis of lamina propria lymphocyte subsets in ulcerative colitis and Crohn's disease. *Dig Dis Sci;* **36**: 1453-1458.

Shanahan F, Brogan M, and Targan S. (1987). Human mucosal cytotoxic effector cells. *Gastroenterology*; **92**: 1951-1957.

Shanahan F, Deem R, Nayersina R, Leman B, and Targan S. (1988). Human mucosal T-cell cytotoxicity. *Gastroenterology*, **94** : 960-967.

Shull MM, Ormsby I, Kier AB, *et al.* (1992). Targeted disruption of the mouse transforming growth factor-β1 gene results in multifocal inflammatory disease. *Nature*; **359**: 693-699.

Siebrecht MS, Hsia E, Spychalski C, and Nagler-Anderson C. (1993). Stimulation of murine intestinal intraepithelial lymphocytes by the bacterial superantigen Staphylococcal enterotoxin B. *Int immunol*; **5**: 717-724.

Spalding D, Williamson SI, McGhee JR, and Koopman WJ. (1984). Peyer's patch dendritic cells: Isolation and functional comparison with murine spleen dendritic cells. *Immunobiology* ; **168**: 380-390.

Spencer J, Dillon SB, Isaacson PG, and MacDonald TT. (1986). T cell sub-classes in fetal human ileum. *Clin Exp Immunol*; **65**: 553- 558.

Spencer J, Isaacaon PG, Walker-smith JA, and MacDonald TT. (1989). Heterogeneity in intraepithelial lymphocyte subpopulation in fetal and postnatal human small intestine. *J Pediatr Gastro Nutr*; **9**: 173-177.

Spencer J, Isaacson PG, Diss TC, *et al.* (1989). Expression of disulfide-linked and non-disulfide-linked forms of the T cell receptor γδ heterodimer in human intestinal intraepithelial lymphocytes. *Eur J Immunol*; **19**: 1335-1338.

Spencer J, Isaacson PG, Diss TC, *et al*: (1989). Expression of disulfide-linked and non-disulfide-linked forms of the T cell receptor γ/δ heterodimer in human intestinal intraepithelial lymphocytes. *Eur J Immunol*; **19**: 1335-1338.

Spencer J, MacDonald TT, Diss TC, Walker-Smith JA, Ciclitira PJ, and Isaacson PG. (1989). Changes in intraepithelial lymphocyte subpopulations in coeliac disease and enteropathy assocciated T cell lymphoma (malignant histiocytosis of the intestine). *Gut*; **30**: 339-346.

Spencer J, MacDonald TT, Finn T, and Isaacson PG. (1986). The development of gut associated lymphoid tissue in the terminal ileum of fetal human intestine. *Clin Exp Immunol;* **64**: 536-543.

Sprinz H. (1962). Morphological response of intestinal mucosa to enteric bacteria and its implication for sprue and Asiatic cholera. *Fed Proc*; **21**: 57-64.

Stamenkovic I, Sgroi D, Aruffo A, Sun Sy M. and Anderson T. (1991). The B lymphocytes adhesion molecule CD22 interacts with leucocyte common antigen CD45RO on T-cells and α2-6 sialytransferase, CD75, on B-cells. *Cell*; **66**: 1133-1144.

Strobel S. (1992). Dietary manipulation and induction of tolerance. *J paediatr;* **121**: S74-S79.

Strober W and James SP. (1994). The mucosal immune system. In: Stites DP, Terr AI, and Parslow GP. eds. *Basic and Clinical Immunology*. London: prentice-Hall International Inc., 541-551.

Strominger JL. (1989). The γ/δ TcR and class 1b MHC-related proteins: enigmatic molecules of immune recognition. *Cell*; **57**: 895-898.

Sykes M. (1990). Unusual T cell populations in adult murine bone marrow prevalence of $CD3^{+}CD4^{-}CD8^{-}$ and $\alpha/\beta TCR^{+}NK1.1^{+}$ cells. *J Immunol*; **145**: 3209-3215.

Tagliabue A, Befus AD, Clark DA, and Bienenstock J. (1982). Characteristics of natural killer cells in the murine intestinal epithelium and lamina propria. *J Exp Med;* **155**: 1785-96.

Taguchi T, Aicher WK, Fuhihashi K, *et al.* (1991). Novel function for intestinal intraepithelial lymphocytes. Murine $CD3^{+}$, γ/δ TcR^{+} cells produce IFN-γ and IL-5. *J Immunol;* **147**: 3736-3744.

Taguchi T, *et al.* (1990). Analysis of Th1 and Th2 cells in murine gut-associated tissues. Frequencies of $CD4^{+}$ and $CD8^{+}$ T-cells that secrete IFN-γ and IL-5. *J Immunol*; **145**: 68-77.

Takahama Y, Sharrow SO, and Singer A. (1991). Expression of an unusual T cell receptor (TCR)-Vβ repertoire by Ly-$6C^{+}$ subpopulations of $CD4^{+}$ and/or $CD8^{+}$ thymocytes. Evidence for a developmental relationship between Ly-$6C^{+}$ thymocytes and $CD4^{-}CD8^{-}$TCR-α/β^{+} thymocytes. *J Immunol*; **147**: 2883-2891.

Takimoto H, Nakamura T, Takeuchi M. *et al* . (1992). Age-associated increase in number of $CD4^{+}CD8^{+}$ intestinal intraepithelial lymphocytes in rats. *Eur J Immunol*; **22**: 159-164.

Targan SR, Deem RL, Liu M, Wang S, and Nel A. (1995). Definition of a lamina propria T cell responsive state. Enhanced cytokine responsiveness of T cells stimulated through the CD2 pathway. *J Immunol*; **154**: 664-675.

Thompson F, Mayrhofer G, and Cummins AG. (1996). Dependence of epithelial growth of the small intestine on T cell activation during weaning in the rat. *Gastroenterology* ; **111**: 37-44.

Tokuyama H and Tokuyama Y. (1989). Bovine colostrum transforming growth factor-β like peptide that induces growth inhibition and changes in morphology of human osteogenic sarcoma cells (MG-63). *Cell Biol Int Rep*; **13**: 251-8.

Tomassini M, Tsicopoulos A, Tai PC, Gruart V, Tonnel AB, Prin L, Capron A, Capron A, and Vapron M. (1991). Release of granule proteins by eosinophils from allergic and non-allergic patients with eosinopholia on immunoglobulin-dependent actvation. *J Allergy Clin Immunol*; **88**: 365-375.

Torres-Nagel N, Kraus E, Brown MH, Tiefenthaler G, Mitnacht R, Williams AF, and Hunig T. (1992). Differential thymus dependence of rat CD8 isoform expression. *Eur J Immunol*; **22**: 2841-2848.

Trejdosiewicz LK. (1992). Intestinal intraepithelial lymphocytes and lymphoepithelial interactions in the human gastrointestinal mucosa. *Immunol Letters*; **32**: 13-20.

Trejdosiewicz LK. (1993). What is the role of human intestinal intraepithelial lymphocytes? *Clin Exp Immunol*; **94**: 395-397.

Trier J. (1991). Structure and function of intestinal M cells. *Gastroenterol Clin North Am*; **20**: 531-547.

Trinchieri G. (1989). Biology of natural killer cells. *Adv Immunol*; **47**: 187-376.

Umesaki Y, Setoyama H, Matsumoto S, and Okada Y. (1993). Expansion of α/β T-cell receptor-bearing intestinal intraepithelial lymphocytes after microbial colonization in germ-free mice and its independence from thymus. *Immunology*; **79**: 32-37.

Vaage JT, Dissen E, Agar A, *et al.* (1990). T cell receptor-bearing cells among rat intestinal intraepithelial lymphocytes are mainly α/β^+ and are thymus dependent. *Eur J Immunol*; **20**: 1193-1196.

Van Garderen E, Van Dijk JE, Van Den Ingh TSGAM. (1991). The biology and pathobiology of the intestinal intraepithelial lymphocyte: a review of the literature. *Veterinary Quartery*; **13**: 225-232.

Versteeg R. (1994). NK cells and T-cells: mirror images? In: Kuby J. eds. *Immunology.* NY: WH Freeman and Company, 356-357.

Vicari A, Abehsira-Amar O, Papiernik M, Boyd RL, and Tucek CL. (1994). MTS-32 monoclonal antibody defines $CD4^+8^-$ thymocyte subsets that differ in their maturation level, lymphokine secretion, and selection patterns. *J Immunol;* **152**: 2207-2213.

Vicari AP and Zlotnik A. (1996). Mouse NK 1.1^+ T-cells: a new family of T-cells. *Immunol Today;* **17**: 71-76.

Vidovic D, Roglic M, McKune K, *et al.* (1989). Qa-1 restricted recognition of foreign antigen by a γ/δ T-cell hybridoma. *Nature*; **340**: 646-650.

Viney J, MacDonald TT, and Spencer J. (1990). Gamma/delta T-cells in the gut epithelium. *Gut*; **31**: 841-844.

Viney JL and MacDonald TT. (1992). Lymphokine secretion and proliferation of intraepithelial lymphocytes from murine small intestine. *Immunology;* **77**: 19-24.

Viney JL, Kilshaw PJ, and MacDonald TT. (1990). Cytotoxic α/β^+ and γ/δ^+ T-cells in murine intestinal epithelium. *Eur J Immunol*; **20**: 1623-1626.

Wade AW and Szewezuk MR. (1984). Aging, idiotype repartoire shifts, and compartmentalization of the mucosal-assoliated lymphoid system. *Adv Immunol*; **36**: 143-188.

Wahl SM, Hunt DA, Bansal G, McCartney-Francis N, *et al.* (1988). Bacterial cell wall-induced immunosuppression. Role of TGF-β. *J Exp Med*; **168**: 1403-1417.

Wakefield LM, Thompson NL, Flanders KC, O'Connor-McCourt MD, and Sporn MB. (1988). Transforming growth factor β: multifunctional regulator of cell growth and phenotype. *Ann NY Acad Sci;* **551**: 290-298.

Walker WA. (1986). Antigen handling by the small intestine. *Clins in Gastroenterology*; **15**: 1-20.

Wang J, Whetsell M, and Klein JR. (1997). Local hormone networks and intestinal T cell homeostasis. *Science;* **275**: 1937-1939.

Watanabe H, Miyaji C, Kawachi Y, *et al.* (1995). Relationships between intermediate TCR cells and NK1.1$^+$ T cells in various immune organs. *J Immunol;* **155**: 2972-2983 .

Watanabe M, *et al.* (1995). Interleukin 7 is produced by human intestinal epithelial cells and regulates the proliferation of intestinal mucosal lymphocytes. *J Clin Invest*; **95**: 2945-2953.

Watkins SG, Dearin JL, Yong LC, and Wilhelm DL. (1976). Association of mastopoiesis with haemopoietic tissues in the neonatal rat. *Experientia*; **32**: 1339-1340.

Weiler IJ, Jickler W, and Sprenger R. (1983). Demonstration that milk cells invade the suckling neonatal mouse. *Am J Reprod Immunol*; **4:** 95-98.

Wilders MM, Sminia T, and Janse EM. (1983). Ontogeny of non-lymphoid and lymphoid cells in the rat gut with special reference to large mononuclear Ia-positive dendritic cells. *Immunology*; **50**: 303-314.

Williams AF, Galfre G, and Milstein C. (1977). Analysis of cell surface by xenogeneic myeloma-hybrid antibodies: Differentiation antigens of rat lymphocytes. *Cell*; **12**: 663-673.

Williams DG. (1994). Immunoreactivity and Inflammation in Renal Epithelium. In: Goldie R. eds. *The Handbook of Immunopharmacology; Immunopharmacology of Epithelial Barriers*. London, San Diego: Academic Press Inc., 225-240.

Winoto A, and Baltimore D. (1989). Separate lineages of T-cells expressing the alpha beta gamma delta receptors. *Nature*; **338**: 430-432.

Woodbury RG and Neurath H. (1978). Purification of an atypical mast cell protease and its levels in developing rats. *Biochemistry*; **17**: 4298-4304.

Woodward JM, Jenkinson E, and Owen JJT. (1998). Lymphocyte ontogeny in the murine intestinal epithelium-An immunohistochemical study. *Gut* (supplement); **42**: F89.

Yamamoto M, Fujihashi K, Amano M, *et al.* (1994). Cytokine synthesis and apoptosis by intestinal intraepithelial lymphocytes:signaling of high density α/β T cell receptor^{+} and γ/δ T cell receptor^{+} T-cells via T cell receptor-CD3 complex results in γ-IFN and IL-5 production, while low density T-cells undergo DNA fragmentation. *Eur J Immunol*; **24**: 1301-1306.

Yamamoto M, Fujihashi K, Beagley KW, *et al.* (1993). Cytokine synthesis by intestinal intraepithelial lymphocytes. Both γ/δ TcR-positive and α/β TCR-positive T-cells in the G1 phase of cell cycle produce IFN-γ and IL-5. *J Immunol*; **150**: 106-114.

Zaitz M, Greene WC, Peffer NJ, and James SP. (1988). Lymphocytes from the intestinal lamina propria of normal non-human primates have increased expression of genes associated with T-cell activation. *Gastroenterology;* **94**: 647-55.

Zeitz M, Quinn TC, Graeff AS, and James SP. (1988). Mucosal T-cells provide helper function but do not proliferate when stimulated by specific antigen in lymphogranuloma venerum proctitis in nonhuman primates. *Gastroenterology*; **94**: 353-66.

Erratum

P48 line 2 "abdomine" → "abdomen"
P50 line 3 "Although it is<u>a</u> very ..."
line 22 : "*glass*" → "glass"
P55 line 4/8 "minutes" → "mins"
P56 line 15 "0.01%" → "0.01% wt/vol"
line 16 "Scott'a" → "Scott's"
P59 line 3 "naphtol" → "naphthol"
P60 line 8 "Alumminium" → "Aluminum"
P61 line 15 "para-corttical" → "paracortical"
P63 line 12 "Cellconts" " → "Cell counts"
P65 line 6 "co-inciding" → "coinciding"
line 16 "Although, much" → "Although much"
P67 line 17 "Crypt cellproageration" → "Crypt cell proliferation"
P73 line 13 "form" → "from"
P74 line 6 "same" → "some"
line 7 "latter" → "refers back to the count of CD4 + CD8 + double-positive IEL with a mixture of antibodies against these two markers.
P82 line 18 "continous" → "continuous"
P87 line 5 "parrafin" → "paraffin"
P101 line2 "*adult*" → "adult"

Data points on figure 5.4 and 6.4, pages 111 and 124 onwards, respectively relate to litters rather than individual pups.

P112 line 10 "*intensity*" → "intensity"
P116 last two lines must be ignored.
P117 last line "was ? " → "was performed"

P137 line 2 onwards is also describing the results for expression of IL-2R on LFV cells. To accomplish this, **<u>Table 7.1</u>** should be added to the end of line 5 "The mean proportion of IL-2R+ cells at day 19 was 55 (±8.2), whereas at day 70 it was 33 (±6.0) (**<u>Table 7.1</u>**).

P138 line 4, (Table 7.1) "∂" → "this symbol means that there was only one value for counting of IL-2R on LFV cells, hence no SE".

P153 line 9 "persented" → "presented"