NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

REGIONAL VARIATION IN EXPRESSION OF MAJOR HISTOCOMPATIBILTY CLASS II ANTIGENS IN ENTEROCYTES OF POSTNATAL AND ADULT MICE

A thesis

Submitted to the Graduate Faculty

in Partial Fulfilment of the Requirements

for the Degree of

Master of Science

in the Department of Anatomy and Physiology

Faculty of Veterinary Medicine

University of Prince Edward Island

Nirmal K. Sidhu

Charlottetown, P.E.I.

April, 1991

ISBN 0-315-62407-8

Canada

SIGNATURE PAGES

iii-iv

REMOVED

ABSTRACT

The constitutive expression and ontogeny of major histocompatibility class II antigens in enterocytes of small intestine of adult and postnatal C3H/He mice were investigated. Cryosections of duodenal, jejunal, and ileal segments from 7-, 14-, 16-, 18-, 19-, 20-, 21-, 23-, 25-, 27-, 28-day-old, and 7-week-old mice were stained for the class II antigens with MRC OX6 monoclonal antibodies by peroxidase-antiperoxidase labelling. In adults, the immunoreaction revealed that the expression of class II antigens was greatest in ileum and decreased proximally towards the duodenum. The villus epithelium of the duodenum showed a granular staining pattern in the apices of some cells. In jejunum, granular immunostaining within apical and basal regions of the cells lining the villus was observed. The cells at the tip of the villus showed strong lateral surface staining. Ileal enterocytes demonstrated an intense cytoplasmic and baso-lateral immunoreaction for the antigens. In the crypt epithelium, the staining also increased in the proximo-distal direction along the length of the small intestine. This regional variation of class II molecules in the enterocytes may influence regional differences in antigen presenting function and immune responsiveness to ingested antigens.

Ontogenic studies revealed that the first appearance (21 days postnatal) of class II antigens in enterocytes coincides with age of weaning, suggesting that immunologic stimulation by ingested antigens after weaning may influence expression of these antigens. There is an age-dependent regional variation in expression of class II antigens in enterocytes, and the expression increases with age. In duodenum and jejunum, the expression in the villus epithelium was first seen 21 days after birth, and the ileal enterocytes were negative. At day 28 after birth, the duodenum and jejunum expressed levels of class II antigens comparable to those of adult levels. The ileum first showed an expression at 28 days after birth but it was far less than that of adult. The crypt epithelium of three regions of the small intestine showed expression similar to that of corresponding regional villus enterocytes. The variation in expression of class II antigens in enterocytes of postnatal mice is attributed to the developmental status of the tissue. The nature of adult and postnatal expression of the antigens is discussed since altered expression of the class II antigens in intestinal epithelium of adults and an early appearance of these antigens may have important implications in oral immunization and autoimmunity, respectively.

DEDICATION

To my parents: with much love and deep respect.

ACKNOWLEDGEMENTS

Many people gave assistance to me during the course of this project but I am most deeply indebted to my supervisory committee: Drs. G. Wright, F. Markham, J. Amend for their invaluable advice and help, and precious time. I would also like to gratefully acknowledge my supervisor and guru Dr. Amreek Singh for his patience, encouragement, and giving me this oppurtunity.

My gratitude to all those friends who helped in a variety of ways: Janice Gillis, Dianne Friesen, Rosemary Hood, Thomas Klein, Hal McRae, Sandra Vokaty, Barry Connell, Mark Powell, Anna Dykeman, and Margaret Horne. Also, I would like to acknowledge Carla Smith, Sharon Gromley, Cheryl Gaudet for their patience and help during preparation of this manuscript. Help from Audiovisual Services of the University of Prince Edward Island, especially Shelley Ebbett, Michelle Gauthier, and Glenda Clements is greatly appreciated.

My special thanks to Agriculture Canada, and to Department of Anatomy and Physiology for the financial assistance.

My most sincere thanks to Amrit Chohan and Yatri Drastini, both dear friends whose moral support and confidence I deeply cherish.

Finally, I owe a special debt of gratitude to my sisters, Kanwalpreet Sarkaria, Simerdeep Nijjer and Rajbir Sidhu, and my cousins Nirmaljit Sra and Devinder Brar for their encouragement and always being their when I needed them.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

1. GENERAL INTRODUCTION

A. EXPRESSION OF MAJOR HISTOCOMPATIBILITY CLASS II ANTIGENS IN ENTEROCYTES

1.1 Introduction

The absorptive epithelium (enterocytes) of the small intestine comprises an extensive area exposed to ingested antigens. Mechanisms that distinguish immunogenic or tolerogenic responsiveness to antigens are important in protection of the host and in pathogenesis of gastrointestinal diseases (1). Peyer's patches and other organized intestinal lymphoid tissues are undoubtedly involved in the regulation of immune responses to intestinal antigenic challenge (2). However, role of enterocytes in induction and regulation of immune responses to ingested antigens in small intestine is underestimated. A variety of immunohistochemical studies have shown that enterocytes express major histocompatibility complex (MHC) class II antigens (3,4,5), which suggests that enterocytes may be involved in initial antigen handling and possible presentation of ingested antigens to immune cells within the lamina propria or intraepithelial lymphocytes (IELs) of the small intestine (6).

1.2 Histological organization of small intestine

In general, the wall of small intestine is made of four concentric layers; serosa, muscularis, submucosa, and mucosa (7). The mucosa is the most important layer of small intestine in relation to absorptive, digestive and secretory functions. Adult mucosal epithelium, having the thickness of a single cell, is arranged into tubular invaginations, crypts, and finger-like projections, known as villi (8). The

proliferative phase of an intestinal crypt cell is approximately one day, and the life span of a villus cell is approximately two days (9). Generally a villus is supplied with cells from approximately three crypts (9). Proliferation of the cells is confined to the base of the crypts, from where the cells migrate to the villi and are finally shed at the tip of the villus. Adult crypt epithelium may descend from a single progenitor cell which may give rise to several stem cells responsible for cell renewal in a fully formed crypt (8).

The epithelium covers the free surface of the mucosa, in which five cell types can be distinguished - enterocytes, M cells (membranous), goblet cells, caveolated cells, and endocrine cells. The enterocytes of villi are tall and columnar, approximately 25 μm in length and 8 μm wide, and contain an oval nucleus. The apical striated or brush border has numerous microvilli that are arranged in a parallel array; they extensively increase the cell surface area exposed to the luminal contents (10). Peyer's patches are separated from luminal contents by a layer of low columnar epithelial cells, M cells; these cells are absent from the villi. The apical surface of M cells has numerous microfolds, and microvilli are wider and less abundant than those of the enterocytes. The M cells have a central invagination at their proximal end which is occupied by B or T lymphocytes; lymphocytes may shuttle across basal lamina between M cells and lymphoid follicle. The M cells may transport macromolecules and microorganisms that cannot penetrate villus epithelium to the underlying lymphoid population (11,12).

1.3 Major histocompatibility complex

The MHC is a polymorphic genetic system possessing genes (Ir) coding the production and expression of cell membrane glycoproteins. These genes in humans (HLA on chromosome 6) and in mice (H-2 on chromosome 17) may control cell-mediated and humoral immune responses. The MHC of the mouse is divided into six regions called K, I, S, D, Qa, and T1a (Fig. 1). The I region codes for class II molecules, the K, D, Qa, and T1a regions code for class I molecules, and S region codes for certain enzymes and some complement components (13).

1.4 Structure of class II antigens

Class II molecules are composed of two polypeptide chains, α- and β-, which have variable (Vα, Vβ) and constant (Cα, Cβ) extracellular protein domains that are maintained by intrachain disulfide bonds as illustrated in Figure 2 (14). The transmembrane portion of the molecule spans the cell membrane and the carboxyl ends extend into the cytoplasm. Molecular weights of α and β polypeptides are approximately 34,000 and 28,000 daltons respectively.

1.4.1 Tissue distribution of class II antigens

Class II antigens are present on a variety of cells participating in the immune response, especially in those cells with antigen presenting capacity. These include Langerhans cells of the skin, macrophages, dendritic cells, B lymphocytes and activated T cells (15,16). Certain parenchymal cells also possess these antigens although exact role of these antigens has not been elucidated. In mice, class II antigens have been identified using immunohistochemical techniques, on enterocytes,

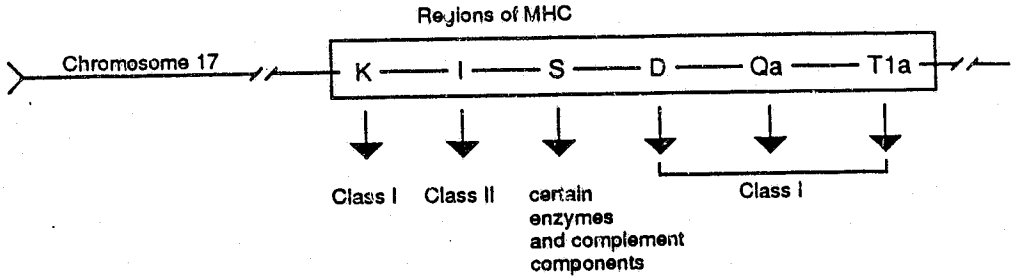


Figure 1. Diagrammatic illustration of major histocompatibility complex (MHC) of mouse. [After Collins, T. et al. (1986) See Reference 13]

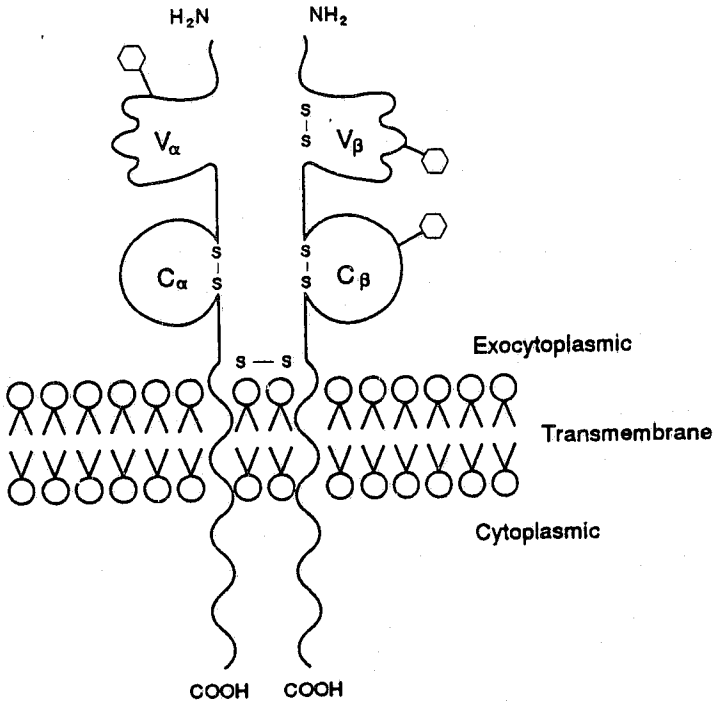


Figure 2. Diagrammatic representation of MHC class II molecule of the mouse. Class II molecules have an α- and β- chain structure, each of which has an exocytoplasmic variable (V_α, V_β), and constant (C_α, C_β) domain. S - S, disulfide bonds maintain domain structure. Class II molecule is anchored by its transmembrane and cytoplasmic parts. ♀, carbohydrate side chain. [Modified from Collins, T. et al. (1986) See Reference 13]

endometrium, thymic reticulo-epithelial cells, capillary endothelia (15), and macrophage-like cells in muscularis externa of small intestine (17). Earlier studies have also shown presence of these antigens on spermatozoa, macrophages, and epidermal cells (18). In guinea pigs, studies using fluorescent antibody technique (FAT) demonstrated the presence of class II antigens in epithelia of small intestine, gall bladder, kidney pelvis, urinary bladder, and ducts of lactating mammary glands (19). In rats, convoluted tubules of kidney (20), dendritic cells of Peyer's patches (21), and enterocytes (5) have been shown to be positive for these antigens.

Expression of class II antigens in human non-lymphoid tissues of different embryonic origin has been demonstrated; these tissues include: epithelium of gastrointestinal tract, urinary bladder, bronchial glands, and thymic reticuloepithelial cells (endodermic origin), epithelium of mammary glands, acinar cells of parotids, and astrocytes (ectodermic origin), and alveolar macrophages, Kupffer cells, glomerular and peritubular renal endothelium, endometrium, and Langerhans cells (mesodermic origin) (4). Class II (HLA-DR-like) antigens were revealed by immunofluorescence in enterocytes of human small intestine (22), and by immunoperoxidase studies of epithelium overlying Peyer's patches (23).

1.4.2 Expression of class II antigens on enterocytes

There is considerable species variation in the distribution of class II antigens in enterocytes. Previous studies of class II antigen expression in enterocytes revealed that they were only present on the surface of the cell (24,3,25). Electron microscopical studies of immunoferritin labelled, dissociated, fixed, mouse intestinal

epithelial cells revealed baso-lateral staining of plasma membrane whereas unfixed cells showed patchy labelling of all regions of the plasma membrane indicating migration of class II antigens through the leaky zonula occludens (25). However, subsequent immunohistochemical studies at the light microscopical level indicated that a high proportion of class II antigens in enterocytes reside intracellularly. The staining of apical cytoplasm of enterocytes was demonstrated by immunofluorescence (26). Immunoperoxidase studies indicated that in mice the baso-lateral membranes of all cells lining the villus were clearly outlined, and extensive granular staining occurred throughout the cytoplasm (5,27). It was noted that the pattern of staining of cells was not consistent along the length of villi. In rats, immunostaining for class II antigens revealed a granular intracellular staining pattern within epithelial cells covering the mid-portion of jejunal villi which became progressively weaker towards the tip of villi, but crypt epithelium and cells lining bases of villi did not stain for the antigens (5). In humans, enterocytes showed predominant staining of the cells lining the villi of jejunum, and the staining appeared to be associated with membrane as well as cytoplasm (28). Immunofluorescent studies indicated a patchy pattern apically in enterocytes, decreasing in intensity from top to base of the jejunal villi (22). The above studies did not sample all regions of the small intestine.

1.4.3 Induced expression of class II antigens

The distribution and intensity of expression of class II antigens can be altered by certain cytokines. Epithelial cell line EC 17 of rat origin, which normally does not express class II antigens, can be induced to express these antigens when

cultured with supernatants obtained from Con A-stimulated lymphocytes (29). Continuous expression of class II antigen required the presence of class II antigen inducing factor in the medium. Characteristics and kinetics of the inducing factor were similar to interferon activity. Gamma interferon (γ-IFN) is a lymphokine secreted by activated T lymphocytes, which can induce the expression of class II antigens by a variety of non-lymphoid cells that may allow them to act as "non-professional" antigen-presenting cells (APC) or accessory cells (30). It was shown that increases in class II antigens after *in vivo* treatment by γ-IFN was 6-to-10 folds in epithelial cells of small intestine of mice (31). Induced expression of class II antigens after γ-IFN treatment *in vivo* has been demonstrated in epithelium of proximal small intestine and crypts which in rats normally express very low levels of class II antigens (16). Induced class II expression was rapidly lost within 24-48 h after cessation of γ-IFN treatment suggesting that induced epithelial expression might be a short-term immunoregulatory event which may be distinct from the function of constitutively class II positive cells. Recently, it has been demonstrated that quantitative induction of class II molecules in rat enterocytes by γ-IFN may influence their antigen presenting function (32). These studies suggest that expression of class II antigens is secondary to release of γ-IFN released by activated T lymphocytes (13). An argument against this phenomenon was provided when expression of class II antigens on enterocytes in nude mice was demonstrated (5), and explained on the basis that other factors such as luminal contents and hormones may be involved in the expression of class II antigens. Evidence for these factors obtained by

transplanting syngeneic grafts of fetal gut under the capsule of the kidney. In this location the graft failed to express class II antigens; however, when the graft was exposed to luminal contents, the expression of class II antigens was evident in the enterocytes (5).

Induced expression of class II antigens was demonstrated in the rat intestinal epithelium and epidermal cells by naturally occurring immunological stimuli. Infection of the gut lumen with *Trichinella spiralis* larvae serves as a normal immune stimulus. It induced antigen expression in otherwise class II negative crypt epithelium 8-12 days after infection and increased the expression in villus epithelium 12-16 days post-infection (33). This delayed increased expression in the villus epithelium may be due to migration of the class II positive crypt epithelium from the zone of proliferation to zone of differentiation and migration. Recently, it has been shown that the amount of class II antigen expression by enterocytes of isolator-reared mice is considerably less than in the mice reared in conventional environment (27). These studies indicate that antigenic challenge or some other components in the gut flora play a role in class II antigen expression which might be involved in the initial antigen handling and their presentation to lymphoid cells present in the lamina propria and IELs.

In many disease conditions, induced or increased expression of class II antigens can be demonstrated. When graft-vs-host disease (GvHD) was induced in rats, the expression of these antigens increased in cells of gut epithelium (34). Various human intestinal disease conditions such as coeliac disease, dermatitis

herpetiformis (35), ulcerative colitis and Crohn's disease (36) exhibit increased expression of class II antigens in the intestinal epithelium. In these diseases, there is also an increase in MHC class II positive subepithelial cells in the lamina propria (36). It is still unknown how the increased expression of class II antigens in gastrointestinal tract may influence local immune regulation, but enhanced antigen presentation might be a possibility (2). This suggested a possible role of the enterocytes in immunopathologic conditions.

1.5 Possible accessory cell function of enterocytes

The main characteristics of an APC or accessory cell are antigen uptake and processing, and the expression of MHC class II antigens (37). The participation of APC is required for antigen-induced stimulation of T lymphocytes. One of the important functions of APC is to process an immunogenic moiety which can be recognized by T lymphocytes. Thus, APC take up antigen, process it to reveal the relevant determinant and present it in context with MHC class II antigens to T lymphocyte expressing the appropriate receptor (38). The class II positive APCs such as macrophages, dendritic cells and B cells cause stimulation of helper T lymphocytes. Similarly, enterocytes have been shown to constitutively express class II antigens, and freshly isolated epithelial cells from human small intestine were demonstrated to take up, process, and present soluble antigens to immunocompetent T lymphocytes causing activation of $CD8^+$ suppressor cells (6). The majority of IELs have been shown to be both Thy-1^+, and Thy-1^- $CD8^+$ $CD3^+$ T lymphocytes in mice (39,40), indicating a cytolytic or non-cytolytic action, respectively. In rats, enterocytes

appear to induce activation of T lymphocytes with suppressor/cytotoxic phenotype (41). T cell receptors (TCRs) composed of α and ß chain heterodimers are expressed on most of the $CD8^+$ T lymphocyte populations (42). Heterodimeric TCRs composed of γ and δ chains were reported to be expressed by $CD8^+$ IEL's (43,44). It is proposed that $CD8^+$ TCR-$\alpha\beta^+$ expressing T lymphocytes might encounter mainly viral antigens, and $CD8^+$ TCR$^-\gamma\delta$ IEL are most likely to encounter large amounts of bacterial antigens in the small intestine (44). However, 16% and 13% of $CD3^+$ $CD4^+$ IEL have been demonstrated in jejunum and ileum, respectively (45,46,47,48). The induction of $CD8^+$-mediated immunosuppression possibly depends on initial activation of IEL $CD4^+$ T lymphocytes (49,50). Therefore, class II positive epithelial cells may function as APC and play an active role in the local immune response. It is still uncertain as to which T lymphocyte type is activated by these antigens of enterocytes.

1.6 Regional variation of small intestine

It is well known that within a species there are morphologic and physiologic differences along the length of the small intestine, although basic histologic structure of the organ remains similar. The duodenum in most domestic animals is distinct from other regions of the small intestine due to the presence of Brunner's glands and shape of villi, which are generally blunt and wide; are subject to variation. Lymph nodules are sparse in this area (51). The jejunum is similar in structure to the duodenum with minor variations. The Brunner's glands are confined to the initial part, or may be absent in the jejunum. Villi are fewer, smaller and thinner than

those in the duodenal portion (51). Ileum is distinguished by the presence of Peyer's patches and abundance of goblet cells (10). Villi of this region are club shaped and may be filled or obliterated by lymph nodules (51). There are substantial differences in the composition of microvillous membrane of enterocytes of different regions of the small intestine. For instance, alkaline phosphatase, lactase, trehalase, and sucrase-isomaltase activities are greater in the proximal than in the distal intestine of rat (52). The receptor for intrinsic-factor-mediated cobalamin in hamsters is located on apical membrane of enterocytes in distal small intestine (53). Also, the cholesterol content of apical membrane of enterocytes ileum is greater and membrane fluidity is less than that of the proximal small intestine in rats (54).

1.7.1 Rationale

An intriguing study stated that "no generalizations are possible regarding the localization of enzymes in the small intestine; each portion of the intestine must be considered separately" (55). This suggests that each region of small intestine has an independent role. Various *in vitro* studies have indicated a possible antigen presenting role of enterocytes in normal and disease conditions. The histologic and physiologic variation in different regions of small intestine lead us to speculate that expression of class II antigens may vary in enterocytes along the length of the intestine. If there is a regional variation in expression of the antigens, and if the enterocytes are shown to be major APCs, then the results may be indicative of regional differences in antigen handling in the small intestine.

The M cells are believed to be the main sites of antigen handling, however,

the importance of enterocytes in this process has been underestimated. Since intestinal epithelium is exposed to a large number and array of antigens, the enterocytes might be more efficient in antigen handling because of their strategic location and number. On surgical removal of Peyer's patches, the mucosal immune response remains active (56). Enhanced antibody responses were observed in a study where all the Peyer's patches in rats were removed, and when lipid-conjugated human serum albumin (HSA) was administered intralumenally (57). The above study suggests that on primary encounter, absorption of antigen into Peyer's patches produces immunosuppression. However, if antigen was directly injected into Peyer's patches and M cells were bypassed, it caused stimulation of germinal centers and antibody production (58,30). Therefore, the route of absorption of antigen and the knowledge of possible APC role of enterocytes may influence the outcome of oral immunization.

1.7.2 Objective of the study

The first objective of the present study is to investigate whether there is a regional variation in expression of MHC class II antigens in enterocytes of small intestine of adult C3H/He mice.

B. POSTNATAL DEVELOPMENT OF ENTEROCYTES

1.8.1 Histogenesis of intestinal epithelium

The histogenesis of intestinal epithelium comprises an early phase of proliferation and morphogenesis, an intermediate phase of cellular differentiation, and a later phase of physiologic maturation of various epithelial cell types (59). At 15 or 16 days of gestation, during epithelial proliferation in duodenum of rats, cilia-like structures, and junctional complexes associated with membrane-bound vesicles appear in deep epithelial layers at 15 or 16 days of gestation (60). The preceding study has described major characteristics associated with villus formation in duodenum of rat fetuses that are: (a) the formation of secondary lumina in the primitive stratified epithelium of 15- and 16-day old fetuses, (b) fusion of secondary lumina with primary lumen by continued growth, and eventual desquamation of superficial layers of epithelium in 17-to-18-day old fetuses, and (c) the upward growth of mesenchyme towards the lumen which takes place in 18-day-old fetuses (60). Tubular invaginations at the base of the villi form crypts, the epithelial cells of which have a high mitotic rate. Cells of a crypt are derived from a single progenitor cell (8).

Cellular differentiation of the epithelium of villi occurs concomitantly with the villus formation (61). The development of microvilli and the appearance of activity of brush border enzymes begins at 16 days of gestation, which proceeds in a cranio-caudal direction (62).

1.8.2 Functional development of small intestine

The enterocytes produced during late fetal and early postnatal life are structurally different from those produced in the adult (63). Striking ultrastructural changes in the epithelium of duodenum and jejunum occur within the first 48 h of birth. In the epithelial cells at birth there is an apical tubule system. Following feeding, large protein filled vacuoles appear. However, within 48 h these structures disappear, and the cells take on the ultrastructural characteristics of adult cells. A loss of tubular system appears to be triggered by colostrum uptake, and an increase in blood levels of glucocorticoids (64).

The entire gastrointestinal tract of the mouse is functionally immature at birth, and the first two weeks of postnatal life. During the third postnatal week, there are considerable changes in digestive capacities, and adult states of digestion are functional by the end of the fourth week (11). One of the marked features of the neonatal rat intestine is its ability to adsorb macromolecules, including proteins (65,66). The enhanced uptake of intestinal antigens during neonatal periods may be attributed to the transient deficiency of intestinal secretory antibodies, affinity of antigens to immature intestinal surfaces, increased pinocytosis of macromolecules, and decreased intestinal proteolysis (67). Pinocytotic activity of the epithelial cells in rodents appears by day 19 or 20 of gestation, remains high during the first two weeks of suckling, and then decreases considerably towards the end of third week (68,69,70). Murine milk constitutes two important classes of proteins, i.e., immunoglobulins and nutritional proteins. In the jejunum, pinocytosis is highly

specific and transport of intact immunoglobulins occurs across the intestinal mucosa to the circulatory system of the neonate intestine (71), whereas the ileum absorbs nutritional proteins because the pinocytosis is non-specific (72). These studies indicate a difference in handling of antigens by different regions of small intestine due to different levels of enterocyte maturity that occurs in a cranio-caudal direction.

1.8.3 Presence of Fc receptors on immature enterocytes of rodents

In the proximal small intestine of neonate rodents, immunoglobulin uptake is receptor-specific and pH-dependent (73). Immunofluorescent studies of duodenum and jejunum indicate that Fc-receptors are present on immature enterocytes before closure, i.e., cessation of antibody uptake by intestinal epithelium. Thus immune complexes are selectively adsorbed in the proximal small intestine, whereas luminal contents in the ileum are non-selectively adsorbed by the enterocytes (73). This study did not indicate which epithelial cell of ileum were involved in non-selective absorption. Immunoperoxidase studies of the Fc-receptors of isolated intact epithelial cells from the proximal small intestine of 10-to-12-day-old rats show that specific binding of rat IgG conjugated with horseradish peroxidase to the cells occurs at pH 6.0 (74). Functionally, similar receptors were found on the luminal and abluminal surfaces of the absorptive cells, indicating that the IgG is transported in the form of IgG-receptor complexes to the abluminal surface and these complexes are exposed to serosal plasma that would cause release of IgG from the receptors in the alkaline medium (74). Thus, the possession of Fc-receptor by immature enterocytes is similar to APC, for example, macrophages.

1.8.4 Postnatal development of small intestine

Ultrastructural and enzymatic studies suggest that the function of enterocytes depends on age of the individual and region of the small intestine. In rats, postnatal development of small intestinal mucosa occurs in a cranio-caudal direction, however, after weaning the mucosal hyperplasia develops equally in all regions of the intestine (75). In mice and rabbits, maturity of villi, intestinal glands, and other structures in the duodenum occurs by day 20 after gestation, and the development occurs in a cranio-caudal direction (76,77). The regional differences in enzymatic activities along the luminal border of the small intestine during fetal, postnatal and adult life are well documented (62,78,79). It is speculated that jejuno-ileal differences in activity of lactase, sucrase, and β-galactosidase might be genetically controlled in fetal small intestine, and other factors *in situ* might be responsible for its magnitude (80).

1.8.5 Ontogeny of MHC class II antigens

The intestinal epithelium of mice during fetal development is negative for MHC class II antigen expression (15,26). The expression of class II antigens was first seen in the jejunal epithelium of 3- and 4-week-old rats in the apical cytoplasm of villi epithelium; 6-week-old animals showed adult pattern of staining and intensity on the epithelium (5). In rats, the first expression of the antigens occurs after weaning.

MHC class II antigen-bearing dendritic cells appear in the lamina propria of the small intestine of rat fetuses a few days before birth (5). The preceding study also documented that by day 18 of gestation, villi formation occurs in small intestine

and many class II positive cells can be observed in the lamina propria. Class II antigen-bearing dendritic macrophages in the propria are large cells with irregular outlines. By day 20 and 21 of gestation, the proximal intestine is well developed, and class II positive cells in lamina propria express adult levels of the antigen. However, the distal small intestine, which lags in development, has fewer class II positive cells in the lamina propria. Immunoperoxidase studies showed that the stained processes of the dendritic cells could be seen passing between the enterocytes (81). Class II antigen positive cells colonize both Peyer's patches and the lamina propria before the appearance of significant numbers of lymphocytes (30).

1.8.6 Possible factors regulating ontogeny of class II APC

Regulation of the development of class II antigen positive APC may be extremely important. The delayed acquisition of class II antigens may be important in aquiring self-tolerance or an inappropriately early expression may terminate self-tolerance, hence possibly risking the induction of autoimmune disease (82). Several factors in neonatal environment have been elucidated which participate in suppression of class II antigen positive APC/macrophages in neonates (82). Many studies have shown that γ-IFN, a T lymphocyte lymphokine, stimulates expression of class II antigens on intestinal epithelium, and in splenic and peritoneal macrophages (16,31,83) allowing them to act as APC. However, intestinal epithelial cells and macrophages of neonatally thymectomized and nude mice (5,82) expressed adult levels of class II antigen, indicating that ontogeny of class II antigens on intestinal epithelial cells and macrophages is independent of mature T lymphocyte activity.

Factors which recruit class II antigen bearing macrophages *in vivo* in adult mice, i.e., intraperitoneal injection of live *Listeria monocytogenes* or γ-IFN, failed to do the same in postnatal mice (82,84). This suggests that the neonatal environment exhibits suppressive influences (82). These suppressive influences have been attributed to the presence of prostaglandin E_2 and alpha-fetoprotein (AFP). Prostaglandin E_2 has been demonstrated to inhibit macrophage class II antigen expression, and thus their antigen presenting function *in vitro* (85). Murine AFP is a 74,000 dalton glycoprotein, and is one of the major fetal and neonatal serum constituents.

Enteroctyes have been assigned a possible APC role; hence the above mentioned factors may also have important functions in ontogeny of class II antigens in these cells.

1.9.1 Rationale

The antigen presenting function of class II positive cells of the mouse spleen reaches levels similar to those in adults by three weeks postpartum, which coincides with the age of weaning (86). The latter study speculated that a maternal factor present in the milk, which is subsequently transmitted to the neonate, might be blocking T-lymphocyte and macrophage maturation. After weaning, APC are allowed to differentiate because the postulated factor is removed. Since enterocytes might be serving as APC, the same phenomenon may apply to them. A study on the ontogeny of MHC class II antigen-bearing accessory cells in autoimmune MRL-lpr mice found that mice had inappropriately early ontogenetic appearance of class II-

bearing splenic accessory cells, which might contribute to the development of autoimmunity (87). Hence, it is important to know the ontogeny of class II antigens in the murine enterocytes of different regions of the small intestine which may be helpful in understanding their role in autoimmune diseases.

1.9.2 Objective of the study

The second objective of the study, therefore, was directed at the ontogeny of MHC class II antigens in enterocytes of C3H/He strain of mice.

2. COMPARISON BETWEEN FLUORESCENT ANTIBODY TECHNIQUE AND PEROXIDASE-ANTIPEROXIDASE TECHNIQUE

2.1 Introduction

Special staining techniques are required to detect MHC class II antigens. Immunocytochemical (ICC) techniques are widely-used, labelling procedures for a vast variety of antigens. The success of ICC techniques depends on its practicality and reliability (88). The specific contributions of each individual factor cannot be determined in ICC studies since ICC procedures are the result of mutual relationships between various factors influencing the final outcome (89). For instance, immunoreactivity is determined by the specificity and potency of the primary antiserum, and, in turn, these are dependent on sensitivity of the type of ICC procedure used.

The initial tissue processing and fixation imposes a compromise between the stabilization of antigens *in situ* without destruction of antigenicity, preservation of cellular morphology, and easy access of antibodies to the antigen. Antigens are required to be fixed or cross-linked chemically in the tissue. Fixation may have adverse effects on antigens, such as chemical alteration of their epitopes, masking of antigen due to steric hinderance caused by immobilization of adjacent molecules, extraction of molecules due to cross-linking or washing, and structural changes resulting in masking of epitopes that may be accessible in native form (90). Temperature, pH, osmolarity, fixative concentrations and type, and rate of

penetration, all play important roles in fixation. Therefore, it is apparent that an ideal immunocytochemical fixative for all antigens currently does not exist, it is necessary to arrive at a fixative procedure adapted to the specific experimental problem.

Various ICC techniques at light and electron microscopical levels have been used to study class II antigens in the mucosal epithelium of the small intestine. At the light microscopic level, the fluorescent antibody technique (FAT) seems to be a common method used for detecting the class II antigens (4,6,15,19,22). Indirect immunoperoxidase (5,91) and peroxidase-antiperoxidase (92) techniques have also been used. Fluorescein emits green to yellowish-green light after ultraviolet excitation. This fluorochrome absorbs light of 490 nm wave length and emits light of higher wavelength, i.e., 520 nm, which is visible. Fluorescein is coupled to protein by an isothiocyanate intermediate (93). The FATs are commonly performed by two methods, i.e., direct and indirect immunofluorescence. The indirect FAT is a more sensitive method (94), and consists of primary antibody directed against the antigens concerned, and a secondary antibody conjugated with fluorescein isothiocyanate directed against the primary antibody (Fig. 3).

The peroxidase-antiperoxidase (PAP) immunocytochemical technique, first demonstrated by Sternberger et al. (1970) (95), requires three different antisera (Fig. 4). The primary antibody and PAP soluble complex must be from the same species. The third reagent, i.e., secondary antibody acts as a bridge between primary antibody and PAP complex, and is produced in another species (Fig. 4). The secondary

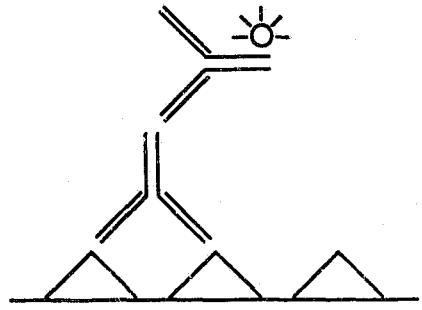


Figure 3. Schematic representation of indirect fluorescent antibody labelling process

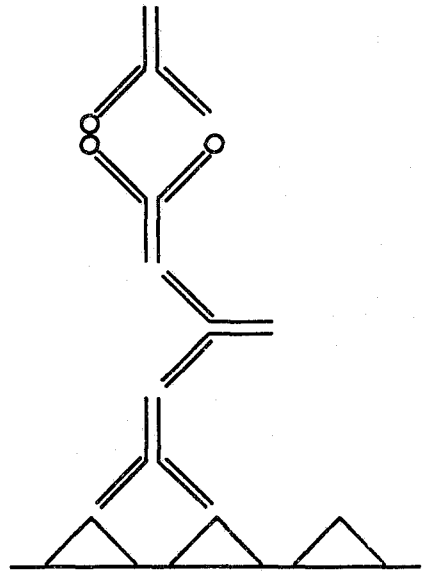
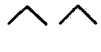


Figure 4. Schematic depiction of peroxidase-antiperoxidase labelling process

Key



Tissue antigen (MHC Class II antigen)

Primary antibody (anti-mouse class II antigen IgG)

Secondary antibody (goat anti-mouse IgG)

Isothiocyanate fluorescein label

Peroxidase-antiperoxidase complex

antibody is used in excess so one epitope is bound to primary antibody and the second is free to bind PAP complex (96). Several advantages of the PAP method over indirect FAT method have been documented (95,96,97,98).

Various controls are used to validate ICC staining (99,100) in order to avoid false positive and false negative results.

Sections prepared by cryomicrotomy were chosen because the tissue is not exposed to harsh fixative and to high temperatures required for paraffin embedding. A mild post-fixation at -20°C was performed, thus preserving antigens in their native form. Acetone, ethanol, and paraformaldehyde were used as post-fixatives. Comparative study on FAT and PAP techniques was performed to establish a sensitive, practical, and reliable method for the demonstration of MHC class II antigens.

2.2 Materials and methods

2.2.1 Animals and management

Seven-week-old mice (C3H/He brown inbred strain of k-haplotype and C57BL/6 of b-haplotype), were obtained from Charles River, Montreal, Quebec. Animals were housed in the animal facility at the Atlantic Veterinary College. The test animals were kept in stainless steel cages, each cage housed five to six animals. The cages were cleaned three times a week, and water was changed every day. Temperature was maintained at 20 ± 1°C within the animal room. The lighting (daylight type) was alternated on a 12 hour basis by an automated timing device. All

mice were fed *ad libitum*, and had free access to water. Animals were kept according to the guidelines of the Canadian Council of Animal Care.

2.2.2 Fixation and processing of specimens

The animals were killed by cervical dislocation. Spleen and small intestine were dissected out and the small intestine was flushed with 0.1M phosphate buffered saline (PBS) solution, pH 7.4. To keep the lumen patent, a solution containing equal amounts of O.C.T., an embedding material (Canlab, Toronto, Ontario), and PBS solution were introduced into the intestine, and both ends of the organ were clamped. The intestine was immediately frozen with Cytocool, a tissue freezing aerosol (Canlab). Samples of duodenum represented areas 1.5 cm posterior to the pylorus; ileal samples were collected 1.5 cm anterior to the ileo-caecal junction and jejunal samples were collected from the mid-region of the small intestine. Segments of 3 mm in length from duodenal, jejunal and ileal regions, and the entire spleen were embedded in O.C.T. compound and snap frozen in liquid nitrogen, and were stored at -80°C, until prepared for staining.

2.2.3 Preparation of clean subbed slides

Microslides were treated (101) with 10% HCl overnight, and washed in running water for 1 h and were treated with 95% ethanol (Fisher Scientific, Nepean, Ontario), to avoid non-specific staining.

Two grams of gelatin (BDH, Darmouth, Nova Scotia) were sprinkled on 200 ml of distilled water and allowed to sink slowly. The solution was placed in an oven at 60°C for 1 h then removed, and stirred. Chromium potassium sulfate (190

mg) was added to the solution. Slides were dipped in the gelatin chrome-alum solution, and were left overnight to dry.

2.2.4 Immunocytochemical techniques

2.2.4.1 Indirect fluorescent antibody technique (FAT)

Embedded intestine specimens of C3H/He mice were placed on cryostat stubs, trimmed and transverse cryosections, 6 μm thick were cut and mounted on treated microslides. Cryosections of duodenum, jejunum, and ileum were dried at -20°C for 1 h and fixed in absolute ethanol (Commercial Alcohols Limited, Montreal, Quebec) for 10 min at -20°C. After rinsing in PBS solution, the tissues were incubated with 20% normal goat serum in PBS solution containing 0.5% bovine serum albumin (BSA) (Sigma, St. Louis, Missouri, USA) for 20 min at room temperature (RT). The PBS solution containing 0.5% BSA was used as diluent for all antisera. The blocking serum was removed by draining off the excess serum with filter paper. The microslides were divided into three sets. The first set of microslides was incubated with 50 μl of anti-mouse MHC class II antigen monoclonal antibodies (MRC-OX6) (Daymar Labs, Toronto, Ontario) diluted at 1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200, 1:4000, 1:5000, 1:6400, 1:12000 for 24 h at 4°C in a humid atmosphere (Daymar Labs, Toronto, ON). The second set received PBS in place of primary antisera and the third set was incubated with an unrelated purified monoclonal antibody at similar dilutions to that of primary antibodies. Peyer's patches in the ileum and jejunum served as positive controls. All sets were brought to RT for 30 min and washed in PBS for 30 min with remaining procedures done at

RT. Subsequently, the sections were incubated with 50 µl of fluorescein conjugated, affinity purified, goat anti-mouse IgG (Bio/Can, Toronto, Ontario) at 1:10, 1:20, 1:40, 1:80, 1:160 dilutions for 30 min at RT. The sections were rinsed and washed with PBS for 20 min at RT. The stained sections were mounted with FA mounting fluid (Difco Labs, Detroit, Michigan, USA), coverslipped, and examined with a Zeiss fluorescent microscope.

2.2.4.2 Peroxidase-antiperoxidase technique (PAP)

This protocol was conducted on C3H/He and C57BL/6 strains of mice. The cryostat sections of 6 µm thickness were air-dried for 1 h at -20°C and post-fixed in absolute ethanol, absolute acetone, or 4% paraformaldehyde for 10 min at -20°C. After rinsing the sections in PBS solution for 15 min, the samples were incubated with 20% normal goat serum for 20 min at RT to avoid non-specific staining. Excess normal goat serum was drained off and gently removed with filter paper. Each trial constituted three groups of two slides each. Samples in the first group were incubated with 50 µl of anti-mouse class II antigen monoclonal antibodies (purified from ascites) (Daymar Labs) at similar dilutions to that of the above method. Samples in the second group received unrelated monoclonal antibody at a dilution similar to that in the preceding group. Samples in the third group were incubated with PBS solution alone. All groups were incubated in a humid atmosphere for 24 hr at 4°C.

All the samples were brought to RT and were rinsed and washed in PBS solution for 20 min. The tissue sections were incubated for 30 min at RT, with 50

µl of affinity purified goat anti-mouse IgG at a 1:25, 1:50, 1:100, 1:200 dilutions. Excessive secondary antibody was removed by rinsing and washing in PBS for 20 min at RT. Fifty microliters of mouse PAP soluble complex (Sigma) diluted at 1:50, 1:100, 1:200 were added to the tissue sections and incubated for 30 min at RT. After washing the tissue in 0.05M Tris-HCl (Sigma), pH 7.6, the tissue bound PAP complexes were revealed by addition of 0.05% 3,3'-diaminobenzidine (DAB) tetrahydrochloride (Polysciences, Barrington, Pennsylvania, USA) and 0.02% hydrogen peroxide in 0.05M Tris-HCl, pH 7.6 for 20 min at RT. After rinsing and washing the tissue in Tris-HCl buffer for 20 min, the sections were counter-stained with Harris' haematoxylin, dehydrated and mounted in Entallen (BDH Chemical Canada Ltd., Nova Scotia). A brown deposit represented the positive immune reaction for the antigens.

Haematoxylin and eosin staining was performed on paraffin and cryosections of small intestine postfixed with 10% neutral buffered formalin and ethanol, respectively. The adequacy of fixation, and retention of cellular morphology were compared in both types of sections.

2.2.5 Controls

I. A method was standardized by testing serial dilutions of all the reagents to best fit our experimental protocol.

II. Spleen sections served as positive controls.

III. Method specificity was determined by using the following deletion controls so as to determine whether reagents other than primary antisera were reacting with the antigenic sites:

(a) The primary antibody, i.e., MRC-OX6 was eliminated from the above protocol, but the concentration, time and dilutions of rest of the reagents remained similar to that in group 1.

(b) The secondary antibody, i.e., goat anti-mouse IgG, was eliminated from the above protocol.

(c) Incubation with PAP was deleted as one of the steps.

(d) Some sections were incubated with only DAB, with deletion of all other steps.

IV. Non-specificity arising due to binding of immunoglobulins to the tissue by mechanisms other than specific antigen-antibody reaction was monitored by:

(a) substitution of primary antisera with an unrelated antisera or PBS solution.

(b) non-specificity may result from secondary antisera binding directly with tissue due to Fc-related interaction resulting in false-positive reaction. This was eliminated by blocking with normal goat serum prior to application of primary antisera.

V. Serum specificty was determined as follows: Monoclonal antibodies used in this study were directed against (k-

haplotype); C57BL/6 strain of b-haplotype were used to determine the serum specificity. It is well established that MRC OX6 monoclonal antibodies are directed against MHC class II antigens in enterocytes and other cells, therefore, absorption controls were not performed.

VI. Irreversible inhibition of endogenous peroxidase was performed prior to the immunostaining in some control sections, and sections stained for class II antigens. Blocking of endogenous peroxidase was achieved by immersing the slides in a bath of 100 ml of absolute ethanol containing 0.2 ml of concentrated HCl for 15 min.

2.2.6 Photography

2.2.6.1 Fluorescent photomicrography

Fluorescent sections were photographed with Kodacolor Gold 200 film (Kodak Canada Inc., Toronto, Ontario) using a Zeiss fluorescent microscope (Zeiss, West Germany).

2.2.6.2 PAP photomicrography

Immunoperoxidase-stained sections were photographed with Kodak Ektar-125 (Eastman Kodak Co., Rochester, New York), EPY 135-136, and EPY-50 (Kodak Canada Inc., Toronto, Ontario) films using a Zeiss photomicroscope III. An daylight correcting filter, and various combinations of neutral density filters were used with Ektar-125 film, whereas only the latter filters were used for EPY films.

2.3 Results

2.3.1 Paraformaldehyde and acetone fixatives

Sections fixed with acetone retained adequate cellular morphology (Fig. 5a). The overall cellular morphology and integrity of the villus of jejunum was disrupted with the use of paraformaldehyde (Fig. 5b). Outlines of cells lining the villus could not be distinguished, and tissue within the lamina propria was disrupted and showed large vacuolations (Fig. 5b). Interpretation of results was difficult although the antigencity was retained by the cells following the use of both fixatives. Both fixatives caused the shrinkage and curling of the edges of the villus thus giving a false enhanced reaction at the luminal side of the cells (Fig. 5a,b).

2.3.2 Ethanol fixative

The cellular morphology and antigencity of the enterocytes were well preserved in specimens fixed with ethanol (Fig. 5c). The cell outlines were distinct. There was no disruption in lamina propria, making interpretation of results convenient.

2.3.3 Haematoxylin and eosin

Cellular morphology of paraffin sections fixed with neutral buffer formalin was comparable to cryosections fixed with absolute ethanol (Compare Fig. 5c and Fig. 6).

2.3.4 Indirect FAT

Immunofluorescence was easily discernable with the characteristic yellowish-green staining distributed in the apical and basal cytoplasm of the

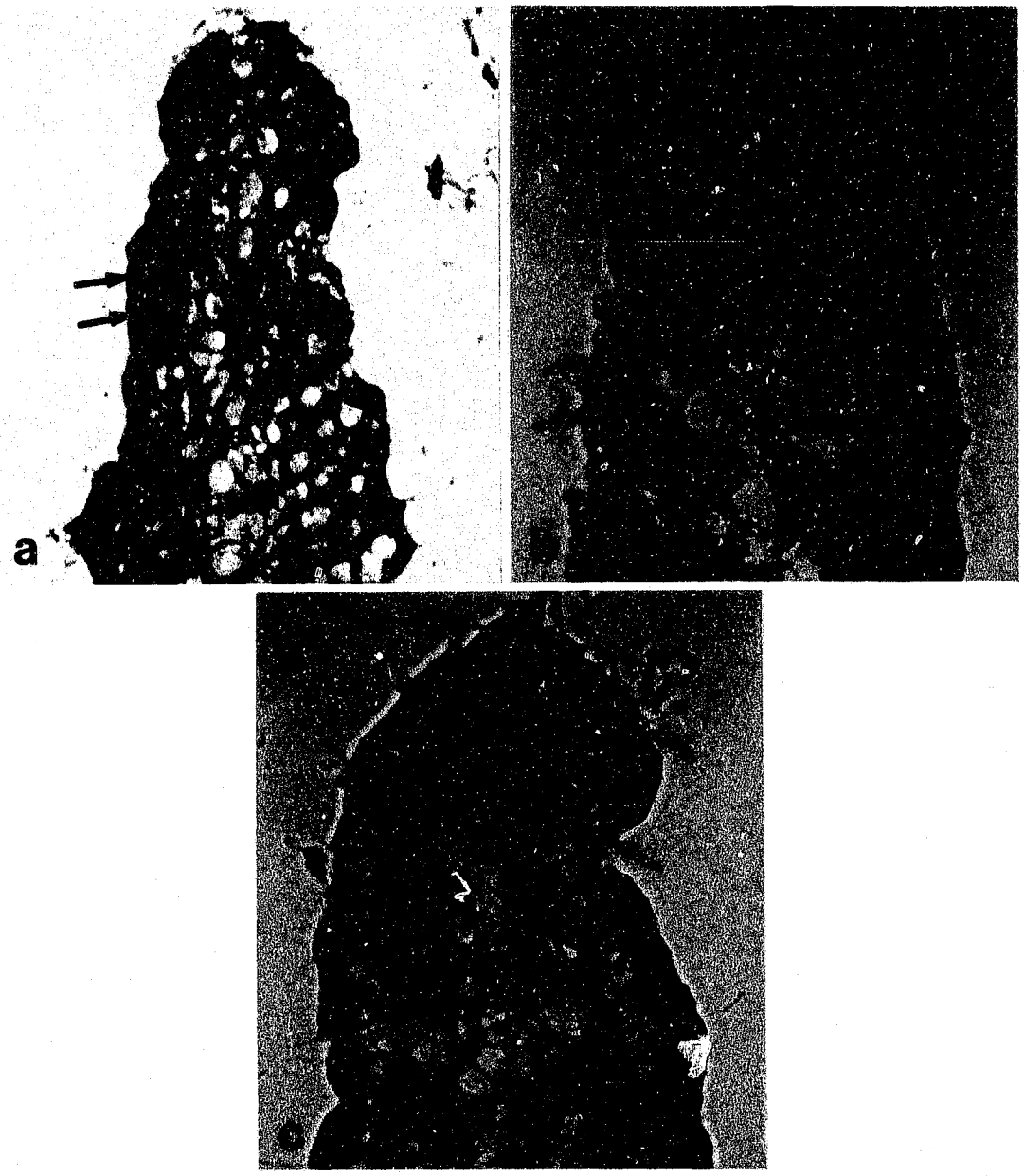


Figure 5 a-c. Photomicrographs of PAP treated cryosections from jejunum of adult C3H/He mice depicting different fixation protocols. a. Absolute acetone: antigenicity and cellular morphology was retained, however, there was overall shrinkage (*arrows*) of villus. b. 4% paraformaldehyde: cell morphology was disrupted *(arrows)*. c. Absolute ethanol: cellular morphology and antigenicity were well retained (*arrows*). X 640.

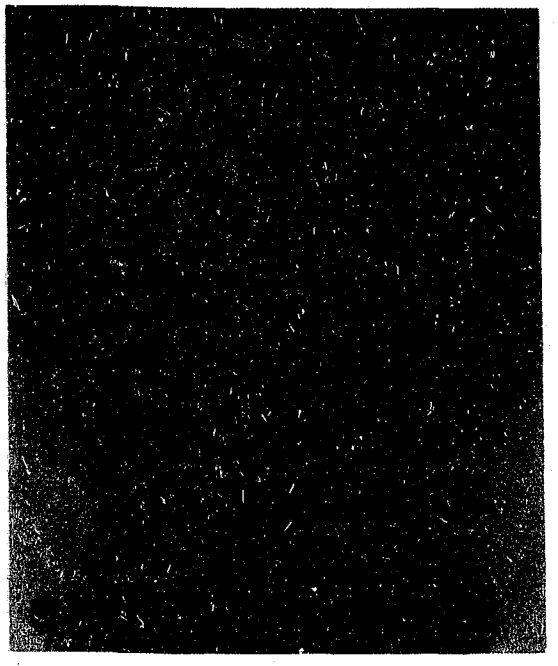


Figure 6. Photomicrograph of ethanol fixed cryosection from jejunum stained by haematoxylin and eosin. Note the characteristic distribution of enterocytes on a villus. X 640.

enterocytes (Fig. 7a). Lateral cell surfaces showed a strong fluorescence. Crypt epithelium stained similar to that of villus epithelium. Cells in lamina propria exhibited intense granular staining. These cells were probably macrophages and other class II antigen positive cells. Exposure of the fluorescence stain to continuous excitation for more than 30 seconds produced a bleaching effect (Fig. 7b). Sections incubated with unrelated monoclonal antibodies showed no immunoreaction in the enterocytes (Fig. 7c).

2.3.5 PAP

In sections of spleen stained for class II antigens certain cells showed strong immunoreaction of their surfaces (Fig. 8a). Spleens from animals of control group showed endogenous peroxidase reaction mainly localized in the cytoplasm (Fig. 8b).

The sections of small intestine (jejunum) from control group of both strains of mice were negative for the antigens in epithelium and lamina propria (Compare Fig. 9a-c with 9d). The granular brown staining in some cells of the lamina propria resulted from endogenous peroxidase reactivity (Figs. 9a-c and 10a), since the reactivity in these cells was eliminated (Fig. 10b) when the sections were subjected to endogenous peroxidase blocking. There was a light brown diffuse staining of sections, but this reaction product was not similar to the reaction seen when staining for class II antigens. The serial dilutions of primary antisera helped to establish the most significant dilution at 1:4000.

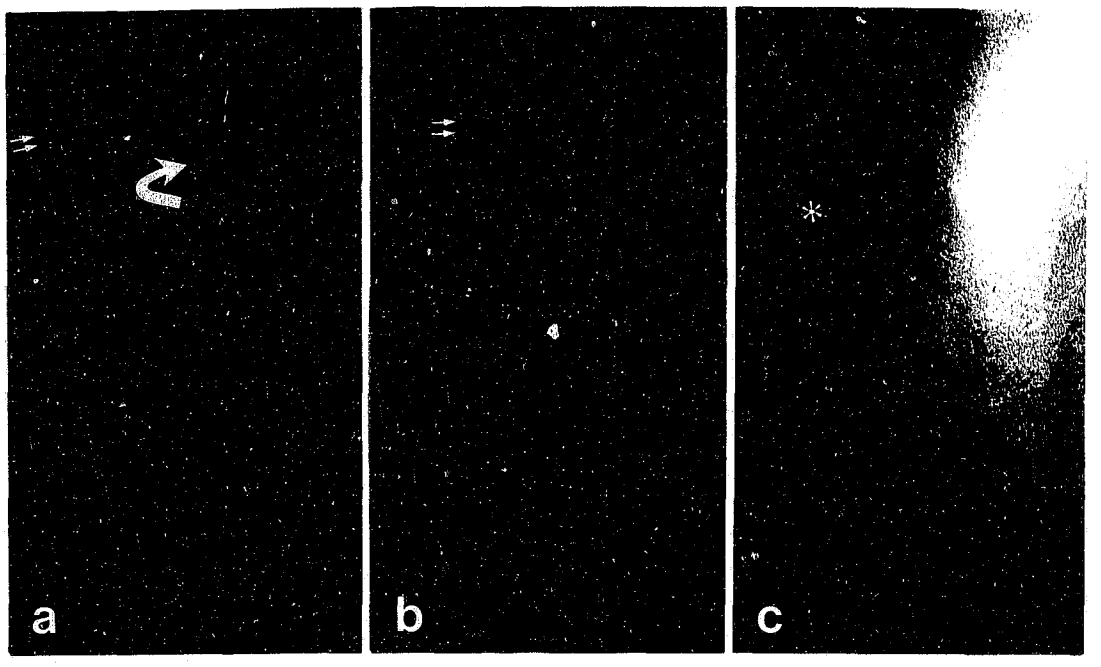


Figure 7 a-c. Photomicrographs of cryosections from jejunum of adult C3H/He mice, stained with FAT method. a. Enterocytes were easily discernable after approximately 30 sec excitation, there was granular apical cytoplasmic (*arrows*) and lateral cell surface staining (*curved arrow*). b. Bleaching of fluorescein occurred after 40 sec - 60 sec excitation (*arrows*). c. Sections incubated with unrelated antibody demonstrated negative staining of enterocytes (*asterisk*). X 400.

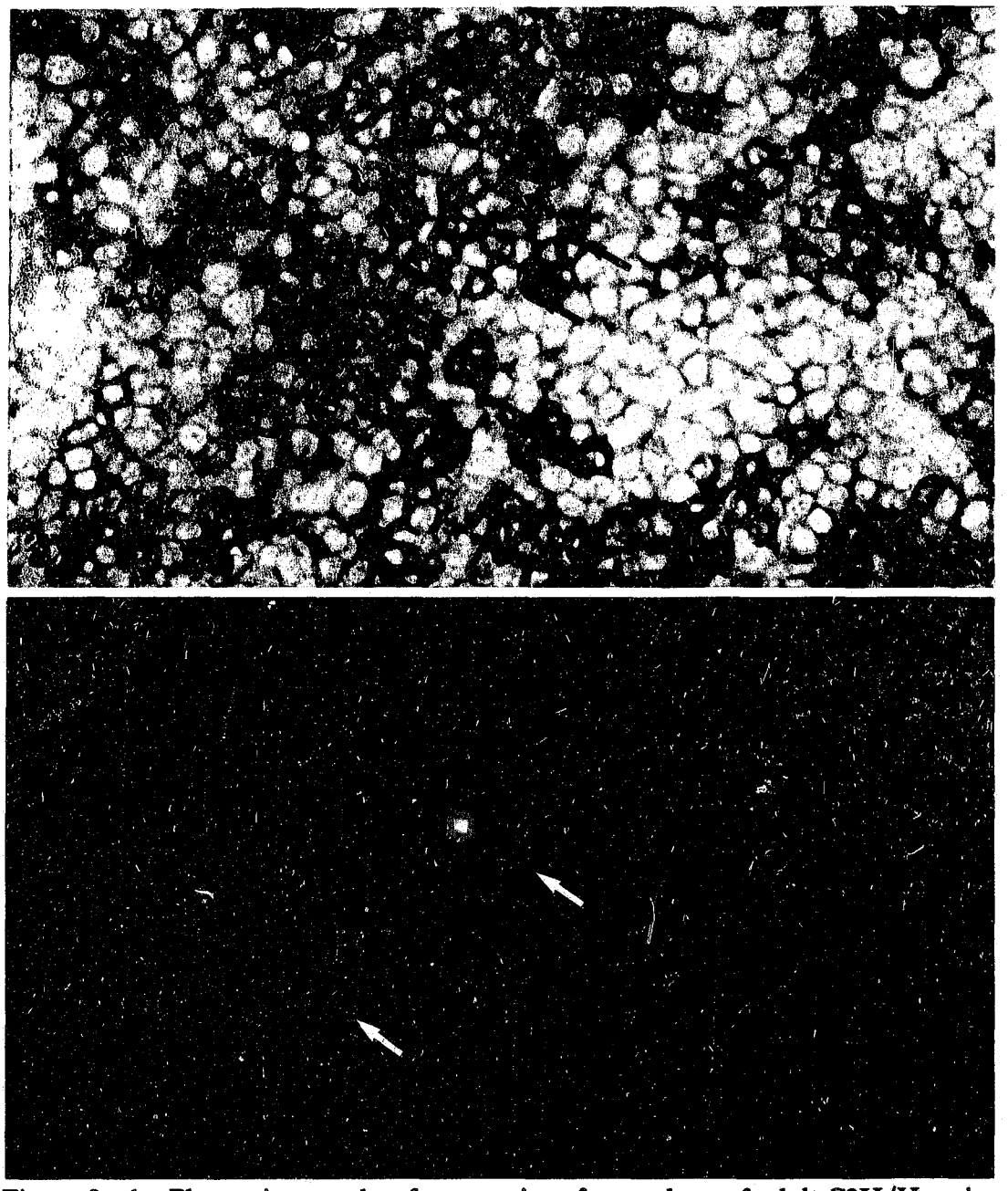


Figure 8 a-b. Photomicrographs of cryosections from spleen of adult C3H/He mice stained by PAP method. a. Many class II antigen positive cells were illustrated in the spleen (*arrows*) when incubated with MRC OX6 monoclonal antibody. b. Section incubated with unrelated antibody showed absence of immunoreaction but showed reaction for endogenous peroxidase (*arrows*). X 640.

Figure 9 a-d. Photomicrographs of cryosections from jejunum stained by PAP method. a. Section depicting serum specificity: section from adult C57BL/6 mice stained for class II antigens showed enterocytes (*asterisk*) stained negative. b-d. Endogenous peroxidase staining was observed in lamina propria (*arrows*) of all control sections. b-d. Method specificity controls used adult C3H/He strain of mice. b & c. Micrographs represented a deletion and substitution control respectively; enterocytes (*asterisk*) and lamina propria cells exhibited absence of an immune reaction. d. Section stained for class II antigens showed granular immunoreactio in enterocytes (*arrows*) and cells of lamina propria. X 640.

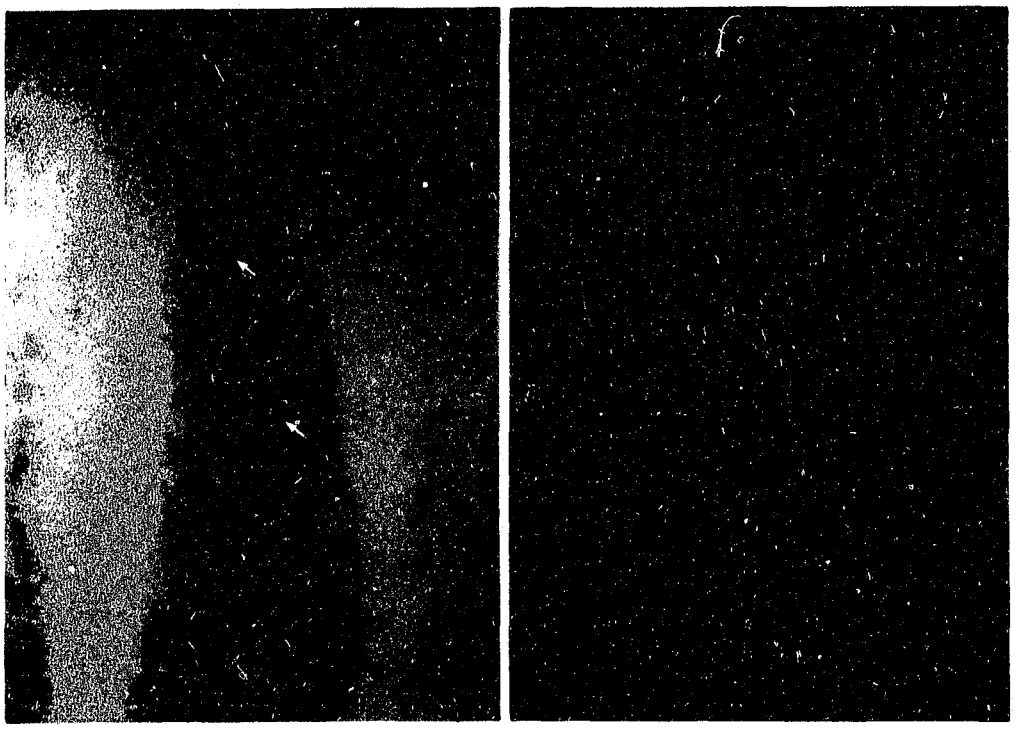


Figure 10 a-b. Photomicrographs of cryosections from jejunum of adult C3H/He mice stained by PAP method with unrelated monoclonal antibody. a. Endogenous peroxidase reaction was observed in lamina propria (*arrows*) in the form of brown granular deposit. b. Blocking of endogenous peroxidase resulted in absence of reaction within lamina propria. X 250.

A positive immunoreaction was characterized by brown staining (Fig. 9d). The staining pattern was similar to that observed in FAT. Furthermore, the stained sections could be retained permanently because the reaction product did not fade.

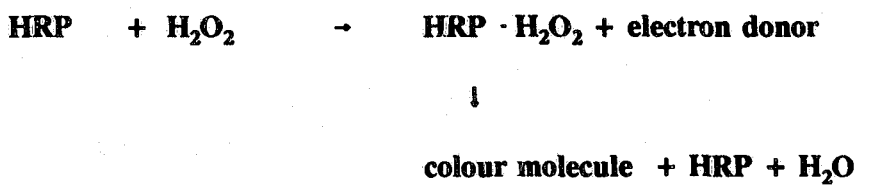
2.4 Discussion

The present study suggests that PAP is a more sensitive and stable method for demonstration of MHC class II antigens. Also, cold-ethanol post-fixation of cryosections preserved the antigencity and cellular morphology for proper interpretation of results, which is in agreement with other studies (102,103). In the controls using C3H/He and C57BL/6 mice, enterocytes of the villus and crypt epithelium, and cells in lamina propria were stained negative after staining for class II antigens, indicating haplotype specificity of the anti-mouse MHC class II monoclonal antibodies.

Fixatives were evaluated with regard to preservation of cellular morphology, intensity and localization of immunostaining and for non-specific staining. Paraformaldehyde did not maintain cellular morphology and, in addition, caused an extensive background staining which made interpretation of antigenicity difficult. A marked shrinkage of tissue was observed, causing the edges to curl and resulting in a putative enhanced reaction. The aldehyde-based fixatives produce intermolecular and intramolecular bridges that formed numerous cross-linkages. There is evidence that the number of bridges formed depends on concentration, temperature, pH, and incubation period of a fixative. These cross-linkages may cause denaturation and masking of antigenic sites due to steric hinderance (104). Acetone

preserved tissue morphology but caused overall shrinkage of the villus producing an appearance of intense reaction. Ethanol proved to be a good fixative in our system, suitably preserving both morphology and antigenicity. Acetone and ethanol immobilize proteins and carbohydrates by precipitation. The denaturing effect of these fixatives is mild and proteins can be preserved in native state after ethanol fixation (104). In our system, frozen sections were used as these sections are not exposed to harsh embedding and fixation procedures or to high temperature. Thus, the cryosections preserve relatively more antigens than in paraffin and epoxy sections (105). Also, in paraffin sections sensitivity of immunoassay was reported to be lower, and to aggravate the problem further, a high background due to nonspecific staining occurred (106). Therefore, when primary antiserum is applied, correspondingly large amounts will bind to the antigens because more antigenic sites will be available in the cells.

PAP appeared to be a more sensitive and stable technique than the immunofluorescence method. The main advantage of PAP method was its high sensitivity because it multiplies and enhances the staining so that even minute amounts of antigen are revealed (105). It has been stated that approximately 20-200 fold higher sensitivity may be achieved by PAP method than that in other immunocytochemical methods (95,107,108). The increased sensitivity of immunoperoxidase techniques over immunofluorescence was due to the amplification factor of enzyme techiques as described by the following reaction (109):



Horseradish peroxidase (HRP) forms a complex with hydrogen peroxide (H_2O_2) which reacts with an electron donor to form a coloured molecule and water. The peroxidase enzyme is not used, and is free to react with H_2O_2 to form more coloured molecules. However, fluorescence occurs following excitation of certain molecules by light energy of a particular wavelength. Electrons are transported to high energy levels in such molecules and when electrons return to lower energy states, the energy is released as light of longer wavelength (110). Hence, only small amounts of light can be given off by fluorescent molecules in comparison with that after treatment with peroxidase enzyme, and thus can produce many coloured molecules. In addition, the PAP technique was more sensitive as more than one secondary antibody could bind to the primary antibody in the indirect method. As three peroxidase molecules were bound in a PAP complex, and only one fluorescein molecule was bound in indirect FAT, obviously an enhanced reaction in PAP was produced as compared to that in the indirect FAT. One disadvantage of PAP method is the possibility of a "false-negative" results due to improper dilution of the primary antisera (105). But in our study, various dilutions of primary antisera were tested to overcome this problem. The most effective dilution selected was 1:4000 out of 10 other dilutions tested.

Although FAT is a widely used method for labelling antigens, the major disadvantage was rapid bleaching of stain due to continuous excitation of fluorochromes. Bleaching was accelerated by use of objectives of high magnification (excessive light) and oxygen, suggesting that it might be a photo-oxidative degradation process. However, reducing agents such as, 1,4-diazobicyclo-(2,2,2)-octane have the ability to quench the excited state of dyes, thereby retarding the bleaching phenomenon (111); furthermore this study demonstrated that over 80% of the initial brightness was maintained during the first 30 sec of continuous exposure with a high magnification (X40) objective. This indicated that only a limited time was available to examine the slides. Due to difficulty in obtaining consistently satisfactory micrographs by FAT, and, as subsequent experiments were to show, a variation in expression of the antigens, and their postnatal ontogeny, we needed a highly sensitive method of labelling. Therefore, FAT was not suitable for the present study.

Incubation time, pH of buffers, and concentration of antisera all play important roles in efficacy of ICC. Thus, combination of cold-ethanol fixation and use of PAP staining method produced the best possible sensitivity and reproducibility in this study.

3. REGIONAL VARIATION IN EXPRESSION OF MHC CLASS II ANTIGENS IN ENTEROCYTES

3.1 Introduction

Villous epithelium of small intestine comprises an extensive area exposed to ingested antigens. Mechanisms that distinguish immunogenic or tolerogenic responsiveness to these antigens are yet to be clearly defined (1). A major portal of antigen entry and its presentation to the enteric lymphoid population is thought to be through the M cells, a specialized epithelium overlying Peyer's patches (112). Both soluble and particulate antigen absorption has been demonstrated in M cells (113). The exposure of the gut epithelium to such a vast quantity and array of antigens makes the M cell portal of antigen entry potentially inefficient, as the number of M cells are limited (6). Upon surgical removal of the Peyer's patches, the mucosal response remains active suggesting that other elements including the enterocytes may contribute to the local immune response in the gut (56). In rats, enterocytes can absorb antigens of low molecular weight and macromolecules (114), and process the absorbed proteins to antigenic peptides (115). Recently, enterocytes were shown to be important components involved in the initial processes of antigen handling *in vitro* (6). Hence, enterocytes might participate in antigen handling *in vivo* because of their strategic location and number.

Enterocytes of the small intestine of guinea pig (19), mouse (25), man (22), and rat (5) express major histocompatibility class II antigens. Class II antigens are generally present on APC, such as dendritic cells, some macrophages, B lymphocytes,

and activated T lymphocytes. Expression of MHC class II antigens is an important characteristic of an APC (37), although class II antigen expression by itself does not ensure APC cell function. Class II antigens bind antigenic peptides in an MHC restricted fashion which allows presentation of the peptides to $CD4^+$ T lymphocytes (116). Certain other parenchymal cells, for example, epidermal cells (18), and tubular epithelium of kidney (91,92) have been shown to express class II antigens constitutively.

In adult mice, immunoferritin labelling of dissociated, fixed, enterocytes for MHC class II antigens at electron microscopic level revealed baso-lateral staining of the plasma membrane (3). Intracellular and cell surface expression of class II antigens has been demonstrated immunohistochemically in enterocytes by light microscopy in humans, mice, and rats (5,15,22). Freshly isolated epithelial cells from human small intestine were observed to take up, process, and present soluble antigens to immunocompetent T lymphocytes, causing activation of $CD8^+$ suppressor cells (6). In rats, enterocytes appear to induce activation of T cells with suppressor/cytotoxic phenotype (41). The majority of intraepithelial lymphocytes (IEL) are also T suppressor cells in rats and mice (117,118), suggesting that IEL differentiate under the influence of the absorptive epithelium (119). Class II positive epithelial cells therefore, may function as effective APC, having an active role in the local immune response.

The efficiency of antigen presentation may be controlled by multiple factors (120), namely: (i) quantitative (121) and qualitative (122) variations in class II

antigen expression by the APC, (ii) differences in antigen processing, (iii) requirements for cytokines synthesized by APC and responding T cells, and (iv) mechanisms involved with T lymphocyte receptors for the class II molecule and antigen complexes.

The class II antigens are present in the enterocytes of the small intestine of mice (3,15,123), however, there has been no indication of regional variation in expression along the length of the small intestine within individual mice. Study reported herein demonstrated a variation in expression of the class II antigens in various regions of the mouse small intestine. This may indicate a difference in antigen handling by enterocytes in different regions of the small intestine.

3.2 Materials and methods

Three approximately seven week-old mice, C3H/He brown inbred strain of k-haplotype, and C57BL/6 of b-haplotype, were used in the present study (Charles River, Montreal, Quebec). Processing of the tissue was similar to that described in Chapter 2.

Fixation, reagents, controls, time, and temperature of incubation of PAP labelling were as described in Chapter 2. However, the fixative of choice was absolute ethanol, and the primary anti-class II antigen and unrelated antibody were used at 1:4000 dilution. The dilutions of secondary antibody (goat anti-mouse IgG), and PAP soluble complex were 1:50 and 1:100, respectively.

3.3 Results

Immunoreactivity to MHC class II antigens in enterocytes was seen along the entire length of the small intestine. However, the cellular distribution pattern and the intensity of the stain on villus (Fig. 11; Fig. 12) and crypt epithelium (Fig. 13) varied in different regions of the intestine. Many cells in the lamina propria also stained positive for the antigens.

In C3H/He mice, the villus (Fig. 11b,d,f; Fig. 12a) and crypt epithelium (Fig. 13a) that was treated with an unrelated monoclonal antibody as primary antibody, and all other tissue sections from control groups were negative for class II antigens. A few cells in the lamina propria showed endogenous peroxidase activity in those controls in which enzyme activity was not quenched (Fig. 11b,d,f)

3.3.1 Duodenum

In C3H/He mice, anti-mouse MHC class II monoclonal antibody produced a scattered staining pattern on the duodenal villi. Many enterocytes showed a negative reaction, however, absence of the stain did not follow a distinct pattern in different villi. The granular intracellular immunoreaction was observed mainly in the apical cytoplasm of the enterocytes (Figs. 11a, 12b). The basal and lateral surfaces of the cells in the lower portion of the villus exhibited a weak immunoreaction. Crypt epithelium showed pattern of staining similar to that in the villus epithelium (Fig. 13b).

The lamina propria possessed many class II positive cells, located in the distal portion of the villus. Some of these cells exhibited abundant cytoplasmic

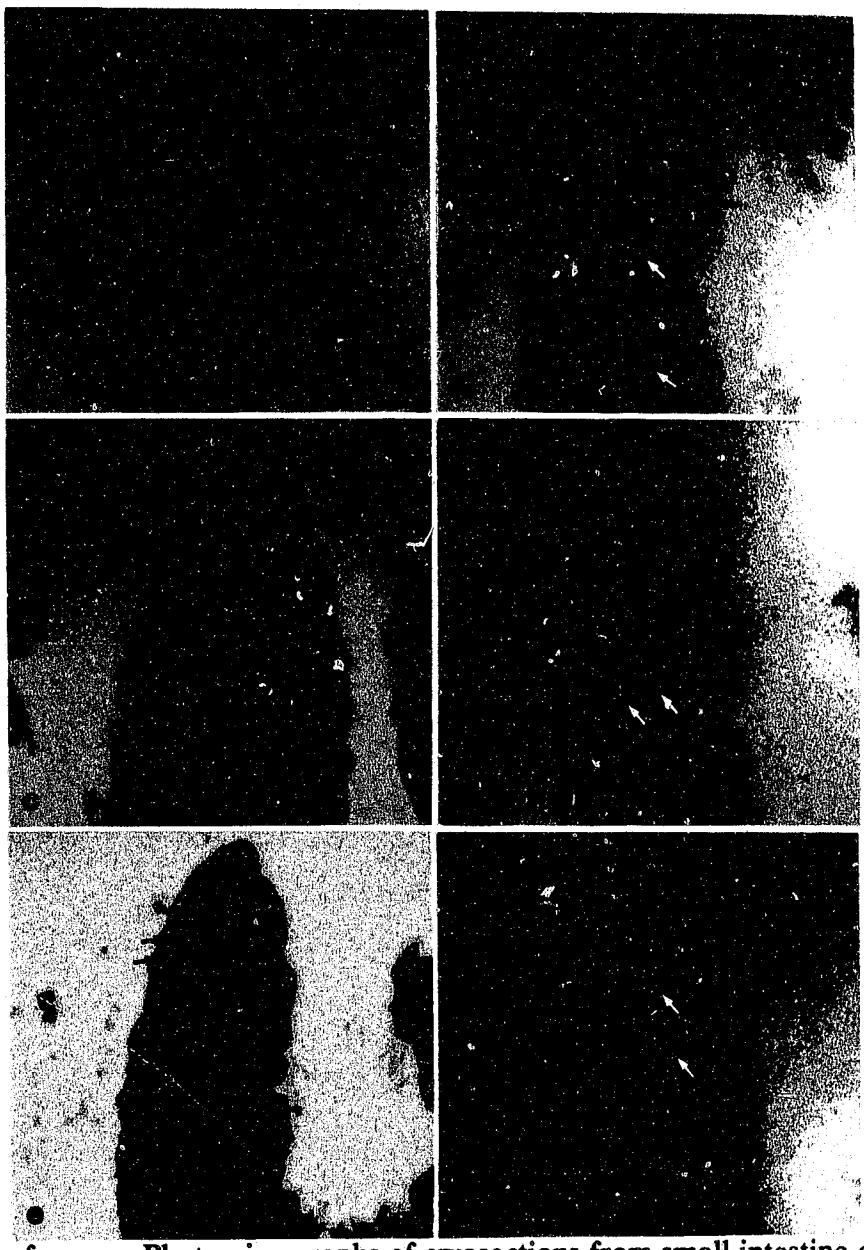


Figure 11 a-f. a,c,e. Photomicrographs of cryosections from small intestine of adult C3H/He mice stained for MHC class II antigens. a. Duodenum showed dispersed immunoreaction on the villus epithelium (*arrows*). c. Jejunal villus showed immunoreaction in enterocytes (*arrows*) and in lamina propria along its length. e. Intense immunoreaction was observed in enterocytes (*arrows*) and in cells of lamina propria of the entire ileal villus. Distinct baso-lateral cell surface staining was observed (*outlined arrows*). b,d,f. Duodenum, jejunum, and ileum, respectively, represented substitution controls, showed absence of immunoreaction in enterocytes, and lamina propria exhibited endogenous peroxidase reaction (*arrows*). X 250.

Figure 12 a-d. Photomicrographs of cryosections from small intestine of adult C3H/He mice stained by PAP. a. Duodenal enterocytes (*asterisk*) were unstained with unrelated primary monoclonal antibody, and cells in lamina propria showed endogenous peroxidase reaction (*arrows*). b-d. Sections stained for class II antigens. b. Apical cytoplasm of few duodenal enterocytes was weakly stained (*arrows*). c. Jejunal enterocytes showed staining in the cytoplasm (*arrows*). Cells at tip of a villus exhibited intense staining of baso-lateral cell surfaces (*outlined arrows*). d. Ileum exhibited intense staining seen in the apical (*arrows*) and basal cytoplasm and baso-lateral cell surfaces (*outlined arrows*). X 640.

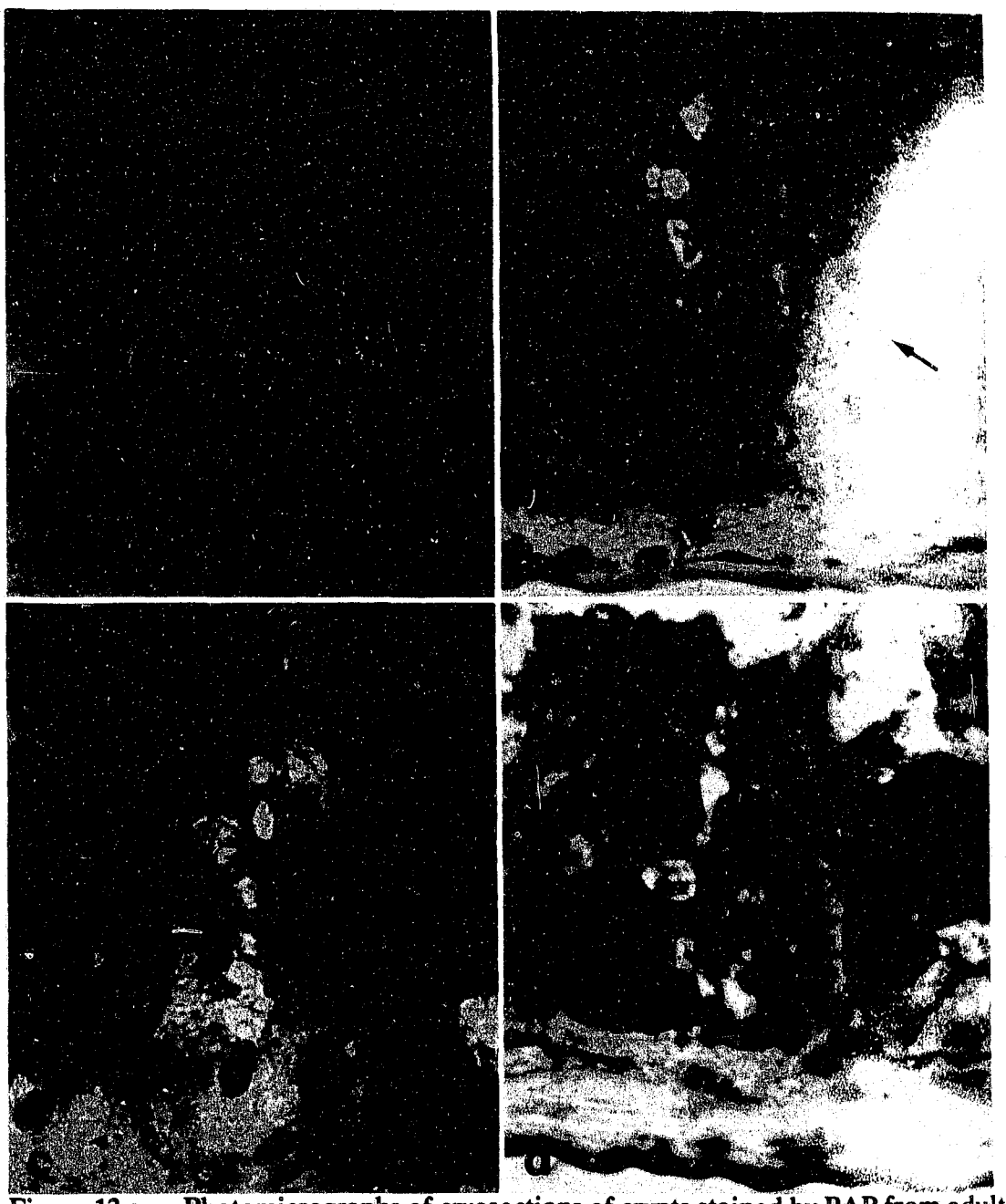


Figure 13 a-c. Photomicrographs of cryosections of crypts stained by PAP from adult C3H/He mice. Epithelium of crypts (*asterisk*) from duodenum stained with unrelated primary monoclonal antibody showed absence of immunoreaction. b-d. Sections stained for class II antigens. b. Duodenal crypts possessed granular staining in apical part of epithelial cells (*arrows*). c. Crypt epithelium of jeunum stained more extensively (*arrows*) than that of duodenum. d. Epithelium of crypts from ileum showed intense immunoreaction (*arrows*). X 640.

processes which, in some instances, extended to the basement membrane and even between the columnar epithelial cells. Small round cells within the lamina propria exhibited distinct immunoreactivity. Elongated cells towards the proximal portion of the villus also showed positive reaction for class II molecules.

3.3.2 Jejunum

Apical and basal intracellular immunoreactivity to class II antigens was more intense in the enterocytes lining the jejunal mucosa (Fig. 12c), when compared to that of the duodenal epithelium staining patterns in the C3H/He strain of mice. This immunoreactivity was seen in cells lining the entire villus (Fig. 11c). Distinct lateral cell surface staining of enterocytes for class II molecules was observed in this region. Enterocytes which formed the tip of the villus showed intense baso-lateral surface staining (Fig. 12c). Crypt epithelium exhibited similar distribution and intensity of the reaction product as did villus epithelium (Fig. 13c). The lamina propria possessed numerous class II positive cells similar to those in the propria of duodenum.

3.3.3 Ileum

Intense intracellular immunoreactivity for the antigens was exhibited in enterocytes lining the villi of ileum (Fig. 11e). There was an obvious increase in the expression of the antigens by these enterocytes when compared to those in duodenum and jejunum (Compare Figs. 12b and 12c, with 12d). Apical and basal intracellular areas, and basal and lateral cell surfaces displayed well-defined staining for the antigens (Fig. 12d). The crypt enterocytes exhibited an intense immunoreactivity in

the apical cytoplasm (Fig. 13d). Lateral cell surfaces of crypt epithelium stained positive for the antigens. The class II positive cells of the lamina propria showed an expression similar to that of the propria cells in the jejunum.

3.4 Discussion

The present report demonstrated that the distribution pattern and the intensity of immunoreaction for MHC class II antigens increases progressively from duodenum to the ileum in adult C3H/He strain of mice. A study on rat enterocytes showed similar class II antigen expression that increased from duodenum to ileo-caecal junction (16). Recently, in a study where adult C57BL/6 mice were used, a regional variation in the expression of class II antigens was not observed in enterocytes of the small intestine within individual animals (27). The variation in the reported (27) and our results may indicate a strain to strain variation in expression of the antigen by the enterocytes in mice.

There may be a number of reasons for the variation in expression of class II antigens in the small intestine. The unidirectional flow of luminal contents is important for ensuring low bacterial/antigen content in the small intestine. This function is accomplished by peristalsis and movement through the ileo-caecal valve. The ileo-caecal valve is not completely effective in allowing the movement of intestinal contents through ileo-caecal opening(30), therefore, the distal ileum has higher counts of viable micro-organisms, than in the jejunum. There is evidence that the expression of class II antigens changes upon immunological stimulation (33). If there is greater stimulation of the ileum by the bacteria and other elements in the

luminal contents, the stimulation may contribute to the increased expression of these glycoproteins. The amount of class II antigen expression by the enterocytes of isolator-reared mice is considerably less than that of the mice reared in conventional environment (27). Since the gut of isolator-reared mice is exposed to a lesser quantity and array of antigens than the mice reared in the conventional environment, the isolator-reared mice expressed lower levels of class II antigens. This hypothesis supports the concept that the concentration of antigens in the gut does influence the level of class II antigen expression by the enterocytes.

The magnitude of response of MHC-restricted T-lymphocyte clones depends on the concentration of foreign antigens and the class II molecules (124). Enterocytes may be involved in the presentation of "non-self" antigens by binding antigenic fragments to MHC class II antigens and presenting this complex to the activated T lymphocytes. The density of the antigen-MHC class II antigen complexes must reach a critical level for T lymphocyte activation and the activation of other cells with which the T lymphocytes interact. The number of antigen-MHC class II antigen complexes generated is proportional to the concentration of both foreign antigens and class II antigens on the cell surface (121). Therefore, another possible explanation for the comparative increase in expression of class II antigens on the ileal enterocytes is, with geater exposure of these cells to antigens, more class II antigens are required to form complexes with these antigens for presentation to activated T lymphocytes.

The low levels of physiologically secreted cytokines may maintain the normal expression of class II antigens (125). The induced expression of class II antigens after interferon-gamma treatment *in vivo* has been demonstrated in the rat intestinal epithelium (16). Gamma-interferon is a lymphokine secreted by activated T lymphocytes which can induce the expression of class II antigen by a variety of non-lymphoid cells that may allow them to act as "non-professional" APC (30). However, studies on nude mice show that expression of class II antigens on enterocytes is not completely dependent on lymphokines secreted by T lymphocytes (5).

Various human intestinal mucosal diseases such as coeliac disease (35), ulcerative colitis and Crohns' disease (126) exhibit increased expression of class II antigens in the enterocytes. In these diseases, there is also an increase in MHC class II positive cells in the lamina propria (36). It is not known how the increased expression of class II antigens in the gastrointestinal tract may influence local immune regulation, but enhanced antigen presentation might be a possibility (2) indicating a possible immune role of the enterocytes in disease conditions.

In conclusion, there is now considerable evidence to speculate that MHC class II antigens on enterocytes are influenced by the level of environmental antigenic challenge. Hence, level of expression of class II antigens varies in different regions of the small intestine of C3H/He mice. This regional variation provides evidence that the enterocytes of ileum may be much more involved and efficient in antigen handling, and subsequent immune tolerance or responsiveness than those in the

duodenum and jejunum because of increased levels of class II antigen expression. Therefore, the route of absorption of antigens through enterocytes of the ileum, and the knowledge of possible APC function of enterocytes may influence the outcome of oral immunization.

4. ONTOGENY OF MHC CLASS II ANTIGENS IN MURINE ENTEROCYTES

4.1 Introduction

The gastrointestinal tract is generally considered a secondary lymphoid organ and has an important role in the immune response to luminal antigens. Enterocytes produced during late fetal and early postnatal life are structurally different from those found in the adult (63). The immature intestinal epithelial cells in rodents have several possible immunologic roles. In the proximal small intestine of neonate rodents, immunoglobulin uptake is receptor-specific and pH-dependent (73). During fetal development, the intestinal epithelium of mice is negative for the class II antigen expression (15,26). The adult levels of expression are reached at approximately one month of age (15). In rats, the initial expression of class II antigens begins at approximately 3-4 weeks postnatally; this age coincides with weaning time (5). The class II antigen-bearing dendritic macrophages appear in the lamina propria of small intestine a few days before birth in rats (5,127). These studies also indicate that by day 18 of gestation, villi formation occurs in the small intestine and many class II positive cells can be observed in the lamina propria. By days 20 and 21 of gestation the proximal intestine is well developed and class II positive cells in the lamina propria express adult levels of the antigen. However, the distal small intestine which lags in development has fewer numbers of class II positive cells in the lamina propria. There is abundant evidence from work done on rats that the postnatal development of mucosa of small intestine is in a cranio-caudal

direction (75). In rabbits, the mucosal development, i.e., villi, intestinal glands and other structures, is initiated in duodenum and proceeds caudally (77). However, mucosal hyperplasia develops equally in all the regions of the intestine after weaning (75).

The regional differences in enzymatic activities along the luminal border of small intestine during fetal, postnatal and in adult mice are well documented (62,78,79). An intriguing ultrastructural and enzymatic study demonstrated that differences in the jejunum and ileum of the activity of lactase, sucrase, and β-galactosidase might be genetically controlled in fetal small intestine, and other factors *in situ* might be responsible for the magnitude of the enzyme activity and differences (80). The study suggests that the functions of enterocytes depends on age of the individual and the region of small intestine.

In man and rats, the extent of class II antigen expression in parenchymal cells depends on the age (5,92). Immunofluorescent studies show that Langerhans cells and gastrointestinal epithelium express class II antigens after birth (15). They speculated that these cells during embryonic life may express a level of class II antigens which is below the sensitivity of the assay used, however, and the expression increases after birth because of increased synthesis of class II antigens by exposure to environmental stimulation. As there is a regional variation in the development of the small intestine, it will be interesting to note whether class II antigens develop concurrently in all regions of the small intestine. Variation in development of antigens in different regions of small intestine, and at different time periods may

contribute to understanding the role of class II antigens in development of immune competence. If the villous epithelium does not express the antigens until weaning, then there may be a difference in antigen handling in postnatal and adult animals; a antigenic stimuli might play an important role in its expression.

4.2 Materials and methods

4.2.1 Animals and management

Adult mice, C3H/He brown inbred strain of k-haplotype, were obtained from Charles River (Montreal, Quebec). The mice were bred at the Atlantic Veterinary College to obtain pups of different age groups to conduct postnatal ontogenic studies of MHC class II antigens. All mice were fed *ad libitum* and had free access to water.

Conception was determined by the appearance of a vaginal plug the morning after a male mouse was placed with four or five females for breeding the previous evening. The female mice were subsequently segregated. The day of finding the vaginal plug was designated as day 0 of pregnancy.

During later phases of this study three pregnant mice of the same strain were purchased to obtain mice pups (Charles River, Boston, Massachusetts, USA). Mice pups were kept with their mothers at all times, and they had access to solid food approximately by day 15 postnatally.

Mice were killed by cervical dislocation. Specimens from the duodenum, jejunum and ileum were obtained from 7-, 14-, 16-, 18-, 19-, 20-, 21-, 23-, 25-, 27- and

28-day-old animals postpartum. Three animals were used for each age group. The pups were raised in similar environmental conditions.

Procedures for fixation and processing of small intestine from 7- to 28- day-old animals were similar to those described in the previous chapter.

4.2.2 Immunocytochemistry

The fixation, reagents, time and temperature of incubation of PAP labelling for postnatal ontogenic studies of MHC class II antigens were similar to those described in the previous chapter. However, the primary anti-class II antigen and an unrelated antibody were used at the following dilutions: 1:50, 1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200 and, 1:4000 for all age groups. The ideal dilution for postnatal studies was 1:100. These dilutions were used to detect the concentration of class II antigens. The controls were similar to those described in the study of adult animals.

4.3 Results

Ontogenic studies of MHC class II antigens on the small intestine demonstrated that when mice pups were reared in conventional environment, the first expression of these antigens was seen at 21 days after birth in the villous (Fig. 14a-d), and crypt (Fig. 15a-d) epithelium of duodenum and jejunum. However, there was a variation in expression of the antigens in different regions of the small intestine with age. A positive reaction was indicated by a brown immunostaining.

4.3.1 Duodenum

The earliest expression of class II antigens was observed on day 21 of postnatal life in C3H/He strain of mice. A weak immunoreaction was observed in

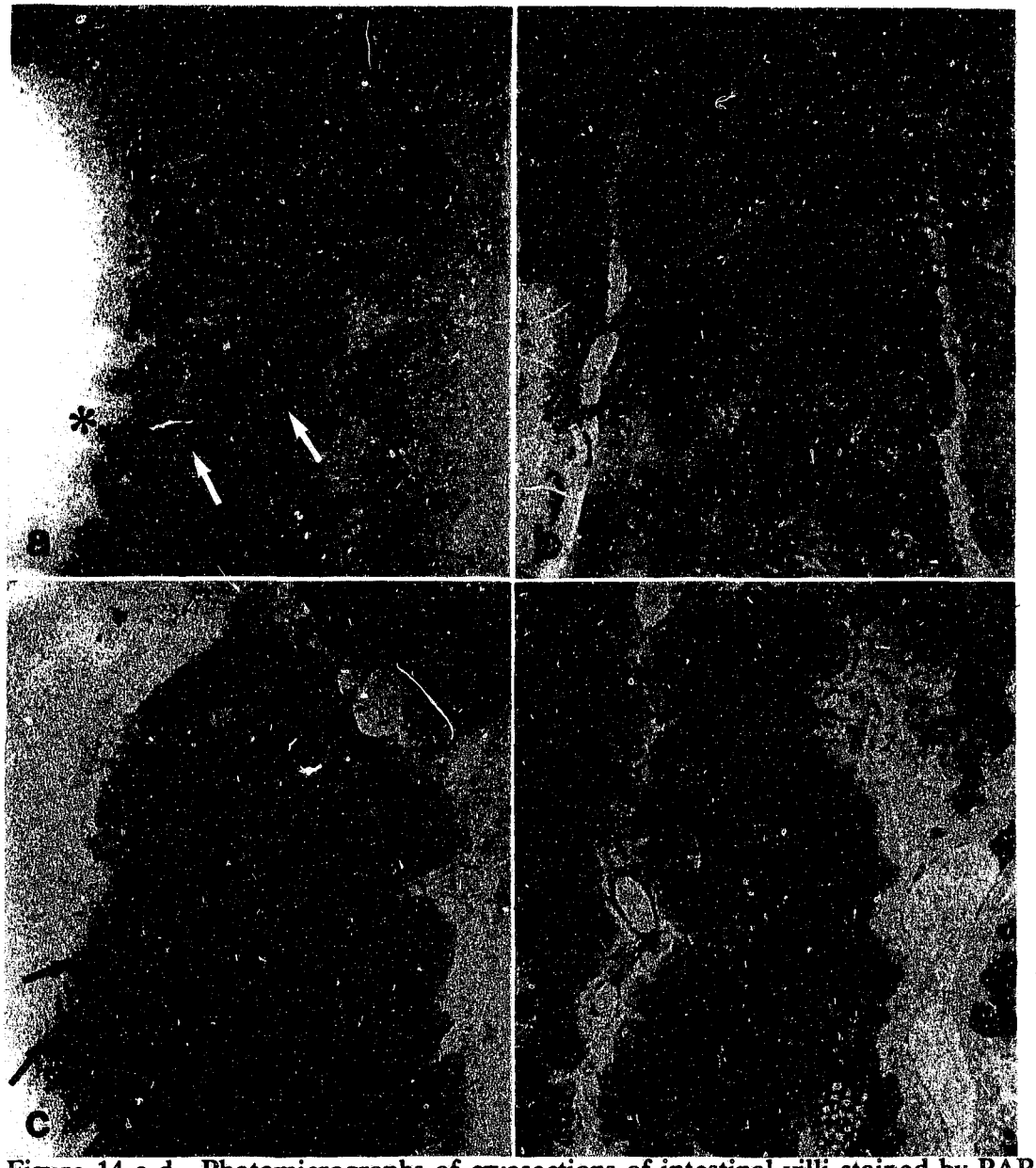


Figure 14 a-d. Photomicrographs of cryosections of intestinal villi stained by PAP from 21-day-old C3H/He mice. a. Duodenum stained with unrelated primary monoclonal antibody showed absence of immunoreaction in enterocytes (*asterisk*). Distinct reaction for endogenous peroxidase was observed in the lamina propria (*arrows*). b-d. Sections stained for class II antigens. b. Cytoplasm of enterocytes exhibited significant immunoreaction (*arrows*) in the duodenum. c. Jejunal enterocytes showed more immunoreaction (*arrows*). Cell surfaces of enterocytes was negative. d. Ileal enterocyte cytoplasm and cell surfaces exhibited absence of immunoreaction (*arrows*). X 640.

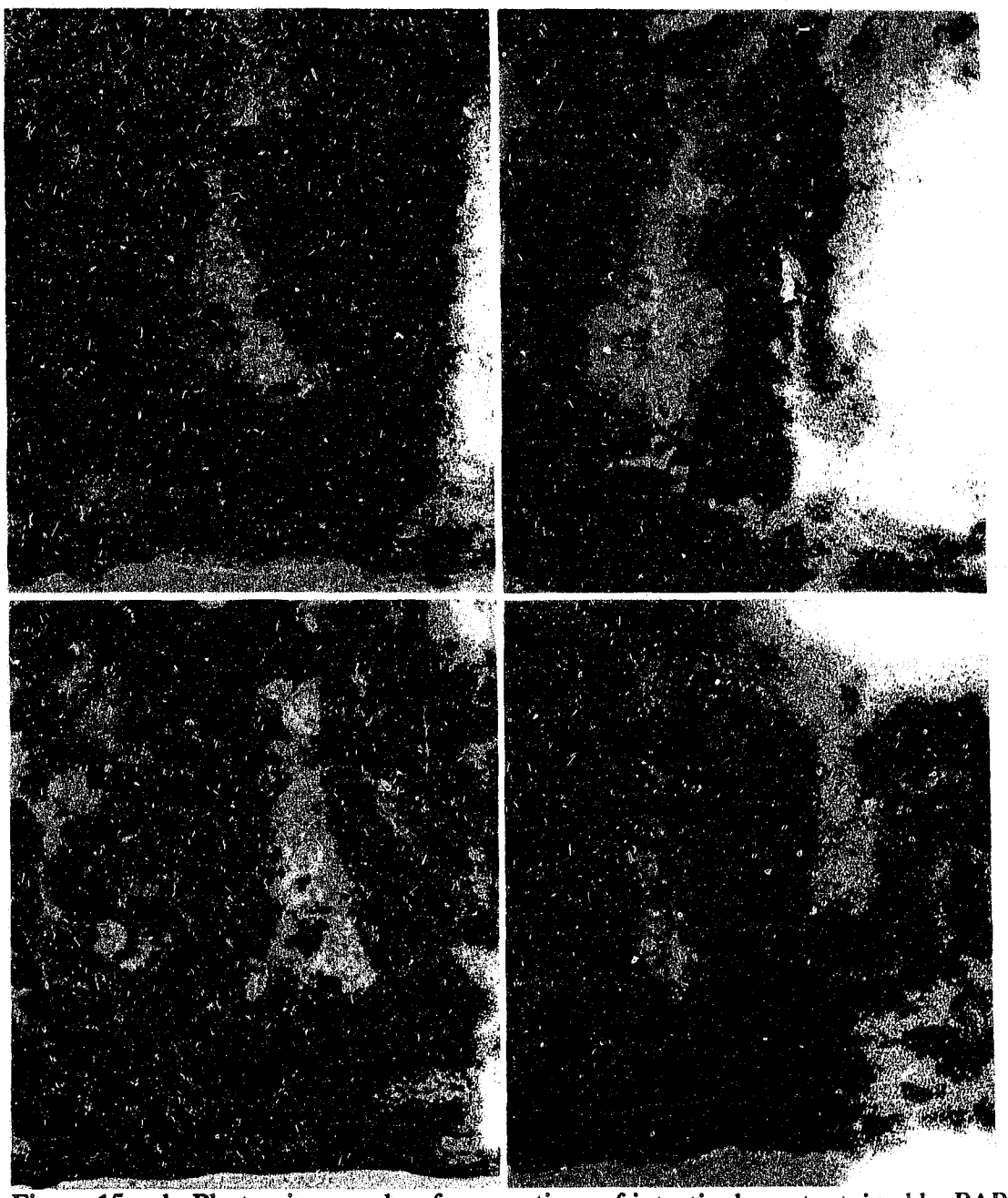


Figure 15 a-d. Photomicrographs of cryosections of intestinal crypts stained by PAP from 21-day-old C3H/He mice. a. Duodenum stained with unrelated primary monoclonal antibody showed absence of immunoreaction in enterocytes (*asterisk*). b-d. Sections stained for class II antigens. b & c. Crypt epithelium of duodenum and jejunum exhibited considerable immunoreaction (*arrows*). d. Cells of crypts (*asterisk*) from ileum revealed no staining. X 640.

the form of granules in apical cytoplasm of some enterocytes lining the villi of the duodenum (Fig. 14b). The majority of enterocytes did not show any immunoreactivity. The crypt epithelial cells showed a staining pattern similar to that of the villous epithelium (Compare Figs. 14b and 15b). Lamina propria contained numerous cells that exhibited positive immunoreaction to class II antigens. Staining pattern of these cells was similar to that in the duodenum from adults. The enterocytes from the control group were negative for class II antigen (Fig. 14a). A few cells in the lamina propria demonstrated an endogenous peroxidase reaction product.

4.3.2 Jejunum

On day 21 of postnatal life, the enterocytes showed a weak immunoreactivity to class II antigens (Fig. 14c). The reaction product was seen mostly in the apical cytoplasm of a few epithelial cells on villi. Basal cell cytoplasm and cell surfaces were negative for the antigens. Immunoreactivity did not follow a distinct pattern along the villus. Intensity and distribution of class II antigens in immature enterocytes was different from that in the enterocytes of the adult mice (Compare Figs. 12c and 14c). Many crypt epithelial cells were positive for class II antigens (Fig. 15c). Expression of the antigens in lamina propria cells was comparatively lower in intensity than that in the duodenum, although the cell types that stained positive for these antigens were similar in both regions.

4.3.3 Ileum

At 21 days postpartum, the ileum was not as developed as the proximal part of small intestine. Cytoplasm and cell surfaces of the enterocytes were negative for class II antigens (Fig. 14d). Crypt epithelium did not show any immunoreactivity (Fig. 15d). Immunoreaction was observed in the cells of lamina propria.

4.3.4 Duodenum, jejunum, ileum (28-day-old mice)

At day 28 of postnatal life, the villous (Fig. 16a-c) and crypt (Fig. 17a-c) epithelium of duodenum, and jejunum showed levels of class II antigen expression comparable to those of adult levels, whereas enterocytes of 28-day-old ileum villi (Fig. 16d), and crypts (Fig. 17d) exhibited weaker expression of class II antigens than those in ileum from adult animals. Lamina propria showed considerable immunoreaction for class II antigens.

The epithelial lining of all regions of small intestine at age groups preceding day 21 of postnatal life did not stain for the antigens. However, animals of age groups of day 23, 25, and 27 showed immunoreaction in duodenal and jejunal epithelium, and the reaction increased with age. The results are summarized in Table I and Table II.

4.4 Discussion

This study demonstrated that development of MHC class II antigens varies in different regions of the small intestine. The antigens were first detected in enterocytes of duodenum and jejunum at 21 days, and in the ileum at 28 days postpartum. The age at which class II antigens were first observed in duodenum and

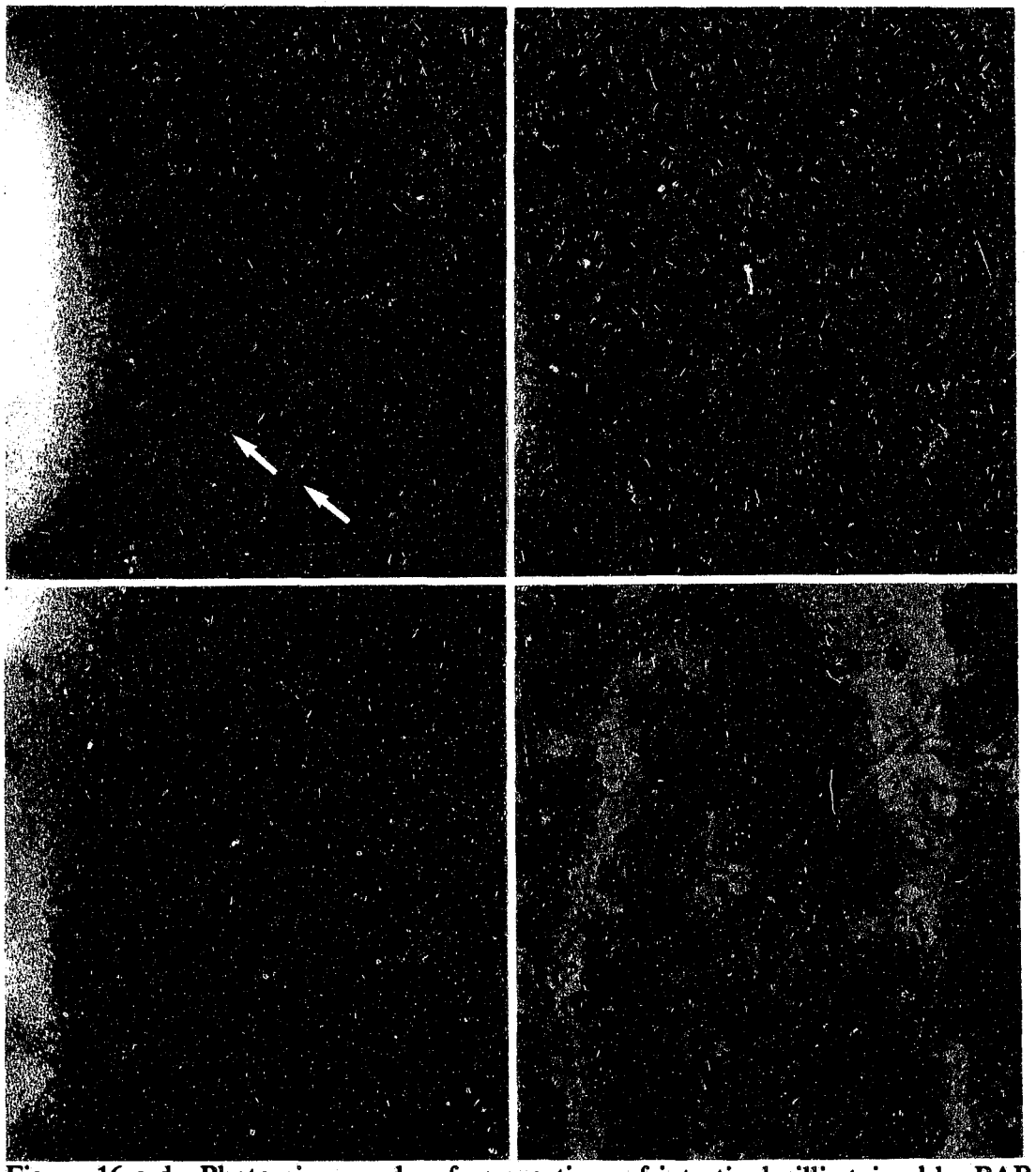


Figure 16 a-d. Photomicrographs of cryosections of intestinal villi stained by PAP from 28-day-old C3H/He mice. a. Duodenum stained with unrelated monoclonal exhibited no immunoreaction in enterocytes (*asterisk*). Some cells in lamina propria showed endogenous peroxidase reaction (*arrows*). b-d. Sections stained for class II antigens. b & c. Duodenal and jejunal enterocytes (*arrows*) and cells of lamina propria exhibited staining comparable to that of villi from adults. d. Ileal enterocytes (*arrows*) and immune cells of lamina propria showed considerable staining but it was not comparable to that of levels in adults. X 640.

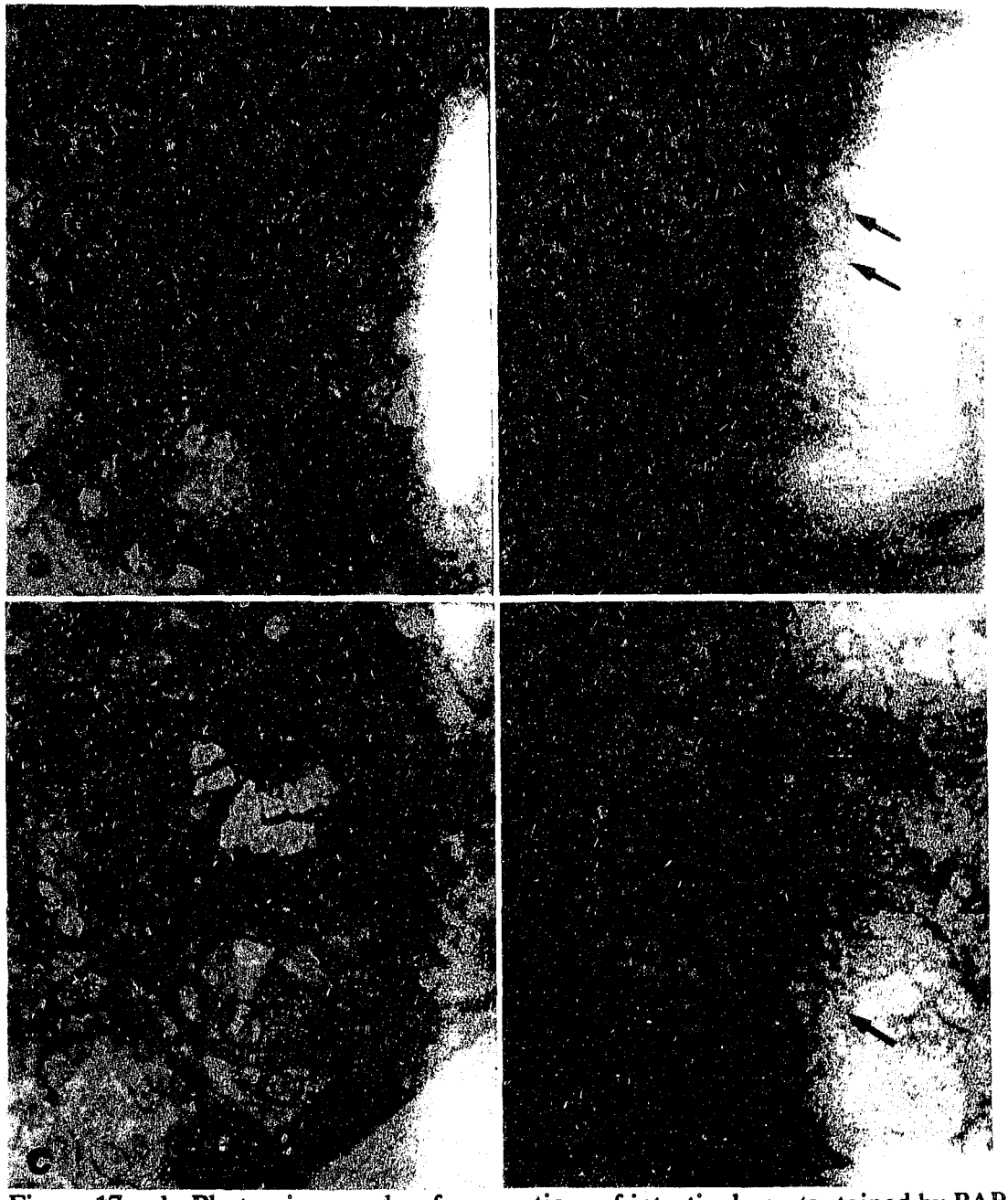


Figure 17 a-d. Photomicrographs of cryosections of intestinal crypts stained by PAP from 28-day-old C3H/He mice. a. Crypt epithelium (*asterisk*) of duodenum stained by unrelated primary monoclonal antibody showed no immunoreaction. b-d. Epithelium of crypts from duodenum, jejunum, and ileum, respectively showed significant immunostaining (*arrows*), however, ileum exhibited levels which were not comparable to those of levels in adults. X 640.

jejunum coincides with the age of weaning in mice, which is approximately at 17-21 days postnatal. Hence, when the intestinal mucosa first comes in contact with environmental antigens, the enterocytes probably begin to express class II antigens.

The expression also appears to relate to the development of small intestine. The postnatal development of mucosa of the small intestine is in cranio-caudal direction (75). Our study has shown that the development of class II antigens might follow the same direction. It has been observed that the expression of class II antigens increases with the maturity of the cells expressing them (128). The immature enterocytes are either incapable of expressing class II antigens, or the antigens are still in their developmental state. Alternatively, the enterocytes during fetal and neonatal life may express a level of class II antigens which is below the sensitivity of the assay. An increased exposure to antigenic stimuli after birth might enhance synthesis of class II antigens (15). However, in mice of C3H/FeJ strain, expression of class II antigens in the enterocytes is detectable at approximately seven days after birth (15). This early expression may be due to exposure of animals to different antigenic environment or a strain to strain variation in level of expression of class II antigens.

The enterocytes of proximal small intestine of rodents possesses Fc receptors for immunoglobulin transport across the mucosal barrier (73). These receptors are pH-dependent and are absent on the mature enterocytes. Therefore, the antibody uptake until weaning, is receptor specific which is important in passive immunity. Closure proceeds in the caudal direction along the small intestine and

appears to be induced in intensity by feeding (30). The development of the class II antigens also proceeds in the same direction, and is induced to the intensity seen in adults by various factors including antigenic challenge. The class II antigen expression occurs shortly after weaning in the proximal small intestine where the antigen uptake might be MHC class II antigen-restricted at this time. Enterocytes of the proximal small intestine select the antibodies from the luminal contents for their subsequent transportation to lamina propria. However, other proteins and those antibodies which proximal enterocytes fail to transport are then non-selectively degraded in the enterocytes of distal portion of the organ (129).

The antigen presenting function of class II positive cells of the spleen reaches levels similar to those in adults by three weeks of age; this period coincides with age of weaning (86). These workers speculated that a maternal factor present in the milk, which is subsequently transmitted to the neonate, might be blocking T-lymphocyte and macrophage maturation. After weaning, APC are allowed to differentiate because the postulated blocking factor is removed. Since enterocytes might serve as APC, they may also be regulated by this maternal factor. Prostaglandin E_2 and AFP in neonates have been shown to inhibit class II antigen expression by APC *in vitro* (82). The immune system early in the ontogeny of an organ is speculated to play an important role in self tolerance and not self defense (82). The delayed expression of class II antigen positive cells in fetal and neonate tissues may have evolved to allow self tolerance as new antigens are still being expressed on differentiating tissue during development. As there is cranio-caudal

development of small intestine, duodenal enterocytes differentiating at an earlier age than those in the jejunum and ileum, and may therefore, express class II antigens at an earlier age in comparison with that of the epithelium in jejunum or ileum.

Ontogenic studies of class II antigens on enterocytes are important to understand autoimmune diseases. A study on the ontogeny of MHC class II antigen-bearing accessory cells in autoimmune MRL-lpr mice found that there was inappropriately early ontogenetic appearance of MHC class II bearing accessory cells in spleen (87); such cells might contribute to the development of autoimmunity in these mice.

MHC class II antigen expression by enterocytes appears to be related to an increase in the number of IEL of suppressor/cytotoxic phenotype in rats (29). Ontogenic studies of these cells have revealed that the number of IELs increases with age. Hence, the onset of MHC class II expression by enterocytes might be related to the number of IEL that are in close proximity of enterocytes at the time. The MHC class II antigen positive cells increase with age in the cranio-caudal direction in the lamina propria of the small intestine, and the presence of these cells might influence the enterocytes to express these antigens (5). Our results are indicative of differences in antigen handling by enterocytes of postnatal and adult mice which might help correlate the onset of immune competence with expression of class II antigens.

TABLE I. **Ontogeny of expression of MHC class II antigens in small intestine of C3H/He strain of mice**

		Region of Gut		
Days Postpartum	Location	Duodenum	Jejunum	Ileum
7	Enterocytes	-	-	-
	Lamina propria	+	+	+
14	Enterocytes	-	-	-
	Lamina propria	+	+	+
21	Enterocytes	+	+	-
	Lamina propria	+	+	+
28	Enterocytes	+	+	+
	Lamina propria	+	+	+
49	Enterocytes	+	+	+
	Lamina propria	+	+	+

\+ Presence of class II positive cells

\- Absence of class II antigens

TABLE II. Cellular distribution of MHC class II antigens in small intestine of postnatal C3H/He mice

Days Postpartum	Location	Region of Gut		
		Duodenum	Jejunum	Ileum
7	Enterocytes	-	-	-
14	Enterocytes	-	-	-
21	Enterocytes	+	+	-
28	Enterocytes	+	++	++
49	Enterocytes	+	++	++

++ All cells lining the villus were positive for class II antigens

\+ Few cells demonstrated class II antigens

\- No cells exhibited immunoreaction

5. GENERAL DISCUSSION

5.1 Selection of technique

When comparing FAT and PAP labelling techniques for the detection of MHC class II antigens, we found that the latter was a more sensitive, efficient and convenient technique.

Decidedly, FAT is a widely used method for labelling antigens, however, a major disadvantage of the technique was a rapid bleaching of the fluorescent dye due to its continuous excitation. The bleaching phenomenon became a difficulty in obtaining consistently satisfactory micrographs, and, especially, to keep permanent record of stained sections. Our experiments showed a variation in expression of class II antigens in adults and in postnates and in initial expression of these antigens, the experiments required detection of minute amounts of antigen. Therefore, an extremely sensitive and stable system was needed, and the PAP method fulfilled the requirements. In summary, sensitivity and stability of PAP staining procedure were significant factors in its selection as our method of choice.

5.2 Regional variation of class II antigens in enterocytes of adults

This study of class II antigen expression in the enterocytes of adult C3H strain of mice showed a variation along the length of the small intestine. The duodenum exhibited the least expression where only a few class II positive enterocytes occurred on villi. The expression was localized in the apical cytoplasm of enterocytes. The cell surfaces appeared negative for the antigens. In the jejunum, the expression was noted over the entire villus. There was an increased reaction in

the apical as well as in the basal cytoplasm of the enterocytes. Ileum showed an intense reaction on cell surfaces and within enterocytes lining the villi. These results are consistent with the observations made in a study on the expression of class II antigens in rat small intestine which also showed an increased expression of the antigens from duodenum to ileum (16). However, a regional variation in the expression was not found in the small intestine of adult C57BL/6 mice (27). The difference between C57BL/6 and C3H/He mice might be due to a strain to strain variation in the level of expression of class II antigens. Such a variation has been observed in the rat enterocytes (5).

A most likely explanation for regional variation of class II antigens in small intestine is that the expression is dependent on antigenic challenge, and time of exposure to the challenge. Due to peristaltic movements of intestine and inefficient function of the ileo-caecal valve, the ileum is exposed to vast array and quantity of antigens. There is evidence that immunologic stimulation, for instance, by parasitic infestations or by other environmental factors, brings about increased expression of class II antigens on gut epithelium (33,27). Indeed, there is a continuous and greater immunologic stimulation of the ileum by its luminal contents causing an enhanced expression to meet the antigenic challenge.

A possible *in vitro* APC role has been assigned to the enterocytes (6). Possible involvement of class II molecules of enterocytes in antigen presentation has been formulated by Bland (1988) (119). A modified version of this theory is given in Fig. 18. If enterocytes are able to function as APC then antigen may be presented

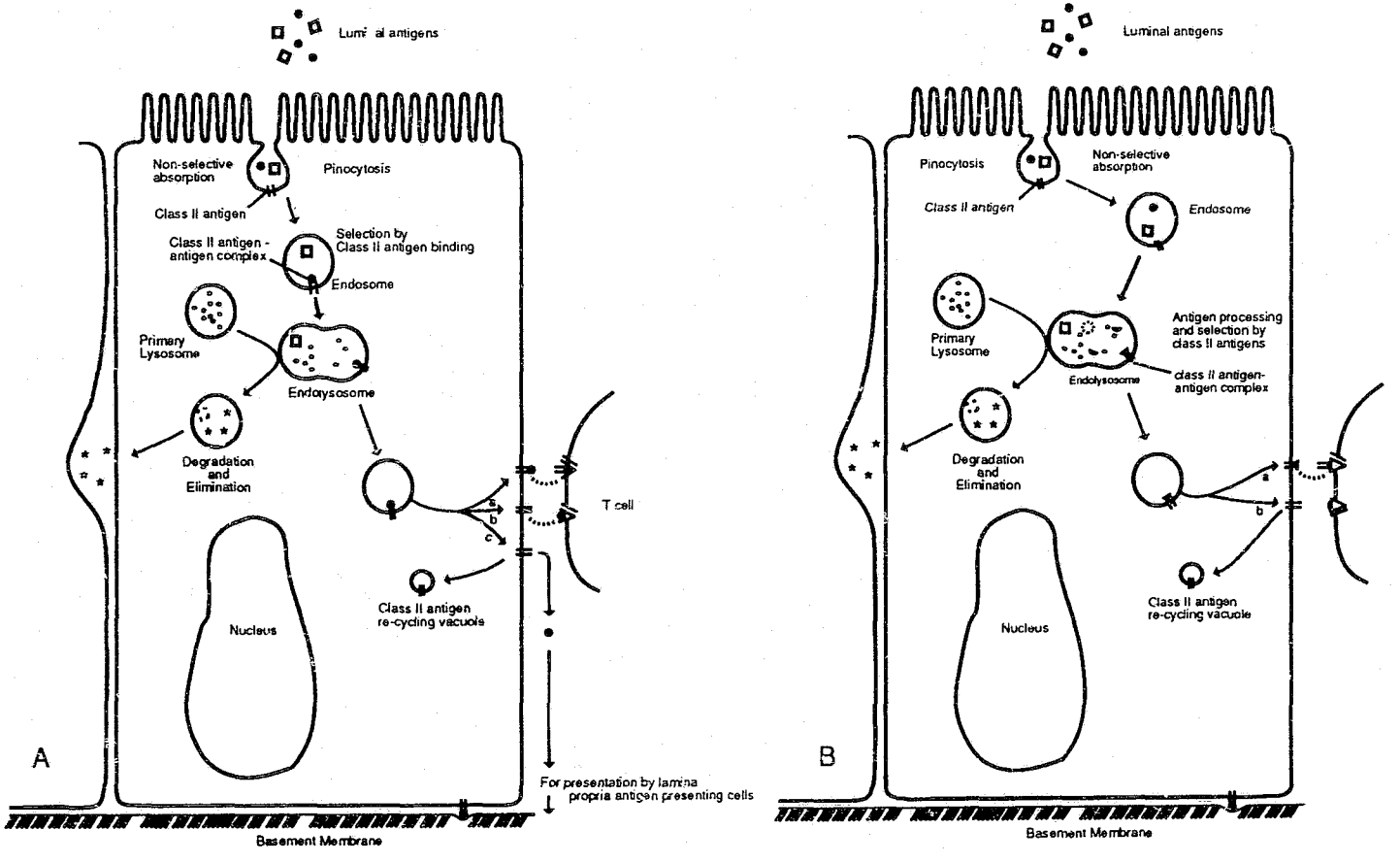


Figure 18. Schematic representation of possible antigen handling by enterocytes. Luminal antigens are non-selectively absorbed by pinocytosis.

A. In endosomes selection by class II antigens takes place to form class II antigen-antigen complex. Primary lysosomes fuse with endosomes to form endolysosomes where non-selected proteins undergo lysosmal degradation.

B. There is no selection by class II antigen in endosomes. Endosomes fuse with primary lysosome to form endolysosomes where antigen processing takes place for class II antigen selection. Class II antigen-antigen complex is then formed, and non-selected proteins undergo lysosomal degradation.

The endolysosomes cleave to form two types of vesicles. One vesicle type contains antigens undergoing further degradation and eventual elimination, and the other vesicle type contains class II antigen-antigen complex which may be presented in three different ways:

a. Entire class II antigen-antigen complex is presented.
b. Presents antigen only, and class II antigens are recycled.
c. Antigen only is released into intercellular space for its presentation by lamina propria antigen presenting cells, and class II antigens are recycled.

in the following manner. Extrinsic antigens are non-selectively absorbed by pinocytosis to form endosomes. *A*. In endosomes selection by class II antigens takes place to form class II antigen-antigen complex (Fig. 18A). Primary lysosomes fuse with endosomes to form endolysosomes where non-selected proteins undergo lysosomal degradation. *B*. There is no selection by class II antigen in endosomes (Fig. 18B). Endosomes fuse with primary lysosomes to form endolysosomes where antigen processing takes place for class II antigen selection. A class II antigen-antigen complex is then formed, and non-selected proteins undergo lysosomal degradation. The endolysosomes in both hypotheses may cleave to form two types of vesicles. One vesicle type contains antigens undergoing further degradation and eventual elimination, and the other vesicle type contains class II antigen-antigen complex which may be presented in three different ways: *a*. The entire class II antigen-antigen complex may be presented. *b*. Antigens alone are presented, and class II antigens are recycled. *c*. Only the antigen is released into intercellular space for its presentation by lamina propria APC, and class II antigens are recycled. The above hypotheses assign a possible antigen presenting role to enterocytes.

The extent of the response of histocompatibility-restricted T lymphocyte clones depends on the product of concentration of extrinsic antigens and MHC class II molecules (124). Also, the density of antigen-class II antigen complexes must reach a critical level for T lymphocyte activation and the activation of other cells with which T cells interact. Another theory that supports the latter study is that the number of antigen-MHC class II antigen complexes generated is proportional to the

concentration of both extrinsic antigens and class II antigens on cell surface (121). A comparative increase in the expression of class II antigens in the ileal enterocytes might occur since these epithelial cells are continuously exposed to large amounts of extrinsic antigens, and, therefore, more class II antigens are required to form complexes with these antigens; hence their presentation to activated T lymphocytes.

This regional variation suggests that epithelial cells of ileum are much more involved and efficient in antigen handling and subsequent immune tolerance or responsiveness than those in the duodenum and jejunum. Therefore, the route of absorption of antigens through enterocytes, and the knowledge of possible APC function of enterocytes may influence the outcome of oral immunization.

5.3 Importance of ontogeny of class II antigens in enterocytes

Ontogenic studies of MHC class II antigens have provided evidence that expression of these antigens depends on maturity of cell structure and function. In the present work, the first expression of class II antigens was seen at day 21 of postnatal life in enterocytes of proximal small intestine. The duodenum of 21-day-old mice expressed level of class II antigens almost comparable to its adult counterparts. Enterocytes of 21-day-old jejunum did show considerable expression of the antigens but the level or density was far less than that seen in adult animals. Enterocytes of ileum did not show any expression of the antigens at day 21, postnatally.

It is well documented that the functional, and structural postnatal development of small intestine occurs in cranio-caudal direction (77,78,80). Perhaps the ontogeny of class II antigens in enterocytes is closely associated with the

development of the organ in a cranio-caudal direction. The ileum may not be structurally and functionally mature at day 21 of postnatal life to express class II antigens. Incidentally, the first expression of the class II antigens also coincides with age of weaning of the mouse pups, i.e., 17-21 days of postnatal life. In our experiments we followed the development of class II antigens until the first expression on the ileum was seen at day 28 of postnatal life. This is the age when the ileum has structurally matured. The expression of the antigens by enterocytes is similar to the age i.e., three weeks at which the antigen presenting function of class II positive cells of spleen is revealed (86). It is speculated that a maternal factor in the milk might be transmitted to neonates and block maturation of T cells and macrophages (86). The APC may be allowed to differentiate when the postulated factor is removed after weaning.

The negative staining for class II antigens by the enterocytes does not neccessarily mean that these antigens are absent from the cells. Their molecular configuration or epitopic fragments may not be recognizable at the time, or the enterocytes might express levels of the antigens which are below the sensitivity level of the assay used. The increased exposure to antigenic stimuli after birth progressively enhances synthesis of class II antigens (15).

It is important to know the age of development of class II antigens because studies on ontogeny of the class II antigen-presenting splenic accessory cells in autoimmune MRL-lpr mice have indicated that these animals have inappropriately early ontogenic appearance of class II positive cells. Therefore, early ontogenic

appearance might contribute to development of autoimmunity in these mice (87). The present study may be indicative of as to when each region of the intestine first expressses class II antigens, and may possibly contribute to autoimmune studies of the gut.

6. REFERENCES

1. BLAND PW, WHITING CV. Antigen processing by isolated rat intestinal villus enterocytes. Immunology 1989; 68: 497-502.

2. BRANDTZAEG P, BAKLIEN K, BJERKE K, ROGNUM TO, SCOTT H, VALNES K. Nature and properties of the human gastrointestinal immune system. In: Miller K, Nicklin S, eds. Immunology of the gastrointestinal tract. Florida: CRC Press, 1987: 3-65.

3. PARR EL, KIRBY WN. An immunoferritin labeling study of H-2 antigens on dissociated epithelial cells. J Histochem Cytochem 1979; 27: 1327-1336.

4. NATALI PG, DE MARTINO C, QUARANTA V, NICOTRA MR, FREZZA F, PELLEGRINO MA, FERRONE S. Expression of Ia-like antigens in normal human nonlymphoid tissues. Transplantation 1981; 31: 75-78.

5. MAYRHOFER G, PUGH CW, BARCLAY AN. The distribution, ontogeny and origin in the rat of Ia-positive cells with dendritic morphology and of Ia antigen in epithelia, with special reference to the intestine. Eur J Immunol 1983; 13: 112-122.

6. MAYER L, SHLIEN R. Evidence for function of Ia molecules on gut epithelial cells in man. J Exp Med 1987; 166: 1471-1483.

7. FAWCETT DW, comp. A textbook of histology. 11th ed. Philadelphia: WB Saunders, 1986: 641-671.

8. PONDER BAJ, SCHMIDT GH, WILKINSON MM, WOOD MJ, MONK M, REID A. Derivation of mouse intestinal crypts from single progenitor cells. Nature 1985; 313: 689-691.

9. THOMSON ABR, KEELAN M. The development of the small intestine. Can J Physiol Pharmacol 1986; 64: 13-29.

10. NEUTRA MR, PADYKULA HA. The gastrointestinal tract. In: Weiss L, ed. Histology: cell and tissue biology. New York: Elsevier Biomedical, 1988: 658-704.

11. MADARA JL, TRIER JS. Functional morphology of the mucosa of the small intestine. In: Johnson LR, Christensen J, Jackson MJ, Jacobson ED, Walsh JH, eds. Physiology of the gastrointestinal tract. 2nd ed. New York: Raven Press, 1987: 1209-1250.

12. OWEN RL, ERMAK TH. Structural specialization for antigen uptake and processing in the digestive tract. Springer Semin Immunopathol 1990; 12: 139-152.

13. COLLINS T, POBER JS, STROMINGER JL. Physiologic regulation of class II major histocompatibility complex gene expression. In: Solheim BG, Moller E, Ferrone S, eds. HLA class II antigens. New York: Springer-Verlag 1986: 14-31.

14. ELSON CO, KAGNOFF MF, FIOCCHI C, BEFUS AD, TARGAN S. Intestinal immunity and inflammation: recent progress. Gastroenterol 1986; 91: 746-768.

15. NATALI PG, NICOTRA MR, GIACOMINI P, PELLEGRINO MA, FERRONE S. Ontogeny of murine I-A^k antigens in tissue of nonlymphoid origin. Immunogenetics 1981; 14: 359-365.

16. STEINIGER B, FALK P, LOHMULLER M, VAN DER MEIDE PH. Class II MHC antigens in the rat digestive system. Normal distribution and induced expressi n after interferon-gamma treatment in vivo. Immunol 1989; 68: 507-513.

17. MIKKELSEN HB, MIRSKY R, JESSEN KR, THUNEBERG L. Macrophage-like cells in muscularis externa of mouse small intestine: Immunohistochemical localization of F4/80, M1/70, and Ia-antigens. Cell Tissue Res 1988; 252: 301-306.

18. HAMMERLING GJ, MAUVE G, GOLBERG E, MCDEVITT HO. Tissue distribution of Ia antigens: Ia on spermatozoa, macrophages, and epidermal cells. Immunogenetics 1975; 1: 428-437.

19. WIMAN K, CURMAN B, FORSUM U, KLARESKOG L, MALMNAS-TJERNLUND U, RASK, TRAGARDH L, PETERSON PA. Occurrence of Ia antigens on tissues of non-lymphoid origin. Nature 1978; 276: 711-713.

20. HART DNJ, FABRE JW. Endogenously produced Ia antigens within cells of convoluted tubules of rat kidney. J Immunol 1981; 126: 2109-2113.

21. WILDERS MM, SMINIA T, PLESCH BEC, DREXHAGE HA, WELTEVREDEN EF, MEUWISSEN SGM. Large mononuclear Ia-positive veiled cells in Peyer's patches: II. Localization in rat Peyer's patches. Immunol 1983; 48: 461-467.

22. SCOTT H, SOLHEIM BG, BRANDTZAEG P, THORSBY E. HLA-DR-like antigens in the epithelium of the human small intestine. Scand J Immunol 1980; 12: 77-82.

23. SPENCER J, FINN T, ISAACSON PG. Expression of HLA-DR antigens on epithelium associated with lymphoid tissue in the human gastrointestinal tract. Gut 1986; 27: 153-157.

24. KIRBY WN, PARR EL. The occurrence and distribution of H-2 antigens on mouse intestinal epithelial cells. J Histochem Cytochem 1979; 27: 746-750.

25. PARR EL, MCKENZIE IFC. Demonstration of Ia antigens on mouse intestinal epithelial cells by immunoferritin labeling. Immunogenetics 1979; 8: 499-508.

26. NATALI PG, DEMARTINO C, PELLEGRINO MA, FERRONE S. Analysis of the expression of I-A^k-like antigens in murine fetal and adult tissues with the monoclonal antibody 10-2.16. Scand J Immunol 1981; 31: 541-546.

27. WILSON AD, BLAND PW, STOKES CR. Expression and distribution of Ia antigens in the murine small intestine: Influence of environment and cholera toxin. Int Arch Allergy Appl Immunol 1990; 91: 348-353.

28. SELBY WS, JANOSSY G, GOLDSTEIN G, JEWELL DP. T lymphocyte subsets in human intestinal mucosa: the distribution and relationship to MHC-derived antigens. Clin Exp Immunol 1981; 44: 453-458.

29. CERF-BENSUSSAN N, QUARONI A, KURNICK JT, BHAN AK. Intraepithelial lymphocytes modulate Ia expression by intestinal epithelial cells. J Immunol 1984; 132: 2244-2252.

30. MAYRHOFER G. Physiology of the intestinal immune system. In: Newby TJ, Stokes CR, eds. Local immune responses of the gut. Florida: CRC Press, 1984: 2-72.

31. SKOSKIEWICZ MJ, COLVIN RB, SCHNEEBERGER EE, RUSSELL PS. Widespread and selective induction of major histocompatibility complex-determined antigens *in vivo* by γ-interferon. J Exp Med 1985; 162: 1645-1664.

32. ZHANG Z, MICHAEL JG. Orally inducible immune unresponsiveness is abrogated by IFN-γ treatment. J Immunol 1990; 144: 4163-4165.

33. BARCLAY AN, MASON DW. Induction of Ia antigen in rat epidermal cells and gut epithelium by immunological stimuli. J Exp Med 1982; 156: 1665-1676.

34. MASON DW, DALLMAN M, BARCLAY AN. Graft-versus-host disease induces expression of Ia antigen in rat epidermal cells and gut epithelium. Nature 1981; 293: 150-151.

35. SCOTT H, BRANDTZAEG P, SSOLHEIM BG, THORSBY E. Relation between HLA-DR-like antigens and secretory component (SC) in jejunal epithelium of patients with coeliac disease or dermatitis herpetiformis. Clin Exp Immunol 1981; 44: 233-238.

36. SELBY WS, POULTER LW, HOBBS S, JEWELL DP, JANOSSY G. Heterogeneity of HLA-DR-positive histiocytes in human intestinal lamina propria: a combined histochemical and immunohistological analysis. J Clin Pathol 1983; 36: 379-384.

37. ERB P, KENNEDY M, WASSMER P, HUEGLI G. Antigen-presenting cells and T cell activation. Agents & Actions 1986; 19: 5-6.

38. GEPPERT TD, LIPSKY PE. Anitgen presentation by interferon-γ-treated endothelial cells and fibroblasts: differential ability to function as antigen-presenting cells despite comparable Ia expression. J Immunol 1985; 135: 3750-3762.

39. PARROTT DMV, TAIT C, MACKENZIE S, MOWAT MA, DAVIES MOJ, MICKLEM HS. Analysis of the effector functions of different populations of mucosal lymphocytes. Ann N Y Acad Sci 1983; 409: 307-319.

40. PETIT A, ERNST PB, BEFUS AD, CLARK DA, ROSENTHAL KL, ISHIZAKA T,BIENENSTOCK J. Murine intestinal intraepithelial lymphocytes. I. Relationships of a novel Thy-1$^-$, Lyt-1$^-$, Lyt-2$^+$, granulated subpopulation to natural killer cells and mast cells. Eur J Immunol 1985; 15: 211-215.

41. BLAND PW, WARREN LG. Antigen presentation by epithelial cells of the rat small intestine. II. Selective induction of suppressor T cells. Immunol 1986; 58: 9-14.

42. KRONENBERG M, GOVERMAN J, HAARS R, MALISSEN M, KRAIG E, PHILLIPS L, DELOVITCH T, SUCIU-FOCA N, LEROY H. Rearrangement and transcription of the β-chain genes of the T-cell antigen receptor in different types of murine lymphocytes. Nature 1985; 313: 647-653.

43. GOODMAN T, LEFRANCOIS L. Expression of the γ-δ T-cell receptor on intestinal CD8$^+$ intraepithelial lymphocytes. Nature 1988; 333: 855-858.

44. LEFRANCOIS L, GOODMAN T. In vivo modulation of cytolytic activity and Thy-1 expression in TCR-γδ + intraepithelial lymphocytes. Science 1989; 243: 1716-1718.

45. SELBY WS, JANOSSY G, JEWELL DP. Immunohistological characterization of intraepithelial lymphocytes of the human gastrointestinal tract. Gut 1981; 22: 169-176.

46. SELBY WS, JANOSSY G, BOFILL M, JEWELL DP. Lymphocyte populations in the mucosa of patients with coeliac disease. Clin Exp Immunol 1983; 52: 219-228.

47. SELBY WS, JANOSSY G, BOFILL M, JEWELL DP. Intestinal lymphocyte subpopulations in inflammatory bowel disease: an analysis by immunohistological and cell isolation techniques. Gut 1984; 25: 32-40.

48. HIRATA I, BERREBI G, AUSTIN LL, KEREN DF, DOBBINS III WO. Immunohistological characterization of intraepithelial and lamina propria lymphocytes in control ileum and colon and in inflammatory bowel disease. Dig Dis Sci 1986; 31: 593-603.

49. BRANDTZAEG P, SOLLID LM, BJERKE K, KETT K, KVALE D, ROGNUM TO, SCOTT H, THRANE PS. Interactions of lymphoid cells with the epithelial environment. Monogr Allergy 1988; 24: 51-59.

50. BRANDTZAEG P, SOLLID LM, THRANE PS, KVALE D, BJERKE K, SCOTT H, KETT K, ROGNUM TO. Lymphoepithelial interactions in the mucosal immune system. Gut 1988; 29: 1116-1130.

51. BANKS WJ. Applied veterinary histology: digestive system I. Baltimore: Williams & Wilkins, 1986.

52. HAURI HP, GREENE JR. The identification of rat intestinal membrane enzymes after electrophoresis on polyacrylamide gels containing sodium dodecyl sulphate. Biochem J 1978; 174: 61-66.

53. DONALDSON RM, MACKENZIE Jr. IL, TRIER JS. Intrinsic factor-mediated attachment of vitamin B_{12} to brush borders and microvillus membranes of hamster intestine. J Clin Invest 1967; 46: 1215-1228.

54. BRASITUS TA, SCHACHTER D. Cholesterol biosynthesis and modulation of membrane cholesterol and lipid dynamics in rat intestinal microvillus membranes. Biochem 1982; 21: 2241-2246.

55. JERVIS HR. Enzymes in the mucosa of the small intestine of the rat, the guinea pig, and the rabbit. J Histochem Cytochem 1963; 11: 692-699.

56. HAMILTON SR, KEREN DF, YARDLEY JH, BROWN G. No impairment of local intestinal immune response to keyhole limpet haemocyanin in the absence of Peyer's patches. Immunol 1981; 42: 431-435.

57. HEATLEY RV, STARK JM, HORSEWOOD P, BANDOUVAS E, COLE F, BIENENSTOCK J. The effects of surgical removal of Peyer's patches in the rat on systemic antibody responses to intestinal antigen. Immunol 1981; 44: 543-548.

58. BIENENSTOCK J, DOLEZEL J. Peyer's patches: lack of specific antibody-containing cells after oral and parenteral immunization. J Immnuol 1971; 106: 938-945.

59. COLONY PC. Successive phases of human fetal intestinal development. In: Kretchmer N, Minkowski A, eds. Nutritional adaptation of the gastrointestinal tract of the newborn. New York: Vevey/Raven Press, 1983: 3-28.

60. MATHAN M, MOXEY PC, TRIER JS. Morphogenesis of fetal rat duodenal villi. Am J Anat 1975; 146: 73-92.

61. CARLSON BM, comp. Patten's foundations of embryology: The digestive and respiratory systems and the body cavities. 5th ed. New York: McGraw-Hill, 1988.

62. CALVERT R, MALKA D, MENARD D. Establishment of regional differences in brush border activities during the development of fetal mouse small intestine. Cell Tissue Res 1981; 214: 97-106.

63. SMITH MW, PEACOCK MA. Anomalous replacement of foetal enterocytes in the neonatal pig. Proc R Soc London B 1980; 206: 411-420.

64. LATSHAW WK, comp. Veterinary developmental anatomy: a clinically oriented approach. Toronto: B.C. Decker, 1987.

65. HENNING SJ. Postnatal development: coordination of feeding, digestion, and metabolism. Am J Physiol 1981; 241: G199-G214.

66. HENNING SJ. Functional development of the gastrointestinal tract. In: Johnson LR, Christensen J, Jackson MJ, Jacobson ED, Walsh JH, eds. Physiology of the gastrointestinal tract. New York: Raven Press, 1987: 285-300.

67. UDALL JN, PANG K, FRITZE L, KLEINMAN R, WALKER WA. Development of gastrointestinal mucosal barrier. I. The effect of age on intestinal permeability to macromolecules. Pediatr Res 1981; 15: 241-244.

68. CLARK SL. The ingestion of proteins and colloidal materials by columnar absorptive cells of the small intestine in suckling rats and mice. J Biophys Biochem Cytol 1959; 5: 41-49.

69. CLARKE RM, HARDY RN. An analysis of the mechanism of cessation of uptake of macromolecular substances by the intestine of young rat ('closure'). J Physiol (Lond) 1969; 127-134.

70. DANIELS VG, HARDY RN, MALINOWSKA KW. The effect of adrenalectomy or pharmacological inhibition of adrenocortical function on macromolecule uptake by the newborn rat intestine. J Physiol (Lond) 1973; 229: 697-707.

71. MORRIS B, MORRIS R. The digestion and transmission of labelled immunoglobulin G by enterocytes of the proximal and distal regions of the small intestine of young rats. J Physiol (Lond) 1977; 273: 427-442.

72. CORNELL R, PADYKULA HA. A cytological study of intestinal absorption in the suckling rat. Am J Anat 1969; 125: 291-316.

73. MORRIS IG. An immunofluorescence study of IgG receptors in rodent enterocytes. Immunol 1980; 40: 273-280.

74. RODEWALD R. Distribution of immunoglobulin G receptors in the small intestine of the young rat. J Cell Biol 1980; 85: 18-32.

75. BUTS JP, DE MEYER R. Postnatal proximodistal development of the small bowel mucosal mass in growing rats. Biol Neonate 1981; 40: 62-69.

76. O'CONNOR TM. Cell dynamics in the intestine of the mouse from late fetal life to maturity. Am J Anat 1966; 118: 525-536.

77. TOOFANIAN F, TARGOWSKI SP. Morphogenesis of rabbit small intestinal mucosa. Am J Vet Res 1982; 43: 2213-2219.

78. MOOG F. The functional differentiation of the small intestine. VIII. Regional differences in the alkaline phosphatase of the small intestine of the mouse from birth to one year. Dev Biol 1961; 3: 153-174.

79. MOOG R, DENES AE, POWELL PM. Disaccharidases in the small intestine of the mouse: Normal development and influence of cortisone, actinomycin D and cycloheximide. Dev Biol 1973; 35: 143-159.

80. KENDALL K, JUMAWAN J, KOLDOVSKY O. Development of jejunoileal differences of activity of lactase, sucrase and acid ß-galactosidase in isografts of fetal rat intestine. Biol Neonate 1979; 36: 206-214.

81. COLLAN Y. Characteristics of non-epithelial cells in the epithelium of normal rat ileum. A light and electron microscopical study. Scand J Gastroenterol 1972; 7: Suppl. 18.

82. LU CY, UNANUE ER. Macrophage ontogeny: Implications for host defence, T-lymphocyte differentiation, and the acquisition of self tolerance. Clin Immunol Allergy 1985; 5: 253-269.

83. STEEG PS, JOHNSON HM, OPPENHEIM JJ. Regulation of murine macrophage Ia antigen expression by an immune interferon-like lymphokine: inhibitory effect of endotoxin. J Immunol 1982; 129: 2402-2406.

84. SNYDER DS, BELLER DI, UNANUE ER. Prostaglandins modulate macrophage Ia expression. Nature 1982; 299: 163.

85. SNYDER DS, LU CY, UNANUE ER. Control of macrophage Ia expression in neonatal mice: role of a splenic suppressor cell. J Immunol 1982; 128: 1458-1465.

86. LU CY, BELLER DI, UNANUE ER. During ontogeny, Ia-bearing accessory cells are found early in the thymus but late in the spleen. Proc Natl Acad Sci (USA) 1980; 77: 1597-1601.

87. LU CY, CHANGELIAN PS. The ontogeny of Ia-bearing accessory cells-regulation by mouse amnionic fluid, abnormal ontogeny in MRL-lpr/lpr mice. 67th Annual meeting of the Federation of American Societies for Experimental Biology, Chicago, IL. Fed Proc 1983; 42: Abst 5441.

88. PETRUSZ P, ORDRONNEAU P, FINLEY JCW. Criteria of reliability for light microscopic immunocytochemical staining. Histochem J 1980; 12: 333-348.

89. POOL CR, BUIJS RM, SWAAB DF, BOER GJ, VAN LEEUWEN FW. On the way to a specific immunocytochemical localization. In: Cuello AC, ed. Immunohistochemistry. Toronto: J Wiley & Sons, 1983: 1-46.

90. LARRSON L. Immunocytochemistry: theory and practice. Boca Raton: CRC Press, 1988.

91. MAYRHOFER G, SCHON-HEGARD MA. Ia antigens in rat kidney, with special reference to their expression in tubular epithelium. J Exp Med 1983; 157: 2097-2109.

92. FUGGLE SV, ERRASTI P, DAAR AS, FABRE JW, TING A, MORRIS PJ. Localization of major histocompatibility complex (HLA-ABC and DR) antigens in 46 kidneys. Transplantation 1983; 35: 385-390.

93. RIGGS JL, SEIWALD RJ, BURCKHALTER JH, DOWNS JH, METCALF TG. Isothiocyanate compounds as fluorescent labeling agents for immune serum. Am J Pathol 1958; 34: 1081-1097.

94. SCHAUENSTEIN K, WICK G. Membrane immunofluorescence. In: Wick G, Trail KN, Schauenstein K, eds. Immunofluorescent technology. New York: Elsevier Biomedical Press, 1982: 28-37.

95. STERNBERGER LA, HARDY PH, CUCULIS JH, MEYER HG. The unlabelled antibody enzyme method of immunocytochemistry: preparation and properties of soluble antigen-antibody complex (horseradish peroxidase-antiperoxidase) and its use in identification of spirochetes. J Histochem Cytochem 1970; 18: 315-333.

96. MARUCCI AA, DOUGHERTY RM. Use of the unlabelled antibody immunohistochemical technique for the detection of human antibody. J Histochem Cytochem 1975; 23: 618-623.

97. PETRALI JP, HINTON DM, MORIARTY GC, STERNBERGER LA. The unlabeled antibody enzyme method of immunocytochemistry: quantitative comparison of sensitivities with and without peroxidase anti-peroxidase complex. J Histochem Cytochem 1974; 22: 782-801.

98. CHILDS GV UNABIA G, ELLISON D. Immunocytochemical studies of pituitary hormones with PAP, ABC, and immunogold techniques: evolution of technology to best fit the antigen. Am J Anat 1986; 175: 307-330.

99. CHILDS GV. The use of multiple methods to validate immunocytochemical stains. J Histochem Cytochem 1983; 31:168-176.

100. PETRUSZ P. Essential requirements for the validity of immunocytochemical staining procedures. J Histochem Cytochem 1983; 31: 177-179.

101. HUMASON GL. Animal tissue techniques: solution preparation. San Francisco: W.H. Freeman & Co, 1979.

102. BOORSMA DM, NIEBOER C, KALSBEEK GL. Cutaneous immunohistochemistry: the direct immunoperoxidase and immunoglobulin-enzyme bridge methods compared with the immunofluorescence method in dermatology. J Cutaneous Path 1975; 2: 294-301.

103. ROGNUM TO, BRANDTZAEG P, ORJASAETER H, FAUSA O. Immunohistochemistry of epithelial cell markers in normal and pathological colon mucosa: comparison of results based on routine formalin and cold ethanol-fixation methods. Histochem 1980; 67: 7-21.

104. BRANDTZAEG P. Tissue preparation methods for immunocytochemistry. In: Bullock GR, Petrusz P, eds. Techniques in immunocytochemistry. New York: Academic Press, 1982: 1-76.

105. BIGBEE JW, KOSEK JC, ENG LF. Effects of primary antiserum dilution on staining of "antigen-rich" tissues with the peroxidase anti-peroxidase technique. J Histochem Cytochem 1977; 25: 443-447.

106. BOSMAN FT, LINDEMAN J, KUIPER G, VAN DER WAL A, KREUNIG J. The influence of fixation on immunoperoxidase staining of plasma cells in paraffin sections of intestinal biopsy specimens. Histochem 1977; 53: 57-62.

107. MORIARTY GC, MORIARTY CM, STERNBERGER LA. Ultrastructural immunocytochemistry with unlabeled antibodies and the peroxidase anti-peroxidase complex: a technique more sensitive than radioimmunoassay. J Histochem Cytochem 1973; 21: 825-833.

108. VANDESANDE F. Peroxidase anti-peroxidase techniques. In: Cuello AC, ed. Immunocytochemistry. Toronto: J Wiley & sons, 1983: 101-119.

109. BOURNE JA. Handbook of immunoperoxidase staining methods. Santa Barbara: Dako Cooperation, 1983.

110. DE TOMA FJ, MAC DONALD AB. Experimental Immunology: a guidebook. New York: Macmillan Publishing Co, 1976.

111. JOHNSON GD, DAVIDSON RS, MCNAMEE KC, RUSSELL G, GOODWIN RD, HOLBOROW EJ. Fading of immunofluorescence during microscopy: a study of the phenomenon and its remedy. J Immunol Methods 1982; 55: 231-242.

112. RICHMAN LK, GRAEFF AS, STROBER W. Antigen presentation by macrophage-enriched cells from the mouse Peyer's patches. Cell Immunol 1981; 62: 110-118.

113. BOCKMAN DE, COOPER MP. Pinocytosis by epithelium associated with lymphoid follicles in the bursa of Fabricius, appendix, and Peyer's patches. An electron microscopic study. Am J Anat 1973; 136: 455-477.

114. WARSHAW AL, WALKER WA, CORNELL R, ISSELBACHER KJ. Small intestinal permeability to macromolecules. Transmission of horseradish peroxidase into mesenteric lymph and portal blood. Lab Invest 1971; 25: 675-684.

115. STERN N, WALKER WA. Food proteins and gut mucosal barrier. I. Binding and uptake of cow's milk proteins by adult rat jejunum *in vitro*. Am J Physiol 1984; 246: G556.

116. YEWDELL JW, BENNINK JR. The binary logic of antigen processing and presentation to T cells. Cell 1990; 62: 203-206.

117. LYSCOM N, BRUETON MJ. The development of intraepithelial and Peyer's patch lymphocyte sub-types in the small intestine of newborn rats. Clin Exp Immunol 1983; 54: 158-162.

118. MOWAT AM, TAIT RC, MACKENZIE S, DAVIES MDJ,PARROTT DMV. Analysis of natural killer effector and suppressor activity by intraepithelial lymphocytes from mouse small intestine. Clin Exp Immunol 1983; 52: 191-198.

119. BLAND PW. MHC class II expression by the gut epithelium. Immunol Today 1988; 9: 174-178.

120. BLAND PW, WARREN LG. Antigen presentation by epithelial cells of the rat small intestine I. Kinetics, antigen specificity and blocking by anti-Ia antisera. Immunol 1986; 58: 1-7.

121. JANEWAY CA, BOTTOMLY K, BABICH J, CONRAD P, CONZEN S, JONES B, KAYE J, KATZ M, MCVAY L, MURPHY DB, TITE J. Quantitative variation in Ia antigen expression plays a central role in immune regulation. Immunol Today 1984; 5: 99-104.

122. CULLEN SE, KINDLE CS, SCHREFFLER DC, COWING C. Differential glycosylation of murine B cell and spleen adherent cell Ia antigens. J Immunol 1981; 127: 1478-1484.

123. CURMAN R, KAMPE O, RASK L, PETERSON PA. Presence of alloreactive Ia antigens on murine intestine epithelial cells. Scand J Immunol 1979; 10: 11-15.

124. MATIS LA, GLIMCHER LH, PAUL WE, SCHWARTZ RH. Magnitude of response of histocompatibility-restricted T cell clones is a function of the product of the concentrations of antigen and Ia molecules. Proc Natl Acad Sci 1983; 80: 6019-6023.

125. GROENEWEGEN G, BUURMAN WA, VAN DER LINDEN CJ. Lymphokine dependence of in vivo expression of MHC class II antigens by endothelium. Nature 1985; 316: 361-363.

126. SELBY WS, JANOSSY G, MASON DY, JEWELL DP. Expression of HLA-DR antigens by colonic epithelium in inflammatory bowel disease. Clin Exp Immunol 1983; 53: 614-618.

127. WILDERS MM, SMINIA T, JANSE EM. Ontogeny of non-lymphoid and lymphoid cells in the gut with special reference to large mononuclear Ia-positive dendritic cells. Immunol 1983; 50: 303-314.

128. SPENCER J, MACDONALD TT, ISAACSON PG. Heterogeneity of non-lymphoid cells expressing HLA-D region antigens in human fetal gut. Clin Exp Immunol 1987; 67: 415-424.

129. RODEWALD R. Intestinal transport of antibodies in the newborn rat. J Cell Biol 1973; 58: 189-211.

7. APPENDIX

1. Phosphate buffered saline 0.01M, pH 7.4

   $Na\ H_2PO_4\ H_2O$ 0.8 g
   Na_2HPO_4 5.68 g
   NaCl 35.2 g
   Dissolve the above ingredients in 4 l of distilled H_2O.

2. Tris-HCl buffer, 0.05M, pH 7.6

   Add 15.76 g of Tris-HCl to 2 l of distilled water.

3. Diaminobenzidine hydrochloride solution

   Dissolve 5 mg of 3,3' diaminobenzidine tetrahydrochloride in 10 ml of 0.05M Tris-HCl pH 7.6. Aliquots of 250 µl were stored at -20°C. To the above aliquots 50 µl of 3% hydrogen peroxide and 2.5 ml of Tris-HCl was added.

4. Harris' haematoxylin

   After PAP immunoreaction the cryosections were placed in:
   i) Harris' haematoxylin (Fisher Scientific), 3 min
   ii) Distilled water, 2 min
   iii) Acid alcohol, 30 sec
   iv) Distilled water, 2 min
   v) Ammonia water, 10 sec
   vi) Distilled water, 2 min
   vii) 70% alcohol, 2 min
   viii) 90% alcohol, 2 min
   ix) Absolute alcohol, 2 min
   x) Hemo-De (Baxter Healthcare Co., McGaw Park IL), 2 min

5. Haematoxylin and eosin staining for cryosections

   After fixation of cryosections in absolute ethonol:
   i) Placed in 95% alcohol, 2 min
   ii) 70% alcohol, 2 min
   iii) Distilled water, 2 min
   iv) Harris' haematoxylin, 10 min
   v) Distilled water, 2 min
   vi) Acid alcohol, 30 sec

vii) Distilled water, 2 min
viii) Ammonia water, 10 sec
ix) Running water, 10 min
x) Eosin, 2 min
xi) 95% alcohol, 2 min
xii) Absolute alcohol, 2min
xiii) Hemo-De, 2 min

6. Preparation of 4% paraformaldehyde fixative

Heated water in a water bath to 60°C, and added 8 g of paraformaldehyde per 100 ml of distilled water. Allowed to mix, and add 2-3 drops of 1N NaOH till solution clears. Cool and pH solution. Add equal volume of 8% paraformaldehyde to 1.8% NaCl.