

KUOPION YLIOPISTON JULKAISUJA D. LÄÄKETIEDE 266
KUOPIO UNIVERSITY PUBLICATIONS D. MEDICAL SCIENCES 266

TIMO ÖRMÄLÄ

Age related changes in the immunology of the intestinal mucosa

Doctoral dissertation

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Department of Paediatrics
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KUOPIO 2002

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P.O.Box 1627
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FINLAND
Tel. +358 17 163 430
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- Series editors:** Professor Esko Alhava, M.D., Ph.D.
Department of Surgery

Professor Martti Hakumäki, M.D., Ph.D.
Department of Physiology
- Author's address:** Department of Paediatrics
Kuopio University Hospital
P.O.Box 1777
FIN-70211 KUOPIO
FINLAND
Tel. +358 17 172 752
Fax +358 17 172 410
- Supervisors:** Docent Erkki Savilahti
Department of Paediatrics
University of Helsinki

Docent Mikko Perkkiö
Department of Paediatrics
University of Kuopio
- Reviewers:** Docent Marja-Riitta Ståhlberg
Department of Paediatrics
University of Turku

Professor Outi Vaarala
University of Lindköping
Sweden
- Opponent:** Docent Jorma Kokkonen
Department of Paediatrics
University of Oulu

ISBN 951-781-866-1
ISSN 1235-0303

Savon kopiokeskus Oy
Kuopio 2002
Finland

Örmälä, Timo. Age related changes in the immunology of the intestinal mucosa.
Kuopio University Publications D. Medical Sciences 266. 2002. 71 p.
ISBN 951-781-866-1
ISSN 1235-0303

ABSTRACT

This thesis was undertaken to evaluate the influence of age and inflammation of the large intestine on the development of mucosal T cells and their subgroups, HLA class II molecules expressing cells, distribution of the IgA expressing lymphocytes, as well as, possible developmental associations between T and B lymphocytes soon after birth. Age related changes on the mucosal immune system were also evaluated in jejunal specimens taken from patients with coeliac disease (CD) or dermatitis herpetiformis (DH).

Fifty nine specimens taken from the large intestines of infants were studied by immunohistochemical methods to evaluate the influence of inflammation and age on the immunology of large intestine. Twenty two jejunal specimens taken from pediatric patients with CD, were supplemented with 78 biopsy specimens taken from 66 patients with CD or DH of different ages described in earlier studies, were evaluated by immunohistochemical methods to evaluate age related changes in jejunal mucosa from patients with CD and DH, as well as controls.

In non-inflamed colon of infants, we found fewer numbers of CD3+ IELs than earlier reported in adults and adolescents. In colonic lamina propria, CD4+, CD8+ and HLA-DR expressing cells declined in number significantly during the first 2.5 months of life. On the basis of knowledge from studies made with fetuses, the proportion of the TCR $\gamma\delta$ + increased and the non-disulphide linked form of TCR $\gamma\delta$ + appeared at the surface epithelium and lamina propria of large intestine after the second trimester of pregnancy.

In inflamed colon, the numbers of CD3+, TCR $\alpha\beta$ +, TCR $\gamma\delta$ + and non-disulphide linked form of TCR $\gamma\delta$ + were significantly higher than in non-inflamed colon.

The density of CD22 positive B cells in lamina propria was high already in young infants and this cell density increased with age. This increase was attributable mostly to the increase in IgA1 positive cells.

In patients with CD and DH consuming a gluten containing diet, there was an age dependent increase in the density of TCR $\gamma\delta$ + cells on the surface epithelium of jejunum, whereas in controls, TCR $\alpha\beta$ + cells showed a corresponding age related increase. Almost all patients with CD or DH had TCR $\gamma\delta$ + cell densities more than two standard deviations higher than controls.

In this study, frequent age related immunological phenomenon in the intestine were described. Our intestinal immune system is in a dynamic interaction with the environment and age is an important modifying factor of the intestinal immune system.

National Library of Medicine Classification: QW 504.5, QW 573

Medical Subject Headings: immunohistochemistry; receptors, antigen, T-cell; receptors, antigen, B-cell; infant; intestines; colitis; celiac disease;

To Ikka, Anni-Maria, Arttu-Kalle, Iris and Jaakko

ACKNOWLEDGEMENT

This study was carried out at the Hospital for Children and Adolescents, University of Helsinki, Helsinki and Department of Paediatrics, Kuopio University Hospital, Kuopio. I wish to express my gratitude to Professor Kari Launiala, M.D., Head of the Department of Paediatrics, Kuopio University Hospital, until November 1997, and Professor Raimo Voutilainen, M.D., head from December 1997, for their support during the study.

I express my deepest gratitude to my principal supervisor Docent Erkki Savilahti, M.D., senior paediatric gastroenterologist, the Hospital for Children and Adolescents, University of Helsinki, Helsinki. He introduced me to this subject, and his wide experience of paediatric gastroenterology and immunology has been essential for this study. It has been a great privilege to work under his supervision. Furthermore, I wish to thank him for the opportunity to practice my clinical skills under his guidance.

I am most grateful to Professor Risto Rintala, M.D., The Head of Department of Paediatric Surgery and Docent Harri Lindahl, M.D., Hospital for Children and Adolescents, University of Helsinki, Helsinki, who made this study possible by helping us to collect the material for the study. Without their contribution this study could not have been done.

For Docent Marja-Riitta Ståhlberg, M.D., University of Turku, and Professor Outi Vaarala, M.D., University of Lindköping. I want to express sincere thanks for their constructive criticism and expert advise during the preparation of the final manuscript of this thesis.

I address warm thanks to my second supervisor Docent Mikko Perkkiö, M.D., for his constructive criticism and encouraging support during the study. I wish to thank Professor Matti Korppi, M.D., who offered me expert advise during the study. His humanity and never ending sense of humour helped me to accomplish this thesis.

My sincere thanks to my collaborators Timo Klemola, M.D., Ph.D and Gabor Hacsek, M.D., Ph.D., who were my room mates during the first years of the study. We had many fruitful conversations concerning different subject. I

am looking back those conservation with warm. I thank all the collaborators, who have participated this study.

Immunohistochemistry was carried out at the Laboratory of Pathology, the Hospital for Children and Adolescents, University of Helsinki, Helsinki. I like to thank especially Mrs. Sirkku Kristiansen and all the staff of the laboratory for their skilful and helpful assistance, which made this study possible.

I express my warmest thanks to Ewen MacDonald, D. Pharm., and Mrs. Leona Gilbert, Ph.M. for revising the language of this manuscript and articles. I am grateful to Pirjo Halonen, M.Sc., for her expertise to give advise on the statistical problem encountered in this study. My sincere thanks also due to Mrs. Liisa Korkalainen for all kind of practical assistance.

I like to express my gratitude to all my colleagues in the Department of Paediatrics, Kuopio University Hospital and the Hospital for Children and Adolescents, University of Helsinki, with whom I have had the opportunity to share friendship and to work in good atmosphere.

Above all, I want to express deepest gratitude to my wife Ikka for her love and support throughout these years, and my dear children Anni-Maria, Jaakko, Arttu-Kalle and Iiris for making life worthwhile. You are the most valuable thing in my life.

Financial support provided by the Finnish Pediatric Research Foundation, The Finnish Medical Foundation Duodecim, University of Kuopio and Kuopio University Hospital is gratefully acknowledged.

Kuopio, January 2002

Timo Örmälä

LIST OF ABBREVIATIONS

APC	antigen presenting cell
CD	coeliac disease
DH	dermatitis herpetiformis
GALT	gut associated lymphoid tissue
GFD	gluten-free diet
GC	germinal centre
HLA	human leukocyte antigen
IL	interleukin
IEL	intraepithelial lymphocyte
IFN	interferon
IgA	immunoglobulin A
IgG	immunoglobulin G
IgM	immunoglobulin M
M-cell	microfold cell
MHC	major histocompatibility complex
PP	Peyer's patch
SED	subepithelial dome
SC	secretory component
SPSS	Statistical Package for the Social Sciences software.
TCR	T cell receptor

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following articles, which are referred to in the text by their Roman numerals.

- I. Örmälä T, Rintala R, Savilahti E. T cells and HLA-class II expression in the large intestine of infants in the early postnatal period. *J Pediatr Gastroenterol Nutr.* 1998 Apr;26(4):422-8.
- II. Hacsek G, Örmälä T, Rintala R, Savilahti E. B cell development in lamina propria of the large intestine: influence of age and T cell densities. *APMIS.* 1999 Jul; 107(7):661-6.
- III. Örmälä T, Rintala R, Savilahti E. T cells of the colonic mucosa in patients with infantile colitis. *J Pediatr Gastroenterol Nutr.* 2001; 33 (2):133-8.
- IV. Savilahti E, Örmälä T, Arato A, Hacsek G, Holm K, Klemola T, Nemeth A, Mäki M, Reunala T. Density of gamma/delta+ T cells in the jejunal epithelium of patients with coeliac disease and dermatitis herpetiformis is increased with age. *Clin Exp Immunol.* 1997 Sep;109(3):464-7.

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1. INTRODUCTION

After birth the newborn infant is faced with major challenge being transferred from the sterile environment of the uterus to an environment containing large numbers of foreign antigens. The intestinal immune system of the human body is regularly exposed to large amounts of ingested antigens. The gut mucosa must both protect the body against infectious pathogens and prevent the entry of excessive amounts of food antigens. It must also regulate the immune response to oral antigens. Physiologic passage of luminal antigens through the mucosal membrane is essential for the development of the immune response but uncontrolled immunity to antigens could however, initiate pathologic processes evoking gastrointestinal disease. Restrictions in the amount of antigens reaching the surface of the epithelium of the intestine and the physical characteristics of the intestine itself, may prevent the development of a pathological immune response. Many barrier mechanisms are still not fully developed at birth and antigen transport is less restricted in the neonatal period than in adults. There are many published studies concerning the ontogeny of the mucosal immune system of the gut with fetuses before the 20th gestational week or later in childhood. Therefore there is a special interest in the development of gut associated lymphoid system (GALT) immediately after birth.

Little information is available concerning the development of the GALT in the large intestine, where in addition to the antigens present in the food, the immune system is challenged by an enormous population of colonic bacteria. In this study, the influence of age on the colonic T cells, distribution of immunoglobulin A (IgA) bearing lymphocytes in distal intestine, as well as possible developmental connections between colonic T and B cells in healthy infants were evaluated. Our purpose was to study, how the immature GALT handles the increased antigenic load and inflammation during the early postnatal period. This question was studied in twelve infants with colitis. Age related immunological changes in the intestinal mucosa were studied in jejunal biopsies from patients with CD and DH at different ages.

2. REVIEW OF LITERATURE

2.1. The structure of gut associated lymphoid tissue

The gut associated lymphoid tissue (GALT) has long been considered as the central mucosa-associated lymphoid tissue based on its mass and its exposure to high amounts of mucosal antigens. The GALT can be divided into inductive and effector sites. GALT consists of lymphocytes scattered throughout the lamina propria of the intestine, between the epithelial cells and organised into aggregates such as those found in Peyer's patches (PPs). These aggregated lymphoid structures are generally considered as the principal mucosal sites where the interaction of antigens from gut lumen and circulating lymphocytes takes place. On the other hand, the main effector sites for intestinal immune responses are the intestinal lamina propria (LP), where mature T and B cells migrate following induction in the PP, and the intestinal epithelium, which contains the intraepithelial lymphocytes (IEL's).

2.1.1. Intestinal epithelium

2.1.1.1. Enterocytes

The epithelial cells have an important role in both B and T cell mucosal immune function. The role of epithelial cells in T cell function relates mainly to their potential ability to act as luminal antigen processing and antigen-presenting cells (APC) (Bland et al. 1986, Bland 1988). Their capacity to express MHC class I and II molecules (Mayer et al. 1987, Kaiserlian 1991) facilitates this potential; well-differentiated enterocytes possess MHC class II molecules, primarily at the apical cell membrane. It has been noted that a high level of MHC class II molecule expression occurs when epithelial cells are exposed to IFN- γ , produced by IEL's (Beagley et al. 1995). This indicates that, under certain circumstances, epithelial cells may present antigens to CD4+ T cells in lamina propria.

With respect to B cell function, enterocytes play an important role in transporting IgA synthesised by plasma cells in lamina propria to the mucosal surface. This transport is mediated by a secretory component; the resultant molecule assumes a new form, secretory IgA (sIgA), which is more efficient at functioning in the mucosal milieu.

2.1.1.2. Microfold cells

The follicle associated epithelium (epithelium overlying PP) differs from the mucosal surfaces in other areas of the gut. This region contains fewer goblet cells and, thus, less mucus, but has an increased density of intraepithelial lymphocytes and unique cells involved in antigen uptake called the microfold (M) cells (Wolf et al. 1984, Rowinski et al. 1984). M cells have poorly developed brush border, reduced enzymatic activity and thin glycocalyx. M cells can form endocytic vesicles, which facilitate transport of micro-organisms, particulates, and soluble antigens from the mucosal lumen to the subepithelial dome (SED) of the PP (Owen et al. 1974, Owen et al. 1983). SED overlie each of the multiple B-cell follicles that contain germinal centres (GC).

Though M cells are clearly specialised for antigen transport to underlying lymphoid tissue, they also have been shown to express MHC class II molecules and to produce interleukin-1 (IL-1) (Nagura et al 1991, Allan et al. 1993, Pappo et al.1993). They could conceivably function as antigen presenting cells.

2.1.1.3. Intraepithelial lymphocytes

The intestinal epithelium layer contains a unique population of T cells that develop independently of the PP; these are called IELs. These cells are prevalent in both small and large intestines, with a mean incidence of the 5-10 IELs/100 epithelial cells in human jejunum.

IELs consist almost exclusively of T lymphocytes whereas only 30-40% of lamina propria lymphocytes are T cells. In humans 80 – 90% of these cells possess the CD8+ phenotype, which is associated with suppressor/cytotoxic activity, while in lamina propria most of the T cells carry the helper/inducer marker (CD4+) (Selby et al. 1981a, Selby et al. 1981b). Lymphocytes recognize foreign antigenic peptides through their T cell receptor (TCR) complex on the cell surface in the context of major histocompatibility complex (MHC) molecules. Antigens presented with class II MHC molecules are recognized by CD4+ T cells with their CD3 α/β TCR whereas CD8+ T cells identify antigens associated with class I MHC molecules.

In humans, the TCR heterodimer consists of either alpha/beta or gamma/delta chains (Brenner et al. 1986) The alpha/beta expressing T cell population predominates in both blood and the gut epithelium (Faure et al. 1988, Spencer et al 1989). A small percentage of T cells in blood and epithelium, however, do not express α/β , instead they have a second type of TCR complex designated as γ/δ . It is found in two forms, a disulphide-linked heterodimer and a nondisulphide-linked heterodimer (Bottino et al. 1988). The latter also exists in two forms that differ in the size of the γ chain (40-kD and 55-D) (Hochstenbach et al. 1988)

In the epithelium in relation to blood and lamina propria, the $\gamma\delta$ TCR expressing lymphocytes are overrepresented. The precise role of the $\gamma\delta$ T cell is still obscure. At least some of these cells appear to recognise the antigen directly without the requirement for antigen processing (Chien et al. 1996). Alternatively, $\gamma\delta$ TCR bearing T cells may play a role in autologous surveillance via removing damaged epithelial cells by recognition of "stress" or heat shock proteins. (Haregewoin et al. 1989, Raulet, et al.1989, Janeway et al.1988).

2.1.2. Peyer's patches

Organised lymphoid aggregates present in the wall of small and large intestine are generally considered to be the principal inductive sites in the mucosa, where the interaction of the luminal antigen and circulating

lymphocytes takes place. In the small intestine, these collections are referred to as PPs and they consist of multiple lymphoid follicles. In humans, PPs are most numerous in the ileum. Lymphoid aggregates similar to PPs are present in high numbers in the wall of the large bowel and appendix/caecum. Their structures are similar to PPs and thus are assumed to have a similar function.

Following ingestion, antigens are transported via M cells, that are scattered among conventional epithelial cells overlying the dome of the PPs follicle, to subepithelial dome (SED) region of the PP (Neutra et al 1996, Davis et al.1997). There antigens encounter professional APC (dendritic cells, macrophages and B cells) which possess MHC class II molecules and are capable of presenting antigens to T cells inducing the activation and differentiation of T cells. After stimulation in the Peyer's patches and other mucosal follicular tissue, these activated T cells migrate out of these inductive sites and traffic to mucosal lamina propria, which is the main effector site of the intestinal immune response. Once in the lamina propria, the T cells lie in a dormant state as resting memory cells. When stimulated by APC, T cells express their definitive function secreting cytokines that are essential for terminal B cell differentiation or induction of a cellular immune response.

2.1.3. Lamina propria

More than 80 % of the normal intestinal lamina propria T cells express the $\alpha\beta$ TCR heterodimer (Ulrich et al. 1990). In normal human intestine lamina propria, T cells are predominantly (60-70%) of the helper/inducer phenotype (CD4+) whereas 30 – 40% of lamina propria T cells have suppressor/cytotoxic phenotype (CD8+) (Brandtzaeg 1985). CD4+ T cells are highly differentiated effector cells that have a poor capacity to proliferate but a great capacity to produce lymphokines. The specific cytokines produced under stimulation consist of a mixture of Th1 (IFN-1) and Th2 (IL-4 and IL-5) cytokines.

Antigens transported into the lamina propria are thought to be taken up by professional APC which are abundant in the lamina propria. These

consist for the most part of nonfollicular dendritic cells that are concentrated beneath the epithelial layer (Pavli et al. 1990, Mayrhofer et al. 1983). The presentation of the antigens to the T cells induces the initial T cell responses. After that stimulation, the T cells in the lamina propria lie in a dormant state as resting memory cells, and only on re-encounter with the antigen do they express their definitive effector function, the production of helper or suppressor cytokines, or mediation of cytotoxicity.

2.1.4. IgA secreting B-lymphocytes

The humoral immune response in the GALT is primarily characterised by the production of secretory IgA (sIgA). This is the principal immunoglobulin present in intestinal secretions, but low numbers of IgG and IgM plasma cells are also present in lamina propria (Perkkiö et al. 1980). Final maturation of IgA B cells occurs in lamina propria. Mature plasma cells produce IgA antibodies which are specific for the antigens. For sIgA to be formed, two IgA molecules are combined with a J chain to form a complex which is further combined with a secretory component (SC) present on the basal lamina of the enterocyte. The ultimate product is sIgA which is transported by a particular receptor-mediated, polymeric immunoglobulin receptor (pIgR), transport mechanism (Mostov 1994) across the cell body into the intestinal lumen (Ahnen et al. 1985, Brandtzaeg 1985a). Moreover sIgM antibody activity is present in the epithelial mucus layer and serous secretions. Secretory antibodies bind antigens, thereby preventing their attachment to the epithelial surface. In addition, secretory antibodies might reinforce immune exclusion by capturing antigens intracellularly during pIgR/SC mediated transport through the epithelial cell, thus avoiding damage to the epithelium through cytolysis (Mazanec et al. 1992, Mazanec et al. 1995).

Two structurally distinct IgA subclasses exist, namely IgA1 and IgA2 (Delacroix et al. 1982, Brown et al. 1985). The IgA2 subclass predominates in the distal part of the gut (Kett et al. 1988). The IgA2 subclass

is more resistant to bacterial proteases (Kilian et al. 1983) and its synthesis may be stimulated by lipopolysaccharide of gram negative bacteria (Brown et al. 1985). The concentration ratios of the two sIgA subclasses in various secretions are similar to the relative distribution of sIgA1 and sIgA2 immunocytes at the corresponding sites. The relative increase of sIgA2 in secretions may be important for the stability of secretory antibodies since sIgA2, in contrast to sIgA1, is resistant to many of the IgA1 specific proteases that are produced by a variety of potentially pathogenic bacterial species (Kilian et al. 1983). The fact that jejunal IgA immunocytes are predominantly of the IgA1 subclass, in contrast to the IgA2 dominance in the colon, may hence reflect the varying exposure to gram negative bacteria in different parts of the intestine.

2.2 Ontogeny of the gut associated lymphoid tissue

2.2.1. Peyer's patch

By using the immunohistochemical techniques Spencer showed that PPs appear at 11 weeks of gestation (Spencer et al. 1986b). PPs "anlagen" that are present at 11 weeks do not contain lymphocytes but consist of cells that strongly express HLA-D region antigens HLA-DR and DP, but not HLA-DQ. They also express the CD4 antigen, which in postnatal tissue is expressed both on the class II restricted T cells (helper/inducer T cell subset) and also on some macrophages.

By 14 weeks of gestation, T cells expressing CD3 associated with the TCR are present within subepithelial clusters. Both CD4+ (class II restricted T cells) and CD8+ T cells (class I restricted T cells) can be seen within the follicles at this time (Spencer et al. 1986b).

By 16 weeks of gestation, the larger aggregates of lymphoid tissue are observed consistently containing both T and B cells but with no cellular zonation. The T cells express CD3 and CD4 or CD8, although CD4 cells predominate over CD8 cells. This is also true in adult PP. B cells expressing

IgM and IgD markers are interspersed amongst the T cells at this time (Spencer et al. 1986b).

Structured PPs, consisting of discrete B and T cell areas, are present at 19 weeks of gestation. The T cells in lymphoid follicles surround the B cell. As in postnatal tissue, the lymphocytes are predominantly CD3+, CD4+ interspersed with CD3+, CD8+ cells. Thus, it appears that in the period from 11 to 19 weeks of gestation, the PPs develop into organised structures that are very similar to those in adult tissue follicles (Spencer et al. 1986b).

There is no evidence of PP GC formation in healthy fetal human small intestine. At birth, the mucosal immune system is stimulated by bacteria that colonise the gut. Although the infiltration by lymphoid cells into PP occurs before birth, the distinct nodular development is a postnatal event. The number of PP increases from approximately 60 at birth to over 200 by 12 to 14 years of age (Cornes 1965), but it is several weeks after birth before activated lymphoid follicles with GC appear in PP (Bridges et al. 1959).

2.2.2. Lamina propria lymphocytes

The main cell population within the fetal lamina propria is strongly HLA-DR positive with a dendritic morphology (Spencer et al. 1987). These cells also express CD4 and CD45 (leukocyte common antigen). This infiltrate, which precedes the appearance of lymphocytes, is present presumably before 11 weeks of gestation (Spencer et al. 1986b, Spencer et al. 1987). At 11 weeks of gestation, very low numbers of CD3+ are present in lamina propria (MacDonald et al. 1994). Scattered B and T cells are present in fetal lamina propria from 14 weeks of gestation (Spencer et al. 1986b). The majority of T cells in the lamina propria are CD4+. At 18 weeks of gestation, all T cells in lamina propria are $\alpha\beta$ TCR+ while $\gamma\delta$ TCR+ T cells are absent at that time (MacDonald et al. 1994).

Since stimulation by exogenous antigens does not occur in the fetus, there is little or no stimulation of the organised lymphoid tissue, and therefore there are no lamina plasma cells. At birth, antigen exposure starts to stimulate the mucosal immune system. It has been demonstrated that

immunoglobulin containing cells are absent from the intestinal lamina propria for the first 12 days of life (Perkkiö et al. 1980). IgM plasma cells, together with some IgG plasma cells, can be identified in PPs a few days after birth, prior to the appearance of IgA. IgA plasma cells begin to appear by day 12 and increase in number thereafter. The numbers of IgM containing cells remain constant between 1 month and 16 years. At first, IgM cells predominate over IgA plasma cells but after 1 – 3 months of age, IgA plasma cells are the most common plasma cells. The IgA containing cells continue to increase with age, being more numerous in children over 2 years of age than at younger ages (Savilahti 1972). In adults, over 80 % of the total plasma cell population are IgA containing cells (Crabbe et al. 1966).

2.2.3. Intraepithelial lymphocytes

It has been shown that lymphocytes appear between the epithelial cells at approximately 11 weeks of gestation, at which time 0.3 IEL/100 epithelial cells were noted (Orlic et al. 1977). CD3+ IEL appear in fetal intestine at 14 weeks of gestation (Spencer et al 1986). In the fetus, only approximately 50% of IELs express CD4 or CD8, the rest being CD3+, CD4-, CD8-. As in the postnatal bowel, CD8+ cells predominate in the epithelium (MacDonald et al 1994). Phenotypically, 75% of adult IELs are CD8+. Approximately 14% of IEL have the phenotype CD7+, CD3-, CD4- and CD8- (CD7 is an antigen present on T cells and null cells). Immunohistological studies have shown that the CD7+, CD3- IEL (non-T cells) component seen in postnatal intestine is absent in the fetus (Spencer et al. 1989b).

In adults, the TCR $\alpha\beta$ expressing T cell population predominates, comprising 95% of CD3+ cells in the blood and gut epithelium (Faure et al 1988, Spencer et al 1989a). The remaining 10 % express the $\gamma\delta$ heterodimer.

TCR $\gamma\delta$ + T cells are found in two forms in man, a disulphide-linked heterodimer (V δ 2) and nondisulphide-linked heterodimer (V δ JV δ 1 and V δ JV δ 2) (Bottino et al. 1988). The latter exists in two forms that differ in the size of the γ chain (40-kD and 55-D) (Hochstenbach et al. 1988). In adults, the non-

disulphide-linked form predominates in the gut epithelium (Spencer et al. 1989). In the fetus, CD4⁻ CD8⁻ IEL are present as a greater percentage of CD3⁺ cells than in the adults (Spencer et al. 1987) but γ/δ TCR IELs are not increased. The non-disulphide-linked form of the γ/δ TCR is absent in fetal IEL and TCR γ/δ ⁺ T cells are exclusively in the disulphide-linked form and constitute approximately 25% of the CD3⁺ population.

2.2.4. Epithelial HLA-D region antigens

In healthy postnatal small intestine, HLA region antigens are present on the epithelial cells of the villi but not on the crypts (Scott et al. 1980, Rognum et al. 1992). They are absent from colonic epithelial cells (Spencer et al. 1986a). HLA region antigens can be induced on crypt epithelial cells by products from the activated T cells (MacDonald et al. 1988). Gamma interferon is known to be a potent inducer of the HLA-D region antigen on epithelial cells. In the fetus, constitutive HLA-D region antigens are present on villus cells from 18 weeks of gestation. It is first seen at the tip of the villi, but in older fetal specimens, it can be present on all villus enterocytes. HLA region antigens can also be prematurely induced on fetal gut epithelium by immunological stimuli. Gamma interferon must bind to a specific receptor on epithelial cells to cause the HLA-D region genes to be expressed. By 16 weeks of gestation, epithelial cells are fully mature and can show this response.

2.3. Intestinal mucosal diseases

2.3.1. Colitis in infancy

The most common symptoms of the colitis in infancy are diarrhea and bloody stools. Colitis in infancy and early childhood comprises a heterogeneous group of conditions including potentially serious diseases such as necrotising enterocolitis, infectious colitis and Hirschsprung's enterocolitis

(Fox 2000). However, in apparently well infants, bleeding is most commonly a secondary symptom of allergic colitis (Odze et al. 1995, Machida 1994).

Hirschsprung's disease or congenital aganglionic megacolon is caused by an abnormal innervation of the bowel. Due to the absence of ganglion cells in the distal segment of the large bowel, the bowel is contracted causing the fecal outlet obstruction. Enterocolitis is the major cause of morbidity and mortality in patients with Hirschsprung's disease. The pathogenesis of the enterocolitis is uncertain – ischaemic causes, bacterial (Thomas et al 1986) and viral (Wilson-Storey et al. 1990) infections have been proposed to be involved. Mucosal immune defence mechanisms may be impaired in patients with enterocolitis complicating Hirschsprung's disease (Wilson-Storey et al. 1989, Imamura et al. 1992).

Allergic colitis due to cow's milk or soy protein in commercially prepared infant formula or the ingestion of breast milk of mothers who themselves are drinking cow's milk is the major type of colitis occurring in the first year of life (Jenkins et al. 1984). Infants most often present with rectal bleeding, at times associated with diarrhea. Infants with allergic colitis usually respond to withdrawal of the offending antigen, by the use of hydrolyzed cow's milk protein formula or more elemental formulas. If the infant has been breast fed, the strict removal of the offending antigen from the diet of the breast-feeding mother may be curative.

2.3.2. Coeliac disease and dermatitis herpetiformis

CD is defined as a permanent sensitivity to gluten associated with mucosal disease in small intestine. It is characterized by small intestinal mucosal damage and nutrient malabsorption after the diet containing prolamins from wheat, rye and barley in genetically susceptible individuals. Gluten sensitivity may also manifest as a skin disease, dermatitis herpetiformis. Both diseases have a strong genetic association with certain HLA DQ genes in chromosome 6. Although most patients with dermatitis herpetiformis show few

or no gastrointestinal symptoms, majority of them have jejunal damage similar to that observed in coeliac disease.

CD and DH are unique among small intestinal disorders because the patients manifest a striking increase in the proportion of $\gamma\delta$ TCR+ relative to $\alpha\beta$ TCR+ IELs in jejunal surface epithelium as a hallmark of disease (Savilahti et al.1992). Moreover, the proportion of $\gamma\delta$ IEL in relation to $\alpha\beta$ IEL remains increased even in CD patients in remission (Halstensen et al. 1989, Spencer et al. 1989a, Savilahti et al.1990, Savilahti et al.1992, Vecchi et al. 1992), which suggests that these cells have an important role in the pathogenesis of the disease, either through protecting the mucosa or by being involved in mucosal damage.

3. AIMS OF THE STUDY

The main purposes of the study were to evaluate developmental aspects of the GALT in normal and inflamed colon, especially soon after birth. We also evaluated a possible connection between the B and T cell development in young infants. In addition, we investigated age dependent changes of the jejunal mucosa in patients with CD and DH in different clinical situations. In particular, TCR, T cell surface antigens and HLA class expressions were studied.

The specific aims were:

To evaluate developmental aspects in the densities of the T cells, CD4, CD8 surface antigen expressing cells, TCR α/β and γ/δ , and mononuclear and epithelial cells expressing HLA class II antigens in colon soon after intraluminal antigenic load by food and bacteria in young infants.

To evaluate changes in cell densities induced by inflammation of the colon among young infants.

To determine the development and distribution of IgA subclasses IgA1 and IgA2 in the distal intestine during the early months of life.

To clarify the developmental association between B and T lymphocytes.

To evaluate possible age dependent relations in TCR α/β and γ/δ positive cells in the jejunal epithelium of patients with CD and DH, as well as control patients.

4. MATERIAL AND METHODS

4.1. Patients

In study I, the densities of T cells, their subsets expressing surface antigen CD4 and CD8 T and cell receptor (TCR) α/β and δ/γ , and the densities of mononuclear and epithelial cells expressing HLA class II antigens as defined by monoclonal antibodies and the peroxidase technique were measured in biopsy specimens taken from the large intestine of infant patients.

Forty seven biopsy specimens were taken for clinical diagnostic purposes from 47 infants, 4 from the colon and 43 from the rectum in the surgical department of the Children's Hospital, University of Helsinki, from 1987 to 1994. The diagnosis and the age distribution of the patients in study I is shown in Table 1.

Table 1. The diagnosis and age distribution of 47 infants with normal morphology in study I.

	n	age ^a (days)
Mb. Hirschsprung (group 1)	11	68 ^b (19;161) ^c
Trisomy 21 ^d	4	
Cartilage-hair hypoplasia	1	
Malformation of anus	1	
Miscellaneous conditions (group 2)	36	95 (51; 148)
Constipation	20	
Malformation of anus	9	
Malrotation of intestine	3	
Meconium plug	2	
Umbilical abscess	1	
Spielmayer-Sjögren syndrome	1	

^aage when biopsy was taken^bmedian^c25 and 75 percentiles^daccessory disease

Eleven patients had Hirschsprung's disease. Of the remainder, 36 infants with miscellaneous conditions 20 underwent colonoscopic examination because of constipation and the samples were taken during the procedure. Nine patients had anorectal malformation for which transversostomy or sigmoidostomy was performed in the neonatal period. The biopsy specimens were taken at the time of the primary operation or when the stomas were closed. Four infants were born prematurely, two in the 30th, one in 25th, and one in 34th gestational week. The age distribution of the patients with Hirschsprung's disease and that of the other patients did not differ. None of the patients had clinical evidence of colitis. All biopsy specimens included in the study had normal morphology.

Thirty six rectal and colonic biopsy specimens collected for clinical purposes in the surgical department of the Children's Hospital from 1987 to 1994 were included in study II in which the development and distribution of mucosal immunoglobulins and possible developmental associations of T and B cells in the distal intestine during the early months of life were evaluated. The

densities of the immunoglobulin positive, IgA-, IgA1-, IgA2-, IgM- and IgG positive cells in lamina propria were studied. In 33 of the patients, the densities of the T cells had been measured in study I. The diagnosis and the age distribution of the patients in study II are seen in Table 2. All biopsies showed normal morphology.

Table 2. The diagnosis and the age distribution of 36 infants with normal morphology in study II

Diagnosis		Age ^a (days)
All patients (n)	36	67 ^b (35, 161) ^c
Mb. Hirschsprung	12	
Trisomy 21 ^d	3	
Constipation	18	
Anorectal malformations	6	

^aage when biopsy was taken

^b median

^c25 and 75 percentiles

^daccessory disease

In study III, twenty-four colonic biopsy specimens taken in the Surgical Department of the Children's Hospital, University of Helsinki, from twelve infants with colitis and twelve age matched control patients were studied for CD4/CD8, $\alpha\beta$ TCR, $\gamma\delta$ TCR and HLA class II expression. Four young infants with rectal bleeding and eight young patients with Hirschsprung's disease and symptoms indicative of enterocolitis were included in the colitis group. The age distribution and the indications for the biopsy in study III are shown in Table 3. Controls of this group were part of the control group from study I.

Table 3. The age distribution and the indications for the rectum or colon biopsy in 8 patients with colitis associated with Hirschsprung's disease, 4 neonatal colitis and 12 controls.

	Colitis			Controls (n=12)
	Mb. Hirschsprung with colitis (n=8)	Neonatal colitis (n=4)	Total	
Age (days) ^a	66 ^b (26; 143) ^c	26(8; 48)	38(23;89)	38 (23; 92)
Indications for the rectum or colonic biopsy				Obstipation (4) Mb. Hirschsprung without colitis (1) Malrotation of intestine(2) Meconium plug (2) Anal atresia (2) Intestinal occlusion (1)

^a age when biopsy was taken

^b median

^c25 and 75 percentiles

The age distribution of the patients with infantile colitis and controls did not differ. In patients with Hirschsprung's disease, five rectal biopsy specimens were taken for diagnostic purposes and three specimens were taken when the stomas were closed. Four out of these eight biopsy specimens showed normal morphology. Four infants with neonatal colitis were studied because of rectal bleeding. The specimens were taken during the diagnostic colonoscopy and all specimens showed inflamed morphology. In the age matched control group, four infants underwent colonoscopy due to constipation and two infants had anal atresia, and had had sigmoidostomy performed in the neonatal period. One rectal biopsy specimen was taken at the time of the primary operation and the other specimen was taken when the stoma was closed. Two infants had meconium plugs, two had intestinal malrotation, and one patient had Hirschsprung's disease without symptoms or signs of colitis. Two samples were colonic and 10 were taken from the rectum. None of the control patients showed any clinical evidence of colitis and all specimens showed normal morphology.

In study IV, 66 biopsy specimens from pediatric patients were used. With respect to the 34 adult patients, 13 came from a family study (Holm et al.

1992) and remaining 21 were patients with DH, 14 out of them were studied before treatment and nine were on a gluten free diet (GFD) (Savilahti et al. 1992). Control biopsy specimens were taken from children studied because of growth failure or from adults suffering from mild gastrointestinal complaints, but with normal biopsy specimens. To the four published series of patients we added 22 patients with CD (Table 4). Of these, 12 were young patients under two years old studied at Huddinge Hospital, Stockholm, because of symptoms suggestive of coeliac diseases. They were found to have total or subtotal villous atrophy and clinically responded to a GFD, thus fulfilling the criteria for coeliac disease.

Table 4. Twenty-two patients in study IV supplemented with 66 patients and 37 controls from four published series

	Patients(n)	Controls (n)
Present study		
Huddinge Hospital, Stockholm	12	
Children's Hospital, University of Helsinki	10	
Savilahti et al. 1990	19	13
Savilahti et al. 1992	21	
Holm et al. 1992	13	24
Klemola et al. 1994	13	
Total	88	37

4.2. Immunohistochemical staining

Rectal and colonic biopsy specimen were divided into two or more parts. One part of the fresh biopsy was embedded in OCT[®], frozen with liquid nitrogen, sealed in an airtight plastic bag with ice and kept at -70°C until cut. Serial cryostat sections cut at $5\mu\text{m}$ were fixed in acetone for 10 min, then in chloroform for 30 min, and washed three times in Tris buffer, pH 7.4. The buffer was removed and the sections were covered with a dilution of monoclonal antibodies in Tris buffer for 1 hour. After washing, endogenous peroxidase was

blocked by incubation in 0.5% peroxide for 30 min. A Vectastain Elite ABC[®] kit (PK-6102, Vectro Laboratories, Burlingham, CA) was used to reveal the binding of the monoclonal antibodies in accordance with the manufacturer's instructions.

The coded specimens were evaluated blinded. The numbers of stained cells were counted with a light microscope through a calibrated graticule at x1000 magnification. The cell density in the lamina propria was determined with a 0.045x0.045 mm graticule fitted to the eyepiece of the microscope; the number of cells in at least 30 graticules between the surface epithelium and muscularis mucosae was counted, and the results were expressed as cells/mm². In the surface epithelium, the cell densities were counted as cells/mm with the same graticule along the basement membrane. Ki67-positive cells in the crypts were calculated as percentages of the crypt epithelial cells. HLA-DR, -DQ and DP expression in surface epithelium and crypts were estimated through the whole specimen by light microscope. If the staining was observed as a mark of HLA-D region expression in the whole cell or only in a tip of the epithelial cells, it was considered as a positive finding.

4.3. Monoclonal antibodies

In studies I, III and IV monoclonal antibodies anti-Leu4 (anti-CD3, Beckton-Dickinson, Mountain View, CA), T4 (anti-CD4, Coulter Immunology, Hialeh, FL) and OKT8 (anti-CD8, Ortho Diagnostic System, Raritan, NJ) were used at a dilution 1:400. Monoclonal antibody TCR δ 1 (T cell Sciences Inc., Cambridge, MA), recognising a constant region of the γ/δ -chain of TCR (Janeway et al. 1988) and all γ/δ + cells (Delovitch et al. 1988) were used at a dilution of 1:100. In addition, antibody δ TCS1 (T cell Sciences Inc. Cambridge, MA), recognising the V δ 1/J δ 1 rearrangement (Wu et al. 1988), and antibody β F1 (T cell Sciences Inc. Cambridge, MA) reacting with virtually all $\alpha\beta$ TCR molecules (Faure et al. 1988), were used at a dilution of 1:100. Monoclonal antibodies to constant fragments of HLA-DR and -DP chains purchased from Becton Dickinson were used at a dilution of 1:1000 and 1:40, respectively. Anti-DQ monoclonal antibody (Serotec Ltd, Oxford, England) reacting with a

monomorphic determinant present on all HLA-DQ molecules was used at a dilution of 1:400. Monoclonal antibody Ki-67 (DAKO-PC, Dakopatts, Glostrup, Denmark) recognising a nuclear antigen present only in proliferating cells (Band et al. 1987, Gerdes et al 1984) was used at a dilution of 1:10.

In study II, the following murine antibodies were used for immunohistochemistry: IgA heavy chain (clone 2D7, dilution 1:800) anti-human IgA2 (clone 2E2, dilution 1:1000) from Oxoid Ltd., Bedford, UK; anti-human IgA1 (clone NIF2, dilution 1:50) and IgM (clone MH15-1, dilution 1:200) from Janssen Bio Chimica, Belgium. Anti-human CD22 (clone To15, dilution 1:50) was from DAKO-PC, Glostrup, Denmark. Antibodies used to measure densities of T cells are the same as those used in study I. A summary of antibodies used in the present study is shown in Table 5.

Table 5. Details of used antibodies

Antibody	Dilution	Specificity	Source
anti-Leu4	1:400	CD3+ cells, pan-T cell marker	Beckton-Dickinson, Mountain View, CA
anti-T4	1:400	CD4+ cells	Coulter Immunology, Hialeh, FL
anti-OKT8	1:400	CD8+ cells	Ortho Diagnostic System, Raritan, NJ
anti-βF1	1:100	TCRαβ+ cells	T cell Sciences Inc. Cambridge, MA
TCRδ1	1:100	all TCRγδ+ cells	T cell Sciences Inc.,
δTCS1	1:100	non-disulphide-linked TCRγδ+ cells	T cell Sciences Inc.
anti-HLA-DR	1:400	constant fragments of HLA-DR chain	Becton Dickinson
anti-HLA-DP	1:400	constant fragments of HLA-DP chain	Becton Dickinson
anti-HLA-DQ	1:10	monomorphic determinant on all HLA-DQ	Serotec Ltd, Oxford, England
IgA	1:800	all IgA+ cells	Oxoid Ltd., Bedford, England
anti-human IgA1	1:50	IgA1+ cells	Janssen Bio-Chimica, Belgium
IgA2	1:1000	IgA2+ cells	Oxoid Ltd., Bedford,
anti-human IgG	1:1000	IgG+ cells	Oxoid Ltd., Bedford,
anti-human IgM	1:200	IgM+ cells	Janssen Bio-Chimica,
anti-human CD22	1:50	B cells with membrane Immunoglobulins	DACO-PC, Glostrup, Denmark

4.4 Statistical analysis

In study I, all cell densities in the colonic and rectal biopsies showed no differences in infants without Hirschsprung's disease and these results were pooled. Comparisons of these results with those from patients with Hirschsprung's disease gave similar results, except for the cells expressing HLA class II antigens in the lamina propria. Thus, all of the biopsy specimens were studied together when evaluating T cell subsets, but densities of the HLA class II antigens expressing cells were analyzed separately. Student's t-test was used to determine differences between the cell groups. A linear correlation was carried out to determine the influence of age on the cell densities of different cell groups. The difference of HLA-DR, -DP and DQ antigen expression between patients with Hirschsprung's disease and other intestinal complaints were evaluated by Fisher's exact probability test.

In study II, the patients had no symptoms or signs of enterocolitis and all studied specimens had normal morphology. Analysis of variance was used to assess, whether patients with Hirschsprung's disease differed from the other patients. Linear correlations were calculated between densities of immunoglobulin positive cells and CD22+ cells and the \log_{10} transformation of age. The correlation coefficients between densities of immunoglobulin-positive and CD22+ cells and T cells with the surface receptors CD3, CD4, CD8 and TCR $\alpha\beta$ and $\gamma\delta$ were calculated. In cases where there was a statistically significant correlation, the correlation coefficient and p values are reported.

In study III, we studied the possible influence of colitis on the mucosal cell densities in the colon of young patients and their age matched controls. Wilcoxon rank sum test was used when comparing age distribution and the different cell densities. The difference of HLA-DR, -DP and DQ antigen expression in epithelial cells between patients and controls was evaluated by Fisher's exact probability test.

In study IV, analysis of variance was used to test the difference between groups. If significant, groups were further evaluated by Student's

t-test. Linear correlation analysis was calculated between the densities of intraepithelial cells and age.

All the calculations were carried out on a PC using the Statistical Package for the Social Sciences (SPSS) software.

5. RESULTS

5.1 Intraepithelial T lymphocytes of the large intestine (I ,III)

5.1.1. Intraepithelial lymphocytes of non inflamed large intestine (I)

Forty-seven large intestinal biopsy specimens from 47 infants in study I had normal morphology. Densities of T cells in lamina propria, surface epithelium and crypts are shown in Table 6.

Table 6. Densities of CD3-, CD4-, and CD8 positive T cells, TCR α/β and γ/δ expressing cells and δ TCS1 positive cells in the large intestine of 47 young infants.

	CD3	CD4	CD8	TCR α/β	TCR γ/δ	δ TCS1
Lamina propria ^a	612 \pm 333 ^c	486 \pm 286	186 \pm 131	479 \pm 303	112 \pm 90	53 \pm 68
Surface epithelium ^b	3.9 \pm 3.8	0.9 \pm 1.1	2.9 \pm 3.1	2.8 \pm 3.2	2.2 \pm 2.9	1.2 \pm 2.4
Crypts ^b	3.3 \pm 3.3	1.2 \pm 2.2	2.3 \pm 1.9	2.5 \pm 1.7	1.4 \pm 1.7	0.8 \pm 1.2

^acells/mm²

^bcells/mm

^cmean \pm sd

It was noticed that densities of the CD3+ IEL in young infants were higher in the surface epithelium than in the crypts. In all, 75 % of the amount of CD3+ cells were CD8+ antigen expressing and less than 20 % were CD4+ cells. The mean density of the TCR α/β + cells accounted for over 70 % of the numbers of CD3+ cells. The density of the TCR γ/δ + was only slightly lower than the density of α/β + cells. Approximately half of the γ/δ + cells were δ TCS1+

having the V δ 1/V δ 2 arrangement. No statistically significant correlation with age was observed in the studied intraepithelial cell densities in non-inflamed colon.

5.1.2. Intraepithelial lymphocytes of inflamed large intestine (III)

Twelve young patients with colitis and their age matched controls were compared. Patients with colitis had significantly higher ($p=0.036$) densities of the intraepithelial CD3+ cells than control patients. Overall, more CD3+ cells were found in the surface epithelium than in crypts of colitis patients but no such difference was discovered in the controls (Table 7). Densities of CD8+ cells were greater than that of CD4+ cells in both patients and controls. The densities of both TCR α/β + ($p=0.023$) and TCR γ/δ + cells ($p=0.027$) were significantly higher in colitis patients than in control patients. There were strikingly more δ TCS1+ IELs found ($p=0.001$) in the colonic epithelium of colitis patients compared to the controls (Figure 1). Nearly all TCR $\gamma\delta$ + IELs reacted with δ TCS1 antiserum in colitis patients whereas 50% of TCR $\gamma\delta$ + were in the non-disulphide linked form in control patients.

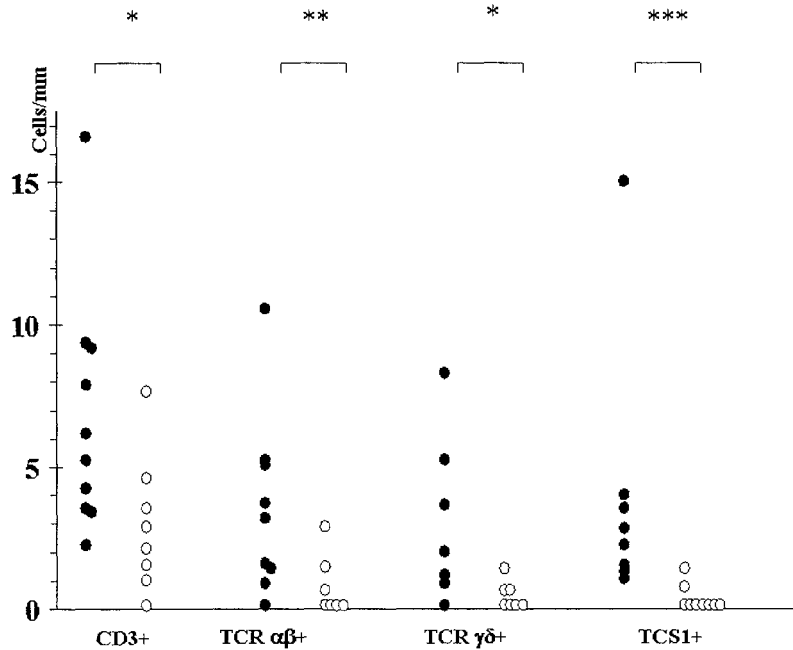


Figure 1. The density of CD3, TCR alpha-beta, TCR gamma-delta and TCS1 positive IELs in the colon of twelve young patients with colitis (dotted circles) and their controls (open circles) (* $p \leq 0.05$, ** $p \leq 0.02$, *** $p \leq 0.001$).

About 80 % of total TCR γ/δ positive cells were reactive with δ TCS1 antiserum. Also in crypts, the densities of the δ TCS1 positive cells were significantly greater ($p=0.047$) in patients with infantile colitis than in controls. Overall, the density of the TCR γ/δ positive cells was low in controls both in surface epithelium and in crypts. Detailed densities of intraepithelial T cells, T cell subgroups and TCR in the twelve patients with colitis and twelve controls are presented in Table 7.

Table 7. Intraepithelial T cells and their subgroups in the surface epithelium and crypts of the colon (cells/mm) of the patients with infantile colitis and controls.

Surface antigen	Surface epithelium of the colon						Crypt epithelium of the colon					
	Patients			Controls			Patients			Controls		
	Median	25 percentile	75 percentile	Median	25 percentile	75 percentile	Median	25 percentile	75 percentile	Median	25 percentile	75 percentile
CD3+	5.5*	3.8	9.0	2.5	1.3	4.8	3.5	3.0	5.8	2.0	1.0	4.8
TCR α/β	3.0*	1.0	5.0	0.0	0.0	1.0	2.0	2.0	4.0	2.0	0.8	4.0
CD4+	1.5	0.3	2.8	0.0	0.0	1.0	1.0	0.0	2.3	2.0	0.3	2.8
CD8+	4.0	1.0	8.0	1.5	0.0	4.0	2.5	1.0	4.8	2.0	1.0	3.0
TCR γ/δ	2.0**	1.0	5.0	0.0	0.0	1.0	1.0	0.0	2.0	1.0	0.0	2.0
TCS1+	2.5***	1.3	4.0	0.0	0.0	0.5	1.0*	0.0	1.0	0.0	0.0	0.0

* $p \leq 0.05$ ** $p \leq 0.02$ *** $p \leq 0.001$

5.1.3. Intraepithelial lymphocytes in jejunum of patients with coeliac disease and dermatitis herpetiformis (IV)

In patients with CD and DH, the density of the TCR α/β + was dependent on diet. In those consuming gluten containing diets, the density of the TCR α/β + IELs was markedly higher in the patients than that of controls but no such difference was seen when patients were consuming a gluten-free diet (GFD). The density of the TCR γ/δ positive IELs was similar in CD and DH patients irrespective of diet. In CD and DH patients, densities of TCR γ/δ positive IELs were markedly higher ($p < 0.001$) than in the controls patients. The density of the δ TCS1+ cells was about 50% of the corresponding mean density of TCR γ/δ + cells in CD and DH patients, irrespective of the diet of the patient. A similar relationship was seen in the controls. The densities of TCR $\alpha\beta$ +, $\gamma\delta$ + and δ TCS1+ (V δ 1/J δ 1+) intraepithelial lymphocytes of patients and controls are shown in Table 8.

Table 8. Densities of TCR $\alpha\beta^+$, $\gamma\delta^+$ and δTCS1^+ ($\text{V}\delta 1/\text{J}\delta 1^+$) intraepithelial lymphocytes in specimens of patients and controls (cells/mm).

Group	(n)	Age (years)	$\alpha\beta\text{TCR}^+$ cells	$\gamma\delta\text{TCR}^+$ cells	δTCS1^+ cells
CD, untreated	43	14 ± 15.9 ^a (0.8-52) ^b	71 ± 29 ^a	34 ± 21 ^a	18 ± 12 ^a
CD, gluten-free diet	19	12.6 ± 4.3 (3.0-18.8)	48 ± 40	25 ± 11	11 ± 7.2
CD, gluten challenge	15	13.4 ± 5.3 (3.2-20.8)	85 ± 38	30 ± 17	12 ± 11
DH, untreated	14	43 ± 18.6 (23.4-88.3)	80 ± 31	41 ± 18	20 ± 12
DH, gluten-free diet	9	38.9 ± 20 (12.5-73.4)	45 ± 11	38 ± 15	22 ± 9.8
CD and DH patients on Gluten containing diet	72	19.5 ± 18.2 (0.8-88.3)	76 ± 31	34 ± 19	17 ± 12
CD and DH patients on Gluten-free diet	28	21.1 ± 17.0 (3.0-73.4)	47 ± 33	29 ± 14	14 ± 9.5
Controls	37	17.9 ± 17.2 (0.6-57)	32 ± 22	2.7 ± 2.2	1.4 ± 2.0

^a mean ± S.D.

^b range

In controls, a positive correlation was found between the TCR α/β^+ IELs and age ($r=0.57$, $r^2=0.33$; $p<0.001$), while no such correlation was noted in patients either on gluten containing diet or GFD. Instead, patients with either CD or DH on normal gluten-containing diet showed a significant positive correlation ($r=0.45$, $r^2=0.20$; $p<0.001$) between the $\gamma/\delta\text{TCR}^+$ cells and age (Figure 2). Patients on GFD or controls did not show any such correlation. Nearly all (97%) of the CD and DH patients had a density of $\gamma/\delta\text{TCR}^+$ above the mean + 2 S.D. of the controls (>7cells/mm) (Figure 3).

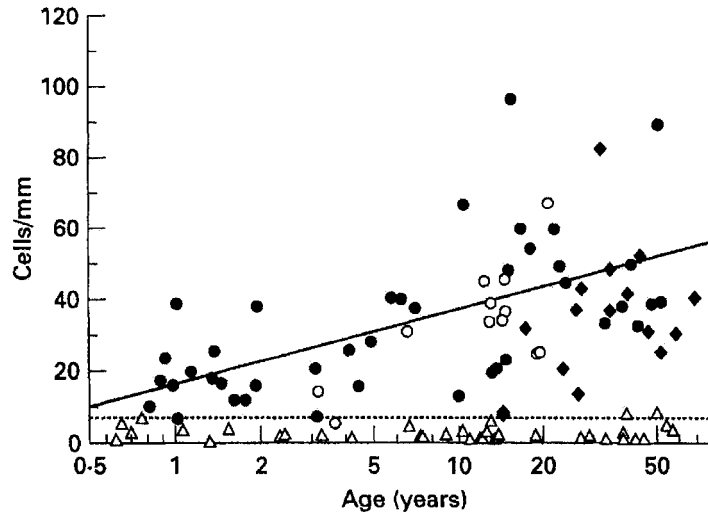


Figure 2. Densities of $\gamma\delta\text{TCR}^+$ cells in surface epithelium of jejunum (cells/mm) of patients with coeliac disease on gluten containing diet, untreated (●) and after gluten challenge (○), of untreated patients with dermatitis herpetiformis (◆), and controls (△). Linear correlation line for patients shown with the solid line and the level of mean + 2 S.D. of controls indicated with the dotted line.

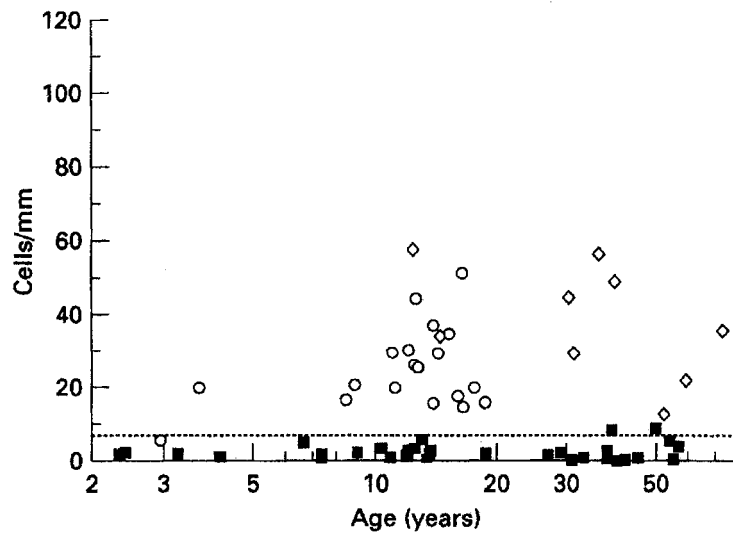


Figure 3. Densities of $\gamma\delta\text{TCR}^+$ cells in surface epithelium of jejunum (cells/mm) of patients with coeliac disease (○) and dermatitis herpetiformis (◇) on gluten free diet and of controls (□). Level of mean + 2 S.D. of controls indicated with dotted line

5.2. T lymphocytes of lamina propria of large intestine (I, III)

5.2.1. T cells of the lamina propria of non-inflamed large intestine (I)

Forty-seven specimens from large intestine without inflammation were studied in study I. In lamina propria, CD4+ cells predominated over CD8+ cells. The T lymphocyte population expressing the TCR $\alpha\beta$ heterodimer accounted for nearly 80% of the amount of CD3 positive cells. The density of TCR $\gamma\delta$ + cells was 18% of the corresponding number of CD3+ cells in lamina propria. Nearly half of the TCR $\gamma\delta$ + were δ TCS1 positive. The densities of T cells, their subsets and T cell receptors in lamina propria are presented in Table 6.

During the first 2.5 months CD3+ ($r=-0.59$, $r^2=0.35$; $p=0.006$,) and CD4+ ($r=-0.64$, $r^2=0.41$; $p=0.002$) lymphocytes in lamina propria showed a significant negative correlation with age. Thereafter the density of the CD3+ cells started to increase, but positive correlations between the densities of cells and age did not reach statistical significance ($r^2= 0.10$ for CD3 and $r^2=0.07$ for CD4+ cells) (Figure 4). Likewise, the density of the T cell subset CD8+ and TCR $\alpha\beta$ also tended to decline during the first 2.5 months, but changes with age were not statistically significant.

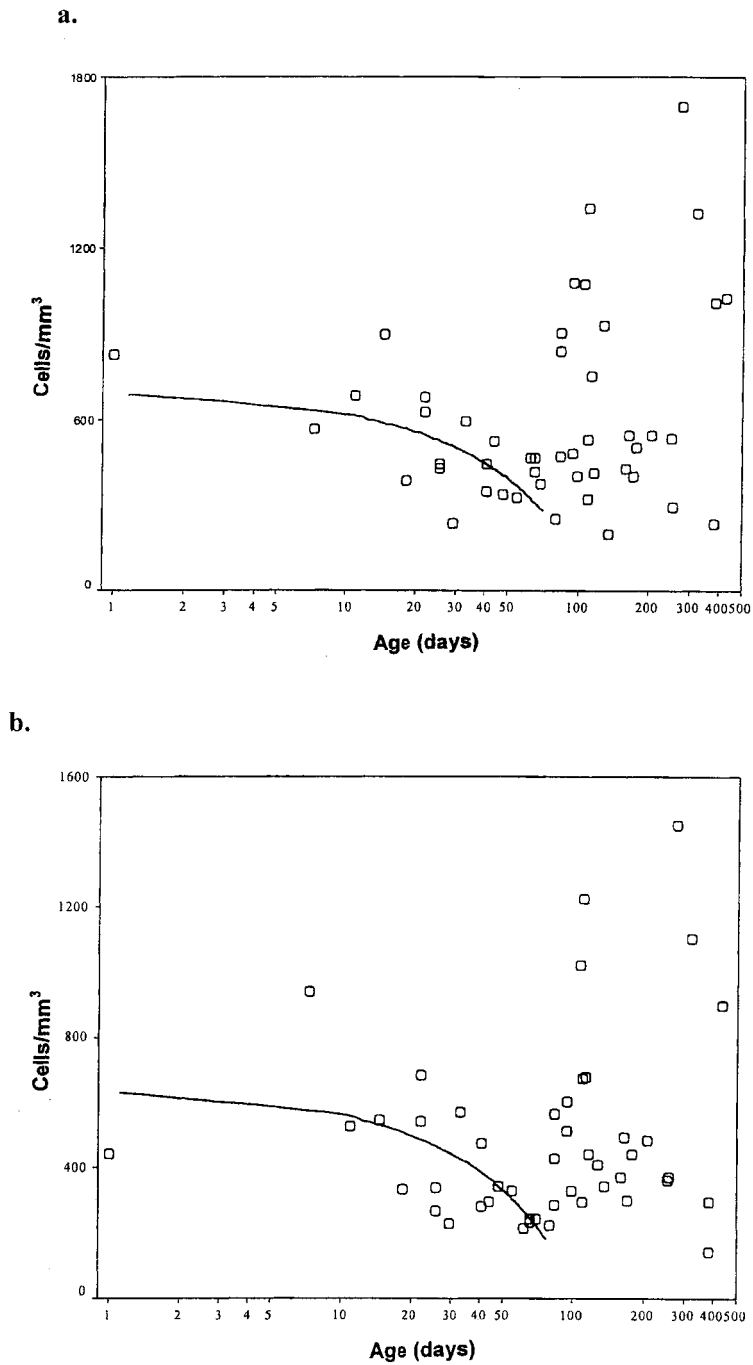


Figure 4. Densities of cells expressing CD3 (figure a) and CD4 (figure b) antigen in lamina propria of the large intestine. The significant correlation line is shown. The X-axis is a logarithmic scale.

5.2.2. T cells in the lamina propria in inflamed large intestine (III)

Twelve infants with colitis showed no statistically significant difference in T cells densities of lamina propria when compared with twelve age-matched controls. The density of T cells, their subsets expressing surface antigens CD4 and CD8 and T cell receptor α/β and γ/δ are presented in Table 9.

Table 9. T cells and their subgroups and HLA region D expressing cells in the lamina propria (cells/mm²) of the patients with infantile colitis and the controls.

Surface antigen	Patients			Controls		
	Median	25 Percentile	75 percentile	Median	25 percentile	75 percentile
CD3+	478,5 ^a	347,9	1009,2	433,9	329,9	608,9
TCR α/β +	485,8 ^a	381,1	718,4	372,6	212,1	558,1
CD4+	610,2 ^a	298,7	964,7	356,7	280,2	588,0
CD8+	127,1 ^a	92,8	285,1	167,2	106,9	179,1
TCR γ/δ +	78,1 ^a	42,3	222,3	88,2	52,9	129,7
δ TCS1+	58,8 ^a	15,5	105,1	32,7	18,3	63,6
HLA-DR	617,5 ^a	320,8	908,5	555,5	386,2	875,0
HLA-DP	441,0 ^a	279,5	811,5	235,0	174,8	576,5
HLA-DQ	377,0 ^a	193,2	745,3	441,0	282,0	626,1

^a not significant

The proportion of TCR α/β was over 80% with TCR γ/δ accounting for approximately 20% of the CD3 positive cells in both groups. Over half of the TCR γ/δ cells were in a δ TCS1 positive form in patients with colitis.

5.3. HLA expression of the epithelial surface of the large intestine (I, III)

5.3.1. HLA expression of the epithelium of the large intestine (I,III)

5.3.1.1. Non-inflamed large intestine (I)

In study I, colonic epithelial cells expressing HLA-D region antigens were examined from 11 patients with Hirschsprung's disease and 36 patients with other intestinal complaints. These groups did not differ statistically. The values for the HLA-D region expression in epithelial cells of the colon are presented in Table 10.

Table 10. Number of specimens with HLA-DR, DP and DQ antigen expression in epithelial cells in patients with Hirschsprung's disease and in patients with miscellaneous intestinal conditions.

Antigen	Surface epithelium		Crypt epithelium	
	Positive	Negative	Positive	Negative
Hirschsprung's disease (n=9)				
HLA-DR	2	7	0	9
HLA-DQ	1	8	0	9
HLA-DP	0	9	1	8
Miscellaneous intestinal conditions (n=27)				
HLA-DR	7	20	1	26
HLA-DQ	2	25	0	27
HLA-DP	2	25	4	23

5.3.1.2. Inflamed large intestine (III)

In study III, the HLA-D region expressing antigens in epithelial cells of the large intestine were examined from twelve young colitis patients and their controls. These groups did not differ significantly with respect to HLA-D region expression in epithelial cells. The epithelial HLA-D region expression figures are shown in Table 11.

Table 11. Number of specimens with HLA-DR, DP and DQ antigen expression in epithelial cells among infants with colitis and controls.

Antigen	Surface epithelium		Crypt epithelium	
	Positive	Negative	Positive	Negative
Patients (n=12)				
HLA-DR	3	5	2	6
HLA-DQ	2	6	2	6
HLA-DP	3	6	2	7
Controls (n=12)				
HLA-DR	3	8	1	10
HLA-DQ	0	10	0	10
HLA-DP	2	9	2	9

5.3.2. HLA expression in the lamina propria of the large intestine (I,III)

5.3.2.1. Non-inflamed large intestine (I)

In study I, eleven patients with Hirschsprung's disease without any signs of colitis had significantly more HLA-DR ($p=0.006$) and -DP ($p=0.003$) expressing cells in the lamina propria when compared with 38 patients with miscellaneous gastrointestinal symptoms. In addition, the density of HLA-DR expressing cells in the lamina propria of the large intestine showed a significant

positive correlation ($r=0.58$, $r^2=0.34$; $p=0.046$) in relation to the age of the patients with Hirschsprung's disease throughout the whole age range (Figure 5). HLA-DQ did not show this kind of association with age. Instead, the density of HLA-DR+ cells of 38 patients with miscellaneous gastrointestinal symptoms showed significant negative correlation ($r=-0.90$, $r^2=0.81$; $p=0.006$) in lamina propria during the first 1.5 months of age. Thereafter these cells tended to increase, but the slope was statistically insignificant. HLA-DQ and -DP expressing cells in lamina propria showed a tendency to behave similarly but the changes were not significant.

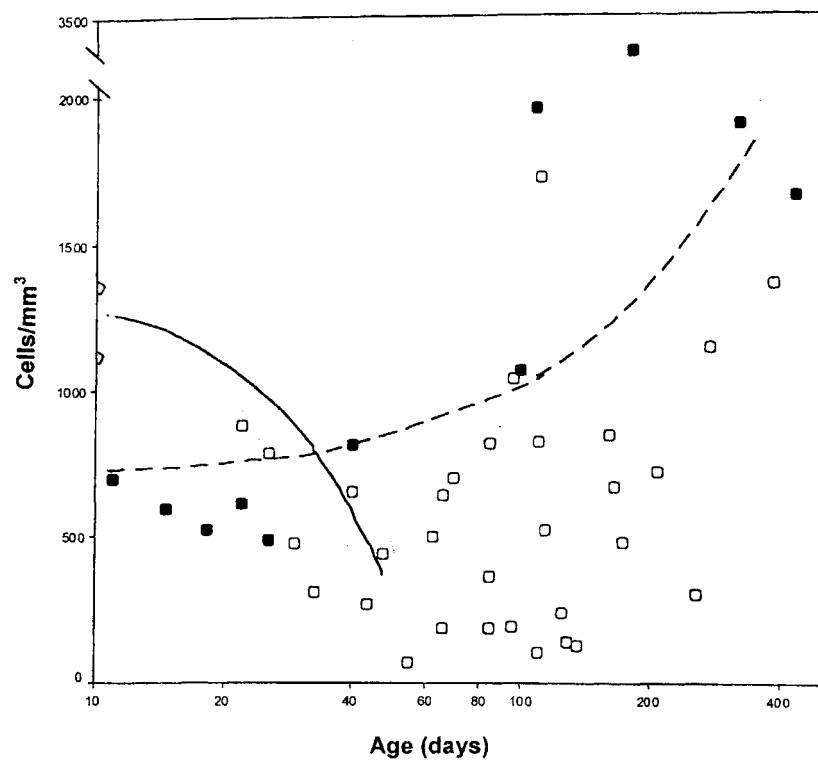


Figure 5. Densities of cells expressing HLA-DR antigen in the lamina propria of the large intestine. ● = patients with Hirschsprung's disease; ○ = patients with other abdominal complaints. Only the significant correlation lines of the density of HLA-DR expressing cells with advancing age are shown. The dotted line is for the patients with Hirschsprung's disease and the solid line is for the other patients. The X-axis is on a logarithmic scale.

5.3.2.2. Inflamed large intestine (III)

In study III, we could not find any difference with the densities of the HLA-DR, HLA-DQ or HLA-DP expressing cells in lamina propria of the colon between patients with colitis and controls. The densities of the HLA-D region antigens expressing cells in lamina propria of young colitis patients are presented in Table 9.

5.4. Immunoglobulin containing cells in the lamina propria of the large intestine (II)

All twelve patients with Hirschsprung's disease had equal densities of IgA, IgA1-, IgA2, IgM-, IgG- and CD22 positive cells as the 24 patients with other clinical problems. Thus, all these patients were included in the study on the association of cell densities and age. As these two groups did not differ in their densities of T Cells (study I), both counts were used for analysis of correlation between the two cell populations.

In this entire study group, cells containing IgA, IgG and IgM were equally frequent in the lamina propria both of colon (n=19; 30%, 33% and 37%) and rectum (n=17; 35%, 28% and 36%). The densities of the IgA1+ and IgA2+ cells were similar and their sum was equal to the density of cells revealed by monoclonal antibody specific for a constant region of alpha chain, which detects all IgA+ cells (Table 12).

Table 12. Densities of CD22, IgA-, IgA1-, IgA2-, IgM-, and IgG-positive cells in lamina propria of the large intestinal mucosa (cell/mm²) of patients divided into three age groups (mean and S.D. given)

Group (age)	Number	CD22	IgA	IgA1	IgA2	IgM	IgG
< 1 month old	9	2360±620	577±485	291±228	274±307	567±202	827±480
1-3 months old	11	2543±317	557±485	268±186	348±160	750±315	764±354
> 3 months old	16	2757±555	1074±524	603±254	494±333	911±340	991±516
Entire range	36	2603±546	792±520	423±276	394±292	783±328	881±462

The densities of the IgA+, IgA1+ and IgA2+ cells were clearly lower in the younger patients than older ones (Table 10). Three out of four patients below the age of two weeks showed no IgA positive cells. The density of the IgA positive cells showed a significant positive correlation with age ($r=0.47$, $r^2=0.22$; $p=0.04$) (Figure 6). The correlation was strongly positive ($r=0.57$, $r^2=0.32$; $p=0.001$) for the IgA1 subclass (Figure 7) and weaker for IgA2 ($r=0.34$, $r^2=0.10$; $p=0.04$) (Figure 8). The densities of the IgG+, IgM+ and CD22+ cells did not show any statistically significant correlation with age. The CD22+ cells seemed to have an insignificant tendency to rise with age.

When studying the connection between B- and T-cells, we found a negative correlation between IgG-containing cells and the density of CD8+ cells in lamina propria ($r=-0.43$, $r^2=0.18$; $p=0.01$). CD4+ cells did not show any such correlation with IgG positive cells.

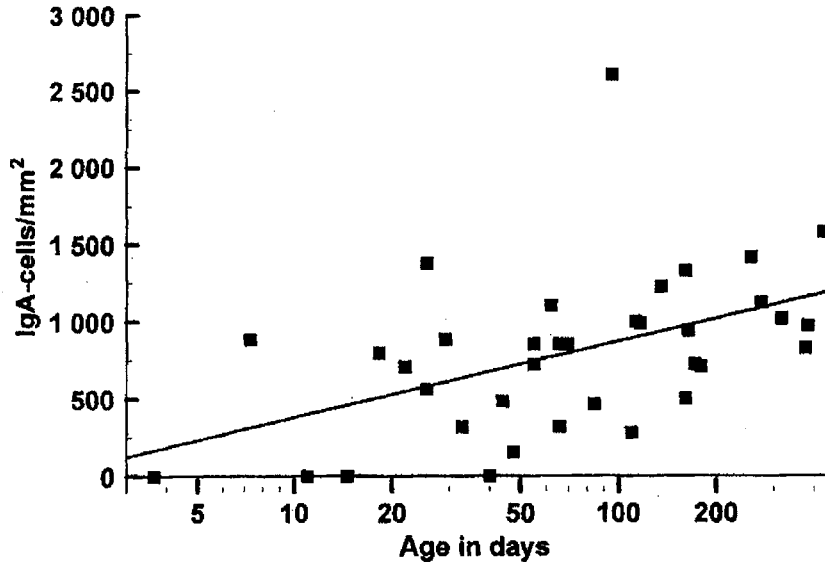


Figure 6. Densities of IgA-positive cells expressed as cell/mm² of lamina propria in specimens from patients. The line shows the significant linear correlation between densities of cells and log₁₀ transformation of ages of the patients.

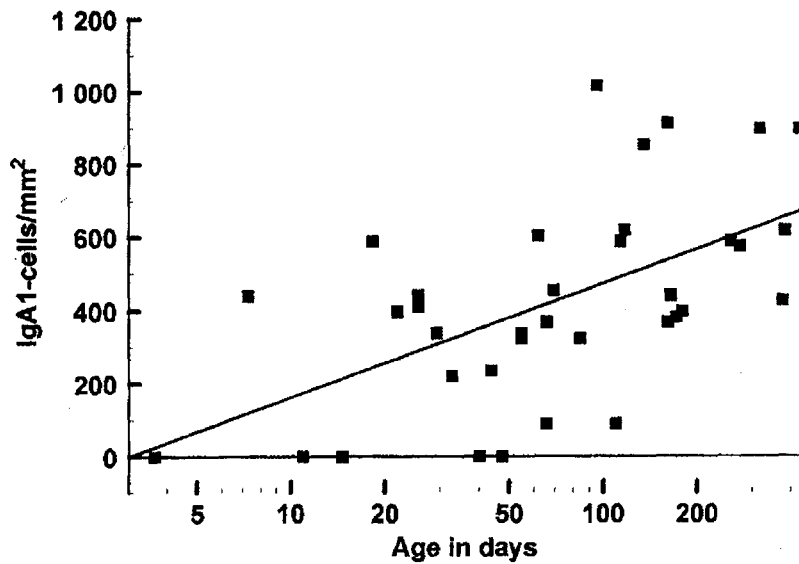


Figure 7. Densities IgA1-positive cells expressed as cells/mm². The line shows the significant linear correlation between densities of cells and log₁₀ transformation of ages of the patients.

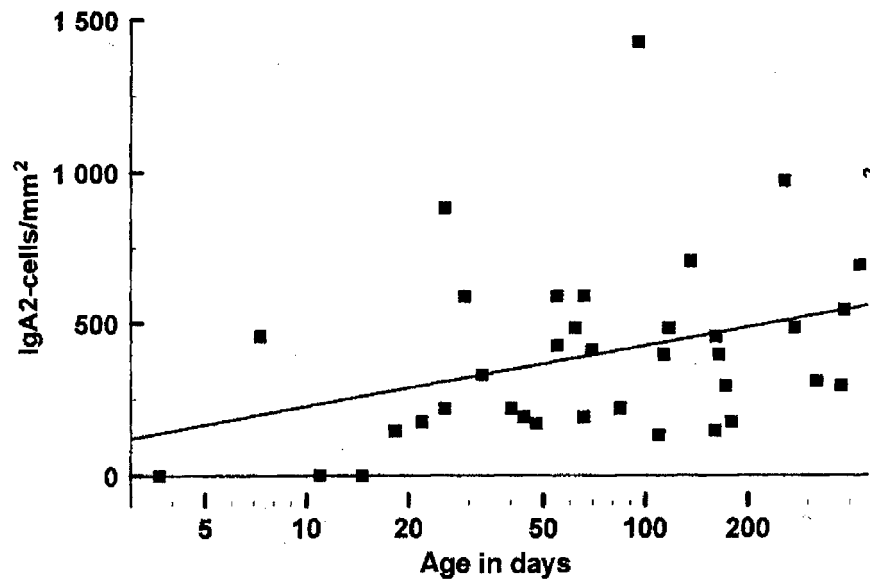


Figure 8. Densities of IgA2-positive cells expressed as cells/mm². The line shows significant linear correlation between densities of cells and log₁₀ transformation of ages of the patients.

6. DISCUSSION

6.1. HLA Class II expression in the epithelium of large intestine

6.1.1. Surface epithelium

HLA-DR does not appear to be expressed in the terminal ileal epithelial cells until 18 to 20 weeks of gestation, although most of the cells in the underlying lamina propria are strongly HLA-DR⁺ from 11 weeks of gestation (Spencer et al. 1987, Macdonald et al 1988). There is no published data of fetal HLA-D region antigens expression in the large intestine.

In healthy postnatal human small intestine, HLA-D region antigens are present on the epithelial cells of the villi (Scott et al. 1980, Rognum et al. 1992), but they have been claimed to be absent from colonic epithelial cells (Scott et al. 1980). Class II MHC expression is increased in colonic epithelium in inflamed rectal and colonic mucosa, and it is probably induced by activated T cells (Arato et al. 1989, Selby et al. 1983). However, some studies have showed (Spencer et al. 1986, Mayer et al. 1991) diminished but clear HLA-DR expression in normal colonic epithelial cells. Also in this study clear HLA-D region expression was found in some cells both in surface epithelium and in crypts both in inflamed and non-inflamed colon, but they did not differ statistically. This expression is probably due the production of γ -interferon by activated T cells induced by colonic bacteria.

6.1.2. Lamina propria

At 11 weeks of gestation, few CD3 positive cells are present in the lamina propria (MacDonald et al. 1994). At 14 weeks of gestation, scattered CD4+ T cells are found in the lamina propria (Spencer et al. 1986b, MacDonald et al. 1988). The main cell populations within the fetal intestinal lamina propria is a strongly HLA-DR positive with a dendritic and macrophage cell morphology (Spencer et al. 1987). The extent of this non-lymphoid infiltrate increases with the age of the fetus.

According to our results, soon after birth an abundance of HLA-DR expressing cells were found in the lamina propria of the large intestine in patients with non-inflamed colon. Subsequently the density of HLA-DR + cells in lamina propria declined during the first 1.5 months which was followed by a slight increase. Possibly HLA-DR+ cells are constitutionally abundant in lamina propria of colon at the time of delivery, as is the case during fetal period. This is followed by a decline in the numbers of HLA-DR+ cells during the first months of life.

HLA-DR and HLA-DP expressing cells in lamina propria were found significantly more abundantly in the specimens taken from the patients with

Hirschsprung's disease than in other patients, perhaps because of chronic constipation. Constipation may predispose the infant to excessive intestinal bacterial growth and fermentation, leading to intense stimulation of the mucosal immune system. However, when we divided our 36 infants with a variety of intestinal symptoms into 20 constipated and 16 non-constipated patients, there was no difference in HLA-D-region expressing cells in lamina propria between these groups. Presumably, patients with Hirschsprung's disease had more severe constipation than the other constipated patients leading to greater immunoactivation of the large intestine than that occurring in the ordinary patients with constipation. In the patients with Hirschsprung's disease, we could observe no decline in the HLA-D region expressing cells in the lamina propria, as was seen during the first 1.5 months after birth among patients with other conditions.

In study III, twelve infants with colitis and twelve age matched control patients were studied. In contrast to study I, we could not find any difference in the densities of the HLA-DR, HLA-DQ or HLA-DP expressing cells in lamina propria of the colon between these groups. We cannot find any plausible explanation for that discrepancy.

6.2. T cells and T cell receptor in the lamina propria of large intestine

The cell population in fetal lamina propria which is strongly HLA-DR positive has dendritic cell and macrophage like morphology (Spencer et al. 1987). The cells also express CD4 and CD45 (leukocyte common antigen). At 11 weeks of gestation, low numbers of CD3+ cells are present in lamina propria (MacDonald et al. 1994). From 14 weeks of gestation, scattered B and T cells are present in fetal lamina propria (Spencer et al. 1986b). The majority of the T cells in the lamina propria are CD4+.

According to our results, CD3+ and CD4+ T lymphocytes in lamina propria of the colon showed a significant decline during the first 2,5 month after birth. Thereafter these cells tended to increase, but the slope was insignificant. In addition, CD8+ T cells, TCR $\alpha\beta$ and TCR $\gamma\delta$ showed a similar tendency which,

however, was statistically insignificant. These findings may be explained by rapid colonisation of the previously sterile large intestine, which induces the rapid increase in the numbers of cells with various immunologic functions soon after birth. Subsequent breast feeding allows protective immunoglobulin A to reach the colon and rectum and may reduce the inflammation (Fitzsimmons et al. 1994). Later, changes in infant feeding may reduce IgA intake leading to the increased antigenic load in the intestine. Consequently, the result is the observed increase in several cell types.

Our results showed a similar postnatal distribution of the T cell subsets in lamina propria of large intestine as has been previously reported in adults (Selby et al. 1984). CD4⁺ cells predominated over CD8⁺ cells. However, the densities of the CD3⁺, CD4⁺ and CD8⁺ cells were lower than those previously reported in adolescents (Arato et al. 1989, Fukushima et al. 1991). The proportion of the TCR $\alpha\beta$ bearing T cells was equal to that described in lamina propria of adults, but the relative density of TCR $\gamma\delta$ ⁺ expressed in 18% of the amount of CD3⁺ cells is more than that observed in lamina propria of the small intestine of adults (Arato et al. 1989, Fukushima et al. 1991). Hence, this may be an age dependent phenomenon which may involve the mucosal defence against colonic bacteria.

At 18 weeks of gestation, all the T cells in lamina propria are TCR $\alpha\beta$ ⁺ while TCR $\gamma\delta$ ⁺ cells are absent at that time (MacDonald et al. 1994). According to the present study, the proportion of TCR α/β bearing cells was approximately 80% of that of CD3⁺ cells soon after birth, whereas TCR γ/δ ⁺ was expressed in close to 20% of the amount of CD3⁺ cells. With regard to γ/δ ⁺ cells, nearly half were δ TCS1 positive. Accordingly, TCR γ/δ ⁺ T cells appear in the lamina propria during the last trimester of pregnancy or during the first days of life. Later in life, the numbers of TCR $\gamma\delta$ ⁺ cells start to decline accounting for less than 15% of cells in adults. Only in CD and allergy (Kokkonen et al. 2000) there are a high frequency of TCR $\gamma\delta$ ⁺ cells found.

6.3. Intraepithelial lymphocytes of large intestine

Our results showed that in infancy the density of the CD3+ intraepithelial lymphocytes was lower than that reported earlier in adult (Fukushima et al. 1991) and adolescent patients (Arato et al. 1989); the difference could be age related. The distribution of the T cell subsets CD4+ and CD8+ was equal to the finding reported earlier (Selby et al. 1981, Brandtzaeg et al. 1989). The CD8+ dominance over CD4+ IELs is less striking in fetal IELs (Macdonald et al. 1994, Spencer et al. 1986). CD3+/CD4-/CD8- cells were frequent after birth, on average 6% of the CD3+ IELs, but this fraction in fetal gut is 35% to 70% (Spencer et al. 1989). By 20 weeks of gestation, approximately only 20% of the total number of CD3+ IELs have been reported to carry TCR γ/δ (Spencer et al. 1989). Non-disulphide linked form of TCR γ/δ , is absent in fetal IEL or lamina propria (Spencer et al. 1989). Over half of the amounts of the CD3+ IELs were TCR γ/δ positive. The density of the γ/δ positive IELs in proportion to CD3+ cells was higher in study I than reported in previous studies. Within TCR γ/δ + IELs, the proportion of cells reactive with δ TCS1 antiserum (56%) and defining the V δ 1/J δ 1 rearrangement specific for mucosal γ/δ + cells was as high as reported previously, thus the proportion of TCR γ/δ and its non-disulphide linked form increases markedly after 20 weeks of gestation. Overall, the density of the IELs in the large intestine during infancy was low (Arato et al. 1989).

In study III, infantile colitis patients showed a clear increase of intraepithelial T cells (CD3+) probably due to the increased antigen handling in inflamed colon. This significant increase involved both TCR α/β + and γ/δ + IELs. The distributions of the T cell subsets CD4+ and CD8+ in colitis patients were similar to those detected in study I and also to those previously reported in adults and young infants (Selby et al. 1981, Selby et al. 1984, Brandtzaeg et al. 1989). Nearly all TCR γ/δ + IELs were reactive with δ TCS1 antiserum in colitis patients, while only 60% of TCR γ/δ + IELs were in the non-disulphide-linked form in control patients, which is equal that noted in study I. The increase in the proportion of the non-disulphide-linked form during the last trimester or soon

after delivery and the intraepithelial accumulation non-disulphide-linked form of TCR γ/δ + cells at the site of inflamed colon supports the proposal that they play important roles in immune defence system in the gut.

6.4. Jejunal intraepithelial T cells in patients with coeliac disease and dermatitis herpetiformis

The increased density of γ/δ + IELs was present in 97% of patients with CD or DH, irrespective of diet. In controls, TCR α/β + IELs showed age-dependent increase, while TCR γ/δ + cells remained equally sparse throughout the age range studied (0.6 – 57 years).

The function of TCR α/β + cells is well known, but the role of TCR γ/δ + cells as well as the antigens they recognize, are still unknown (Haas et al. 1993). In the mucosa, TCR γ/δ + cells may be important in modulating the growth of epithelial cells (Boismenu et al. 1994), or in modulating immune responses of TCR α/β cells with respect to sensitisation (Askenase et al. 1995), to tolerance (Fujihashi et al. 1992) and to secretion of IgA (Fujihashi et al. 1996).

In patients with CD and DH on gluten a containing diet, an age-dependent increase on the density of TCR γ/δ + IELs was found. The density of these cells is not dependent on the presence of gluten in the diet, which is in contrast to intraepithelial α/β + cells which are related to gluten ingestion. The correlation of the density of the γ/δ + cells with age was significant in the whole group of patients. It may be anticipated that inflammation which continues in the jejunal epithelium in patients with CD and DH, in spite of all dietary manipulations, could result an increased generation of fibronectin (Campbell et al. 1993). The V δ -1 subset of TCR γ/δ + cells is apt to localise to fibronectin, because these cells frequently bear the VLA5 receptor (Nakajima et al. 1995); about half of the intraepithelial γ/δ + cells in study IV also displayed this V- δ region.

Intraepithelial T cells, particularly $\gamma/\delta+$ cells, are thymus independent and may proliferate locally (Poussier et al. 1994, Gross et al. 1994). The expansion of local clones may take place with increasing age, accounting for the increase of the IELs with age.

6.5. Immunoglobulin containing cells in the lamina propria of large intestine

In the present study we found several times more immunoglobulin-positive cells than in the study done on paraffin sections with the direct immunoperoxidase method (Perkkiö et al. 1980). In the technique used in the present study, the binding of the antibody was amplified by biotin-avidin. This kind of binding is much more sensitive than in the direct peroxidase technique used in Perkkiö's study. Therefore the present technique also revealed surface immunoglobulin positive B cells.

The lamina propria of very young infants showed a high density of CD22 positive B cells, which increased little with age. IgM- and IgG positive cells were found abundantly even in the youngest infants, suggesting an intense local response of B cells in the large intestine (Haneberg et al. 1994). The increase in the density of CD22 positive cells is attributable exclusively to the increase in IgA positive cells. In this study, we found an equal number of IgG and IgA positive cells in large intestine, whereas, according to earlier studies, the density of IgG secreting plasma cells is lower in rectum compared with the density of IgA cells (Savilahti 1972, Brandtzaeg et al. 1974). A proportion of the IgG positive cells may switch to IgA-positive cells (Brinkmann et al. 1992), accounting for the high IgG positive cell count.

B cells express the CD22 surface antigen until the cells mature into immunoglobulin secreting plasma cells (Uckun 1990). B cells with membrane immunoglobulins carry this antigen. In our study the density of the cells with this receptor represented 80 – 95% of the combined density of the IgA, IgM and IgG positive cells and only a small percentage of immunoglobulin-positive cells were mature plasma cells.

The differentiation of immunoglobulin secreting cells is dependent on the cytokines secreted by T cells (McIntyre et al. 1995, Mosmann et al. 1996) and direct T-B cell interactions. Nonetheless, we found little association between the densities of the immunoglobulin positive cells and T cells; in lamina propria there was negative correlation between CD8 + and IgG + cells, suggesting that CD8+ T cells at a higher density may suppress IgG class-switch and proliferation. The majority of the CD8+ cells secrete cytokines, which suppress immunoglobulin synthesis (Mosmann et al. 1996).

7. SUMMARY AND CONCLUSION

Our study was undertaken to evaluate the influence of age and inflammation of the large intestine on the development of mucosal T cells and their subgroups, as well as HLA-D region expressing cells soon after birth, when the previously sterile intestinal mucosa is challenged by large amount of food and bacterial antigens. In addition, the development and distribution of IgA and its subgroups were of interest, as well as the possible developmental associations between T and B cells of the GALT. The influence age on the mucosal immune system was also studied in jejunal specimens taken from patients with CD and DH of different ages and clinical situations.

Our data showed, that in non-inflamed colon of young infants, CD3+ IELs are found in lesser amounts than earlier reported in adults and adolescents, which might be an age related phenomenon. During the last trimester of pregnancy or soon after delivery, the proportion of TCR $\gamma\delta$ + cells increased and the nondisulphide linked form of TCR $\gamma\delta$ appeared on the surface epithelium and lamina propria of the large intestine.

In patients with colitis CD3+, TCR $\alpha\beta$, TCR $\gamma\delta$ and especially non-disulphide linked TCR $\gamma\delta$ bearing IELs were found significantly more frequently than in controls, which reflects the immunological response of GALT to inflammation and gives a good reason to assume that TCR $\gamma\delta$ bearing IELs may have an important role in mucosal immune defence. Instead, the distributions

of the CD4+ and CD8+ bearing cells in inflamed and non-inflamed large intestine were similar as have been described earlier.

By 18 weeks of gestation, TCR $\gamma\delta$ + cells are not seen in the lamina propria, but they were noted abundantly in the postnatal period. In the lamina propria of colon, CD4+ and CD8+ antigen bearing lymphocytes as well as HLA-DR antigen expressing cells declined significantly during the first 2.5 months in patients. This phenomenon was noted only in normal large intestine, i.e. patients with Hirschsprung's disease and colitis did not exhibit such a decline. This may be due to the relative decrease of new antigen and protective action of IgA in healthy controls without colitis. Patients with Hirschsprung's disease may have more severe constipation than other constipating patients leading to immunoactivation, although biopsy specimens taken from Hirschsprung's disease patients did not show signs of enterocolitis.

The density of CD22 positive B cells in lamina propria was high already in young infants and this cell population increased marginally little with age. This increase was caused exclusively by an increase in the numbers of IgA positive cells, particularly of IgA1 positive cells. The observed high density of IgG positive cell count may be explained by IgG+ switch to IgA+. Only a small percentage of immunoglobulin containing cells were mature plasma cells.

There was evidence of cross-talk between T and B cells in lamina propria. We found a negative correlation between CD8+ and IgG+ cells, suggesting that T cells at higher density may suppress the IgG class-switch and proliferation

Patients with CD and DH were found to have an age dependent increase in the density of $\gamma\delta$ TCR+ cells on the surface epithelium of the jejunum when they are consuming a gluten containing diet. In controls, TCR $\alpha\beta$ + cells showed an age related increase, while the density of TCR $\gamma\delta$ + cells remained sparse. In all, 97% of the patients with CD or DH showed a density of $\gamma\delta$ + cells above the mean plus two standard deviation of the controls, irrespective of their diet, making this a good marker of the disease.

In summary, in this study many age related immunological changes in gastrointestinal tract were described. This is evidence of how the immune

system is in dynamic interaction with the environment, where the age is one of the important contributing variables.

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9. ORIGINAL PUBLICATIONS (I-IV)

