Defence Capabilities of Human Intestinal Epithelial Cells

by

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ABSTRACT

The epithelial cells lining the intestinal mucosa separate the underlying tissue from components of the intestinal lumen. Innate immunity mediated by intestinal epithelial cells (IECs) provides rapid protective functions against microorganisms. Innate immunity also participates in orchestrating adaptive immunity. Key components in innate defence are defensins.

To study the production of defensins and how it is affected by intestinal inflammation IECs were isolated from the small and large intestines of patients suffering from ulcerative colitis (UC), Crohn’s disease (MbC), celiac disease (CD), and from controls, and analyzed by quantitative RT-PCR (qRT-PCR) and immunoflow cytometry. Defensin expressing cells were also studied by in situ hybridization and immunohistochemistry.

Normally, only small intestinal Paneth cells express human α-defensin 5 (HD-5) and HD-6. In UC colon IECs, HD-5, HD-6, and lysozyme mRNAs were expressed at high levels. In Crohn’s colitis differentiation in UC colon was primarily responsible for the expression of the antimicrobial components. Human β-defensin 1 (hBD-1) mRNA was more abundant in large than in small intestine of controls, and remained unchanged in UC and MbC. hBD-2 mRNA was barely detectable in normal intestine and was induced in UC IECs but not in MbC IECs. mRNAs for the recently discovered hBD-3 and hBD-4 were detected in IECs from both small and large intestine. Both hBD-3 and hBD-4 mRNA were significantly increased in IECs from UC patients but not of MbC patients. Bacteria and IL-1β induced hBD-2 but not hBD-1 mRNA in colon carcinoma cell lines. IFN-γ but not TNF-α or IL-1β, augmented hBD-3 expression in these cells, while none of the agents induced hBD-4. High antimicrobial activity of IECs in UC may be a consequence of changes in the epithelial lining, which permit the adherence of microorganisms.

Unexpectedly, in situ hybridization revealed expression of hBD-3 and hBD-4 mRNAs by numerous lamina propria cells in colonic tissue from UC patients. These cells were identified as plasma cells (CD138+). hBD-3 and hBD-4 mRNAs were also demonstrated in the plasmacytoma cell line U266. This is the first demonstration of defensins in plasma cells.

The four prominent constituents of the intestinal glycocalyx, carcinoembryonic antigen (CEA), CEA cell adhesion molecule 1 (CEACAM1), CEACAM6 and CEACAM7 all seem to play a critical role in innate defence of the intestinal mucosa by trapping and expelling microorganisms at the epithelial surface. The inducibility of these molecules in colonic epithelial cell lines was analyzed by qRT-PCR, immunoflow cytometry, and immunoelectron microscopy. IFN-γ but not bacteria, LPS, TNF-α, or IL-1β modified the expression of CEA, CEACAM1 and CEACAM6. None of these agents modified CEACAM7 expression. IFN-γ was shown to have two effects: a direct effect on CEACAM1 transcription, and promotion of cell differentiation resulting in increased CEA and CEACAM6 and decreased CEACAM7 expression.

Scanning electron microscopy of jejunal biopsies from children with CD revealed the presence of rod shaped bacteria in ~40% of patients with active CD, but only in 2% of controls. 19% of treated CD patients still had adhering bacteria. Presence of bacteria is not due to lack of antimicrobial factors. In fact, HD-5, HD-6, and lysozyme mRNA levels were significantly increased in IECs of patients with active CD. hBD-1 and hBD-2 were unchanged. Lack of induction of hBD-2 may reflect disturbed signalling in IECs of CD patients. Analysis of CEA and CEACAM1 mRNA/protein expression showed no differences between CD patients and controls. Analysis of the mucins MUC2 and MUC3 revealed significantly increased MUC2 levels in active disease and unchanged MUC3. Immunohistochemistry demonstrated goblet cell metaplasia as well as staining of the apical portion of absorptive cells. Glycosylation status of proteins was studied by lectin histochemistry. Goblet cells in the mucosa of CD patients were stained by the lectin UEAI. This was not seen in controls. The lectin PNA stained the glycocalyx of controls but not that of CD patients. Thus, unique carbohydrate structures of the glycocalyx/mucous layer are likely discriminating features of CD patients and may allow bacterial binding.

We conclude that the intestinal epithelium is heavily involved in the innate defence of the mucosa and that its reactive pattern is affected by intestinal inflammation.
### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>ADCC</td>
<td>Antibody dependent cell mediated cytotoxicity</td>
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<tr>
<td>Ag</td>
<td>Antigen</td>
</tr>
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<td>AMP</td>
<td>Antimicrobial peptide</td>
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<td>APC</td>
<td>Antigen presenting cell</td>
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<tr>
<td>BeR</td>
<td>B cell receptor</td>
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<td>CD</td>
<td>Celiac disease</td>
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<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
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<tr>
<td>CEA</td>
<td>Carcinoembryonic antigen</td>
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<tr>
<td>CEACAM</td>
<td>Carcinoembryonic antigen cell adhesion molecule</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocyte</td>
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<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>FDC</td>
<td>Follicular dendritic cell</td>
</tr>
<tr>
<td>hBD</td>
<td>Human beta defensin</td>
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<tr>
<td>HD</td>
<td>Human alpha defensin</td>
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<tr>
<td>HNP</td>
<td>Human neutrophil peptide</td>
</tr>
<tr>
<td>IBD</td>
<td>Inflammatory bowel disease</td>
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<tr>
<td>IEC</td>
<td>Intestinal epithelial cell</td>
</tr>
<tr>
<td>IEL</td>
<td>Intraepithelial lymphocyte</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>LPL</td>
<td>Lamina propria lymphocyte</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MbC</td>
<td>Crohn’s disease</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen associated molecular patterns</td>
</tr>
<tr>
<td>PC</td>
<td>Plasma cell</td>
</tr>
<tr>
<td>PP</td>
<td>Peyer’s patches</td>
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<tr>
<td>PRR</td>
<td>Pattern recognition receptors</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>TcR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>UC</td>
<td>Ulcerative colitis</td>
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PAPERS IN THIS THESIS

This thesis is based on the following articles and manuscripts, which will be referred to in the text by Roman numerals (I-V).


V. Fahlgren A, Baranov V, Danielsson Å, Hammarström S, Hammarström M-L. Human intestinal plasma cells produce defensins. 2003. (manuscript)

* Both authors contributed equally.
1. INTRODUCTION

The immune system is a sensory and effector system that has evolved to protect the host from pathogenic microorganisms or other harmful antigens. It can be divided into two major branches, innate (or natural) immunity and adaptive (or acquired) immunity, which work together to induce powerful responses. The effector mechanisms of these branches include both specific and non-specific molecules as well as specialized cells. Innate immunity effector molecules are either constitutively expressed or are triggered in immediate response to highly conserved structures on invading microorganisms without a requirement of previous exposure. It limits the number of infecting microorganisms through different effector mechanisms and provides time for the more effective adaptive response to develop – a process that usually takes three to five days. Physical barriers, phagocytic cells (macrophages and neutrophil granulocytes), the complement system, and natural killer cells (NK-cells) are elements in the innate immune response. Induction of innate immunity does not result in increased protection to subsequent infections or memory. Adaptive immunity is characteristic of higher animals and is divided into humoral and cell-mediated immune responses. Humoral responses are mediated through specific antibodies produced by plasma cells derived from specific B-lymphocytes and cell-mediated responses are mediated by specific T-lymphocytes. The cells are activated by a nominal antigen (Ag) and expand clonally for effective recognition of the specific Ag. The specificity is created by somatic rearrangement of the encoding genes and theoretically generates a pool of at least $10^{11}$ different B cell receptors (BcR) and $10^{15}$ different T cell receptors (TcR). The adaptive immune system is characterized by memory of previously encountered antigens, adaptation of the response with time, and diversity and specificity generated by the high number of different BcRs and TcRs. Contact of microorganisms with the host tissue results in: a) the elimination of the microorganism by host defences without activation of the adaptive response or an inflammatory response, or b) a situation where the microbe outgrows the innate immune response. This results in induction of innate immune effector molecules that are antimicrobial, but also mediates induction of adaptive immune response which will eliminate the microbe effectively. The expansion of specific T cells results in cytokine production, cytotoxicity, regulation of humoral responses, and recruitment of immune cells.

1.1 Adaptive immunity

Monocytes/macrophages, dendritic cells, activated B cells and follicular
Dendritic cells have to a variable degree the ability to present foreign antigens and can be defined as antigen presenting cells (APCs). They take up Ag and present processed Ag in the highly polymorphic major histocompatibility complex (MHC) class I (one polymorphic α-chain and β2-microglobulin) or MHC class II (a heterodimer of the two polymorphic α- and β-chains) molecules on their surface. The cells then travel to secondary lymphoid tissues where the Ag is recognised by naïve T cells bearing a TcR specific for the Ag and by B cells. MHC class I molecules are found on the surface of virtually all nucleated cells and present processed peptides from intracellular proteins. MHC class II molecules are present on APCs and present processed extracellular Ags. Monocytes circulate in the blood and when recruited into the tissue they develop to the larger macrophages that have increased phagocytic capacities and move towards a site of infection. Additionally, almost all tissues in the body contain stationary macrophages. In the liver these are called Kupffer cells and in the brain these are called microglial cells. Beside Ag presentation and phagocytosis, macrophages also release pro-inflammatory cytokines such as interleukin-1 (IL-1), IL-6, IL-8, IL-12 and tumor necrosis factor-α (TNF-α) that are important in the inflammatory process. Dendritic cells (DC) are the professional APCs and are characterised by long cytoplasmic processes allowing intimate contact with several cells. They express surface molecules CD80/CD86 (B7-1/B7-2) and CD40, which are important accessory molecules for T cell activation. Follicular DCs (FDC) are stromal cells that reside in primary and secondary lymphoid follicles in tonsils, spleen, lymph nodes, and mucosal tissues. The Ag in the tissue is transported to the lymph nodes by the lymphatics as free antigen or as processed antigen on dendritic cells. Ags in the blood are carried to the spleen (1).

T cells originate in the bone-marrow and mature through positive and negative selection processes in the thymus where T cells learn to discriminate between self and non-self Ags. T cells that recognise self-Ags are eliminated. T cells also mature extra-thymically for example in the small intestine (2). On the cell surface, mature T cells bear the cluster of differentiation 3 (CD3) complex, which is involved in intracellular signalling. Processed Ag is recognised via the TcR, a transmembrane glycoprotein that can consist of one α and one β chain (αβ-T cells) or one γ and one δ chain (γδ-T cells). In blood, 60-90% are TcR-αβ T cells while only 1-10% are TcR-γδ T cells (1). αβ-T cells are usually either CD4+CD8- or CD4-CD8+. CD8+ TcR-αβ T cells recognize Ags in association with MHC class I molecules, and CD4+ TcR-αβ T cells recognise processed Ags presented by MHC class II molecules. T cells require two signals for their activation and subsequent proliferation: First, recognition of the MHC class
I/II presenting the Ag and the CD3/TcR-complex. Secondly, the interaction between co-stimulatory molecules present on the T cell and the APC. Lack of the second signal leads to anergy or apoptosis in CD4+ T cells. The most important co-stimulatory signals are mediated via CD28 and CD40L on the T cell that in turn bind to CD80 (B7-1), CD86 (B7-2), and CD40 on the APC (1).

T cells that have not yet encountered Ag are called naïve T cells (CD45RA+). When these cells are activated by an Ag they proliferate and differentiate into different types of effector T cells (see below) depending on the type stimuli and cytokine milieu. Activated dendritic cells are the most important APC type for the stimulation of naïve T cells. The activated T cells are short-lived but a fraction remain as memory T cells (CD45RO+) and respond to challenge by the same Ag by efficient clonal expansion.

_T-hel_pher (Th) cells_ (MHC class II restricted CD4+ TcR-αβ+ cells) can be subdivided into Th0, Th1, Th2 and Th3 cells. These cells secrete cytokines that affect different immune responses (1): Th1 cells secrete interleukin-2 (IL-2), interferon γ (IFN-γ), TNF-α and lymphotoxin β (LTβ) which induce cell-mediated responses (e.g. cytotoxicity). Th2 cells secrete IL-4, IL-5, and IL-13 which affect the humoral response (antibody production). Th3 cells secrete transforming growth factor β (TGF-β) and appear to be involved in induction of oral tolerance (3). Th0 cells secrete IL-2, IL-4, and IFN-γ, and these cells are thought to be an intermediate stage in Th differentiation (1). DCs can direct Th1/Th2 polarisation, e.g. Th1 polarisation via IL-12 and Th2 polarization by IL-4 and IL-10 (4).

_Cytotoxic T cells (CTLs)_ are usually CD8+ TcRαβ+ and are important in the defence against viruses and intracellular bacteria. They kill infected cells by the perforin/granzyme pathway or via Fas-FasL interactions.

_γδ T cells (TcRγδ+)_ are normally CD4+CD8- T cells that develop early in the thymus and, while rarely found in lymphoid organs, they are frequent in the epithelial compartments where they are part of the “first line of defence”. γδ T cells are large granular lymphocytes that secrete IL-2, IFN-γ, and IL-10, and they store cytotoxic molecules such as perforin, granzymes, granulysin, and Fas-ligand within their granula (5). γδ T cells generally exert MHC-independent cytotoxicity and thus recognize a variety of Ags. These include small non-peptidic ligands and larger protein Ags such as the MHC class Ib-related molecules MICA and MICB expressed on stressed cells (6).

_NKT cells_ are T cells that express typical NK cell receptors such as NK1.1 (mouse) or CD94/NKG2 and KIRs (man). Activated NKT cells produce IFN-γ, TNF-α, and IL-4, and exert lytic activity by using Fas- and
perforin pathways (7). In humans they are found in the liver, peripheral blood (8), decidua (9), and in the small intestine (10).

Regulatory T cells (T\textsubscript{Reg}), T cells with down-regulatory properties, have been found in almost every lymphocyte subpopulation including CD4\textsuperscript{+}CD8\textsuperscript{−}\text{TeRαβ}\textsuperscript{+}, CD8\textsuperscript{−}\text{TeRαβ}\textsuperscript{+}, γδ T cells, NK cells, and NKT cells, but the CD4\textsuperscript{+}CD25\textsuperscript{+}TeRαβ\textsuperscript{+} subset has been shown to have regulatory properties independent of experimental systems (11). The T\textsubscript{Reg} secrete TGF-β and IL-10, which act to down-regulate immune responses by turning them off.

B cells are responsible for humoral immunity. B cells develop into plasma cells that produce immunoglobulins (Ig) [antibodies (Ab)]. Abs neutralise toxins, prevent adhesion of pathogens to mucosal surfaces, activate the complement system, opsonize microorganisms for phagocytosis, and are involved in antibody dependent cell mediated cytotoxicity (ADCC). The B cells develop in the bone marrow. Antigen-driven maturation of naïve B cells occurs in peripheral lymphoid organs. B cells recognize antigens with the B cell receptor (BcR). The heterogenous BcRs consists of a membrane bound Ig molecule in complex with two disulfide-linked heterodimers, Ig-α/Ig-β that contain immunoreceptor tyrosine based activation motifs (ITAMs) that transduce intracellular signals upon crosslinking of surface Ig with the appropriate Ag. The naïve cells express surface bound isotypes IgM and IgD. For proper activation and affinity maturation, B cells require several interactions: a) Ag presented in MHC class II with TcR on the T cell, b) CD40 on the B cell and CD40-ligand (CD40L) on the T cell, and c) CD80/CD86 on the B cell and CD28 on the T cell (12). Cytokines from the T cell lead to proliferation, differentiation, and maturation into blastocysts that leave the lymphoid tissue and fully mature to plasma cells (PC) in the tissue. PCs are short-lived, but a fraction of these cells become memory cells (13).

### 1.2 Innate immunity

The term “innate immunity”, or “natural immunity”, refers to a non-clonal system of recognition and defence that we are born with. It constitutes the first line of host-defence and controls the initial steps of the immune response in multicellular organisms. Innate immunity is ancient, involving elements that are as old as the oldest multicellular organisms, and is found in plants, invertebrates, and vertebrates. The innate immune system provides a fast response to microbial pathogens. In mammals, it plays a direct role in the activation and orchestration of the subsequent adaptive immune response. Key effector mechanisms/cells involved in innate defense are: phagocytosis by macrophages and neutrophils, activation of the complement system by the alternative or lectin pathways which results in lysis of
infected cells, direct or indirect killing of the intruder by NK-cells, γδ-cells, and epithelial cells.

**Natural killer cells (NK-cells)** are large, granular lymphocytes that exert MHC-independent responses and kill their targets (i.e. virus infected cells or tumor cells). Killing can be achieved by ADCC via the Fcγ-receptor on the surface of NK cells. Alternatively NK cells can kill target cells with low expression of MHC class I antigens in an antibody-independent manner. This lytic activity is tightly regulated by stimulatory and inhibitory receptors to avoid lysis of self-cells (14).

The three types of granulocytes (**neutrophils, eosinophils and basophils**) have different cellular morphologies and effector functions. While not normally present in the tissue, neutrophils are abundant in the blood (50-70% of the white blood cells) and are recruited as the first cell type to the site of inflammation in the tissue by chemotactic factors such as IL-8 from locally activated macrophages, leukotrienes from mast cells, and C5a from complement activation. In the tissue they are short-lived. The main physiological role of neutrophils is to phagocytize and kill microorganisms. These phagocytic cells contain primary (azurophilic) granulae with peroxidase, hydrolytic enzymes and defensins, and secondary (specific) granulae with collagenase, lactoferrin, and lysozyme. Eosinophils and basophils constitute minor populations in the blood and do not have phagocytic capacity. **Eosinophils** exert IgE mediated ADCC and are important effector cells in the defence against parasites (1). They are also part of the pathology in allergic reactions (1). **Basophils** bear high-affinity receptors for IgE (FcεRI) and release histamine and other inflammatory molecules involved in the late response to allergens. **Mast cells**, which can be considered as the stationary counterparts to basophils, are involved in defence against parasitic infections and in inflammation, angiogenesis, and tissue remodelling. Like basophils the mast cells are activated by crosslinking of IgE bound to the IgE receptors on the cell surface. This causes the release of inflammatory mediators, e.g. histamine, serotonin, leukotrienes, prostaglandins, platelet activating factor, cytokines and proteoglycans (1).

The rapid innate responses rely on non-clonal receptors that recognise conserved molecular patterns on the surface of infectious microorganisms. By 1989, the “father of innate immunity” Charles Janeway, correctly predicted that pattern-recognition receptors (PRRs) allow cells of the innate immune system to recognize pathogens directly. PRRs recognise pathogen-associated molecular patterns (PAMPs) present not only on pathogens but on all microorganisms (15). The recognition event triggers the cells to express co-stimulatory molecules, which together with processed antigen presented on MHC are necessary for the initiation of
an adaptive response by naïve T cells. PAMPs represent conserved molecular patterns that are essential for the survival of the microbes (such as peptidoglycan and lipopolysaccharide [LPS]). The PRRs fall into three groups: those that induce endocytosis with subsequent antigen-presentation; secreted PRRs that act as opsonins; and those initiating cell activation via nuclear factor κB (NF-κB). The families of toll receptors (Drosophila) and toll like receptors (TLRs in vertebrates) are prominent examples of the last group (16, 17). TLRs of vertebrates are expressed in different cell types including epithelial cells and immune cells. As of today ten different TLRs have been identified. TLR2 recognise a broad range of bacterial products. Ligands for TLR2 include peptidoglycan from Gram-positive bacteria, phenol-soluble modulin from Gram-positive bacteria, bacterial lipoprotein, and lipoarabinomannan from mycobacteria, as well as yeast cell wall particle zymosan. TLR4 is a receptor for LPS on Gram-negative bacteria. TLR3 is a receptor for viral double stranded RNA. TLR5 recognizes bacterial flagellin, and TLR9 bind to unmethylated CpG motifs that are prevalent in bacterial but not vertebrate genomic DNAs. TLR1 and TLR6 function as co-receptors for TLR2. TLRs appear to be able to generate a combinatorial repertoire to discriminate among different pathogens for example the combined expression of TLR2 and TLR6 for the recognition of peptidoglycan (18). Microbes that breach the outer mucosal barrier and reach the phagocytes are hazardous to the host, and therefore, the TLRs of the phagocytes do not need to (and can not) discriminate between commensal and pathogenic microorganisms. For the intestinal epithelial cells the ability to distinguish a pathogen from the sea of commensals is a true challenge and this may be controlled by regulation of TLR expression on IECs by specific bacterial stimuli. In Drosophila it has been described that different classes of microorganisms activate specific receptors of innate immunity resulting in a host defence response aimed at the specific microorganism (19). This hypothesis of an adaptive innate immune response with antimicrobial peptides (AMPs) as effector molecules has yet to be proven for vertebrates.

A major defence mechanism of innate immunity is the generation of antimicrobial substances such as inorganic disinfectants (e.g. hydrogen peroxidase and nitric oxide), antimicrobial proteins (e.g. lysozyme, azurocidin, cathepsin G, phospholipase A2 and lactoferrin), and small antimicrobial peptides. The latter will be dealt with in detail in section 1.6.

1.3 Cross talk

Although there is a clear division between the adaptive and innate immune systems, they can not be considered as separate entities since these systems
work closely together to elicit an effective defence. Innate immunity shapes and induces the adaptive immune response and adaptive immunity affects the innate response through several mechanisms. One key to communication is the release of cytokines and chemokines. These are small molecules that primarily work as highly regulated messengers between cells of the immune system and are produced by immune cells, epithelial cells, fibroblasts and endothelium. They act in an autocrine, paracrine, or endocrine fashion on cell activation, proliferation, inflammation, and differentiation in a complex network and can have pleiotropic, synergistic, or antagonistic effects. Cytokines act by binding to specific receptors and induce intracellular signal transduction (1). Chemokines (with chemoattractant activity), interleukins (mainly affecting leucocytes), interferons (interfere with viral replication), and colony-stimulating factors (cause stem cell differentiation and proliferation) are subgroups of cytokines (1). IFN-γ, TNF-α, and IL-1β are referred to as pro-inflammatory cytokines. IFN-γ is produced by T-, NK- and NKT cells. It activates intracellular killing in macrophages and neutrophils, acts in a positive feedback loop in stimulation of Th0 cells to become Th1 cells instead of Th2 cells, stimulates NK-cell functions, increases MHC class II expression on APCs and certain epithelial cells (1), and plays a role in apoptosis (20) and in innate immunity (21).

Monocytes/macrophages are the main producers of IL-1β but other cell sources include fibroblasts, peripheral neutrophils, T cells, B cells and epithelial cells. IL-1β has various effects including being a strong chemoattractant for leucocytes, stimulation of Th cells, and stimulating B cell proliferation. TNF-α is produced by macrophages, neutrophils, T cells (mostly CD4+), and NK cells following stimulation by LPS. TNF-α in combination with IL-1β has effects on the endothelium where it promotes inflammation. TNF-α is also a promoter of angiogenesis, enhances proliferation of T cells, and stimulates the expression of MHC class I and II in leukocytes.

### 1.4 The mucosal immune system in the intestine

The mucosa of the gastrointestinal tract covers ~400m² (which is 200 times the surface area of the skin). As a consequence of the high exposure of microbes, two-thirds of the whole immune system is located in the intestine. The immune system of the gut has a central role since there is constant exposure not only by microbes but also by food Ags.
1.4.1 The human intestine

The human intestine consists of the small intestine (duodenum, jejunum and ileum) and the large intestine (caecum, colon ascendens, colon transversum, colon descendens, colon sigmoideum and rectum) (Fig. 1). The mucosa of the intestinal wall is built up of an epithelial layer, the lamina propria (LP), and muscularis mucosae. Underneath the mucosa muscularis are the submucosa, circular and longitudinal muscles, and the serosa. The mucosal surface in the small intestine consists of invaginations (crypts of Lieberkühn) and villi (finger-like projections), while the large intestine has crypts but lacks villi. The human intestine harbours a large community of microorganisms. In the gastrointestinal tract about $10^{14}$ bacteria are present, which is approximately ten times the number of human cells in the body.

The microbial flora has a weight of 1.5-2 kg and thus can be considered one of the largest “organs” of the body. At least 400 different bacterial species are believed to be present in the intestine. The numbers of bacteria in the gastrointestinal tract vary dramatically by anatomical region; the proximal small intestine has about $10^5$-$10^7$ bacteria per ml of fluid, the distal part of the small intestine contains greater numbers of bacteria ($10^9$/ml) and the bacteria in the colon reach levels of $10^{11}$-$10^{12}$/ml feces. In healthy individuals the stomach and proximal small intestine contain relatively few aerobes and facultative anaerobes. In contrast, the colon literally teems with bacteria, predominantly strict anaerobes. One reason for the great abundance of bacteria in the colon is the relatively slow passage of luminal contents through this region of the intestine. Between these two extremes is a transitional zone, usually the ileum, where moderate numbers of both
aerobic and anaerobic bacteria are found. Frequently identified anaerobic microbes are *Bacteroides*, *Bifidobacterium*, *Fusobacterium*, *Eubacteria*, and *Lactobacillus* (22). The differences in the composition of the flora between individuals are influenced by age, diet, cultural conditions, and the use of antibiotics. The microflora has several beneficial effects for the host. It affects gut maturation and integrity and has antagonistic effects on pathogens (23).

### 1.4.2 Gut associated lymphoid tissue (GALT)

The GALT consists of several lymphoid structures. The *Peyer’s patches* (*PP*) are groups of small lymphoid follicles – ranging from a few to several hundred – located in the small intestine (24). *Solitary follicles* are present in the small and large intestine and contain a germinal centre with B cells and FDCs surrounded by a mantle zone with T cells and DC (25). Both PP and the solitary follicles are inductive sites for the specific immune response (26). The main effector site is the *lamina propria* (LP), which contains high numbers of T cells, B cells, and macrophages, as well as mast cells, plasma cells, and dendritic cells, and occasionally neutrophils, and eosinophils. Plasma cells in LP produce mainly dimeric IgA that is secreted into the lumen by transcytosis through epithelial cells via the J-chain reactive polymeric Ig receptor located basolaterally on the epithelial cells (27). The IgA is important in the “first line of defence” as it blocks the adhesion and entry by pathogens. Approximately 0.3-1g of sIgA is produced every day.

The T cells are mainly TcR\(^{\alpha\beta}\) with activated/memory cell phenotype (CD45RO\(^{+}\)) (28) where ~55% are CD4\(^+\) T cells and ~45% are CD8\(^+\) T cells (29), while γδ T cells are scarce in LP (30). *Intraepithelial lymphocytes* (*IEL*) reside within the epithelial layer in close contact with epithelial cells (sometimes also with the basal membrane), and are more frequent in the small intestine compared with large intestine (10). There are ~10-20 IELs per 100 villus enterocytes in the small intestine (10) and thus the IEL comprise a large fraction of the body’s T cells. IELs exhibit cytotoxic activities including virus-specific CTL activity and spontaneous cytotoxicity, activities consistent with a role in the first line of defence but also in induction and maintenance of oral tolerance, in surveillance of the IECs, and in immune protection (29, 31). IELs are mainly T cells and most display an activated/memory phenotype (CD45RO\(^{+}\)). The majority of the IELs in the small intestine are CD8\(^+\) TcRαβ\(^+\), while in the large intestine there are equal populations of CD8\(^+\) and CD4\(^+\) as well as CD4\(^-\)CD8\(^-\) TcRαβ\(^+\) cells (10). ~10% of IELs are γδ-cells and almost all are CD4\(^-\)CD8\(^-\) (10) which preferably use V\(\delta1\) and V\(\gamma8\) chains (10, 32) as opposed to peripheral blood γδ-cells which use V\(\delta2\) and V\(\gamma9\) chains. The V\(\delta1\) γδ T cells
have the ability to kill stressed cells (6) and tumor cells of epithelial origin (33). In mice, other roles of intestinal γδ T cells include the regulation of proliferation and differentiation of epithelial cells (34).

1.5 The intestinal epithelium

The intestinal epithelium is a monolayer of cells that separates the lumen from the underlying mucosal tissue. The main site for the absorption of food components is in the small intestine where the absorptive surface is greatly increased by its convolution. Moreover, the mucosa is covered by villi and each cell in turn is covered by microvilli. In the colon villi are absent. Four major intestinal epithelial cell types are found - the absorptive cells (enterocytes), goblet cells, Paneth cells (only small intestine), and enteroendocrine cells. These cell lineages are derived from a pluripotent stem cell situated at the very base of the colonic crypts and at cell position 4-6, i.e above the Paneth cells in small intestinal crypts (35). The enterocytes, goblet cells, and enteroendocrine cells migrate upward, while the Paneth cells migrate to the base of the crypt where they complete their differentiation. The most mature cells face the lumen where they are shed off or eliminated by other means and replaced by new cells. The lifespan of the epithelial cells is 4-6 days, and thus there is a continuous renewal of these cells. The goblet cells secrete mucins, the Paneth cells secrete antimicrobial compounds, and the enteroendocrine cells release hormones and neuropeptides in response to external stimuli.

Intestinal epithelial cells (IECs) “sitting” on the basal lamina and held together by tight junctions provide a protective barrier between the lumen and underlying tissue. IECs actively participate in the modulation of the mucosal responses. IECs 1) secrete and respond to a variety of cytokines, chemokines, and other immunomodulatory molecules, 2) are in intimate contact with T cells, DCs, and PMNs, 3) transmit polymeric Ig from the mucosal tissue to the lumen, 4) transcytose and process luminal peptides, and function as non-professional antigen presenting cells, 5) select IELs by presentation of self antigens, 6) have a role in oral tolerance, 7) express some of the TLRs, 8) and express and secrete AMPs (36, 37).

The intestinal epithelium is in a unique position and can receive and transmit signals from cells in the underlying tissue and from microbes in the gut lumen. Follicle associated epithelium (FAE) covers PP’s and solitary follicles. It consists partly of specialized epithelial cells called microfold cells (M-cells) which lack microvilli and a thick mucous layer. The M-cells take up and transport Ags to APCs in the underlying tissue, which present the Ag to T cells in the lymphoid follicles and induce immune responses. T cells, B cells and macrophages reside within the M-cell-pockets. The FAE
differs from the other epithelia because it has lower levels of digestive enzymes and a less pronounced glycocalyx (38). Ags can also pass the epithelial layer by a transcellular or paracellular route (by fluid phase or receptor mediated endocytosis) (36). While the M cells preferentially take up particulate Ags, the enterocytes take up soluble Ags, and studies of induction of oral tolerance have shown that soluble Ags are the most potent tolerogens while particulate Ags fail to elicit tolerance. This means that the enterocytes probably play a more important role in induction of tolerance (36). The Ag uptake, processing and presentation to T cells is facilitated through IEC expression of both classical MHC class I and II and nonclassical MHC class Ib molecules including CD1d, MICA and MICB (36). Enterocytes express MHC class I and in the small intestine also express class II constitutively. However, they normally lack costimulatory molecules such as CD80 and CD86 and ICAM-1 (39). This probably accounts for induction of tolerance - by induction of anergy - rather than activation of local T cells (40). CD86 (but not CD80) is induced in IECs in inflammatory bowel disease (IBD) and may contribute to the activation of T cells (39). This also suggests that the ability of IECs to stimulate T cells is dependent on the underlying level of inflammation. IECs expressing non-classical MHC class I molecules MICA and MICB are able to stimulate Vδ1 TcR+ T cells in the intestine (6). In a similar manner to professional APCs, IECs were also reported to release exosome-like vesicles with MHC class I, MHC class II, CD63, CD26/dipeptidyl-peptidase IV, and A33 antigen (41). The release was significantly increased in the presence of IFN-γ.

IECs of normal intestinal mucosa constitutively express TLR3 and TLR5, while TLR2 and TLR4 are barely detectable (42). The absence of TLR4 causes hyporesponsiveness to LPS in human IECs (43). TLR2 and its coreceptors TLR1 and TLR6 are expressed at very low levels in IEC lines and the IECs are weakly responsive to known TLR2 ligands (44, 45). However, TLR2-4 were all expressed in intestinal epithelial cell lines (44). The data for TLR4 is somewhat contradictory but may be explained by the observation that, while not present on the surface, TLR4 resides in the Golgi apparatus in a murine small intestinal cell line (m-IC(cl2)) after infection and colocalizes with internalized lipopolysaccharide (46). Thus, LPS do not normally gain access to cytoplasmic TLR4 and may constitute one mechanism of regulation. TLR5 seems to be expressed on basal/lateral surfaces of IECs (47) and thus, under normal physiological conditions, the access to TLR5 by flagellin is limited through tight junctions – a situation that is changed upon inflammation.

The Paneth cells are believed to be sentinels of the crypts and react to bacteria by releasing defensins in a quantity sufficient to kill the bacteria
The secretory granules also contain lysozyme (an antimicrobial enzyme that dissolves the cell walls of bacteria), and type II phospholipase A₂ (an enzyme that lyses bacterial phospholipids) (49). This may be of great importance in order to keep the small intestinal crypts sterile and to protect the stem cells from being infected. However, the importance of Paneth cells is debated. While Paneth cells are found in man, rats, and mice, the intestines of other successful species such as dogs, cats, and racoons lack them. The ablation of Paneth cells in mice had no detectable effects on the distribution of normal gut microflora or on the distribution of cells forming the GALT (50).

1.5.1 The Carcinoembryonic antigen family and the glycocalyx layer

The apical surface of the epithelial cells is covered by two layers of glycoprotein molecules – an inner layer called the glycocalyx or “fuzzy coat” and an outer mucin layer made up of secreted mucins from goblet cells. These two layers protect the epithelial cells from direct contact with pathogenic microbes. In the small intestine the glycocalyx contains various enzymes, disaccharidases, peptidases, receptors, and transport proteins – all of which are important for digestion and absorption of nutrients. Other major components of the glycocalyx in small and large intestines are molecules belonging to the carcinoembryonic antigen (CEA) family. CEA itself was first identified by Gold and Freedman in colorectal cancer (51) and was initially considered to be an oncofetal protein but has now been conclusively demonstrated to be a normal adult tissue component (52). The CEA family molecules are highly glycosylated proteins belonging to the Ig superfamily. The CEA gene family (located at chromosome 19q13.2) contains 18 expressed genes; 7 belong to the CEACAM subgroup and 11 to the pregnancy specific glycoprotein (PSG) subgroup (52, 53). The name CEACAM stands for CEA-related cell adhesion molecules (CEACAMs) (54). The CEACAMs are cell surface glycoproteins attached either via a glycosyl-phosphatidylinositol (GPI) anchor (CEA, CEACAM6-8) or through a transmembrane domain (CEACAM1, CEACAM3 and CEACAM4). All members have a membrane distal IgV-like N-domain and a variable number of IgC-like domains (53).

Four members of the CEA subclass are expressed by colonic epithelial cells: CEA, CEACAM1, CEACAM6 and CEACAM7 (55-57). mRNAs of all four members were expressed at high levels in the mature enterocytes facing the lumen and in the differentiated enterocytes in the crypt mouth. CEA and CEACAM6 mRNAs were also expressed at low levels in the mid and lower levels of the crypts and expressed by goblet cells. Studies at the protein level revealed the same pattern of expression as
with the mRNAs. All four molecules are localized to the glycocalyx, and ultrastructurally they are localized to the microvesicles and filaments of the enterocytes that constitute the fuzzy coat by vesiculation of the microvilli (55-57). While CEA is present only over the tips of the microvilli, CEACAM6 and CEACAM7 is present both on the sides and over the tips of the microvilli and CEACAM1 is mainly present between the microvilli. In the small intestine CEA is only produced by goblet cells while CEACAM1 is expressed by absorptive epithelial cells (our own observations). Fig. 2 shows that there is a large difference in CEA and CEACAM1 mRNA expression levels between colon and jejunum (unpublished). CEACAM6 and CEACAM7 are not expressed in the small intestine (58).

![Figure 2](image)

**Figure 2.** CEA and CEACAM1 mRNA expression levels in IECs isolated from the small intestine vs the large intestine.

Considering the strategic position of CEA, CEACAM-1, CEACAM-6, and CEACAM-7 in the apical glycocalyx of the normal mucosa, and their ability to bind various microorganisms, (59-61) our group has previously suggested that these molecules play a role in innate immunity by facilitating protection from microbial invasion (62). This hypothesis states: A) that CEA family molecules in the glycocalyx/mucin layers bind and trap microorganisms preventing them from penetrating down and invading the epithelial cells. B) If microorganisms do reach the microvilli of epithelial cells, binding to CEACAM1 induces a pinching off process which releases a microvesicle with the bound microorganism thus preventing their further penetration.
This hypothesis is based on several observations: a) the strategic position of the molecules, b) the ability of several different groups of bacteria to bind to CEA family molecules (either to the peptide or carbohydrate moiety), c) CEA and CEACAM6 are released by goblet cells and, as such, are also constituents of the mucus layer, d) the expression and release of these molecules can be regulated by pro-inflammatory cytokines, and e) the rapid evolution of these molecules is consistent with their co-evolution with the intestinal microbiota (62). CEACAM1 is considered to play a key role in this process because of its location in the microvillus subdomain, its ability to transduce signals to the cell interior (the cytoplasmic domain of CEACAM1 has a functional ITAM motif allowing tyrosine phosphorylation) (63), and its evolutionary conservation between species. A recent finding is that CEA and CEACAM1 are also present on M-cells of the FAE covering colonic solitary follicles and likely play a role in selective binding of bacterial and viral pathogens (Baranov and Hammarström, 2003). Another view is that CEA, CEACAM1, CEACAM6, and CEACAM8 function as intercellular adhesion molecules (63, 64).

1.5.2 Mucins

A key component of the intestinal barrier is the mucus layer that lines the surface epithelium and together with the glycocalyx serves to protect from infection, dehydration, and physical or chemical injury. The mucus is a mixture of mucin, free protein, salts, and water, and this viscous, sticky layer traps particles, bacteria, and viruses which are expelled by the peristaltic process of the gut. In combination with the glycocalyx this prevents potential pathogens and Ags from gaining access to the underlying epithelium. The mucus layer increases in thickness from the duodenum to the colon (65). This may at least partly explain why Ags are more easily taken up in the small intestine.

The mucins (MUCs) are heavily glycosylated proteins (more than 80% of their mass is carbohydrate) and consist of a polypeptide backbone dominated by serine and threonine residues substituted with O-linked oligosaccharide side chains. The carbohydrates on mucins provide binding sites for microorganisms (both commensal and pathogenic) and as such the mucus layer is another niche for microbial colonization. Four mucins, MUC1-4 are expressed by IECs. MUC2 is secreted by goblet cells of crypts and villi in both the small and large intestine (66), while MUC3 exists in both a secreted and a membrane bound form and is expressed both in goblet cells and enterocytes of the villous epithelium (small intestine) and superficial epithelium (large intestine) (67, 68). MUC1 and MUC4 are...
expressed in the colon and ileum and are membrane associated in both goblet cells and enterocytes (69).

1.6 Antimicrobial peptides

Antimicrobial peptides (AMPs) are effector molecules that provide fast and energy-effective protection against infectious agents (70-72). AMPs have a molecular mass <10kDa (larger molecules are referred to as antimicrobial proteins) and today over 800 different AMP sequences have been reported from plants, insect haemolymph, mammalian phagocytic granules, epithelial cells of frog skin and intestine, bovine trachea and tongue, mouse intestine as well as in various human epithelial tissues (70-74). In 1956, Hirsch described the first characterized AMP, phagocytin, in polymorphonuclear leukocytes (75). The expression by different cell types suggests that AMPs have served and still serve important functions during the evolution of species.

The AMPs are a highly diverse group of peptides, chemically ranging from linear alpha-helical peptides to disulfide-bonded beta-sheet-containing peptides. Based on their size, three-dimensional structure, or predominant amino acid structure, AMPs can be subdivided into three groups (76) (Table 1). Despite these differences, most AMPs are cationic (polar) peptides with spatially separated charged and hydrophobic regions and this amphipathic design allows the peptides to kill microorganisms by disruption of the microbial cell membrane. Other mechanisms have also been reported, including interference with intracellular processes.

Table 1. Antimicrobial peptides can be divided into three subgroups based on molecular characteristics.

<table>
<thead>
<tr>
<th>Peptide characteristics</th>
<th>Example</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>cecropin</td>
<td>Pigs, insects</td>
</tr>
<tr>
<td>Linear α-helical peptides without cysteines</td>
<td>magainins</td>
<td>frogs</td>
</tr>
<tr>
<td></td>
<td>LL37/hCAP-18</td>
<td>humans</td>
</tr>
<tr>
<td>Group II</td>
<td>defensins</td>
<td>mammals</td>
</tr>
<tr>
<td>Peptides with cysteins linked by disulfide bridges</td>
<td>protegrins</td>
<td>pigs</td>
</tr>
<tr>
<td>Group III</td>
<td>histatins</td>
<td>humans</td>
</tr>
<tr>
<td>Unusual high proportion of specific amino acids</td>
<td>PR-39</td>
<td>pigs</td>
</tr>
</tbody>
</table>
There are now examples of how bacteria counteract AMP attacks. For example, some bacteria modify cell wall or plasma membrane proteins to make them less negatively charged, thus preventing binding of the cationic peptides (77, 78). These bacterial features have broad relevance for pathogenicity.

There is interest in the therapeutic potentials of AMPs because of the growing problem with antibiotic resistance among microbial species. Reasons for this include the fact that bacterial resistance to AMPs is a rare phenomenon, that they are endogenously produced effector molecules, and that they represent a “superfamily” with broad-spectra activities and different specificities.

1.6.1 Defensins

Defensins are small cationic antimicrobial peptides found in mammals, birds, insects, and plants (78-81). The human α-defensin genes have a conserved structure with two exons; the first exon encodes a signal peptide (which targets the peptides to the secretory pathway) and an anionic pro-piece (probably required for correct folding and/or stabilization of charge interactions (79)). The second exon encodes the end of the pro-piece followed by the mature cationic peptide (Fig. 3).

Figure 3. Structure and genomic organization of defensins on chromosome 8p22-p23.

The inactive precursors (~100 amino acids) are activated by posttranslational proteolytic removal of the anionic pro-segment, creating a cationic peptide that is antimicrobial (Table 2). The β-defensins differ somewhat from the α-defensins in that they have a shorter propiece or even lack the propiece (79). The mature peptides have a characteristic six-cystein motif and many basic residues (Fig. 4). The cystein residues form three
disulfide linkages. In the α-defensin family, the intrachain disulfide bond pattern is 1-6, 2-4, 3-5. For β-defensins the pattern is 1-5, 2-4, 3-6. Despite the differences in the pairing of the cysteins, the tertiary structure is highly similar between α- and β-defensins with three anti-parallel beta-sheets secured by the three disulfide-linkages that give an amphipathic feature. The most recently identified subtype of defensin was found in rhesus monkey neutrophils where post-translational ligation of two truncated α-defensins forms a circular mini-defensin named θ-defensin (82). So far, this type of defensin has not been found in man. In the human genome, all well-characterized defensin genes cluster to a <1 Mb region of chromosome 8p22-p23. It is also suggested that β-defensins predate the α-defensin family (83).

Table 2. Molecular size of prepro-form and mature form of human defensins. Size is given as number of amino acids (aa).

<table>
<thead>
<tr>
<th></th>
<th>preproprotein</th>
<th>mature peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>HD-5</td>
<td>94 aa</td>
<td>32 aa</td>
</tr>
<tr>
<td>HD-6</td>
<td>100 aa</td>
<td>30 aa</td>
</tr>
<tr>
<td>hBD-1</td>
<td>68 aa</td>
<td>36-47 aa</td>
</tr>
<tr>
<td>hBD-2</td>
<td>64 aa</td>
<td>41 aa</td>
</tr>
<tr>
<td>hBD-3</td>
<td>67 aa</td>
<td>45 aa</td>
</tr>
<tr>
<td>hBD-4</td>
<td>72 aa</td>
<td>50 aa</td>
</tr>
</tbody>
</table>

Due to the amphipathic, cationic nature of the defensins, it has been postulated that their mode of action is by disruption of microbial cytoplasmic membranes rich in anionic phospholipids. The polar topologic features of defensins with separated hydrophobic and charged regions allows the hydrophobic part to be inserted into the lipid bilayer and the charged/cationic part to interact with anionic phospholipids head groups and water (84). Two models have been suggested: one in which defensin monomers assemble to form pores within the microbial membrane (85), and a second where the defensins disrupt the membrane by electrostatic interactions with the polar head groups of the bilayer (86). The fact that host cells are spared may be due to the fact that their cell membranes are rich in cholesterol- and neutral phospholipids.

All currently identified defensins can kill and/or inactivate microorganisms. They generally exhibit broad antimicrobial activity towards Gram-positive and Gram-negative bacteria, fungi, and some enveloped viruses (74, 79). In many animals the highest concentration (>10mg/ml) is found within the granules of neutrophils (79). After microbial
Figure 4. Amino acid sequences of the defensins with gene localization in the chromosome 8p22-23 cluster.

HD-5: MRTIALAILLVALQAESILQERADEATTQKQGSDNQDLAISFAGNLISALRTSGQR--------ATCCRTGRCATRESLSGVCEISGRLYRCC
HD-6: MRTLITAVILVALQAKAEFLQAEDEIFQAKAYEADAQERQGANDQDFAVSAEADASSLIRAGSTRPTCHRER-EYSTIGICTVGNINHRCCL
HNP-1/3: MRTIALAILLVALQAESILQERADEATTQKQGSDNQDLAISFAGNLISALRTSGQR--------CYCRPACAGERRYGTICYQGRILWACCC
HNP-4: MRTIALAILLVALQVRAGPLQARGDEAPQEDEQPEDQDISISFADOKSSLQVSGSTRG--------VCSCRLVFEPGETRXVNGDIOGSFTYCCTRVD

The sequences of mature peptides are underlined.
stimulation, the Paneth cells of the intestine can secrete \(\alpha\)-defensins at the level of mg/ml which are eventually flushed into the gut lumen (48). Barrier and secretory epithelia produce defensins constitutively or upon infection at an average concentration of 10-100 µg/ml and since the peptides are not evenly distributed, the local concentrations are higher (79). *In vitro*, antimicrobial activity is observed at concentrations of 0.1-100 µg/ml (µM range) under low salt concentrations (less than 150 mM NaCl) (74, 79, 87). For several of the \(\alpha\)- and \(\beta\)-defensins, increasing salt concentrations to physiological concentrations (i.e. 150 mM NaCl) reduces or competitively inhibits defensin activity (74, 88). Therefore it is likely that the defensins exert their antimicrobial activities inside the phagocytic vacuoles of phagocytes or on the surface of skin and mucosal surfaces where there are low ionic concentrations.

The molecular mechanisms by which defensins are induced are not fully understood, but PRRs together with inflammatory effector molecules probably play the most important roles in this process as exemplified by the CD14/TLR4-mediated induction of hBD-2 by lipopolysaccharide in tracheobronchial epithelium (89).

**\(\alpha\)-defensins**

The \(\alpha\)-defensins are exclusively found in mammals (humans, monkeys, and rodents). They are present at multiple sites in the body and are thought to play a major role in host defence. Six \(\alpha\)-defensins are described in humans and the first human \(\alpha\)-defensin to be described was isolated from neutrophils (90). Human neutrophil peptides (HNP) 1-4 are stored as mature peptides in the dense azurophilic granules of neutrophils (90), where HNP1-3 are major components and HNP4 is much less abundant. They are 29-33 amino acids long and constitute 5-7% of the total protein content of neutrophils and 30-50% of the azurophilic granules. Human \(\alpha\)-defensins 5 and 6 (HD-5 and HD-6) are expressed in epithelial cells, mainly in Paneth cells of the gut (91, 92), but also in the female reproductive tract (93). Defensin concentrations in the phagocytic vacuoles of neutrophils are in the mg/ml range, a concentration that should be sufficient to overcome inhibition by extracellular ion concentrations. Similar concentrations have also been measured in the narrow (5-10 mm diameter) intestinal crypts into which Paneth cells secrete their defensin-containing granules (48).

**HD-5 and HD-6**

In the intestine, the source of HD-5 and HD-6 is the Paneth cells, located at the bottom of the crypts of Lieberkühn throughout the small intestine (91, 92). HD-5 and -6 are found in the lysozyme-rich secretory granules of the Paneth cells and are thought to be involved in local host defence. They are
thought to regulate the density of the microbial population in the small intestine and to protect the epithelial stem cells of the crypts by keeping the local environment sterile.

HD-5 (and likely HD-6) is stored as a prepropeptide and upon release, Paneth cell trypsin acts as the processing enzyme (94). The processing enzyme in mouse is the metalloproteinase matrilysin (MAT) (95). MAT⁻/⁻ mice lack mature cryptdins and intestinal peptide preparations from these mice show decreased antimicrobial activity. Paneth cells in mouse small intestinal crypts secrete granules rich in AMPs when exposed to bacteria or bacterial antigens (48). The dose-dependent secretion occurs within minutes and α-defensins account for 70% of the released peptide activity. Gram-negative and Gram-positive bacteria, lipopolysaccharide, lipoteichoic acid, lipid A, and muramyl dipeptide elicit cryptdin secretion. Live fungi and protozoa, however, do not stimulate degranulation. Thus the intestinal Paneth cells contribute to innate immunity by sensing bacteria and bacterial antigens and releasing AMPs at effective concentrations (48).

The genes for HD-5 and HD-6 (DEFA5 and DEFA6) both contain AP-2 and NF-IL-6 response elements (96). However, inducibility of transcription has not been proven. HD-5 and -6 are active against Gram-positive and Gram-negative bacteria, fungi and some viruses, but the activity is inhibited in the presence of salt (97, 98). In urine, multiple N-terminally processed forms of HD-5 are present, while HD-6 is only present as the 69-100-residue form (99).

**β-defensins**

All examined mammals have been shown to express β-defensins. The inducible tracheal antimicrobial protein from bovine epithelial cells (100) and 13 defensins from bovine neutrophils (101) were the first β-defensins to be isolated. β-defensins have later been found in sheep, mice, rats, and pigs (102). In other species than cattle, β-defensins are ubiquitously expressed by epithelial cells lining different organs such as the skin, lung, middle ear, oral mucosa, genitourinary tract, and gastrointestinal tract (103-109). Thus, β-defensins seem to be more widely expressed than α-defensins. The best characterized β-defensins are human β-defensins hBD-1 (108, 110), hBD-2 (111), hBD-3 (112-114), hBD-4 (115), and HE2β1 (112) which are all found on chromosome 8p23-p22. Recently Schutte et al identified a total of 28 new putative human and 43 new putative mouse β-defensin sequences located in five syntenic chromosomal regions with a genomics based method (116). The authors showed that in humans, putative β-defensin coding regions reside not only on chromosome 8p23-p22 but also on 6p12, 20p13 and 20q11.1. Within each syntenic cluster, the gene sequences and
organization were similar, suggesting that each cluster pair arose from a common ancestor and was retained because of conserved functions. Preliminary analysis indicates that at least 26 of the predicted genes in human are transcribed (116, 117), which together with the characterized genes would result in a total of 31 β-defensins.

**hBD-1**

The first human β-defensin to be characterized, hBD-1, was isolated from hemodialysate fluid in nanomolar concentrations (110). The source of hBD-1 was proven to be urine (10-100 ng/ml) and the highest concentrations were found in the kidney and female reproductive tract (108). hBD-1 was initially isolated as a mixture of different forms, varying in length from 36-47 amino acids due to proteolytic cleavage, and the 36-residue hBD-1 was found to be the most potent form (108). hBD-1 has now been found in virtually all tissues examined including the trachea, salivary gland, pancreas, prostate, placenta, thymus, testis, intestine respiratory epithelia, ear, gingiva, and mammary gland (103, 109, 118-121). hBD-1 appears to be constitutively expressed by epithelial cells (119) and is neither upregulated by the presence of bacteria nor by inflammatory stimuli (74, 105, 107, 119). The genomic sequence contains transcription factor regulatory elements for NF-IL-6 and IFN-γ but not for nuclear factor kappa B (NF-κB) and activator protein-1 (AP-1) (74). hBD1 is microbicidal towards Gram-negative bacteria as well as adenoviruses in micromolar concentrations, but this activity is inhibited by high salt concentrations (108, 118).

**hBD-2**

hBD-2 was first discovered in skin and isolated from psoriatic scale extracts (111). The concentration in inflamed skin is in the range of 10 mg/ml and thus sufficient to inhibit or kill many microbes. Other sites of expression include the respiratory tract, and the gastrointestinal tract (74, 79). The promoter region contains AP-1, AP-2, NF-IL-6 and NF-κB responsive elements (122, 123). hBD-2 is inducible by challenge with Gram-negative and Gram-positive bacteria, fungi, and by the pro-inflammatory cytokines TNF-α and IL-1β in skin, lung, intestine, and stomach (103, 105, 111, 122, 124, 125). There is also a provocative report on hBD-2 induction by the essential amino acid L-isoleucine (126). hBD-2 shows salt-sensitive antimicrobial activity against many Gram-negative bacteria (including Escherichia coli and Pseudomonas aeruginosa) and the yeast Candida albicans (~10µg/ml), but show only bacteriostatic effects against the Gram-positive bacterium Staphylococcus aureus (107, 111). hBD-2 is about ten times more potent than hBD-1 against E. coli (107).
Besides the ubiquitous expression in epithelial cells, both hBD-1 and hBD-2 are moderately expressed in blood monocytes (127).

The synthesis and secretion of hBD-2 is regulated by dual circuitry: 1) directly through an epithelial response to LPS and other microbial stimuli, most likely mediated by epithelial CD14/TLR/NF-κB (89). A high threshold characterizes this response. 2) Indirectly through a cytokine-mediated epithelial response triggered primarily by the encounter of microbes with local macrophages that then produce IL-1α/β, and other cytokines that in turn act on epithelial cytokine receptors to increase epithelial defensin synthesis. The threshold for this response is lower. This scheme avoids promiscuous activation by low concentrations of inhaled non-invasive microbes while retaining the ability to activate in response to a large bolus of microbes or epithelial penetration by fewer invasive microbes.

hBD-3

Human beta defensin 3 (hBD-3) was isolated from epidermal keratinocytes of patients with psoriasis (113) and the gene was independently described by two groups (112, 114). Sites of expression include skin, tonsils, airway epithelia, heart, skeletal muscle, placenta, esophagus, testis, cornea, oral mucosa, endometrium, and the gastrointestinal tract (113, 114, 128, 129). hBD-3 is a dimer in solution (130) and has a more potent antimicrobial activity than hBD-1 and hBD-2. The latter two defensins are monomers in solution. In contrast to hBD-1 and hBD-2, hBD-3 efficiently kills Gram-positive bacteria such as *Staphylococcus aureus* (113). Unlike hBD-1 and hBD-2 the antimicrobial activity of hBD-3 towards Gram-negative and Gram-positive bacteria, and the yeasts *Saccharomyces cerevisiae* and *Candida albicans* is salt-insensitive (113, 114).

The inducibility status of hBD-3 is unclear. TNF-α and *Pseudomonas aeruginosa* were shown to induce hBD-3 in primary tracheal cells and keratinocytes (113) and IL-1β was shown to induce hBD-3 in fetal lung explants and gingival keratinocytes (112). Another group reported induction by IFN-γ but not by TNF-α or *Pseudomonas aeruginosa* in lung epithelia and skin keratinocytes (114). Rhinovirus (common cold virus) caused upregulation of hBD-3 in cultured bronchial epithelial cells (124). Several consensus sequences for NF-IL-6, IFN-γ, and AP-1 response elements (but no NF-κB consensus element) are found in the promoter suggesting gene regulation by inflammatory stimuli (112).

hBD-3 can chemoattract monocytes with a maximal response at 50 nM (114). In contrast to hBD-1 and hBD-2, hBD-3 is not expressed by blood monocytes, macrophages, or dendritic cells (131)(our own observations).
**hBD-4**

Human beta defensin 4 (hBD-4) was cloned from lung cDNA and was shown to be expressed in testis, stomach, uterus, neutrophils, thyroid, lung and kidney, and endometrium (115, 128). The promoter region contains several AP-1 and GATA1 binding sites but no NF-κB consensus elements (115). hBD-4 mRNA expression is induced by Gram-negative and Gram-positive bacteria and phorbol 12-myristate 13-acetate (PMA) in respiratory cells, but does not respond to pro-inflammatory cytokines (115). Synthetic hBD-4 inhibits the growth of both Gram-negative and Gram-positive bacteria as well as of the yeast *Saccharomyces cerevisiae* (115). Furthermore, it is a chemoattractant to human blood monocytes but inactive towards neutrophils and eosinophils (115).

**HE2, hBD-5, hBD-6, hBD-7, hBD-8**

The human epididymis secretory protein (HE2) was found 17 kb upstream from the hBD-3 gene (112). One splice variant consisting of three exons, HE2β1, encodes a β-defensin consensus cystein motif, suggesting it represents a new defensin variant. Expression of hBD-5 through hBD-8 were found in testis/epididymis (117, 132). The hBD-6 gene contains the NF-κB consensus sequence, while the gene for hBD-5 does not.

### 1.6.2 Other antimicrobial peptides/proteins

In 1921 Fleming discovered lysozyme. He observed that his own nasal mucus inhibited the growth of *Staphylococci* cultured from the same mucus. Lysozyme acts by breaking the bond between N-acetylglicosamine and N-acetylmuramic acid in the peptidoglycan layer of bacterial cell walls. While lysozyme is highly active against many Gram-positive species (e.g. *Bacillus megaterium*, *Micrococcus lysodeicticus*, and many *Streptococci*) it is ineffective against Gram-negative bacteria unless potentiated by certain cofactors such as lactoferrin, antibody-complement, or hydrogen peroxide-asorbic acid (133). These cofactors disrupt the outer membrane of Gram-negative bacteria which gives the enzyme access to the peptidoglycan layer. Lysozyme is a component of both phagocytic and secretory granules of neutrophils and is also produced by monocytes, macrophages, and Paneth cells (134).

Peptides of the cathelicidin family have conserved N-terminal precursor structures (~100 amino acid residues) but highly heterogeneous C-terminal domains (12-100 amino acid residues) (135). Like many other AMPs, most cathelicidins undergo extracellular proteolytic cleavage for activation. LL-37/hCAP18/FALL39 is the only human cathelicidin discovered so far (136-138). It was initially cloned from human bone
marrow (138). The gene is localized to chromosome 3 and consists of four exons. LL-37/hCAP18 is found in neutrophil specific granules at levels about one third of that of lactoferrin or lysozyme. Exemplifying the overlap of phagocytic and epithelial host defences, mRNA for the human cathelicidin is also found in the testis, in inflamed human keratinocytes (139), in squamous epithelia of the mouth, tongue, esophagus, cervix, and vagina (140), in epithelia of the airway (141) and in luminal enterocytes in the colon and ileum (142). In vitro, the human peptide LL-37 displays both LPS-binding (143) and broad-spectrum antimicrobial activities (144).

Lactoferrin is an iron-binding glycoprotein that was first isolated from milk but has since been found in most exocrine secretions as well as in the secondary granules of neutrophils. This protein’s antimicrobial and anti-inflammatory activities in vitro identify lactoferrin as being important in host defense against infection and excessive inflammation (145).

RNase7 is a ribonuclease with antimicrobial activity isolated from skin (146). It shows broad spectrum antimicrobial activity and was detected in skin keratinocytes, the respiratory tract, and the genitourinary tract, but also at low levels in the gut.

Thrombocidins TC-1 and TC-2 are antimicrobial proteins found in blood platelets similar to two CXC chemokines but with C-terminal truncations of the two last amino acids (147).

Histatins are histidine-rich peptides expressed in human saliva (76).

Granulysin is a broad-spectrum antimicrobial peptide in the granules of cytotoxic T cells and NK-cells. It is produced late after T cell activation, i.e. after 3-5 days. The combination of granulysin and perforin equips T cells to kill intracellular pathogens (148).

Hepcidin is an acute phase protein synthesized by the liver, but is also a broad-spectrum antimicrobial peptide (149).

CAP37/Azurocidin is an antimicrobial molecule found in azurophilic granules of neutrophils (150).

Angiogenins are heparin-binding plasma proteins with angiogenic and ribonuclease activity belonging to the RNase superfamily (151). They exhibit salt-sensitive antimicrobial activities against pathogens and their levels are increased by inflammation in serum during the acute phase response. Mouse angiogenin Ang4 is a bactericidal protein in Paneth cells regulated by the normal intestinal flora (151).

1.6.3 Versatility and importance of antimicrobial peptides

The relative importance of defensins in immune protection in vivo has been a matter of much debate. However, several recent mouse models have taught us that AMPs are indeed important in the protections against
microorganisms. Indirect \textit{in vivo} evidence for a host defence function of AMPs came from a study of mice with a disrupted gene for metalloproteinase 7 (MMP-7), the activating enzyme of Paneth cell cryptdins in mice. MMP-7\textsuperscript{-/-} mice only expressed the precursor form and were more susceptible to infections by enteropathogens (95). Mice deficient of mouse \(\beta\)-defensin-1 (mBD-1) exhibited delayed clearance of \textit{Haemophilus influenzae} from lung tissues (152). Mice with deleted CRAMP (CRAMP\textsuperscript{-/-}), the murine homologue of LL-37, were more susceptible to prominent infection after cutaneous inoculation of bacteria (153). Over-expression of LL-37 by viral gene transfer augmented the innate host defence in a bronchial xenograft model of cystic fibrosis and also in murine animal models of pneumonia and septic shock (154, 155). A recent study shows that transgenic mice expressing HD-5 have increased protection against enteric salmonellosis (156). The importance of LL-37 became clear when it was found that patients with Morbus Kostmann (a maturation arrest at the promyelocyte stage were shown to lack LL-37 completely in both neutrophils and saliva (157). These patients die from infections and treated patients still suffer from recurrent infections.

Besides acting as important antibiotic-like effector molecules of innate immunity, defensins clearly have other roles in innate immunity and also function by alerting the adaptive immune system. HNPs can enhance or suppress the activation of the classical pathway of complement by binding to solid-phase or fluid-phase C1q molecule \textit{in vitro} (158, 159). HNPs from humans are capable of enhancing the phagocytic activity of mouse macrophages (160). HNPs and hBD-2 can induce activation of mast cells which result in release of histamine and prostaglandin D\textsubscript{2} (161, 162). HNP1-3 stimulate bronchial epithelium to release more IL-8, which attracts more neutrophils that in turn produce more defensins (163). HNP1-3 stimulate the release of TNF and IL-1 and decrease the production of IL-10 in monocytes which leads to amplification of the local immune responses (164). HNP1-3 can be produced by NK, B, and \(\gamma\delta\) T cells upon stimulation with cytokines and therefore may be direct effector molecules in cell-mediated defence (165). HNPs have also been described to directly increase binding of bacteria to lung epithelial cells (166).

Interestingly, several studies have also shown that defensins have chemoattractant properties. HNP1 and HNP2 are chemotactic for monocytes, CD\textsuperscript{4} CD45RA\textsuperscript{+} or CD8\textsuperscript{+}CD45RO\textsuperscript{+} T cells, and immature dendritic cells (167, 168). hBD-2 is chemotactic to CD45R0\textsuperscript{-} memory T cells and immature dendritic cells because it binds to a chemokine receptor known as CCR6. Naïve T cells (CD4\textsuperscript{+}CD45RA\textsuperscript{+}), mature dendritic cells or monocytes were not attracted to hBD-2 (169). CCR6 is a receptor that interacts with the chemokine CCL20 and stimulates the migration of B cells.
immature dendritic cells, and a subset of memory T cells through its interaction with the CCR6 receptor. The same observation as with hBD-2 was reported for the binding of murine beta defensins mbd-2 and mbd-3 to murine CCR6 (170). The binding of mbd-2 to TLR4 was shown to induce DC maturation necessary for the antigen presentation capacities (171). hBD-3 and hBD-4 chemoattract monocytes and macrophages (114, 115). Since monocytes do not express CCR6 (169), hBD-3 must use an additional chemokine receptor. The chemotactic activities of α- and β-defensins suggest that they play a role in recruitment of neutrophils, monocytes, and T cells to the sites of infections and thereby facilitate the initiation of the adaptive response. Yang et al suggest a model of how defensins could contribute to the adaptive immune response: First, defensins are likely to recruit immature DCs to the site of inflammation. Secondly, although not yet proven, defensins bind to some part of the microbial membrane and this defensin-Ag-complex would then facilitate uptake by the DC. Third, defensins might promote DC maturation either directly or via the induction of TNF and IL-1 in monocytes/macrophages. Finally, defensins recruit memory T cells to the site of infection (172).

Interestingly, the structure of defensins is reminiscent of some chemokine gene products that share functional and structural features with β-defensins although there is no apparent homology at the amino acid level. Both have a structural core of three anti-parallel β-sheets secured by disulfide linkages (173). hBD-2 and the chemokine CCL20 (MIP-3α) both engage the CCR6 receptor present on immune effector cells (169). NMR solution structures and x-ray crystallography revealed that hBD-2 is a simplified form of MIP-3α/CCL20 structurally (172). Notably, some chemokines exhibit antimicrobial activity. The IFN-γ-inducible chemokines monokine induced by IFN-gamma (Mig/CXCL9), IFN-gamma-inducible protein of 10 kDa (IP-10/CXCL10), and IFN-inducible T-cell alpha chemoattractant (I-TAC/CXCL11) are microbicidal against Escherichia coli and Listeria monocytogenes (174). The antimicrobial activities of these chemokines were inhibited by 50-100mM NaCl, just like several human defensins. Similarly, neutrophil-activating protein-2 (NAP-2/CXCL7) and platelet factor 4 (PF4/CXCL4) show antimicrobial activities against Escherichia coli, Staphylococcus aureus, Cryptococcus neoformans (CXCL7 only) and Bacillus subtilis (CXCL4 and CXCL7) (147). However, receptor-sharing with chemotactic factors is not unique to defensins; human LL-37 binds to the chemotactic receptor formyl peptide receptor like 1 (FPRL1) which is a G-protein coupled receptor expressed on a variety of cells including neutrophils, monocytes, and T cells. By activation of the receptor, LL-37 attracts neutrophils, monocytes, and CD4 T cells, and also activates mast cells (162, 175).
There are also roles for AMPs not directly linked to innate or adaptive immunity. For example, LL-37/hCAP-18 is implicated in wound healing and angiogenesis (135).

### 1.7 Inflammatory bowel disease

The inflammatory bowel disease (IBD) comprises two chronic disorders of the intestine, *ulcerative colitis* (UC) and *Crohn’s disease* (MbC). Although UC and MbC share some clinical and demographic characteristics, they differ in the type of tissue damage, symptoms, and localisation which suggests they have different etiopathogenic processes (Table 3). The prevalence rates are 150-200 cases for UC and 100-150 cases for MbC per 100,000.

*Ulcerative colitis* (UC), described by Wilks 1859, is a relapsing, chronic inflammatory disease of the colon where the inflammation tends to be restricted to the mucosa. Crypt abscesses are common. The main feature of ulcerative colitis is marked infiltration of lymphocytes and plasma cells (PC) as well as neutrophils and macrophages into the tissue. Another typical feature is the patchy destruction of the epithelium causing ulcerations.

*Crohn’s disease* (MbC) was described by Crohn and coworkers more than 70 years ago. It is a chronic inflammation that can affect any part of the gastrointestinal tract, but is primarily located in the ileocecal and proximal colonic regions. The main characteristic of MbC is the segmental transmural granulomatous inflammation often leading to strictures and/or fistula formation.

The etiology of these diseases is still unknown, but numerous studies point to the involvement of genetic as well as environmental factors, including microbes. The importance of genetic factors is evident from the findings that first degree relatives have 4-20 times higher risk of developing IBD than the general population, and that there is higher disease concordance in monozygotic than dizygotic twins (176). Genetic factors seem to be more important in MbC than in UC. Several confirmed IBD susceptibility regions have been reported, e.g 16p12-q13 (IBD1), 12p13.2-q24.1 (IBD2), the MHC complex region on chromosome 6 (IBD3), 14q11-12 (IBD4), and the 5q31 cytokine gene cluster (IBD5) (177). However, most associations have not been useful in terms of understanding the pathogenesis or for diagnosis. Attempts to identify candidate genes have been performed with microarray analysis of expressed genes (178, 179). They show that the expression profiles of UC and MbC are quite different and establish each form of IBD as a distinct entity. Significant differences in the expression profiles from healthy people were found for 170 genes, 20% of which were common to both diseases. Polymorphisms and mutations...
Table 3. Features of ulcerative colitis and Crohn’s disease.

<table>
<thead>
<tr>
<th>FEATURE</th>
<th>ULCERATIVE COLITIS</th>
<th>CROHN’S DISEASE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fever</td>
<td>Uncommon</td>
<td>Common</td>
</tr>
<tr>
<td>Abdominal pain</td>
<td>Varies</td>
<td>Common</td>
</tr>
<tr>
<td>Diarrhea</td>
<td>Very common</td>
<td>Fairly common</td>
</tr>
<tr>
<td>Rectal bleeding</td>
<td>Very common</td>
<td>Fairly common</td>
</tr>
<tr>
<td>Weight loss/ signs of malnutrition</td>
<td>Fairly common</td>
<td>Common</td>
</tr>
<tr>
<td>Perianal disease</td>
<td>Absent</td>
<td>Fairly common</td>
</tr>
<tr>
<td>Site</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rectum</td>
<td>Involved</td>
<td>Infrequent</td>
</tr>
<tr>
<td>Colon</td>
<td>Exclusively</td>
<td>1/3 of patients</td>
</tr>
<tr>
<td>Ileum</td>
<td>Never</td>
<td>1/3 of patients</td>
</tr>
<tr>
<td>Jejunum</td>
<td>Never</td>
<td>Infrequent</td>
</tr>
<tr>
<td>Stomach or duodenum</td>
<td>Never</td>
<td>Infrequent</td>
</tr>
<tr>
<td>Esophagus</td>
<td>Never</td>
<td>Infrequent</td>
</tr>
<tr>
<td>Intestinal complications</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strictures</td>
<td>Rare</td>
<td>Common</td>
</tr>
<tr>
<td>Fistulas</td>
<td>Absent</td>
<td>Fairly common</td>
</tr>
<tr>
<td>Cancer</td>
<td>Increased risk</td>
<td>Probably increased risk</td>
</tr>
<tr>
<td>Histological</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Distribution</td>
<td>Diffuse/ continuous</td>
<td>Segmental/ discontinuous</td>
</tr>
<tr>
<td>Depth of inflammation</td>
<td>Mucosal</td>
<td>Transmural</td>
</tr>
<tr>
<td>Ulceration</td>
<td>Superficial</td>
<td>Deep, submucosal extension</td>
</tr>
<tr>
<td>Fissure</td>
<td>Absent</td>
<td>Common</td>
</tr>
<tr>
<td>Thickened bowel wall</td>
<td>Rare</td>
<td>Common</td>
</tr>
<tr>
<td>Granulomas</td>
<td>Absent</td>
<td>Present</td>
</tr>
<tr>
<td>Pseudopolyps</td>
<td>Common</td>
<td>Fairly common</td>
</tr>
<tr>
<td>Crypt abscesses</td>
<td>Common</td>
<td>Rare</td>
</tr>
<tr>
<td>Dysplasia</td>
<td>Common</td>
<td>Rare</td>
</tr>
</tbody>
</table>


within the NOD2/CARD15 gene have been associated with the development of MbC in a subgroup of patients (180, 181), and persons homozygous for a variant NOD2 may have a 20-fold higher susceptibility to MbC (182). NOD2 encodes an intracellular protein involved in the recognition of bacterial LPS, and participates in the signalling pathways for NF-κB activation or apoptosis. Initially, expression of this gene was thought to be restricted to macrophages, but IECs also express NOD2 and expression in IECs is significantly increased in IBD-patients (183-185). Moreover, stimulation with key proinflammatory cytokines, TNF-α alone or in
combination with IFN-γ, induces NOD2 expression in colonic epithelial cell lines (183, 185). The expression of an MbC-associated NOD2 variant (3020insC) in CaCo-2 cells was associated with loss-of-LPS recognition and loss of the protective function against internalization of *S. typhimurium* (183). This suggests that NOD2 is important in the maintenance of the innate mucosal reaction by mediating epithelial pattern recognition response against pathogens, and that mutants of NOD2 may increase the risk of developing MbC.

Several environmental factors have been studied. For instance the use of anti-inflammatory drugs (nonsteroidal, NSAIDs) can lead to disease flares, early appendectomy reduces the incidence of UC, and smoking seems to protect against UC while increasing the risk for MbC. However, the luminal flora may be the major factor in the development of IBD. It is now generally believed that IBD results from abnormal/dysregulated responses (loss-of tolerance) to harmless Ags of the commensal flora of the intestine (can be thought of as self-Ags), or alternatively to pathogenic microbes. Over 30 animal models of experimental IBD have been described (186) and many of these do not develop, or develop only mild inflammation, when they are raised under germ-free conditions. Examples of this are the IL-2−/− mice, IL-10−/− mice, and TcR mutant/deficient mice. Several features suggest that a clean environment with a low exposure to microbes is a risk factor for the precipitation of MbC. There is a north-to-south gradient in incidence of IBD - in developing countries intestinal infectious diseases are common whereas IBD is uncommon - and good hygiene in childhood seems to be a risk factor for MbC but not for UC (187, 188). There is a higher incidence of MbC in small families, since a single child is likely raised under more hygienic conditions and intrafamilial transmission of common pathogens is not as common as in a big family (189). Another aspect is the rather frequent association of recent intestinal infections with the first appearance of MbC and prevalence of superinfections in established IBD (190). There are several reports on increased numbers of bacteria close to the epithelial lining in IBD (191, 192). Swidsinski’s findings indicate that this is caused by a primary defect in the chemical barrier which would allow bacteria to adhere/penetrate. *Mycobacterium paratuberculosis*, *Listeria*, specific adherent *E. coli*, *Streptococcus*, persistent measles virus, and cytomegalovirus (193-197) have all been suggested to be associated with MbC. UC patients have increased numbers of microorganisms, but decreased numbers of “protective” bacteria such as *Bifidobacteria* or *Lactobacilli* and also display increased levels of mucosal IgG directed against the commensal flora (198). In animal models, antibiotics have been proven successful in the protection against IBD, especially if administered before the onset of inflammation (198). In humans these types of treatments
The abnormal immune responses in IBD may be due to excessive T cell responses or dysregulated T cell functions (199). Patients with IBD demonstrate immunological reactivity to their own commensal flora which is indicative of a breakdown of tolerance (200, 201). There is also a possibility of continued stimulation resulting from a change in the epithelial barrier because barrier abnormalities could lead to the direct access of luminal bacteria to the lamina propria. Epithelial barrier defects have been demonstrated in UC (202) and leaky epithelium in moderate-to-severe disease correlated with epithelial erosion/ulcer-type lesions or crypt abscesses. The enhanced permeability across the epithelium in IBD patients may be due to marked downregulation of junctional proteins and is likely to be a secondary phenomenon of the inflammation (203). Trefoil factors, known to be important for mucosal protection and repair after mucosal damage, are other important players (204). Moreover, bactericidal/permeability-increasing protein (BPI) is increased in colonic mucosa of UC (205).

The importance of T cells in IBD is shown in animal studies in which gut inflammation can be induced and transferred by T cells (206). There are increased numbers of activated T cells in blood and intestinal mucosa of IBD patients, but the pattern of cytokine production in MbC and UC differs. The mucosa of patients with established MbC is dominated by CD4⁺ lymphocytes with a Th1 phenotype and characterized by the production of TNF-α, IFN-γ and IL-2. MbC patients have been treated successfully with anti-TNF-α mAb (176). In contrast, the mucosa of UC patients shows decrease in Th1 cytokines (207) and a Th2 phenotype is suggested based on the observations that elevated local Ab production is seen in UC (208, 209). However, a role of Th2 cytokines has not been established (210). A majority of the increased numbers of lymphocytes in UC are located in basal lymphoid aggregates (25). The most prominent T cell subset in the aggregates is of the suppressive phenotype CD4⁻CD28⁻ TcR-αβ⁺. Furthermore, IL-10 is elevated in CD4⁺ cells of lamina propria in UC (207). This cytokine is mainly known for its regulatory functions (211, 212), but also has described pro-inflammatory capacities (213, 214). The balance between these two functions is likely to be dose-dependent. IL-10 may downregulate the Th1 cytokines in UC, but may also act in a pro-inflammatory manner. Interestingly, IL-10 was recently shown to be able to promote plasma cell differentiation of memory B cells in vitro (but not naïve cells) (215), which could explain the massive increase of PCs seen in UC without the need for a Th2 response. Early studies describe an increase in the number of immunoglobulin producing cells (mainly IgG producing...
cells) (216, 217) in IBD. PCs of all subclasses are increased in UC, but the
greatest percentage increase is found in the IgG1 and IgG3-secreting
populations, while in MbC these show a particular increase of IgG2
producing cells (218). These features may partly reflect the differences in
pathophysiological mechanisms between UC and MbC. A marked reduction
in apoptosis of lamina propria T cells in patients with IBD suggests that
apoptosis might be a mechanism which turns off mucosal T cell responses
to environmental Ags in healthy subjects (219). Resistance to apoptosis
could be an important cause of mucosal immune dysregulation and tissue
inflammation in colitis.

The antigen presenting capacity of IECs is increased in IBD, e.g
MHC class II (220) and CD86 (39). TLR4 is upregulated in both MbC and
UC, while TLR3 is downregulated in MbC but not in UC (42). The increase
in CD86 on IECs is likely a consequence of TLR upregulation and
recognition of luminal PAMPs since CD80 and CD86 are upregulated by
engagement of PRRs. TLR2, TLR4, and TLR5 are all up-regulated in
inflammation-associated macrophages (221). In infection/inflammation the
APCs are more likely to induce activation rather than suppression. Pro-
inflammatory cytokines upregulate antigen presenting molecules and co-
stimulatory molecules, and increase Ag transport across the gut. IFN-γ
disrupts tight junctions and increases paracellular transport (222). TNF-α
increases permeability of HT-29-19A monolayers and alter tight junctions
(223). These effects may be important in the pathogenesis of MbC.
Receptors of the innate immune system may also be critically involved since
the receptors and molecules of innate immunity also provide a bridge
between the innate and adaptive immune system.

5-aminosalicylic acid (5-ASA)-based agents and corticosteroids are
still the current treatment, but antibiotics and immunosuppressive agents are
also used (176, 224). 5-aminosalicylic acid is useful in the treatment of
patients with mild to moderately active UC and acts in an anti-inflammatory
manner by blocking the production of prostaglandins and leukotrienes and
inhibiting neutrophil chemotaxis (176). Corticosteroids are given to both
UC and MbC patients to control acute inflammatory activities, but can cause
many, often serious side effects with long-term use such as osteoporosis,
hypertension, and diabetes (176). Immunomodulatory drugs (azathioprin or
6-mercaptopurin) are generally given to UC and MbC patients who cannot
taper their use of corticosteroids, allowing a gradual decrease in the use of
corticosteroids and a prolonged remission. These effects require several
weeks or months and such drugs should not be used for the control of acute
inflammation. Antibiotics seem to have a limited effect in a subgroup with
MbC, and they are not completely convincing as a therapeutic option. (225).
In MbC the exogenously administered antibiotics may compensate for the
downregulation of endogenous antibiotics in response to infection (see Results and Discussion). Antibiotics have a very limited use in treatment of UC which suggest different roles for the luminal flora in IBD (176). Probiotics seem to have promising potential in both UC and MbC, although conclusive results are lacking (176, 225). Use of the anti-TNF-α agent Infliximab has proven to be effective for selected groups of patients with MbC and is thought to act by neutralizing circulating TNF-α and subsequently binding to cell surface TNF-α (on macrophages), inducing apoptosis.

1.8 Celiac disease

Celiac disease (CD), or gluten sensitive enteropathy, is a T cell-mediated chronic inflammatory disease of the small intestine that is precipitated by a loss of tolerance to wheat gluten or related prolamines from barley or rye (226). Exposure to gluten causes an inflammatory response and usually leads to increased numbers of IELs and LPLs together with partial or total destruction of the villous structure (called villous atrophy) and crypt hyperplasia. CD is diagnosed with the assessment of the villous structure by biopsy examination in combination with serological testing of anti-gliadin antibodies and autoantibodies to tissue transglutaminase and/or endomysium. The columnar epithelial cells in CD are pseudostratisfied with a reduction in height (227). The crypt hyperplasia is associated with increased turn-over of epithelial cells, accelerating migration to the villous tip. CD can arise at any age but the clinical symptoms may differ with age - in early childhood CD typically causes chronic diarrhea, abdominal distension, and a failure to thrive. In older children other symptoms, including extra-intestinal manifestations such as short stature, delayed puberty, anemia or neurological symptoms are more common (226, 228). Some patients experience only mild or no symptoms – silent celiac disease - which explains why many adult patients are undiagnosed. CD provides an outstanding model of mucosal inflammation since i) it has a defined antigen, ii) the genetic predisposition is partly known, and iii) there is a treatment - withdrawal of gluten from the diet results in regression of the disease. Untreated CD is associated with high morbidity (229) and autoimmune disorders (230). There is a clear genetic predisposition for CD with HLA-DQ2 (DQA1*05/ DQB1*2) expressed in ~90% of the patients or HLA-DQ8 (DQA1*0301/ DQB1*0302) (less frequent) (231). The sibling recurrence risk for CD is 10% (232) and in identical twins there is 75% concordance (233) which suggest that other genes are involved and also makes it very likely that environmental factors contribute to the disease risk. Other susceptibility regions on chromosomes
5q31-33 (including for example the Th2 cytokine gene cluster and CD14) and 11q have also been linked with CD (228).

By serologic screening, the worldwide prevalence has been estimated to be 1 in 266 and thus establishes CD as one of the most common genetically based diseases (234). From 1984, CD suddenly became one of the most commonly diagnosed chronic diseases in Swedish children with a cumulative incidence of at least four per 1000 live births (235). From 1995 the incidence declined to a level similar to that prior to the increase, possibly indicating an infectious component (236) or changes in dietary patterns (235) in the pathogenesis of the disease. Duration of breastfeeding, the commencement of gluten intake (237), and viral infections that promote secretion of IFN-α (238) are environmental factors that may contribute to the disease onset. Adenovirus infection has been suggested as a contributing factor because of its immunological cross reactivity with A-gliadin (239), but the relevance of this finding has been questioned. *Candida albicans* was recently suggested as a trigger in the onset of CD because its hyphal wall protein contains amino acid sequences identical with gliadin epitopes (240).

The development of anti-gluten specific T cells in the intestine is specific to CD patients (226). Changes in intestinal permeability or in the processing of gluten are potential mechanisms for the disease process (226, 241). Gliadin is a substrate for tissue transglutaminase which deamidates the positively charged glutamines into negatively charged glutamic acid residues (242). In active CD, tissue transglutaminase is expressed at the epithelial brush border and in the subepithelial region (243). Anti-gluten reactive CD4⁺ T cells recognise deamidated gluten peptides (243, 244). The immunodominant peptides were recently shown to be part of a 33mer gliadin peptide resistant to digestion by gastric and pancreatic enzymes (245). The negatively charged deamidated peptides allow for interaction with the positively charged binding pockets of HLA-DQ2 or -DQ8 on APCs, and subsequent presentation to CD4⁺ T cells will lead to production of IFN-γ (226). There is a massive increase in IELs in CD which display an increased IFN-γ and IL-10 production with a shift in IFN-γ from LP to the epithelial compartment (246). Most IFN-γ⁺ IELs are CD8⁺ (247). In CD patients both TcRαβ⁺CD8⁺ and TcRγδ⁺ T cells are increased among IEL (248). IFN-γ induces production of chemoattractants in IECs (249, 250), the ability to present Ag by IECs (40), and increases the permeability of epithelial monolayers (222, 251). Changes in gastrointestinal permeability in celiac disease have been reported (252), and an IEL supernatant from a celiac disease patient was shown to cause changes in transepithelial electrical resistance (TER) in T84 epithelial cells (251). IFN-γ can cause down-regulation of ZO-1 (a tight junction protein) leading to a broken barrier (253). The up-regulation of zonulin, which is involved in tight
junction regulation, seems to be partly responsible for the increased gut permeability in CD (254). It is thus possibly that IELs regulate tight junctional permeability directly by secretion of IFN-γ. MHC II is induced in celiac disease (255). Enterocyte apoptosis is increased in CD (256), which may be partly responsible for the villous atrophy. Intestinal trefoil factor, which has an important role in mucosal protection and repair, is reduced in goblet cells of CD patients but recovers to normal levels in treated patients (257). The T cell mediated responses are not only directed towards gluten, but also towards epithelial cells that may have induced expression of MICA and MICB caused by cell-stress and/or IFN-γ (228). The increased level of tissue transglutaminase is due to wounding, and the fact that the tissue transglutaminase promoter contains response elements for the pro-inflammatory cytokines IL-6 and TNF-α. Inflammation as induced by an infection may breach the epithelial barrier and lead to high influx of gluten (226).
2. AIMS OF THE STUDY

Epithelial cells are important players in the protection of the intestinal mucosa. Their location, in a single layer between the lumen and the underlying mucosa, provides a physical barrier, but also a chemical barrier. The specific aims of this thesis were to study how the epithelial cells and their specific protein products participate in mucosal defence. More specifically the aims were:

- To study the production of defensins in human small and large intestinal epithelial cells.
- To determine how their expression is affected by inflammation in the large intestine as manifested in UC and MbC and small intestine as seen in MbC and CD.
- To investigate the effect of pro-inflammatory cytokines and bacteria on production of CEA family members and defensins.
- To investigate how expression and composition of glycocalyx components, such as mucins and CEA family members, are affected in children with celiac disease.
- To study whether there is a relationship between occurrences of certain defined bacteria and different epithelial cell characteristics in CD.
3. RESULTS AND DISCUSSION

3.1 Defensin expression in normal intestinal epithelial cells (Paper I and IV)

In order to determine the levels of defensins in normal IECs, we have isolated epithelial cells from human operation specimens. The tissues were from patients with different types of cancers or other non-inflammatory benign conditions but resected distant to macroscopically detectable lesions. None of these patients had been subjected to radio- or chemotherapy, long-standing antibiotic medication or steroid treatment. The isolation procedure yields two subpopulations of IECs; one which is released after dithiotreitol treatment and shaking of tissue pieces and contains epithelial cells mainly from the luminal/villous part (l/v-IEC), and one with cells released upon collagenase treatment. This second subpopulation contains epithelial cells primarily from the crypts (c-IEC). The two populations were further enriched for epithelial cells by Percoll gradient centrifugation, which separates cells by density. The IEC populations were then subjected to treatment with paramagnetic beads charged with anti-CD45-mAb to deplete the IEC fractions from any residual leukocytes. Immunoflow cytometry revealed <1% CD45\(^+\) cells after this isolation procedure. The mRNA levels in each fraction were measured using the real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) with mRNA copy standards for each assay. The assays cover five log units. The mRNA levels in individual samples were normalized to the housekeeping gene 18S rRNA.

Table 4 summarizes the results of the qRT-PCR mRNA studies of normal intestines. The number of plus signs indicates relative expression levels. mRNA for the \(\alpha\)-defensins HD-5 and HD-6 showed similar expression patterns with high expression levels in ileum and jejunum and occasional expression in colon ascendens. Paneth cells of small intestinal crypts produce HD-5 and HD-6. Consistent with this was the finding that c-IEC contained significantly higher mRNA levels of both defensins than in l/v-IEC (paper I). The latter results indicate that the method used here is able to at least partly separate IECs of cryptal origin from IECs present on the villus. hBD-1 mRNA was found in the epithelium of both small and large intestines with the highest expression level in colon. hBD-2 mRNA was barely detectable in IECs from normal intestine. In contrast, hBD-3 and hBD-4 mRNAs were expressed in jejunum, ileum, and colon with hBD-3 being expressed approximately 10 times more abundantly than hBD-4. Lysozyme mRNA was expressed throughout the intestine and most abundant in jejunum. In situ hybridization revealed that hBD-3 and hBD-4 mRNAs were expressed in IECs of jejunum, ileum, and colon with a high
intensity signal in crypt IECs and a barely detectable signal in the most mature cells facing the lumen.

**Table 4.** Defensins and lysozyme mRNA expression levels in human intestinal epithelial cells.

<table>
<thead>
<tr>
<th></th>
<th>jejunum</th>
<th>ileum</th>
<th>colon</th>
</tr>
</thead>
<tbody>
<tr>
<td>HD-5</td>
<td>+++<em>a</em></td>
<td>+++</td>
<td>+/-</td>
</tr>
<tr>
<td>HD-6</td>
<td>+++</td>
<td>+++</td>
<td>+/-</td>
</tr>
<tr>
<td>hBD-1</td>
<td>+</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>hBD-2</td>
<td>-</td>
<td>-</td>
<td>+/-</td>
</tr>
<tr>
<td>hBD-3</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>hBD-4</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>lysozyme</td>
<td>+++</td>
<td>++</td>
<td>+</td>
</tr>
</tbody>
</table>

*a* - , no expression; +/-, some samples with expression; +, low expression; ++, intermediate expression; +++, high expression.

Results are based on specific qRT-PCR assays for each component.

hBD-3, HD-5 and lysozyme were also studied at the protein level using specific antibodies. Immunohistochemistry revealed HD-5 and lysozyme in Paneth cells of the small intestine and proximal colon. In addition, goblet cells in the colon and small intestine were stained with the anti-lysozyme mAb. hBD-3 staining was seen in the entire epithelium and immunoflow cytometry analysis of isolated IECs from all three compartments revealed positive intracellular staining for the hBD-3 peptide in a large fraction of the cells.

HD-5, HD-6, hBD-1, and lysozyme have previously been demonstrated to be expressed in human intestine (91, 92, 105, 134), and Garcia et al recently found hBD-3 mRNA in jejunum (114). The same group was the first to identify hBD-4 (115), but did not find it in the intestine most probably because they extracted RNA from whole tissue instead of from isolated IECs. However, this is the first time that the relative mRNA expression levels of six defensins and lysozyme have been determined in the three major compartments of the intestine. It shows that the intestine is well protected by epithelial derived defensins. hBD-3 and non-inducible hBD-1 seem to be the main defenders of the colon while the small intestine seem to have even better protection since Paneth cell derived HD-5 and HD-6 are abundantly expressed in addition to hBD-3. The finding that hBD-3 is
present in all three intestinal compartments is reassuring in light of its broad antimicrobial spectrum and strong bactericidal effect.

### 3.2 Defensin expression in epithelial cells from IBD-patients (Paper I and IV)

Isolated IECs from colons of ulcerative colitis (UC) patients and from the small intestine and colon from Crohn’s disease (MbC) patients were analyzed for defensin and lysozyme mRNA expression and compared with the corresponding mRNA levels of normal colon and small intestine. For clarity the result is shown as increased, decreased, or unchanged levels of mRNA (Table 5).

**Table 5. Changes in mRNA expression levels of defensins and lysozyme in ulcerative colitis (UC), Crohn’s disease (MbC) and celiac disease (CD).**

<table>
<thead>
<tr>
<th></th>
<th>UC</th>
<th>MbC large intestine</th>
<th>MbC small intestine</th>
<th>CD*</th>
</tr>
</thead>
<tbody>
<tr>
<td>HD-5</td>
<td>↑↑</td>
<td>↑</td>
<td>↔</td>
<td>↑↑</td>
</tr>
<tr>
<td>HD-6</td>
<td>↑↑</td>
<td>(↑)</td>
<td>↔</td>
<td>↑↑</td>
</tr>
<tr>
<td>hBD-1</td>
<td>(↓)</td>
<td>↔</td>
<td>↔</td>
<td>(↓)</td>
</tr>
<tr>
<td>hBD-2</td>
<td>↑↑</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
</tr>
<tr>
<td>hBD-3</td>
<td>↑↑</td>
<td>↔</td>
<td>↔</td>
<td>ND</td>
</tr>
<tr>
<td>hBD-4</td>
<td>↑↑</td>
<td>↔</td>
<td>↔</td>
<td>ND</td>
</tr>
<tr>
<td>lysozyme</td>
<td>↑↑</td>
<td>↑</td>
<td>↔</td>
<td>↑↑</td>
</tr>
</tbody>
</table>

* Active disease, e.g. untreated and challenged CD

As can be seen there was a highly significant increase in HD-5, HD-6, hBD-2, hBD-3, and hBD-4 mRNA levels in colonic IECs from UC patients compared with controls. hBD-1 mRNA levels, in contrast, were unchanged or even slightly decreased. At the protein level, we observed that HD-5 was present in small groups of cells located mainly at the crypt base of UC colons. These cells also tested positive for the presence of lysozyme. The cells are considered to be metaplastic Paneth cells induced by the disease process. Presence of the hBD-3 peptide was confirmed by immunohistochemical staining of UC colon tissue sections and by immunoflow cytometry of isolated IECs from UC colons. The entire epithelium and scattered lamina propria cells (see section 3.8) were intensely stained by the anti-hBD-3 Ab. Immunoflow cytometry revealed that hBD-3 was present intracellularly. Our results confirm and extend a
recent study on whole biopsies of UC colons by Wehkamp et al (258). They also confirm a study by Cunliffe et al on the expression of HD-5 in UC (259). Unchanged expression of hBD-1 and induction of hBD-2 are well in line with the findings in other chronic infections or inflammatory conditions (107, 111, 260) and an initial immunohistochemical observation by O’Neil et al (105). It can be concluded that epithelial cell derived innate immune components are engaged in the chronic intestinal inflammation characteristic of UC.

MbC can be localized either to the small or large intestine. The two subgroups were therefore considered separately. As can be seen from Table 5, HD-5 and lysozyme mRNA levels were increased in MbC colon (Crohn’s colitis) samples although to a lesser degree than in UC colon samples. However, the mRNA levels of all four β-defensins were essentially unaffected by Crohn’s colitis. Analysis of the MbC small intestinal samples revealed that none of the seven anti-microbial components were affected compared to controls. Thus, elevated defensin production in IECs seems to be a feature of UC but not of MbC. This knowledge may be used in the differential diagnosis of the two diseases by analysis of biopsies. Recently Wehkamp et al (258) confirmed our finding that hBD-2 and hBD-3 mRNA is not elevated in MbC.

We have recently studied the expression of mouse β-defensins (mBD) 1-4 mRNAs by semiquantitative RT-PCR in the IL2−/− mouse model of IBD. mBD-4 mRNA was significantly increased in IL2−/− mice (unpublished data). Since mBD-4 shows high sequence similarity with hBD-2 at the amino acid level, these results further validate the IL2−/− mouse as an experimental model for UC. This mouse model may be very useful in understanding the interactions between innate and adaptive components in chronic mucosal inflammation of the type seen in UC.

The increased expression of β-defensins in UC may be explained by the inducibility of these peptides by cytokines and/or bacteria (probably via TLRs). Changes in the glycocalyx/mucus layers allow bacterial penetration and adhesion by pathogenic and/or commensal microorganisms. This will induce the production of pro-inflammatory cytokines as well as stimulate directly via PRRs on IECs. The lack of induction of hBD-2, hBD-3, and hBD-4 in MbC compared with UC, and decrease of hBD-1 in both UC and MbC, indicates different responses of the mucosal immune system and/or a deficient mucosal barrier function in MbC. One possibility is that the inductive stage is defective in MbC leading to an inadequate antimicrobial defense barrier. Alternatively there is an excessive induction of defensins in UC. Deficiency in AMPs in MbC may be one of the explanations for a break of the antimicrobial barrier in IBD, and may lead to persistent bacterial invasion trigging the inflammatory and adaptive responses. Lack
of induction may be explained by mutations in the NOD-2 gene, although this is not the whole explanation since only ~20% of MbC patients have this genetic background (176). An altered NOD-2 would lead to unresponsiveness to intracellular LPS by both macrophages and IECs and lack of induction of genes directed by the transcription factor NF-κB. However, at present there are no data to support a link between NOD-2 and defensins. The increased production of defensins in UC is most likely a sign of active defence by the IECs. It should be very interesting to study the defensin response longitudinally in biopsies in individual patients as they pass through phases of relapse and relative health.

Increased concentration of salt has been suggested to inactivate defensins in cystic fibrosis and to account for the recurrent pulmonary infections (118, 261). hBD-2 and hBD-3 inactivation by the cystein proteases cathepsin B, L, and S has also been reported in cystic fibrosis (262). Thus the problem remains to resolve the state of activity of the released defensins in UC, and to determine the reasons behind the lack of induction of defensins in MbC.

3.3 Presence of bacteria in jejunal biopsies of CD patients, and expression of defensin and lysozyme (Paper III)

When jejunal biopsies from children with CD were analyzed by scanning electron microscopy, numerous bacteria were seen on the flattened mucosa in about 40% of children with untreated CD or challenged CD (i.e. active disease), while controls generally had no adhering bacteria (1/59 control samples were positive). Interestingly, 19% of treated CD patients (with normal villous architecture) had bacteria. The difference in incidence between active and inactive disease was not statistically significant, indicating that presence of bacteria was a trait of individuals with CD. The bacteria were rod shaped, and coccoid bacteria were seen only in a few samples. The bacteria seemed to adhere with one end between or on the microvilli of the epithelial surface and were often adhering in groups like bouquets. The bacterial identity has not been determined but ongoing studies using PCR for the universal bacterial 16S rRNA on biopsies will hopefully reveal their identity.

Presence of bacteria in the jejunal mucosa of CD patients raised the question of whether defects in innate immune functions of intestinal epithelial cells are predisposing factors for bacterial colonization and possibly CD. To explore this possibility we analyzed two aspects of innate immunity: production of AMPs, and changes in glycocalyx/mucous composition (see section 3.6). The mRNA expression levels of HD-5, HD-6, hBD-1, hBD-2, and lysozyme were investigated by qRT-PCR using mRNA
from v-IEC and c-IEC of jejunal mucosa from CD patients. The results are summarized in Table 5. Contrary to what our hypothesis predicted, HD-5, HD-6, and lysozyme mRNA levels were actually increased in the jejunum mucosa of CD patients with active disease (untreated and challenged CD) as compared to controls. Expression levels were higher in c-IEC than in v-IEC indicating increased synthesis in the Paneth cells. In treated CD the levels were comparable with controls. HD-5 and lysozyme protein analysis by immunohistochemistry revealed positively stained cells both at the mid-crypt region as well as at the bottom of the crypts in active CD. These results are also supportive of metaplastic Paneth cell differentiation in CD. Only low levels of hBD-1 and hBD-2 mRNA were detected in all three groups of CD patients. In fact, hBD-1 mRNA was actually slightly decreased in v-IEC of active CD compared to that of treated CD. This tendency was also seen for hBD-1 in UC (Paper I), suggesting that inflammation of both the small and large intestine can result in decreased expression of hBD-1. It is interesting to note that hBD-2 mRNA expression was unaffected despite the fact that about 40% of the samples of jejunal mucosa from CD patients with active disease contained bacteria. This lack of hBD-2 induction could reflect defects such as NOD2 mutations, defective TLR signaling, or alternatively that the observed bacteria are constituents of the normal flora lacking the ability to signal via TLR. 17 of the biopsies analysed for defensins and lysozyme (evenly distributed between untreated, treated, and challenged CD) had previously been analyzed for IFN-γ mRNA expression in CD3⁺ IEL (246). Since IFN-γ is significantly increased in active CD, we analysed whether IFN-γ /GAPDH ratios in IEL showed any correlation with defensin or lysozyme mRNAs in IECs isolated from the same biopsies. We found that the expression levels of HD-5, HD-6, and lysozyme in v-IEC correlated with the expression levels of IFN-γ. Of the biopsies studied by scanning electron microscopy, 26 samples had previously been analyzed for IFN-γ mRNA in CD3⁺ IEL. There was no correlation between IFN-γ mRNA and presence of bacteria.

In summary, there appears to be no defect in epithelial cell α-defensins or lysozyme production in CD. Rather, there was increased expression of these factors, which could be linked to differentiation of epithelial cells into Paneth cells (and goblet cells?) as driven by the increased IFN-γ levels in the epithelium. The lower level of hBD-1 in active CD and the lack of induction of hBD-2 may be factors contributing to persistence of bacterial adherence in CD.

3.4 Inducibility of defensins in intestinal epithelial cells (Paper I and IV)

Colon carcinoma cells were analysed by qRT-PCR for expression of
defensin mRNA before and after stimulation with bacteria or pro-inflammatory cytokines. The results are based on the findings in three cell lines, LS174T, HT-29, and T84, and are schematically summarized in Table 6. Before stimulation, T84 expressed all six defensins, LS174T expressed all defensins except HD-6, and HT-29 cells expressed only the β-defensins.

Table 6. Effect of bacteria and cytokines on the expression levels of six defensins in colon carcinoma cell lines LS174T, T84 and HT-29 (summary of Papers I, IV and unpublished results).

<table>
<thead>
<tr>
<th>Inducing agent</th>
<th>hBD-1</th>
<th>hBD-2</th>
<th>hBD-3</th>
<th>hBD-4</th>
<th>HD-5</th>
<th>HD-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>live E. coli</td>
<td>↔*</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↔</td>
<td>↔</td>
</tr>
<tr>
<td>live B. megaterium</td>
<td>↔</td>
<td>↔</td>
<td>ND</td>
<td>ND</td>
<td>↔</td>
<td>↔</td>
</tr>
<tr>
<td>live M. luteus</td>
<td>↔</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↔</td>
<td>↔</td>
</tr>
<tr>
<td>live S. typhimurium</td>
<td>↔</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↔</td>
<td>↔</td>
</tr>
<tr>
<td>LPS (from E. coli O55)</td>
<td>↔</td>
<td>↔</td>
<td>ND</td>
<td>ND</td>
<td>↔</td>
<td>↔</td>
</tr>
<tr>
<td>IL-1β</td>
<td>↔</td>
<td>↑</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
</tr>
<tr>
<td>TNF-α</td>
<td>↔</td>
<td>↔</td>
<td>(↑)</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>↔</td>
<td>↔</td>
<td>↑</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
</tr>
</tbody>
</table>

* ↔, unchanged expression; ↑, increased expression.

Stimulation of colon carcinoma cells in vitro with live Escherichia coli, Bacillus megaterium, Micrococcus luteus, or Salmonella typhimurium for 1h followed by 5h incubation in bacteria-free media revealed constitutive expression of hBD-1 and induction of hBD-2 mRNA with S. typhimurium, E. coli and M. luteus but not with B. megaterium. Live E. coli, M. luteus, and S. typhimurium also induced expression of hBD-3 and hBD-4, mRNA but not of the α-defensins (unpublished). In contrast, purified LPS did not induce hBD-2. Cytokine induction of defensins in colon carcinoma cells was studied by incubating the cells for different periods of time (2, 4, 6 and 24 hrs). As can be seen in Table 6, IL-1β induced hBD-2 mRNA expression but not mRNA for the other defensins. TNF-α only displayed a weak inductive effect on the expression of hBD-3 mRNA while IFN-γ showed a clear inducing effect of mRNA for this defensin.

The inducibility of hBD-2 by bacteria and IL-1β is due to an NF-κB responsive element in the hBD-2 promoter (123). IL-1 has a major role in inflammatory and immunological responses and increased levels have been reported in experimental colitis and in patients with active UC (263). One possibility for the increased hBD-2 expression in UC IECs may be penetrating bacteria that are phagocytosed by macrophages. These will
secrete elevated IL-1β and induce hBD-2 in neighbouring IECs. Another alternative is direct induction via TLR engagement. Intestinal epithelial cells are normally hyporesponsive to LPS, and this has been explained by the fact that they are missing CD14 expression (44), as well as by downregulated TLR4 and MD-2 (the co-receptor for TLR4 signalling) (43). hBD-3 was reported to contain an IFN-γ responsive element (112) and IFN-γ has been shown to induce hBD-3 in lung and skin epithelial cells (114). None of the pro-inflammatory agents induced hBD-4 mRNA expression although TNF-α showed a tendency to do this in some experiments. The unresponsiveness of hBD-4 to pro-inflammatory cytokines was also reported for lung epithelial cells (115). HD-5 and HD-6 were not upregulated in any of the colon carcinoma cell lines in response to individual bacteria or proinflammatory cytokines. The reverse was argued by Wehkamp et al who reported induction of HD-5 and HD-6 in the colon carcinoma cell line CaCo-2 using a cocktail of IFN-γ, IL-1β, and TNF-α for 24h (264).

3.5 Effect of pro-inflammatory cytokines and bacteria on expression of CEACAMs (Paper II)

To determine if bacteria and/or pro-inflammatory cytokines made by IEL, macrophages or epithelial cells could affect the expression of CEA, CEACAM1, CEACAM6, and CEACAM7 (CEA family members known to be glycocalyx components of colonic epithelial cells), colon carcinoma cells were incubated with these agents for various times. mRNA expression was assessed by qRT-PCR and protein expression by immunoflow cytometry and immunoelectron microscopy. IL-1β and LPS incubated with LS174T-, HT-29- and T84 cells for 1h, 4h, or 96h had no inductive effect on the expression of any of these molecules. Log-phase S. typhimurium, E. coli, M. luteus, and B. megaterium incubated with LS174T- or HT-29 cells for 1h followed by 5h incubation in media deprived from bacteria also failed to induce mRNA expression.

In contrast, IFN-γ proved to be an effective inducer. LS174T-, HT-29- or T84 cells incubated with IFN-γ displayed a significant mRNA upregulation of CEACAM1-3/4L (the long cytoplasmic form with 3 or 4 Ig domains) after 4h. Further increase was seen after 96 h of incubation. CEACAM1 protein was present on the surface of LS174T (~40%) and HT-29 (~50%) and, after 96h IFN-γ treatment increased to ~55% and ~80%, respectively. Immunoelectron microscopy revealed that the intensity of the surface staining varied among cells, and was also present in so called intracellular lumina, particularly in HT-29 cells. IFN-γ also induced CEA- and CEACAM6 mRNA in HT-29 and T84 cells after 96 h, whereas no
effect was seen in LS174T cells. The reason for the latter finding was probably that untreated LS174T express maximally high levels of both CEA and CEACAM6. CEA protein was present on the surface of LS174T (45%) and increased to ~70% after IFN-γ treatment for 96h. Intracellular staining, as analyzed by immunoflow cytometry, showed that 80% of untreated and 90% of treated LS174T cells contained CEA. Untreated HT-29 had almost no CEA present on the cell surface (~2%). Surface staining increased to ~17% after 96h IFN-γ treatment. Immunoelectron microscopy showed CEA-positive material in intracellular lumina of both cell lines and also in untreated HT-29 despite the low/absent surface expression. CEACAM6 protein was present in very low levels on the cell surface of LS174T and increased from 4% to ~18% upon 96h IFN-γ treatment. CEACAM7-2 mRNA levels were unchanged after 1 h and decreased after 4 h and 96 h of IFN-γ treatment. The other isoform of CEACAM7-1, which is expressed at ~100 times lower concentration, was also unaffected by IFN-γ.

Induction of CEA family molecules by IFN-γ has been reported before (265, 266), but this is the first study in which all four members are analyzed quantitatively in the same cells, giving a complete picture of their expression levels and inducibility pattern. This is also the first study in which the inducibility of CEACAM7 has been investigated.

CEA, CEACAM1, CEACAM6 and CEACAM7 are specifically located to the apical glycocalyx of normal colonic epithelium. Their function(s) in normal colon mucosa is still unknown. It has, however, been suggested that these CEACAMs play a role in innate immunity by trapping bacteria in the glycocalyx and thereby preventing them from invading the IECs (62). If bacteria reach down to the microvilli and bind to CEACAM1, a pinching off process is probably initiated leading to the release of microvesicles with bound bacteria. CEACAM1-3/4L has an intracellular domain containing ITAM and ITIM motifs for phosphorylation (63) and is probably directly involved in signaling. The shedding of the molecules via vesiculation from microvilli is thought to be mediated by increased intracellular Ca+ concentrations. Loss of CEACAMs upon shedding requires new synthesis of these molecules. Bacteria themselves, cytokines released from immune cells, or IECs could affect this synthesis. Bacterial treatment, LPS, or IL-1β (prominent epithelial cytokine) failed to induce mRNA of these CEACAMs, whereas IFN-γ was a potent inducer. The rapid effect on CEACAM1 expression was a direct effect on transcription, while for CEA and CEACAM6 the upregulation was likely an effect of induction of cellular differentiation. The inability of live bacteria to induce these CEA family molecules may be a consequence of the lack of cellular polarity. Cell lines in culture are not polarized as in the normal physiology of the
intestine. Further studies using polarized monolayers of T84 and organ cultures of colonic mucosa will hopefully clarify the question of whether or not bacteria can directly induce CEACAMs.

In an inflammatory state (where IFN-\(\gamma\) levels can be elevated in IELs) the mucous layer may be diminished and the barrier function weakened, which would increase the accessibility of the IEC surface for luminal bacteria. Thus, a higher pinching-off rate would be obtained with a requirement of new synthesis. This could be accomplished through IFN-\(\gamma\) produced by IELs sensitized for the higher load of bacteria. The CEACAMs would be upregulated on the apical surface of the epithelial cells and act by efficient binding and pinching off as one important effect function in innate defence. This would be consistent with the increased resistance to bacterial infections for IFN-\(\gamma\) treated epithelial cells (21).

3.6 CEA molecules and mucins in CD (Paper III)

To study whether an abnormal glycocalyx and/or mucus composition was a predisposing factor for CD, and/or for the occurrence of bacteria in CD, we analyzed IECs from CD patients for the expression of MUC2, MUC3, CEA, and CEACAM1 mRNA by qRT-PCR and immunohistochemistry. MUC2 was investigated because it is a prominent secreted mucin exclusively produced by goblet cells in the normal intestine. MUC3 occurs both in a membrane bound and a secreted form and was studied because it is produced both by absorptive epithelial cells and goblet cells. MUC2 and MUC3 mRNA were expressed at similar levels in the \(v\)-IEC and \(c\)-IEC cell fractions of normal jejunum. However, MUC3 mRNA levels were unaffected by the disease but MUC2 levels were significantly increased in active CD compared to controls. The MUC2 mRNA levels returned to normal in treated patients. Immunohistochemical staining for MUC2 in active CD showed staining of the apical portion of enterocytes in many crypts and the cytoplasm of goblet cells, while in controls only goblet cells showed positive staining. Moreover, the MUC2 staining was related to disease activity. Taken together, these results indicate metaplastic development of goblet cells in CD (compare expression of HD-5). Levels of IFN-\(\gamma\) mRNA in IEL correlated with MUC2 mRNA in \(c\)-IEC of individual samples. Thus it is possible that the increased MUC2 levels in CD caused by aberrant epithelial cell differentiation are also the consequence of high IFN-\(\gamma\) production in intraepithelial T cells. Another explanation for MUC2 upregulation is that it is caused directly by the bacteria, as was reported for MUC2 in the lung (267).

Analysis of mRNA for the long form of CEACAM1 (a component of absorptive epithelial cells in the small intestine as well as large intestine),
and of mRNA for CEA (exclusively expressed by goblet cells in the small intestine), revealed that there was no significant difference between CD patients and controls for either of them although challenged CD tended to show slightly elevated CEA mRNA values. Immunohistochemistry for CEACAM1 revealed the expected expression at the apical surface of villous and upper crypt IECs in controls and in CD patients with staining of the luminal epithelium surface in flat mucosa. Staining for CEA was positive in goblet cells in the small intestine of CD patients with no obvious difference in staining intensity from controls. Based on the data from colon carcinoma cell lines (see section 3.5), upregulation of CEA and CEACAM1 was predicted since IFN-γ in IEL of CD patients is strongly upregulated (246). We have no explanation for this discrepancy other than that the situation may be totally different given the more “sterile” milieu in the small intestine compared to the large intestine.

3.7 Glycosylation patterns in IECs from children with CD (Paper III)

The presence of bacteria in all three patient groups with celiac disease further raised the question of whether altered glycosylation patterns of epithelial products such as mucins and glyocalyx components could be a predisposing factor. To adress this question we used a panel of 21 biotinylated lectins to stain jejunum from untreated (active CD) and treated CD, as well as control jejunum. Lectins are carbohydrate-binding proteins isolated from plants, prokaryotes, and animals that have been proven useful for localization of glycoconjugates in vitro and in vivo (268-271). The lectin panel consisted of Concanavalin A (Con A), Datura stramonium lectin (DSL), Dolichos biflorus agglutinin (DBA), Erythrina cristagalli lectin (ECL), Griffonia simplicifolia lectin I (GSL-I), Griffonia simplicifolia lectin II (GSL-II), Jacalin, Lens culinaris agglutinin (LCA), Lycopersicon esculentum lectin (LEL), peanut agglutinin (PNA), Phaseolus vulgaris leucoagglutinin (PVL), Phaseolus vulgaris erythroagglutinin (PVE), Pisum sativum agglutinin (PSA), Ricinus communis agglutinin I (RCA-I), Solanum tuberosum lectin (STL), Sophora japonica agglutinin (SJA), soybean agglutinin (SBA), Ulex europaeus agglutinin I (UEA-I), Vicia villosa lectin (VVL), wheat germ agglutinin (WGA), and succinylated wheat germ agglutinin (sWGA). Absorptive cells were evaluated for staining of Golgi complex, cytoplasm, and glyocalyx, and Goblet cells for mucin staining. The majority of the lectins showed no demonstrable difference in staining patterns between CD patients and controls. However, five lectins showed a difference and were chosen for an enlarged study. They were: GSLI, PNA, SBA, UEA-I, and sWGA. Three interesting patterns arose: 1) UEA-I, which is an L-fucose-binding lectin, displayed intensive goblet cell staining in
jejenum from 15/16 CD patients but no staining in controls. Glycocalyx of absorptive cells of CD patients was also more intensely stained by UEA-I than of controls. 2) PNA with main specificity for galactosyl (β-1, 3) N-acetylgalactosamine showed staining of glycocalyx of control jejunum but not of CD patients. 3) Golgi-complex staining of absorptive cells with GSLI, SBA (GalNAc specificity), and sWGA (GlcNAc specificity) was weak or absent in jejunum from patients with active CD in comparison to treated CD and controls. Similar observations were reported for WGA and SBA brush border staining in CD compared to controls (272). It is interesting to note that the staining patterns obtained with UEA-I and PNA in CD were independent of disease activity. Thus treated CD patients with normal villus architecture showed the same staining pattern as patients with active disease. Since we have previously noted that CD patients with no clinical symptoms still had significantly more bacteria than controls, one might speculate that individuals who develop CD have abnormal glycosylation of the mucous/glycocalyx layers allowing bacterial binding to the epithelium.

Lectins are used for microbial adhesion to tissue glycoproteins, e.g. *Escherichia coli* and *Salmonella typhimurium* adhere to mannose residues on host cells via their type 1 fimbriae. The carbohydrate moieties of glycoproteins and lipids not only serve as receptors for binding of bacterial surface proteins, toxins, etc but also serve as nutrients for the commensal microflora. There are several examples where microorganisms themselves have been shown to induce the production of certain required glycoconjugates or a change in the glycosylation of luminal membranes and cytoplasmic glycoconjugates of small intestinal epithelial cells (269, 271, 273-278). *Bacteroides thetaiotamicron* has the ability to metabolize fucose and can apparently induce the expression of a host α-1, 2-fucosyltransferase in epithelial cells without binding to the cells (273). In our case it is not possible to distinguish between the possibilities that altered glycosylation is an inherited property among CD patients or that bacteria themselves influence glycosylation. However, the presence of adherent bacteria in jejunal mucosa of CD patients may be related to unique carbohydrate structures of the glycocalyx/mucous layers of CD patients. These glycosylation differences could facilitate bacterial adhesion. As in IBD, luminal Ags (in this case gluten) contribute to maintenance of the inflammatory process by continual stimulation of immunocompetent cells in the mucosa. Besides the Ag gluten, resident bacteria could play an important role in maintenance of the inflammation. If bacteria themselves influence the glycosylation, the question is why these bacteria influence the glycosylation only in CD patients. This could indicate presence of pathogenic bacteria as a primary effect in the disease or that the changed
milieu in CD mucosa offers a new niche for commensal bacteria that in turn modulates glycosylation. Another possibility is that individuals who develop CD (genetically or due to the allergic inflammation) primarily adopt changes in glycosylation which allow binding of bacteria. An interesting parallel is that UEA-I does not stain small intestinal goblet cells of germ-free mice but strongly stains goblet cells in conventional mice (271).

3.8 Defensin expression in plasma cells (Paper V)

When colon tissue from UC patients was subjected to *in situ* hybridization with antisense probes for hBD-3 and hBD-4, we observed positive staining not only of the epithelium but also of scattered cells in the lamina propria. Such cells were found in high numbers in colons from UC patients and in low numbers in control colons. Moreover the two probes seemed to reveal the same cells. To determine the nature of this cell type we performed *in situ* hybridization and immunohistochemistry on consecutive sections. Staining with mAbs against CD3 (T cells), CD19/CD20/CD22 (B cells), CD68 (monocytes/macrophages), CD14 (monocytes/macrophages), CD13 (neutrophils/monocytes), CD15 (neutrophils), CD80 and CD86 (monocytes/dendritic cells/activated B cells), and KiM4 (follicular dendritic cells) all failed to overlap with hBD-3 and hBD-4 mRNA positive cells. However, staining with a mAb directed against the plasma cell marker CD138 correlated well with hBD-3 and hBD-4 mRNA positive cells. The specificity of the anti-CD138 Ab for intestinal plasma cells in LP was evaluated and we could confirm by immunoelectron microscopy that the positively stained cells indeed were plasma cells.

To further substantiate the notion that plasma cells produce defensins, we subjected one normal colon sample to fractionation on anti-CD138-coated paramagnetic beads to enrich for plasma cells. The cells that bound to the column expressed hBD-3 and hBD-4 mRNA. We have further analyzed the plasmacytoma cell line U266 (279) by qRT-PCR and demonstrated the presence of hBD-3 (qRT-PCR and cloned) and hBD-4 as well as hBD-1 (qRT-PCR only). Finally, the mouse myeloma cell line SP2/0 was studied for defensin expression by RT-PCR and sequencing of the amplified products. SP2/0 cells expressed mbd-2, mbd-3, and mbd-4 mRNAs (unpublished data) indicating that plasma cells in mouse also have this property. This is the first observation that plasma cells actively participate in innate immunity as well as in adaptive immunity by producing defensins and antibodies. It is tempting to speculate that defensins released by plasma cells mainly act as chemoattractants, as has been suggested for hBD-2.
4. CONCLUSIONS

- Colonic IECs from UC patients express significantly higher levels of mRNA for HD-5, HD-6, hBD-2, hBD-3, hBD-4 and lysozyme compared with epithelial cells from control colon.

- For MbC with colonic localization, the levels were somewhat increased although not to the same levels as for UC. MbC with ileal localization showed no increase of mRNA for any of the defensins.

- The marked induction of HD-5, HD-6 and partly for lysozyme in UC is explained by the emergence of metaplastic Paneth cells in colon.

- The increased expression of β-defensins is explained by the inducibility of these peptides by pro-inflammatory cytokines and bacteria (probably via TLRs).

- Plasma cells in LPL of UC patients produce hBD-3 and hBD-4. This finding gives further support to the recent suggestions that defensins not only have a role as antimicrobial effector molecules but also have an important role in the initiation of the adaptive response, e.g by attraction of immune cells.

- IFN-γ acts upregulating on CEACAM1 in colonic epithelial cells and induces differentiation of the epithelial cells so that the expression of CEA and CEACAM6 are increased while CEACAM7 is downregulated.

- Scanning electron microscopy showed presence of rod-shaped bacteria on the jejunal mucosa surface of CD patients. Bacteria were present in about 40% of children with active CD and in about 20% of treated, clinically symptom–free CD compared to 2% of controls. Presence of bacteria seems to be a trait of CD patients.

- mRNAs for MUC2, HD-5, HD-6 and lysozyme were increased in active CD but levels were retained to normal in treated patients. The increased production was a consequence of goblet- and Paneth cell metaplasia, which in turn correlated to the increased IFN-γ from IEL in CD.

- A difference in glycosylation of certain glyocalyx/mucin proteins exposes unique carbohydrate structures that is predisposing for the binding of the rod-shaped bacteria seen.
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6. REFERENCES


